Examining Reelin Expression and Neural Plasticity in Animal Models of Depression

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ABSTRACT

Stress is an important risk factor for the development of depression, but little is known about the neurobiological mechanisms by which stress might promote depressive symptomatology. The hippocampus and amygdala are susceptible to the detrimental effects of prolonged elevated stress hormone levels and neuroplastic changes within these brain regions have been linked to the onset of depression. Some of the neurobiological changes associated with prolonged elevated glucocorticoids include decreased neurogenesis, synaptic plasticity, dendritic morphology, and spine density within the hippocampus and increased dendritic morphology and spine density in the amygdala. Interestingly, recent evidence has described a regulatory role for the extracellular matrix protein reelin in synaptic plasticity, hippocampal neurogenesis, dendritic arborization, and spine density. Moreover, reelin has been shown to be decreased in neuropsychiatric disorders, such as schizophrenia, bipolar disorder, and depression. Combined, these results suggest that reelin may be an interesting protein to examine in regard to the pathogenesis of depression and to further elucidate potential therapeutic targets for the treatment of this disorder.

The goal of the current research was to provide a comprehensive examination into the role of repeated stress on reelin and neural plasticity in the pathogenesis of depression through multiple preclinical studies. Given the association between reelin and hippocampal plasticity, in chapter 2 the effects of repeated exposure to corticosterone (CORT) or physical restraint on reelin expression in specific hippocampal regions were examined. Results revealed that there was a significant decrease in the number of reelin-positive cells in the CA1 stratum lacunosum and the subgranular zone of the dentate gyrus in rats that received CORT, but not in rats that

received restraint. Interestingly, these results parallel our laboratory's previous observation that CORT increases depression-like behavior but physical restraint does not. As reelin was decreased in the subgranular zone, it suggests that this protein is in a prime location to influence neurogenesis. Accordingly, chapter 3 focused on assessing the effects of different durations of CORT on behavior, hippocampal reelin expression, and neurogenesis, by subjecting rats to 7, 14 or 21 days of repeated CORT injections (40 mg/kg, s.c.) or vehicle injections. Results revealed that both the 14-day and 21-day CORT-treated rats showed increased depressive-like behavior in the forced swim test, significantly fewer reelin-positive cells and decreased neurogenesis compared to the control rats. In chapter 4, mice with a genetic deficit in reelin expression were used to examine their vulnerability to the depressogenic effects of CORT. We hypothesized that heterozygous reeler mice (HRM), with approximately 50% normal levels of reelin, would be more sensitive to the depressogenic effects of CORT than wild-type mice (WTM). Mice received injections of either vehicle, 5 mg/kg, 10 mg/kg, or 20 mg/kg of CORT, and then were assessed for changes in depression-like behavior, reelin expression, and neurogenesis. The effects of CORT on behavior, the number of reelin-positive cells, and hippocampal neurogenesis were more pronounced in the HRM than in the WTM, providing support for the idea that mice with impaired reelin signaling are more vulnerable to the deleterious effects of glucocorticoids. As reelin is expressed in GABAergic interneurons and our previous studies consistently revealed decreases in reelin number following CORT exposure, in chapter 5 the effects of repeated CORT and restraint stress on GABAergic and glutamatergic markers in the hippocampus and amygdala were examined. Western blotting analyses revealed that CORT significantly decreased the GABAergic markers, GAD65 and the α2 receptor subunit, and increased the vesicular glutamate transporter VGLUT2 within the hippocampus. We also found that corticosterone decreased the

GABAergic markers, GAD67 and the α2 receptor subunit, in the amygdala. Restraint stress had no significant effect in either of these areas. These findings suggest that the depressogenic effects of CORT may be related to alterations in GABAergic and glutamatergic neurotransmission within these structures. Together these results support a relationship between glucocorticoid-induced depressive-like behavior and decreases in reelin, neurogenesis and GABAergic signaling and provide support for investigating reelin as a novel therapeutic target for the treatment of depression.

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DEDICATION

I would like to dedicate this dissertation to my Mother-in-law (Rhonda Ross), to my Mémère (Eva Lussier), and to my Grandpa (Kenneth Campbell) who were there when I started this journey and who I wish could be here with me to celebrate the completion of my Doctorate. Each of them supported and inspired me to dedicate my life to the pursuit of knowledge and to find a vocation that makes me happy.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin hormone
ApoER2	apolipoprotein E receptor 2
AVP	arginine vasopressin
BDNF	brain-derived neurotrophic factor
BLA	basolateral amygdaloid complex
BrdU	bromodeoxyuridine
CA	Cornu Ammonis
CA1sl	CA1 stratum lacunosum
CA1so	CA1 stratum oriens
CA1sr	CA1 stratum radiatum
CA3so	CA3 stratum oriens
CA3sr	CA3 stratum radiatum
CDK5	cyclin-dependent kinase 5
CMS	chronic mild/unpredictable stress
CORT	corticosterone
CREB	cyclic AMP-response element binding protein
CRH	corticotropin-releasing hormone
CRF_1	corticotropin-releasing factor 1
DAB1	Diabled-1
DCX	doublecortin
ECL	enhanced chemiluminescence
FGF-2	fibroblast growth factor-2

FST	forced swim test
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
GCL	granule cell layer
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
GSK3β	glycogen synthase kinase 3β
HPA	hypothalamic pituitary adrenal
HRM	heterozygous reeler mice
IML	inner molecular layer
LIS1	lissencephaly 1
MAPK	mitogen-activated protein kinase
MML	middle molecular layer
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NeuN	neuron-specific nuclear protein
NMDA	N-methyl-D-aspartate
NSFT	novelty suppressed feeding test
OML	outer molecular layer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PFC	prefrontal cortex
PI3K	phosphatidylinositol-3-kinase

Prox1	Prospero-homeobox 1
PSA-NCAM	Polysialylated neural cell adhesion molecule
PSD-95	postsynaptic density protein 95
PVN	paraventricular nucleus
SDS	sodium dodecyl sulfate
SFKs	SRC family tyrosine kinases
SSRIs	selective serotonin reuptake inhibitors
RELN	reelin gene
RMS	rostral migratory stream
SGZ	subgranular zone
TCA	tricyclic antidepressants
TK	thymidine kinase
TUJ1	neuron-specific class III β-tubulin
V_{1b}	vasopressin 1b
VEGF	vascular endothelial growth factor
VGLUT2	vesicular glutamate transporter 2
VGCV	valganciclovir
VLDLR	very low-density lipoprotein receptors
WTM	wild-type mice

CHAPTER 1

Introduction to the Relationship between Stress and Depression and Associated Neurobiological Alterations

An integral aspect of the nervous system is its ability to maintain homeostasis while under the influence of alterations in the external environment. The internal regulation of basic functions of an organism is imperative for survival. For example, our bodies have the ability to engage in thermoregulation, osmoregulation, and immune and endocrine regulation. All of these adaptive processes are essential for the proper maintenance and functioning of an organism. Physiological mediators such as glucocorticoids, adrenalin, and cytokines act on receptors in various tissues and organs to produce effects that are adaptive over the short term, but that can be damaging over the longer term if the mediators are not terminated when they are no longer needed. Allostatic load is a term used to describe a situation in which mediators are maintained outside of their normal range due to insufficient or extreme/prolonged adaptive responses, when the individual survives but suffers adverse consequences (McEwen and Stellar, 1993; McEwen, 1998; McEwen, 2003). From these central ideas, an important question has emerged in behavioral neuroscience: What are the biological and behavioral adverse consequences of allostatic load?

Psychiatric disorders such as depression provide a good example of allostatic load. Stressful life events can be a precursor to depression, and depression is a disorder that affects the entire body, including cardiovascular, metabolic, immune, and nervous system function (McEwen, 2003). Elevated and prolonged exposure to stress hormones, such as cortisol, result

in a series of cellular and molecular changes that involve dramatic alterations in the structure and function of neurons throughout the central nervous system. However, the exact mechanisms and processes by which elevations in glucocorticoid levels contribute to the pathogenesis of depression are not fully understood.

In depression and other psychiatric disorders, stress hormones are involved in psychopathology, which is reflected by behavioral alterations such as emotional arousal and cognitive dysfunction (Carroll et al., 2007; Erickson et al., 2003; Kirschbaum et al., 1996; McEwen, 2008; Newcomer et al., 1999; Sachar et al., 1970). Support for a direct role of glucocorticoids in cognitive functioning also comes from reports showing that increased glucocorticoid levels in non-depressed participants can cause deficits in learning and memory (Dresler et al., 2010; Erickson et al., 2003; Kirschbaum et al., 1996). In addition, reduced depressive symptomatology in patients with depression who are treated with antidepressants is associated with decreases in stress hormone levels and increases in glucocorticoid receptors (Anacker et al., 2011a; Calfa et al., 2003; Himmerich et al., 2007; Nemeroff, 1996; Rojas et al., 2011). The above results support the role of stress hormones in the symptomology and behavioral changes seen in depressed patients.

The present dissertation includes a collection of studies that attempt to further our understanding of the relationship between repeated stress, neurobiological alterations in the hippocampus (a structure known to be involved in the stress response), and the related changes in emotional behavior. To begin addressing this question, I will examine the differences in stress paradigms [corticosterone (CORT) injections and restraint stress], duration of stress (1, 2, and 3 weeks of CORT exposure), and intensities of stress used (different doses of CORT) on

changes in depressive-like behavior. I have also investigated the effects of these stressors on reelin expression and neurogenesis in the hippocampus. In addition, I have examined morphological alterations in newly generated granule cells in the hippocampus following repeated stress. Furthermore, I have assessed whether reelin is a susceptibility factor for corticosterone-induced depression and alterations in neurogenesis. Finally, I have examined the effects of two different stress paradigms (CORT exposure and restraint) on inhibitory (ϒ-aminobutyric acid; GABAergic) markers in the hippocampus and amygdala in hopes of providing a potential mechanism to account for the changes in neurogenesis, reelin cell number, and affective behavior seen with exposure to stress.

The subsequent sections of this chapter will provide an introduction to the field of depression, neurogenesis, and reelin. First, I will provide a short discussion of the hypothalamic-pituitary-adrenal (HPA) axis and its relation to stress and depression. Second, I will provide an overview of the clinical factors related to depression and how the use of animal models has greatly enhanced our understanding of the pathological mechanisms responsible for the manifestation of depression-like behaviors. Third, I will discuss the influence of stress on hippocampal synaptic plasticity and neurogenesis in relation to the development of depression. I will also provide a short review of the role of the amygdala in stress and depression. Finally, I will give a brief overview of the role of reelin and GABA in the development of depression. This chapter will conclude with a discussion of specific questions that remain unanswered in the context of stress and depression research.

Chapters 2, 3, 4, and 5 describe experimental data that investigate the specific objectives outlined in Chapter 1. Chapter 6 provides an overview and discussion of the

implications of these findings. As well, areas that potentially need further investigation are proposed.

Hypothalamic-Pituitary-Adrenal axis

The major circuitry responsible for the stress response and ultimately the release of the major stress hormone glucocorticoids (cortisol in humans; corticosterone (CORT) in rodents) is the hypothalamic-pituitary-adrenal (HPA) axis (see Figure 1-1). As a consequence of a stress, the paraventricular nucleus of the hypothalamus releases corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP), among other hormones, into the hypophyseal portal system. In response to AVP and CRH, proopiomelanocortin producing cells within the anterior pituitary secrete adrenocorticotropic hormone (ACTH) into general systemic circulation. The adrenal gland is a major target of ACTH, which regulates the release of steroids and glucocorticoids into the circulatory system. The negative feedback of glucocorticoids on the hypothalamus and the pituitary gland cause these structures to stop releasing their related hormones, thus limiting the effects of glucocorticoids on metabolism, immunosuppression, and central nervous system function. There are two types of glucocorticoid receptors in the central nervous system: the mineralocorticoid receptor (MR; type I), which responds to low concentrations of glucocorticoids; and the glucocorticoid receptor (GR; type II), which responds to both basal and stress levels of glucocorticoids. The negative feedback control of CRH and ACTH release is mediated through the GR (Chrousos and Gold, 1992; Chrousos, 2007; Habib et al., 2001). The high concentration of GR in the medial prefrontal cortex (PFC) and hippocampus further provide higher-ordered inhibitory inputs into the hypothalamus.

Glucocorticoids not only regulate peripheral functions in the body, such as immunity and metabolism, but they also have profound effects on the brain. Numerous studies have linked the hippocampus and amygdala with the inhibition and activation of the HPA axis, respectively (Herman and Cullinan, 1997; Jacobson and Sapolsky, 1991; Sapolsky et al., 1984a; Sapolsky et al., 1986). For example, stimulation of the hippocampus or amygdala has been shown to decrease or increase glucocorticoid secretion, respectively (Dunn and Orr, 1984; Dunn and Whitener, 1986; Matheson et al., 1971; Redgate and Fahringer, 1973; Rubin et al., 1966), which suggests a direct link of these brain regions in the control of HPA activation. Moreover, many studies indicate that total hippocampectomy, fimbria-fornix lesions or hippocampal excitotoxic lesions increase the release of corticosterone and ACTH (Fendler et al., 1961; Knigge, 1961; Knigge and HAYS, 1963; Sapolsky et al., 1986), whereas large amygdaloid lesions or lesions to the medial or central nuclei of the amygdala reduce ACTH and/or corticosterone (CORT) secretion after stress (Dayas and Day, 2002; Feldman et al., 1994; Van de Kar et al., 1991). The effects of hippocampal lesions are most pronounced during the recovery phase of stress-induced glucocorticoid secretion (Herman et al., 1995; Sapolsky et al., 1984b). The high density of glucocorticoid receptors within the hippocampus in combination with the above mentioned findings suggest an important role for the hippocampus in negative feedback control of the HPA axis (Jacobson and Sapolsky, 1991; Sapolsky et al., 1986). In addition, the central and medial nuclei of the amygdala express GR and MR (Ahima and Harlan, 1990; Aronsson et al., 1988; Arriza et al., 1988; Fuxe et al., 1985). Although the MR expression is less than GR in the medial amygdala, the presence of both receptor subtypes suggests that the medial and central nuclei are involved in both basal and stress levels of glucocorticoids.

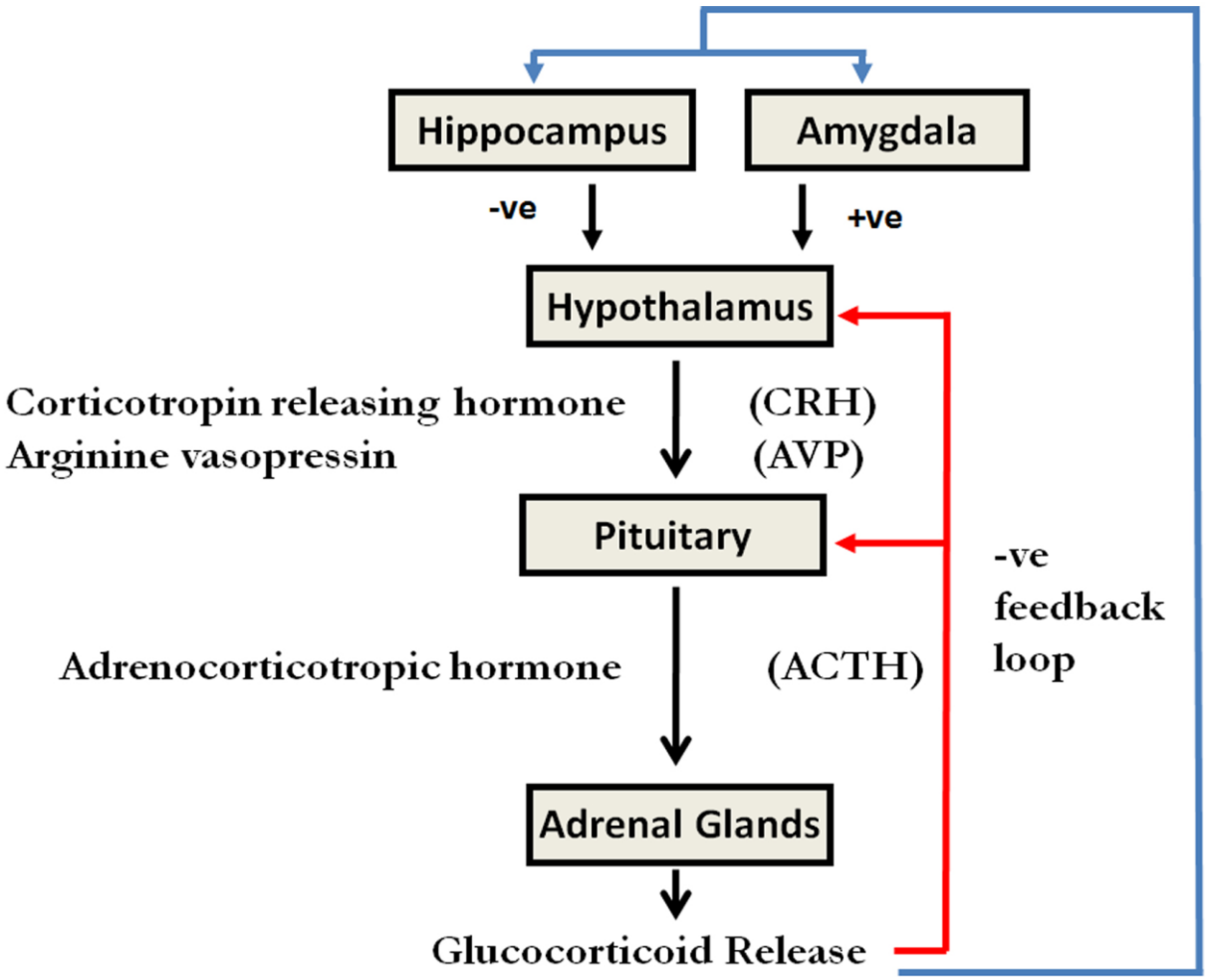

Figure 1-1. Regulation of the Hypothalamic-Pituitary-Adrenal Axis. The parvocellular neurons of the paraventricular nucleus of the hypothalamus release corticotropin releasing hormone (CRH) when influenced by stress. Important neural inputs consist of inhibitory efferents from the hippocampus and excitatory efferents from the amygdala. CRH acts on the anterior pituitary to release adrenocorticotropin hormone (ACTH) into the bloodstream. ACTH reaches the adrenal cortex where it stimulates the release of glucocorticoids. In addition to its many functions, glucocorticoids suppress CRH and ACTH synthesis and release through negative feedback inhibition onto the pituitary and hypothalamic neurons (red arrows). At elevated levels, glucocorticoids impair and/or damage the neurons, or alter neuronal function. For example, in pyramidal neurons of the hippocampus sustained exposure to high levels of corticosteroids results in dendritic arbor retraction and cell loss. Conversely, sustained exposure to high levels of corticosteroids promotes dendritic growth and expansion of the dendritic arbor within basolateral amygdaloid neurons (blue arrows). –ve below hippocampus represents a decrease in activation of the HPA axis, while +ve below amygdala represents an increase in HPA axis response when activated by these structures.

When the stressor becomes chronic or long-term, the negative feedback loop can become overwhelmed and no longer function properly. The end result is a sustained high level of glucocorticoids throughout the brain and periphery. These sustained elevated levels of glucocorticoids can bring about maladaptive changes. For example, in the hippocampus, prolonged exposure to glucocorticoids results in increased excitotoxic cell death, aberrant dendritic structure and spine morphology, and reduced neurogenesis (Dranovsky and Hen, 2006). Importantly, several studies have shown that these hippocampal alterations are associated with behavioral changes such as decreased learning and memory and increased depressive-like and anxiety-like behaviors in rodents (Duman et al., 2001; Duman, 2002; Jacobs, 2002; McEwen, 1999; McEwen and Magarinos, 2001; Nestler et al., 2002a).

Depression

Depression is a prevalent individual lifetime disorder estimated to affect 16.6% of the general population (Kessler and Wang, 2008). Characteristic symptoms of depression include decreased mood, anhedonia, decreased energy, altered appetite and weight, irritability, feelings of worthlessness and guilt, disturbances in sleep patterns, nervousness, and cognitive deficits (American Psychiatric Association, 2000; Nemeroff, 1998; Nestler et al., 2002a). In addition to these devastating symptoms, depressed individuals suffer from more physical illness and have a higher mortality rate than the general population (Nemeroff, 1998). Moreover, the high comorbidity rate of depression with other neuropsychiatric disorders, such as anxiety (Kessler and Wang, 2008; McEwen and Magarinos, 2001), makes it difficult to understand the pathology of this disease and makes it challenging to develop effective treatments for this disorder. The devastating consequences of depression not only affect the patient and his/her

family and friends, but it also places an enormous financial burden on society. However, despite the vast impact of this disease on the public, the precise neurobiological mechanisms involved in the etiology of depression still remain undefined.

One commonly accepted risk factor for the development of depression is stress. Many different areas of research support a role of stress as a risk factor for depression. For example, research has shown that the onset of depression is often preceded by a stressful or traumatic life event (Keller et al., 2007). In addition, patients with depression exhibit alterations in cortisol rhythmicity, and amplified and/or extended release of cortisol following stress (Belmaker and Agam, 2008; Burke et al., 2005; Checkley, 1996; de Kloet et al., 1998; Sapolsky et al., 1984a). Moreover, metyrapone, an antidepressant that acts on neuroendocrine substrates that control cortisol production shows efficacy in treating depressed patients (O'Dwyer et al., 1995). Furthermore, many different antidepressants that act on various neurotransmitter systems can also regulate HPA axis functioning (Heuser et al., 1996; Linkowski et al., 1987; Schule et al., 2009). The importance of the stress response in depression is further supported by the finding that failure of dexamethasone to normalize ACTH and cortisol secretion in depressed patients is associated with poor outcome and early relapse after antidepressant treatment (Nestler et al., 2002a; Southwick et al., 2005) suggesting that dysregulation of the HPA axis is indicative of treatment resistance. Overall, there is strong support for HPA axis dysfunction in the development, potential treatment efficacy, and relapse potential of depressed patients.

In accordance with the large concentration of GR in the hippocampus (McEwen et al., 1968; McEwen et al., 1969), depressed patients have shown decreases in hippocampal volume

that are related to the duration of depressive episodes (Sheline et al., 1996; Sheline et al., 2003). The increased and disrupted cortisol responses in depressed patients are suggested to be involved in the hippocampal alterations seen in these patients. In addition, cognitive disruptions in depressed patients are associated with these changes in hippocampal volume and potentially hippocampal functioning (Channon and Green, 1999; Erickson et al., 2003; Gualtieri et al., 2006; MacQueen et al., 2003; McEwen and Magarinos, 2001; Rubinow et al., 1984; Sternberg and Jarvik, 1976). Moreover, individuals with no neuropsychiatric disorders show increased memory disruption following increased glucocorticoid levels (Kirschbaum et al., 1996; Newcomer et al., 1999). The hippocampus has been linked to learning and memory in numerous human studies (Broadbent et al., 2004; Manns et al., 2003; Reed and Squire, 1997; Shrager et al., 2007; Starkman et al., 2003). The above results link elevated glucocorticoid levels, the hippocampus, and cognitive and memory functioning, which supports the idea that the hippocampus is an important structure to examine in regard to depressive symptoms and associated behavior.

Although the hippocampus is commonly studied when examining brain structures involved in depression, other structures have also been implicated. For example, several neuroimaging studies have shown that the amygdala is altered in depressed patients (Hastings et al., 2004; Mervaala et al., 2000). In addition, the amygdala has been implicated in the activation and negative feedback of the HPA axis (Dayas et al., 1999; Herman et al., 2005; Matheson et al., 1971; Mervaala et al., 2000; Roozendaal et al., 2009). Moreover, the amygdala is well known to be involved in emotional processing and fear responding (Ledoux, 2003; Ledoux, 2007; Phelps and Ledoux, 2005), both of which have been shown to be disrupted in

depressed patients (Murphy et al., 1999; Murphy et al., 2011; Phelps and Ledoux, 2005; Rubinow et al., 1984; Rubinow and Post, 1992).

Animal Models of Depression

Understanding the neurobiological changes associated with depression is critically important for understanding the course of the disease, developing biomarkers to guide diagnosis, and finding efficacious treatments for this debilitating disease. As clinical opportunities for examining neurobiological alterations in patients are limited to imaging studies and post-mortem examinations, preclinical models have been developed to further understand the pathogenesis of repeated stress in the development of depression-like behavior. Preclinical researchers have been utilizing animal models of depression for many years. Of the number of different paradigms that have been used, the most common ones include chronic mild/unpredictable stress (CMS), repeated restraint stress, repeated glucocorticoid exposure, learned helplessness paradigms, olfactory bulbectomy, and maternal separation (Cryan and Slattery, 2007). The validity of these models stems from them incorporating factors involved in the initiation of the disorder (i.e., chronic exposure to life stressors), and producing behaviors similar to those observed in depressed patients (e.g., anhedonia). It is also important that there are similar neurobiological changes between the model and the disorder (i.e., face validity), and that the consequential behavioral and neurobiological alterations in these models can be reversed by antidepressants and therapies effective in treating depression (i.e., predictive validity) (McKinney, Jr. and Bunney, Jr., 1969; Willner and Mitchell, 2002). Another criterion important in the development of a model is that it should be developed using a rational theory that is created to explain and test theories regarding the etiology of the disorder (i.e., construct

validity) (Willner and Mitchell, 2002). In addition, the reliability of an animal model is of great importance, as it is related to the reproducibility of the model being used. It is imperative that these models can be reproduced in many laboratories, numerous times: For example, replication using similar environmental influences (i.e., repeated restraint stress) to create consistent depressive-like behavior and/or neurobiological alterations (i.e., anhedonia and/or decreased hippocampal morphology), and reversal of these behavioral and neurobiological alterations by antidepressants are important for the reliability of these models (McKinney, Jr. and Bunney, Jr., 1969).

As depression is a heterogeneous disorder, it is hard to find an animal model that encapsulates all the symptoms of this disease. In addition, there are a few symptoms that preclinical researchers simply cannot recreate in a laboratory animal, such as feelings of guilt and suicidal ideation. Most of the animal models examine one or two symptoms of depression; the most common symptoms/behaviors that are measured are anhedonia and inferences of depression-like behavior (or learned helplessness) as measured by the forced swim test or the tail suspension test. For example, to infer depressive-like behavior in the forced swim test, there needs to be an increase in immobility behavior and decrease in energetic behavior such as struggling or swimming (Porsolt et al., 1977; Porsolt et al., 1978). Indeed there are numerous studies showing increases in immobility in the forced swim test in animals exposed to repeated stress (Gregus et al., 2005; Hill et al., 2003; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009; Mineur et al., 2006; Sterner and Kalynchuk, 2010; Strekalova et al., 2004). Moreover, many different types of antidepressants that have efficacy in treating human depression have been shown to decrease the amount of time a rodent spends immobile in the

forced swim test (Armario et al., 1988; Cryan et al., 2005; Porsolt et al., 1977; Porsolt et al., 1978).

Dr. Bruce McEwen's group started using CORT injections to examine the effects of glucocorticoids on GR occupancy (Sapolsky et al., 1984b; Sapolsky et al., 1985a). It was shown that serum CORT levels were increased 4 and 8 hrs after injection and decreased back to baseline by 24 hrs after injections (Sapolsky et al., 1985a). Since these pivotal studies showing that glucocorticoid administration produce similar levels of CORT as physical and psychological stressors (Sapolsky et al., 1984b; Sapolsky et al., 1985a; Tornello et al., 1982), glucocorticoid administration has started being used as a paradigm to study the pathogenesis of depression. The different types of administration of glucocorticoids includes pellet implantation, injections and drinking water (Brummelte et al., 2006; Brummelte and Galea, 2010b; Gourley et al., 2008a; Gregus et al., 2005; Hill et al., 2003; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009; Zhao et al., 2008a; Zhao et al., 2009; Murray et al., 2008; David et al., 2009), which show increases in depressive-like behavior in the forced swim test as inferred from an increase in immobility behavior and decreases in latency to immobility and swimming behavior (Cryan et al., 2005). In addition, CORT administration has shown anhedonia behavior such as decreased sucrose preference (David et al., 2009; Gorzalka et al., 2003; Gourley et al., 2008a), decreased grooming (David et al., 2009), supressed sexual behavior (Gorzalka and Hanson, 1998; Gorzalka et al., 2001; Hanson and Gorzalka, 1999), and decreased food reinforced responses (Gourley et al., 2008a; Gourley et al., 2008b). Repeated CORT administration also shows increases in anxiety behavior as measured by the predator odor test (Kalynchuk et al., 2004), the light/dark box (Murray et al., 2008), escape behavior (Stone et al., 1988), the open field test (David et al., 2009; Skorzewska et al., 2006) and the

novelty suppression feeding test (David et al., 2009). The high comorbidity of anxiety and depression in the human population (Kessler and Wang, 2008; McEwen and Magarinos, 2001) and the development of anxiety- and depressive-like behavior in the CORT administration paradigms supports the face validity of this preclinical model. Importantly, many of these CORT-induced behavioral changes can be reversed with antidepressant treatment (Ago et al., 2008; David et al., 2009), which supports the predictive validity of CORT administration paradigms as models of depression. CORT administration also produces physiological changes indicative of depression such as decreased weight gain (Brummelte and Galea, 2010b; Coburn-Litvak et al., 2003; Gregus et al., 2005; Johnson et al., 2006; Magarinos et al., 1998; Bush et al., 2003; Meijer et al., 1997), decreased adrenal weight (Bush et al., 2003; Meijer et al., 1997; Murray et al., 2008), and dysregulation of HPA axis function (Johnson et al., 2006).

CORT administration has been shown to have dose and time-dependent effects on depressive-like behavior and neurobiological changes (Johnson et al., 2006; Zhao et al., 2008a; Zhao et al., 2009). For example, our laboratory has previously shown that CORT produces a dose-dependent increase in depressive-like behavior in the forced swim test, changes in HPA axis functioning, and decreased weight gain following 21 days of injections (Johnson et al., 2006). Interestingly, a single injection of CORT has no significant effect on behavior in the forced swim test or weight (Johnson et al., 2006). Moreover, others have reported a decrease in depressive-like behavior in the forced swim test following smaller doses of CORT administered over a shorter duration (Stone and Lin, 2008; Zhao et al., 2009). In addition to the behavioral changes seen in CORT administration paradigms there are also neurobiological alterations seen in these models. For example, CORT administration has been shown to decrease neurogenesis (David et al., 2009; Hellsten et al., 2002; Karishma and Herbert, 2002;

Mayer et al., 2006; Murray et al., 2008), cause dendritic atrophy in the hippocampus (Magarinos et al., 1998; Magarinos et al., 1999; Sousa et al., 2000; Watanabe et al., 1992; Woolley et al., 1990) and PFC (Cerqueira et al., 2005; Seib and Wellman, 2003; Wellman, 2001), increases in dendritic morphology in the amygdala (Mitra and Sapolsky, 2008), and decreased brain derived neurotropic factor (BDNF) (Dwivedi et al., 2006; Jacobsen and Mork, 2006; Nitta et al., 1997).

Restraint stress is one of the oldest repeated stress models used in research and is favored because of its simplicity and ease of use. Restraint stress was first described in the context of gastric lesions studies (Selye, 1976) and later used for the purposes of examining the behavior, neurobiological and physiological responses to stress (Buynitsky and Mostofsky, 2009). Restraint paradigms range from one episode to multiple weeks of restraint and from a duration of 5 minutes to 6 hrs or more per restraint session (Buynitsky and Mostofsky, 2009; McLaughlin et al., 2007; Pare and Glavin, 1986). In addition, there are a number of different types of restrainers. For example, there are rigid plastic tubes, wire mesh restrainers, restraint cage or box, and taped or restricted limbs (Buynitsky and Mostofsky, 2009; Pare and Glavin, 1986). Restraint stress has been shown to alter both behavior and neurobiology. For example, restraint stress decreases learning and memory, increases ACTH and CORT secretion, increases depression-like behavior, CA3 dendritic atrophy, and decreased body weight gain (Abidin et al., 2004; Barha et al., 2011; Bauer et al., 2001; Conrad et al., 1996; Galea et al., 1997; Glavin et al., 1994; Grissom et al., 2008; Luine et al., 1994; Luine et al., 1996; Magarinos et al., 1997; Mitra et al., 2005b; Watanabe et al., 1992). Although there have been numerous studies showing alterations following repeated restraint stress, there have been discrepancies in findings depending on which restrainer and what duration and number of

restraint episodes are being used (Buynitsky and Mostofsky, 2009; Pare and Glavin, 1986). One of its major limitations is that repeated exposure to restraint produces stress response habituation that can result in inconsistent behavioral and neurobiological outcomes (Galea et al., 1997; Gregus et al., 2005; Grissom et al., 2008; Nestler et al., 2002b; Sterner and Kalynchuk, 2010).

The most valuable advantage that animal models have given researchers when studying depression-like behavior is the ability to examine associated post-mortem alterations following repeated stress and antidepressant treatment. Preclinical animal research has elucidated neurobiological alterations in areas such as the hippocampus, amygdala, and prefrontal cortex following many forms of repeated stress. Consistent changes include decreases in adult hippocampal neurogenesis, dendritic morphology, synaptic plasticity within the hippocampus of animals exposed to repeated stress (see Duman 2002; Jacobs et al. 2000; McEwen 2005; McEwen and Magarinos, 1997; Nestler et al. 2002; Nemeroff 1998; Sapolsky et al. 1985; Sterner and Kalynchuk 2011 for review). Decreases in dendritic morphology and synaptic protein alterations have also been found in the prefrontal cortex following repeated stress and glucocorticoid exposure (Akana et al., 2001; Cerqueira et al., 2005; Hastings et al., 2004; Radley et al., 2005). In addition, alterations in synaptic protein expression and increased dendritic morphology have been shown in the amygdala following repeated stress (Dayas and Day, 2002; Hunter et al., 2007; Mitra et al., 2005a; Mitra and Sapolsky, 2008; Mitra et al., 2009; Thompson et al., 2004; Vyas et al., 2002; Vyas et al., 2003; Vyas et al., 2004; Vyas et al., 2006). Moreover, animal models allow researchers to lesion specific brain structures and examine consequential outcomes. For example, amygdala lesions have been linked to decreases in HPA axis hormone release (Knigge, 1961; Prewitt and Herman, 1994; Roozendaal

et al., 1996). In addition, techniques have been developed to alter specific genes and proteins in different ways in order to directly examine their effects on behavior (Hommel et al., 2003; Mizumatsu et al., 2003). Characterizing the neurobiological effects in different repeated stress models of depression have given scientists new insights in the neuropathology of depression and offers insight into new ways to treat and potentially prevent the onset of depression.

The Hippocampus and Depression

The hippocampus has become very important in the study of depression for a number of reasons. First, decreased hippocampal volume has been shown in patients suffering from depression (Sapolsky, 2001; Sheline et al., 1996; Bremner et al., 2000; Sheline et al., 2003). Second, there is a high concentration of GR and MR throughout the hippocampal formation suggesting that this structure is involved in regulating stress responses (Arriza et al., 1988; Chao et al., 1989; Jacobson and Sapolsky, 1991; Sapolsky et al., 1985b). Third, several preclinical stress models have consistently revealed reduced hippocampal neurogenesis and increased dendritic atrophy within the CA3 and CA1 regions of the hippocampus (Brummelte and Galea, 2010b; Galea et al., 1997; Gould et al., 1998; Magarinos and McEwen, 1995; Magarinos et al., 1996; Magarinos et al., 1997; Nestler et al., 2002a; Watanabe et al., 1992; Wong and Herbert, 2006; Woolley et al., 1990). And finally, chronic treatment with antidepressants has been shown to reverse the deleterious consequences of repeated stress on neurogenesis and hippocampal atrophy (Czeh et al., 2001; Magarinos et al., 1999; Malberg et al., 2000; Malberg and Duman, 2003; Mayer et al., 2006), which importantly is associated with a reduction in depressive behavior.

Hippocampal Neurogenesis and Depression

Historically, scientists believed that you were born with the number of neurons that you would have for the rest of your life. They believed that there was no neurogenesis in the adult brain. However, pioneering studies from Altman and colleagues demonstrated that neurogenesis does occur in the young and adult brain in different structures including the olfactory bulb and dentate gyrus in rodents (Altman, 1962; Altman and Das, 1965; Altman, 1969). Neurogenesis was also shown in adult song birds during song-learning behavior and it was found that these newborn neurons became functionally integrated (Goldman and Nottebohm, 1983; Nottebohm, 1980; Nottebohm, 1981; Nottebohm, 1989; Burd and Nottebohm, 1985; Kirn et al., 1994; Kirn et al., 1999). Further support for adult neurogenesis in mammals comes from research examining tree shrews, macaque monkeys and marmoset monkeys (Gould et al., 1997; Gould et al., 1998; Gould and Tanapat, 1999; Gould et al., 1999; Leuner et al., 2006). However, one of the most integral neurogenesis studies came from Eriksson and colleagues when they found that neurogenesis occurred in the adult human brain (Eriksson et al., 1998). Although initially viewed with considerable scepticism, the recent acceptance of neurogenesis in adult mammals by the scientific community has encouraged researchers to examine the potential roles of this process in many different diseases, including depression.

How is neurogenesis studied in the adult mammalian brain? One method involves the use of 5-bromo-2'-deoxyuridine (BrdU), which is a thymidine analogue that can be incorporated into the DNA of dividing cells during the S-phase of mitosis. The incorporation of BrdU into dividing cells allows researchers to identify and birth-date cells that are

undergoing mitotic division. In combination with standard immunohistochemical techniques, the use of BrdU-labeling along with cell-type specific markers can permit investigators to examine the phenotype of newly formed cells (e.g., neuron vs. astroglia vs. endothelia). Utilizing BrdU labeling, researchers have demonstrated mammalian adult neurogenesis and examined the migration and integration of these newly generated neurons into the granule cell layer (GCL) of the dentate gyrus (Gage et al., 1998; Kempermann et al., 1997a; Kempermann et al., 1998; Kronenberg et al., 2003; Kronenberg et al., 2006; Kuhn et al., 1997) and olfactory bulbs (Fasolo et al., 2002; Kato et al., 2001; Kuhn et al., 2005; Suhonen et al., 1996). This new technique has been used to characterize the different stages of neurogenesis.

Stages of Neurogenesis

The different stages of neurogenesis include proliferation, followed by differentiation, migration, then maturation and integration (Kempermann and Gage, 2000). Within the hippocampus, precursor cells are produced and exist in the subgranular zone (SGZ), an area between the GCL and hilus. After dividing, the proliferating cells become postmitotic, migrate into the GCL, differentiate into excitatory neurons that become firmly integrated into hippocampal circuitry (Kempermann and Gage, 2000; van Praag et al., 2002) (see Figure 1-2).

Stem cells are multipotent cells that go through asymmetric division and are capable of long-term self-renewal. Radial glial cells are the most frequently found stem cells in the adult brain and are commonly referred to as type-1 cells. Radial glia are similar to mature astrocytes in that they have the same electrophysiological properties and morphological appearance (Doetsch, 2003; Seri et al., 2001), and express glial fibrillary acidic protein (GFAP) (von Bohlen Und Halbach, 2007) and the precursor cell marker nestin (Filippov et al., 2003; Fukuda

et al., 2003). These radial glial cells produce all three neuronal lineages: astrocytes, oligodendrocytes, and neurons. Radial processes extending from radial glial cells provide scaffolding for maturing precursors to migrate on (Doetsch, 2003). Another group of adult stem cells give rise to oligodendrocytes and GABAergic interneurons and expresses chondroitin sulfate proteoglycan (Belachew et al., 2003). It is important to note that the rate of proliferation of type-1 cells does not seem to be influenced by environmental factors (Encinas and Enikolopov, 2008; Kronenberg et al., 2003).

Type-1 cells give rise to progenitor cells identified as type-2 and type-3 cells. Type-2 cells express nestin but not GFAP and have short plump processes that are oriented parallel to the SGZ. These cells have a high probability of being BrdU-positive, suggesting that they are highly proliferative (Filippov et al., 2003; Kronenberg et al., 2003). There are two subtypes of type-2 cells: type-2a and type-2b. Type-2a cells are nestin-positive but doublecortin (DCX)-negative; while type-2b cells are both DCX- and nestin-positive (Kronenberg et al., 2003). DCX expression indicates the beginning of neuronal differentiation and migration (Francis et al., 1999). Polysialylated form of the neural adhesion molecule (PSA-NCAM) is highly co-expressed with DCX (von Bohlen Und Halbach, 2007). Importantly, type-1 cells are always negative for PSA-NCAM and DCX. Developing granule cells go through a transient stage of PSA-NCAM and DCX expression (Brandt et al., 2003; Rao and Shetty, 2004). Type-2b cells show signs of neuronal lineage determination by expressing neuron-specific nuclear (NeuN) protein and Prospero-homeobox 1 transcription factor (Prox1). At this stage, external environmental influences such as environmental enrichment (Veena et al., 2009b; Veena et al., 2009a), exercise (Wu et al., 2008; van Praag et al., 2005; van Praag et al., 1999), and contextual learning (Kee et al., 2007) can encourage neuronal progenitor cell survival. It has

been suggested that the selection of progenitors for survival could be amplified though elevations in GABA levels (Ge et al., 2006) or induction of NMDA receptor subunits NR2B and NR1 (Nacher et al., 2007). Antidepressant exposure increases progenitor population (Perera et al., 2007; Anacker et al., 2011b; Malberg et al., 2000; Kee et al., 2007), which has been suggested to be influenced by upregulating the vascular endothelial growth factor (VEGF) (Greene et al., 2009; Warner-Schmidt and Duman, 2007) and other growth factors (e.g., fibroblast growth factor-2, FGF-2; and brain-derived neurotropic factor, BDNF) (Huang and Reichardt, 2001; Palmer et al., 1995; Palmer et al., 1999).

Type-3 cells are positive for DCX, but negative for nestin (von Bohlen Und Halbach, 2007). Cells in this stage migrate into the GCL via radial migration. In this migrating phase of development, newborn neurons begin demonstrating lengthened morphology and migrate to their final destination in the granule cell layer, which is usually within the inner third of the GCL (Kempermann et al., 2003). It is thought that migrating cells reach their final destination early, seemingly during a progenitor cell stage. Complete exit from the cell cycle occurs at the type-3 stage, which coincides with the transient expression of the calcium-binding protein calretinin (Brandt et al., 2003). These cells express Prox1, which remains expressed throughout granule cell development and is found in all adult granule cells (Kronenberg et al., 2003). Once these newborn granule cells reach the postmitotic phase, they also express Hu (Tonchev et al., 2003) and neuron-specific class III β-tubulin (TUJ1) (von Bohlen Und Halbach, 2007).

Immature neurons that express DCX and calretinin begin projecting more extensive apical dendrites toward the outer molecular layer where they begin to form synapses and axons with mossy fiber boutons begin to project toward the CA3 pyramidal cell layer. This is the

most critical period for these immature neurons; it is the phase of maturation where they are selected for activation by external stimuli from learning episodes (Kee et al., 2007) and environmental enrichment (Brown et al., 2003; Kempermann et al., 1997b). When compared to mature neurons, immature newborn neurons are more excitable and exhibit greater long-term potentiation and decreases in induction threshold (Ge et al., 2007b; Ge et al., 2006; Wang et al., 2000). Importantly, these immature neurons are favourably recruited over mature neurons during activation of the dentate gyrus because of this greater plasticity (Kee et al., 2007).

Neurons develop mature morphology and become integrated into the surrounding circuitry, at which time there is decreased excitability due to increases in GABAergic inhibition (Piatti et al., 2006). As a result, novel stimuli cannot activate these cells, although the neuron can be reactivated by the same stimuli that originally recruited them (Becker and Wojtowicz, 2007). Newborn neurons begin integrating at 4-10 days after birth and are synaptically integrated by 4-8 weeks following birth (van Praag et al., 2002), whereas they reach a mature morphology 4 months after generation (Song et al., 2002). A marker for mature neurons, NeuN, becomes expressed during this phase and calretinin is exchanged for the calcium-binding protein calbindin (Brandt et al., 2003), and DCX and PSA-NCAM cease being expressed (Brown et al., 2003).

Regulating Factors of Adult Neurogenesis

Numerous studies have shown that the different stages of adult neurogenesis can be regulated by different environmental influences (Brown et al., 2003; Cameron et al., 1998; Kempermann et al., 1997b; Kempermann and Gage, 2000; van Praag et al., 2002). For example, repeated stress has been shown to decrease different stages of neurogenesis. Some researchers

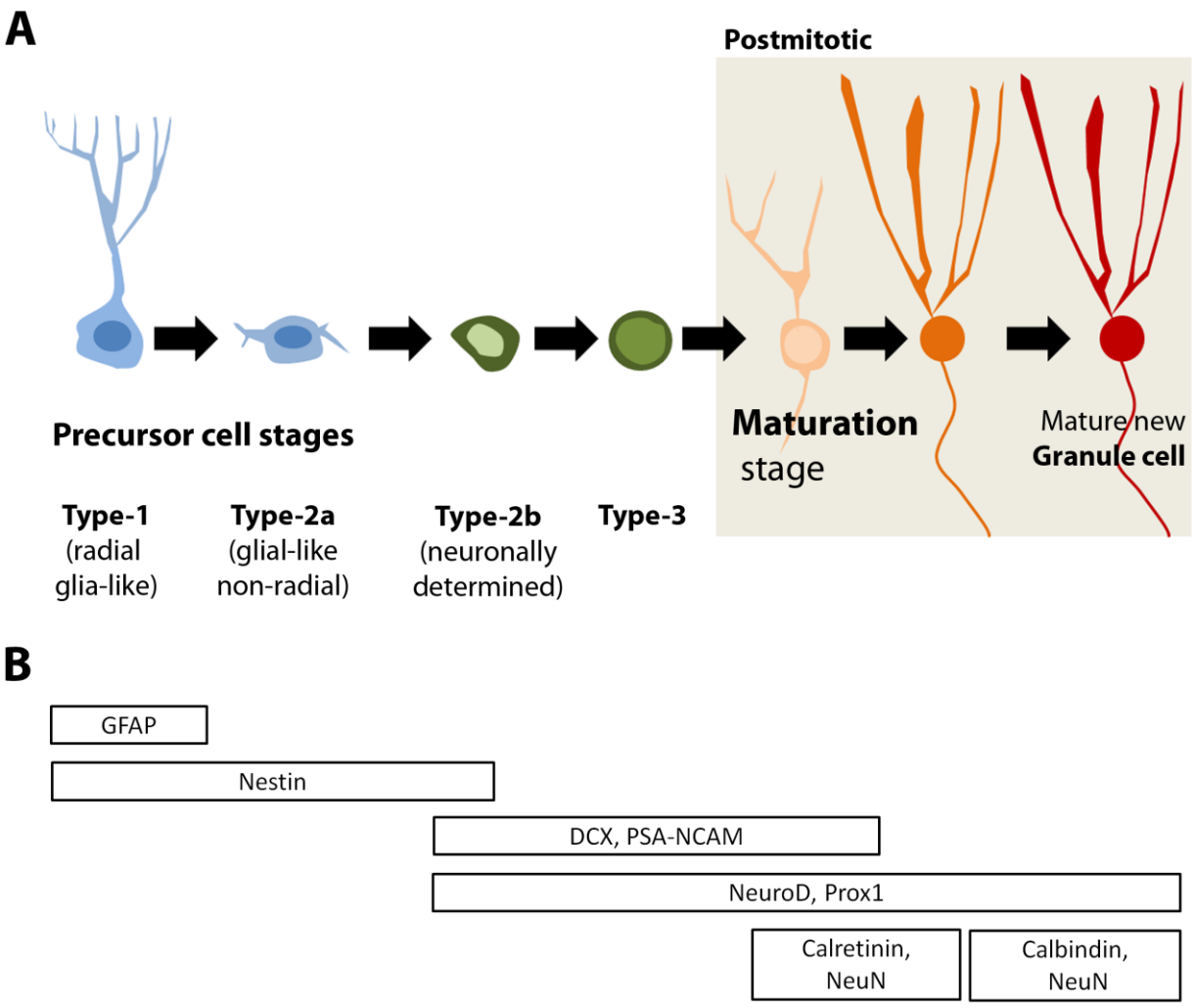

Figure 1-2. (A) Diagram of Adult Hippocampal Neurogenesis. This model of neurogenesis in the adult dentate gyrus showing proliferation and differentiation of stem cells in the SGZ into immature neurons. Immature neurons migrate ino the GCL of the dentate gyrus, and then become integrated into the existing circuitry. See text for more detail. (B) Markers of the different stages of neurogenesis.

have suggested that repeated stress or chronic glucocorticoid exposure decreases neurogenesis by suppressing progenitor proliferation (Brummelte and Galea, 2010a; Duman et al., 2001; Gould et al., 1997; Gould and Tanapat, 1999; Pham et al., 2003). Other research has suggested that stress has no impact on proliferation, but that it decreases the differentiation and survival stages of neurogenesis (Lee et al., 2006). On the other hand, environmental enrichment (Brown et al., 2003; Veena et al., 2009b; Veena et al., 2009a), exercise (Brown et al., 2003; van Praag et al., 1999; Wu et al., 2008), spatial learning (Kee et al., 2007), and seizures (Parent et al., 1997; Parent et al., 2006) all increase adult hippocampal neurogenesis. In addition, antidepressant increased VEGF levels are involved in increasing the number of progenitors (Warner-Schmidt and Duman, 2007) whereas BDNF is a mediator of neuronal fate determination (Huang and Reichardt, 2001). Understanding the regulation of neurogenesis by environmental factors and/or internal states is imperative for insights into the functional role of neurogenesis and also how this process can contribute to the pathophysiology of neuropsychiatric disorders, such as depression.

With advancements in technology, the role of neurogenesis can be directly measured in regard to behaviors and function. For example, research using a conditional knock-out approach with the herpes simplex virus thymidine kinase (TK) under the control of the GFAP promoter, suppressed neurogenesis with valganciclovir (VGCV) for 12 weeks and then measured corticosterone levels after exposure to a 15 min novel environment stressor (Schloesser et al., 2009). They found that compared to the wild-type controls, the neurogenesis supressed mice (TK mice) had increased levels of corticosterone following stress exposure. When examining corticosterone levels in animals left in their home cage and not exposed to stress, they found no significant differences between wild-type mice and neurogenesis

supressed mice (Schloesser et al., 2009). In support of these findings, another group has recently examined the stress response in these same TK mice after one and 17 days of restraint stress (30 min/day) at 0, 30 min, and 60 min following the restraint (Snyder et al., 2011). They found that 30 min after the restraint stress the TK mice had higher corticosterone levels than the wild-type mice after both one and 17 days of restraint stress and that the TK mice had impaired dexamethasone suppression of corticosterone following restraint stress (Snyder et al., 2011). To further examine the role of hippocampal neurogenesis, this same study examined the effected of decreased hippocampal neurogenesis through irradiation while sparing subventricular zone neurogenesis. They found similar elevated levels of corticosterone during the recovery period following restraint stress in hippocampal irradiated mice. Under basal conditions, the TK mice and wild-type mice displayed similar approach-avoidance behaviors in the novelty suppressed feeding test (NSFT). However, following acute restraint stress, the TK mice show an increased latency to feed compared to the wild-type mice suggesting that stress exposure is necessary to observe changes in anxiety-like behavior following reductions in hippocampal neurogenesis. Interestingly, these authors also reported that the TK mice showed higher levels of immobility in the forced swim test and decreased preference to a sucrose solution compared to wild-type control mice, suggesting that suppressing neurogenesis can produce a pro-depressive phenotype, reflected in higher levels of behavioral despair and anhedonia under basal conditions (Snyder et al., 2011). Taken together, these studies support a role for hippocampal neurogenesis in HPA axis function and stress response, and they also highlight a complex role of hippocampal neurogenesis in mediating anxiety- and depressive-like behavior.

Other researchers have also examined the role of hippocampal neurogenesis in the behavioral actions of antidepressants. For example, a pivotal study suppressing hippocampal neurogenesis with X-irradiation examined the role of hippocampal neurogenesis on the behavioral effects of fluoxetine and imipramine on the latency to feed in the NSFT. It was found that blocking hippocampal neurogenesis prevented the effects of fluoxetine in the NSFT (Santarelli et al., 2003). In addition, this study found that suppressed hippocampal neurogenesis prevents the influence of fluoxetine on grooming behavior and state of coat after chronic unpredictable stress exposure (Santarelli et al., 2003). However, this same group further examined the role of hippocampal neurogenesis in antidepressant action by examining the effect of monoamine antidepressants (e.g., imipramine, fluoxetine) and two non-monoaminergic HPA acting antidepressants SSR125543 (a corticotropin-releasing factor 1 (CRF_1) antagonist) and SSR149415 (a vasopressin 1b (V_{1b}) antagonist) on the NSFT (Surget et al., 2008). Interestingly, the authors showed that although both classes of antidepressant drugs (monoamine acting vs. non-monoamine acting) increased levels of hippocampal neurogenesis, irradiation only blocked the behavioral effects of the monoaminergic acting antidepressants (e.g., fluoxetine and imipramine) and not HPA acting antidepressants on the novelty suppressed feeding test and splash test in mice exposed chronic unpredictable stress. These important results suggest that although hippocampal neurogenesis appears to be required for the behavioral action of monoaminergic antidepressants, therapeutic effects can still be achieved, even in the absence of antidepressant-induced neurogenesis, by directly targeting the HPA axis and related neuropeptides. Further support for neurogenesis-independent effects of antidepressants comes from a recent study by David and colleagues (2009). In this study, hippocampal irradiation and chronic corticosterone exposure both blocked the beneficial

effects of fluoxetine treatment in the NSFT but not in the open field or forced swim tests (David et al., 2009). Taken together, these findings highlight the role of both neurogenesis-dependent and –independent pathways in underlying the therapeutic effects of antidepressant drugs.

The research described above demonstrates a complicated relationship between hippocampal neurogenesis, anxiety- and depressive-like behavior, and antidepressant efficacy. There are important drawbacks of the methods described above, such as the nonspecific effects of X-irradiation that could involve other areas beyond the hippocampus and potentially more importantly the lack of temporal specificity of ablation. This is an important point because there is increasing evidence that stages of neurogenesis are differentially affected in depression and that these different stages are functionally important to specific behaviors. For example, some research suggests that proliferation is decreased following repeated stress (Brummelte and Galea, 2010b; Gould et al., 1997; Duman et al., 2001; Pham et al., 2003; Gould and Tanapat, 1999), whereas others suggest that proliferation is not affected but it is differentiation and maturation that is decreased with repeated stress (Lee et al., 2006). Interestingly, there is a critical period between ~2-6 weeks after birth that neurons are preferentially incorporated into the existing circuitry and become functional (Ge et al., 2007b; Kee et al., 2007; Zhao et al., 2006a). Therefore, if repeated stress alters differentiation and maturation of newly born cells then the cells that are ~2-6 weeks of age may be important in understanding the behavioral alterations seen following repeated stress or elevated CORT levels (Conrad et al., 1996; Kirschbaum et al., 1996; Luine et al., 1994). The above results suggest that examination of the different stages of neurogenesis is an important step for understanding the importance of environmental influences on hippocampal functioning.

Amygdala and Depression

The amygdala is a structure known to be involved in mood and emotion (Ledoux, 2007; Phelps and Ledoux, 2005; Ledoux, 2003) and thought to be involved in depression. In support of this idea, the amygdala has been shown to be altered in patients with depression (Sheline et al., 1998; Hastings et al., 2004). Moreover, patients with amygdala damage show deficits in the recognition of facial emotions (Adolphs et al., 1994; Adolphs et al., 1997; Adolphs and Tranel, 2004). Similar deficits have also been shown in depressed patients, with a strong tendency to ascribe neutral faces with negative emotions (Rubinow and Post, 1992). Interestingly, this impaired assignment of appropriate emotional valence to neural facial images has been linked to increased relapse potential (Bouhuys et al., 1999).

Importantly, in preclinical studies, the amygdala has been shown to be involved in the neurobiological response to numerous types of stressors (Van de Kar et al., 1991; Prewitt and Herman, 1997; Prewitt and Herman, 1994; Dayas et al., 1999). For example, studies of repeated stress have shown an increase in dendritic complexity and length, and spine density within the amygdala (Mitra et al., 2005a; Mitra and Sapolsky, 2008; Vyas et al., 2002; Vyas et al., 2003; Vyas et al., 2004; Vyas et al., 2006). Moreover, research has also implicated the amygdala as an important structure involved in the antidepressant effects of neurosteroids in a learned helplessness animal model of depression (Shirayama et al., 2011). In further support of the role of the amygdala in stress, previous work has shown that electrical stimulation of the amygdala increases glucocorticoid secretion (Gallagher et al., 1987; Mason, 1959; Redgate and Fahringer, 1973). Recently, an interesting study using optogenetics to stimulate the basolateral amygdala (BLA) terminals within the central (Ce) nuclei produced anxiolytic effects in the

open field test and elevated-plus maze (Tye et al., 2011), suggesting that this circuit is important in regulating anxiety behavior. Moreover, increased activation of the BLA has been found to amplify fear conditioning, whereas focal inactivation of the BLA with musimol impaired fear conditioning (Conrad et al., 2004). These results indicate that the amygdala may be an important structure for understanding the mechanisms involved in the pathophysiology of depression.

Reelin

Reelin is an extracellular matrix glycoprotein that is important for cell migration during embryonic development and for synaptic plasticity in adulthood. Although the cellular functions of reelin in the adult brain are not fully understood, numerous reports implicate reelin dysfunction in the etiology of several neuropsychiatric disorders, such as schizophrenia, bipolar disorder, autism, epilepsy, and depression (Eastwood and Harrison, 2006; Fatemi et al., 2000; Fatemi et al., 2001; Guidotti et al., 2000; Hong et al., 2000; Impagnatiello et al., 1998; Saez-Valero et al., 2003). Mice with a spontaneous deletion of the reelin (RELN) gene, called reeler mice, show significant disruption in cortical lamination that includes abnormal positioning of neurons and aberrant orientation of neuronal cell bodies and fibers (Badea et al., 2007; Caviness, Jr. and Sidman, 1973; Caviness, Jr., 1976; Caviness, Jr., 1982; D'Arcangelo, 2005; Hamburgh, 1963). Reeler mice also display behavioral abnormalities, such as a reeler gait, ataxia, and tremor, which can be explained to some extent by their maldeveloped cerebellum and inferior olivary complex (Blatt and Eisenman, 1985; Goffinet, 1983). Anatomical studies examining the cortex of these reeler mice indicated that all of the major

structures were present; however, they seemed to be disorganized in an inside-out lamination (Badea et al., 2007; Caviness, Jr., 1976).

Reelin signalling occurs when it binds to very-low density lipoprotein (VLDLR) and apolipoprotein E receptor 2 (ApoER2) receptors (D'Arcangelo et al., 1999; Trommsdorff et al., 1999). The binding of reelin to these receptors results in the phosphorylation of the adapter protein Disabled-1 (DAB1) by the Src family of tyrosine kinases (SFKs)/Fyn kinase (Hiesberger et al., 1999) (see Figure 1-3). Increases in the concentration of SRKs activate the cytosolic kinase cascade, which starts with phosphatidylinositol-3-kinase (PI3K) and ends with the inhibition of glycogen synthase kinase 3β (GSK3β). GSK3β is one of the main kinases involved in the phosphorylation of the microtubule-stabilizing protein tau. In addition, the phosphorylation of DAB1 leads to the recruitment of the lissencephaly 1 (LIS1) complex, which is involved in the regulation of cortical lamination and neural migration during development (Assadi et al., 2003). The interaction of ApoER2 with postsynaptic density protein 95 (PSD-95) is critical for the coupling of the reelin signaling complex to the N-methyl-D-aspartate receptor (NMDAR) (Beffert et al., 2005; Chen et al., 2005). Reelin activates the NMDAR on NR2 subunits by SFKs tyrosine phosphorylation, which results in the influx of NMDAR-mediated Ca2+. Increases in intracellular Ca2+ activate the transcription factor cyclic AMP-response element binding protein (CREB) via phosphorylation, which initiates gene expression important in dendritic growth, spine formation, and synaptic plasticity (Chen et al., 2005).

In the adult hippocampus, reelin is synthesized and released by a subset of GABAergic interneurons, where it contributes to synaptogenesis, dendritic arborization and

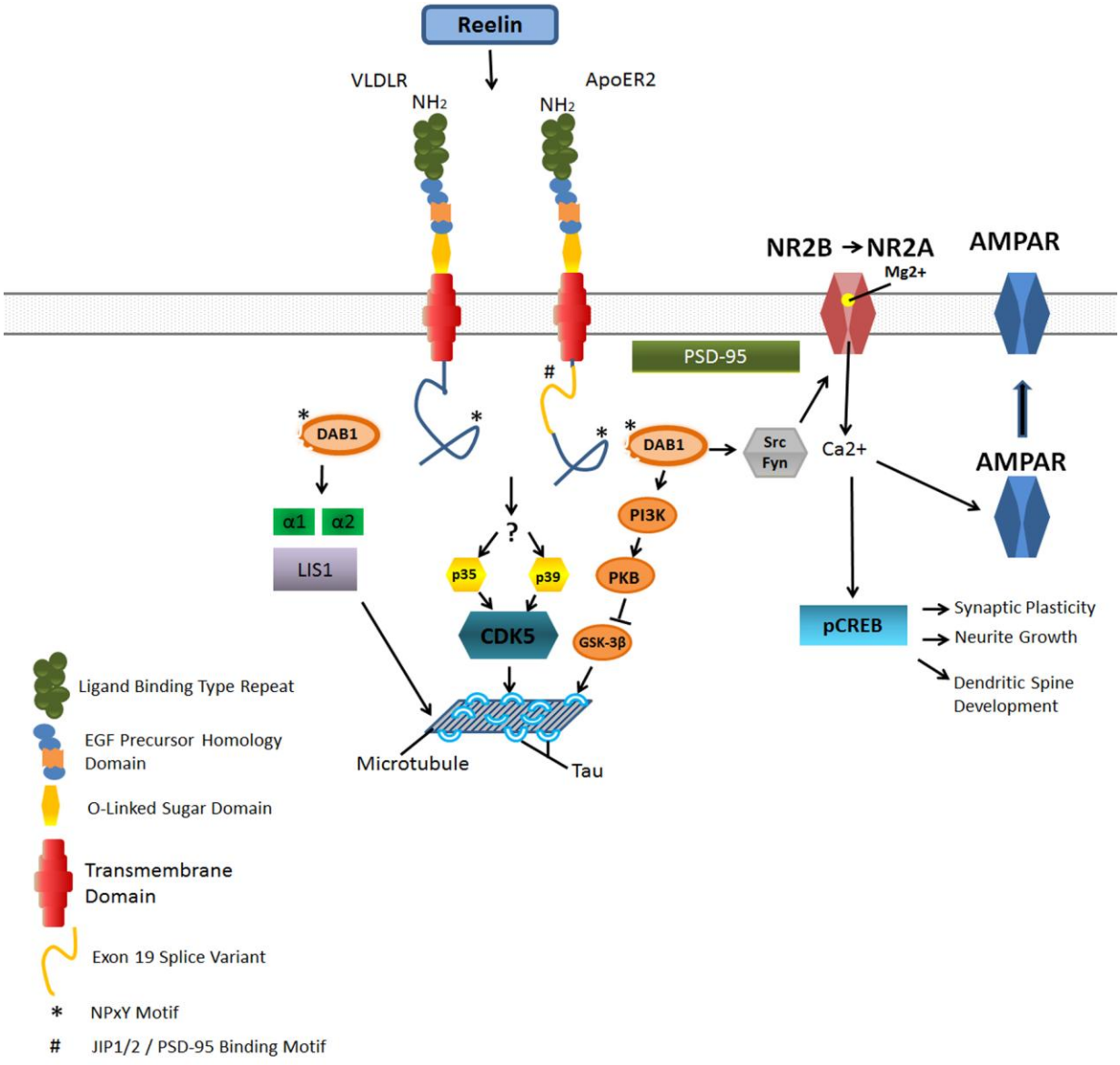
Reelin
VLDLR
ApoER2
NH2
NH2
NR2B → NR2A
Mg2+
AMPAR
PSD-95
#
*
DAB1
DAB1
Src
Fyn
Ca2+
AMPAR
α1
α2
LIS1
?
PI3K
p35
p39
PKB
CDK5
GSK-3β
pCREB
Synaptic Plasticity
Neurite Growth
Dendritic Spine
Development
Microtubule
Tau
Ligand Binding Type Repeat
EGF Precursor Homology
Domain
O-Linked Sugar Domain
Transmembrane
Domain
Exon 19 Splice Variant
* NPxY Motif
# JIP1/2 / PSD-95 Binding Motif

Figure 1-3. Schematic representation of reelin signalling pathway. Reelin binds to ApoER2 and VLDLR, which activates src-family tyrosine kinase (SFKs), which in turn potentiates tyrosine phosphorylation of DAB1. DAB1 binds to the NPxY motif of both receptors. Phosphorylation of DAB1 further activates phosphatidylinositol-3-kinase (PI3K) and subsequently protein kinase B (PKB). PKB activation inhibits the activity of glycogen synthase kinase 3β (GSK3β) (Beffert et al., 2002). As a result, phosphorylation of Tau is reduced, promoting microtubule stability. Tyrosine-phosphorylated DAB1 also binds to LIS1, which is associated with α-subunits (α1 and α2) to form a Parfah1b complex, which is involved in microtubule dynamics (Assadi et al., 2003; Zhang et al., 2007). Cyclin-dependent kinase 5 (CDK5) acts in parallel with reelin on numerous substrates, including microtubules. p35 and p39 can activate subunits of CDK5 (Beffert et al., 2004). The reelin receptor ApoER2 associates with postsynaptic density protein 95 (PSD-95), a scaffolding protein in the PSD, through an alternatively spliced exon. This interaction is crucial for the coupling of the reelin signalling complex to the NMDAR receptor (Qiu et al., 2006b). Reelin-activated SFKs tyrosine phosphorylate the NMDAR on NR2 subunits, resulting in the potentiation of NMDAR-mediated Ca^{2+} influx. Elevated intracellular Ca^{2+} can activate CREB, thereby potentially initiating the expression of genes that are important for synaptic plasticity, neurite growth, and dendritic spine development.

spine formation, and synaptic plasticity (Forster et al., 2006; Gong et al., 2007; Niu et al., 2008; Pesold et al., 1998). Recent research has shown that alterations in reelin levels can influence synaptic plasticity. For example, haploinsufficient HRM show decreased dendritic spine density in the cortex and hippocampus (Liu et al., 2001). In addition, cultured reeler neurons lack spines but incubation with recombinant reelin protein can increase dendritic spine number (Matsuki et al., 2008; Niu et al., 2008). Moreover, recent evidence has shown that intraventricular infusions of recombinant reelin enhances spatial learning and memory in the Morris water maze, increases CA1 LTP, and increases CA1 dendritic spine density (Rogers et al., 2011). Conversely, knocking down reelin expression in the medial prefrontal cortex of rats impairs cognitive performance on several tests, such as the prepulse inhibition task, object recognition test, and elevated t-maze (Brosda et al., 2011).

Reelin and Adult Neurogenesis

In the adult brain, reelin is expressed in GABAergic basket cells of the hippocampus that synapse with pyramidal cells, thus reelin participates in the GABAergic circuits that exert inhibitory control over hippocampal functioning (Pesold et al., 1998). Although the role of reelin in dendritic growth and spine formation has been demonstrated many times (Beffert et al., 2004; Liu et al., 2001; Niu et al., 2008; Qiu et al., 2006a; Rodriguez et al., 2002; Weeber et al., 2002), reelin has also been shown to influence neurogenesis. For example, reeler mice have fewer proliferating cells in the adult dentate gyrus (Zhao et al., 2007; Won et al., 2006). In addition, reeler mice have fewer immature neurons compared to wild-type mice (Won et al., 2006). Moreover, the number of cells co-expressing BrdU and NeuN is decreased whereas the number of cells co-expressing BrdU and GFAP cells is increased in reeler mice, indicating that

proliferating cells favourably differentiate into astrocytes in the absence of reelin (Zhao et al., 2007). In contrast, mice that have a threefold increase in hippocampal reelin expression (reelin-OE) show increased adult neurogenesis (Pujadas et al., 2010). Moreover, reelin-OE mice not only show increased number of proliferating cells, but they also show a higher number and immature neurons with hypertrophic dendritic growth (Pujadas et al., 2010), indicating that these cells were more likely to become functionally integrated into the existing circuitry. Furthermore, there were more BrdU/NeuN double-labeled cells in the reelin-OE mice compared to the wild-type mice (Pujadas et al., 2010), showing that newborn cells were more likely to become neurons in mice with increased reelin. In conclusion, these results support a role for reelin in the proliferation and differentiation of newborn cells, whereas deficits in reelin expression in the hippocampus decreases the number of proliferating cells and supports preferential astrocyte differentiation.

How reelin influences neuronal differentiation is not fully understood; however, a few possibilities exist. As reelin has been shown to activate the mitogen-activated protein kinase (MAPK) and PI3K cascades (see Figure 1-3), and these cascades have been shown to influence differentiation and cell survival (Beffert et al., 2002; Simo et al., 2007), this interaction could be a mechanism by which reelin influences differentiation of cells. In addition, reelin and cyclin-dependent kinase 5 (CDK5) have been shown to act in parallel on migration and synaptic plasticity (Beffert et al., 2004) (see Figure 1-3). CDK5 has also been linked to the maturation of newborn cells in the hippocampus of adult mice (Jessberger et al., 2008). Using retrovirus techniques, Jessberger and colleagues (2008) found that knocking down CDK5 in newborn granule cells altered maturation and migration patterns in this cell population. The relationship between reelin and CDK5 suggests another interaction of signaling cascades that

may influence granule cell migration. Another possible interaction that may be involved in the regulation of cellular differentiation during neurogenesis is that reelin activates Notch-1 signaling, which can increase radial glia process extension (Keilani and Sugaya, 2008). Support for the role of reelin and Notch-1 comes from results showing that Notch-1 is decreased in reeler mice, DAB1 co-localizes with Notch-1, and reelin and Notch-1 can interact to influence the formation of radial glial scaffolding during dentate gyrus development (Sibbe et al., 2009). Further research is needed to understand the relationship between these cascades and differentiation during neurogenesis.

The role of reelin in migration was first identified in reeler mice as they show abnormal lamination in the cortex, cerebellum and hippocampus (Curran and D'Arcangelo, 1998; D'Arcangelo et al., 1995; D'Arcangelo, 2001; Stanfield and Cowan, 1979). In addition, mice lacking DAB1 or reelin receptors have abnormal hippocampal lamination (Forster et al., 2002; Weiss et al., 2003). As reelin is expressed by cells within and adjacent to the subgranular zone of the dentate gyrus, and this area is the location of hippocampal neurogenesis (Pesold et al., 1998), it has been suggested that reelin is in a privileged location for influencing neurogenesis. In addition, DAB1 messenger ribonucleic acid (mRNA) has been shown to be expressed in GFAP-positive radial glial cells and neuronal progenitors, which suggests that these cells may respond to reelin signaling (Forster et al., 2002; Rice et al., 1998). Moreover, dentate granule cells express VLDLR and ApoER2 mRNA (Haas et al., 2002; Kim et al., 1996). As such, recent research has started focusing on reelin in regard to migration of newly generated cells in the adult hippocampus and rostral migratory stream (Frotscher et al., 2003; Courtes et al., 2011; Hack et al., 2002; Zhao et al., 2004; Kim et al., 2002). Support for the role of reelin in the migration of newly generated cells comes from animal models of epilepsy. For example,

aberrant distribution of the GCL is associated with decreased reelin expression in animal models of epilepsy (Gong et al., 2007; Heinrich et al., 2006; Muller et al., 2009; Fournier et al., 2010). Moreover, the aberrant dispersion of the GCL associated with chronic seizures can be prevented with hippocampal infusions of recombinant reelin (Muller et al., 2009). In addition, transplantation of human neural stem cells into the ventricles of reeler mice failed to show normal migration patterns such as those seen in wild-type mice (Kim et al., 2002). Moreover, the deficits in lamination in the dentate gyrus of reeler hippocampal cultures can be rescued by co-culturing hippocampal neurons with reelin-expressing cells (Zhao et al., 2004; Zhao et al., 2006b). The above results suggest that reelin is important in appropriate migration processes. However, reelin-OE mice have shown aberrant migration of proliferating cells within the rostral migratory stream and SGZ of the dentate gyrus (Pujadas et al., 2010). This aberrant migration was more severe in the rostral migratory stream (RMS) than in the hippocampus of the reelin-OE mice; which is not surprising given that the expression of reelin is 7 times the amount of that in wild-type mice in the RMS and only 3 times the level in the hippocampus (Pujadas et al., 2010). These combined results indicate that both too much and too little reelin can be maladaptive for proper migration of newborn neurons and perhaps an optimal level of reelin is required for proper migration of newly generated cells.

Another way that reelin can influence migration in the adult hippocampus is through increasing GFAP-positive fiber length, which maintains radial glial scaffolding and thereby supports the migration of postnatal granule cells (Zhao et al., 2007). Interestingly, radial glial scaffolding has been shown to be altered in reeler mice (Weiss et al., 2003; Frotscher et al., 2003). Indeed, multiple studies have suggested at least an indirect influence of reelin on lamination in the dentate gyrus by affecting formation of radial glial scaffolding, which dentate

neuroblasts use to migrate (Forster et al., 2002; Forster et al., 2006; Weiss et al., 2003; Zhao et al., 2004; Zhao et al., 2006b). Other research has suggested a direct effect of reelin on radial glial scaffolding through brain lipid-binding protein and DAB1 (Hartfuss et al., 2003). It has been suggested that the abnormal reelin signaling in the dentate gyrus can lead to neuroblasts forming chains and migrating aberrantly into the hilus or molecular layer (Gong et al., 2007). Indeed, Hack and colleagues have demonstrated that reelin acts as a detachment signal for chain-migrating neuroblasts in the adult subventricular zone. Once in the olfactory bulb, neuroblasts come into contact with reelin that is expressed by mitral cells, detach from the chains, and migrate radially into the granule and periglomerular layers of the olfactory bulb (Hack et al., 2002). Consistent with decreases in reelin expression shown in epilepsy patients and animal models of epilepsy (Fournier et al., 2009; Gong et al., 2007; Haas et al., 2002; Heinrich et al., 2006), a pilocarpine model of epilepsy also shows neuroblast chains in the dentate gyrus that are not found in controls, which supports the function of reelin in the aberrant migration of progenitor cells into the GCL in epilepsy (Parent et al., 2006). In addition, *in vitro* studies demonstrated that reelin facilitates the detachment of dentate gyrus chain-migrating neuroblasts and blocking reelin signaling increases chain-migrating neuroblasts and alters the location of these proliferating progenitors (Gong et al., 2007).

The last stages of development in newborn neurons, in which the dendritic processes extend into the molecular layer, spines develop, and cells become functionally integrated are likely to be influenced by reelin because of its role in synaptic plasticity and dendritic and spine development (Beffert et al., 2006; Herz and Chen, 2006; Niu et al., 2004; Qiu et al., 2006a; Rogers et al., 2011; Weeber et al., 2002). Within the adult brain, reduced levels of DAB1 or reelin are associated with reduced dendritic growth and decreased dendritic

branching (Niu et al., 2004). Interestingly, supplementation of reelin can reverse the reduction in dendritic growth seen in cell cultures lacking reelin (Niu et al., 2004; Qiu et al., 2006a).

Adult neurogenesis is a dynamic process in which all stages of neurogenesis are occurring at any given time point. Each transient stage of neurogenesis depends on the previous stage's outcome; therefore any influences by reelin on the different stages can have lasting alterations and functional implications. The above section has reviewed research that has supported the role of reelin in a number of these stages; however, the exact mechanisms that are involved in this relationship are unknown.

GABA in Depression

As previously mentioned, reelin is expressed in GABAergic interneurons in the adult brain (Abraham and Meyer, 2003; Pesold et al., 1998). Given the decreases in reelin seen in many different psychiatric disorders (Fatemi et al., 2000; Fatemi et al., 2001; Guidotti et al., 2000; Impagnatiello et al., 1998; Knable et al., 2004) it is not surprising that some of these disorders are also associated with lower GABA levels. For example, GABA levels are decreased in patients with depression (Gerner and Hare, 1981; Gerner et al., 1984; Gold et al., 1980; Kasa et al., 1982; Petty et al., 1992; Roy et al., 1991). In addition, an enzyme involved in the conversion of GABA from glutamate, glutamate decarboxylase (GAD), is also decreased in depressed patients (Fatemi et al., 2005b). As such, it is not surprizing that plasma levels of glutamate and its metabolites have been shown to be increased in patients with major depression (Altamura et al., 1993; Levine et al., 2000; Mauri et al., 1998; Mitani et al., 2006). In addition, decreased GABA concentrations in depressed patients can be reversed by treatment with SSRIs (Sanacora et al., 2002) and electroconvulsive therapy (Sanacora et al.,

2003a). Moreover, GABA receptor modulators have also been co-administered with selective serotonin reuptake inhibitors (SSRIs) to increase the efficacy of SSRIs in treating depression (Fava et al., 2006; Fava et al., 2011; Krystal et al., 2007). Furthermore, different GABA receptor subtypes have recently begun to be suggested as novel targets for the treatment of depression (Cryan and Slattery, 2010; Ghose et al., 2011; Luscher et al., 2011; Vollenweider et al., 2011). For example, the $GABA_A$ α1 receptor subunit is associated with anxiety, whereas the α2 receptor subunit has been shown to be involved in both anxiety and depressive-like behavior (see Smith and Rudolph 2011 for review). These findings suggest a dysfunction in the balance between excitatory and inhibitory neurotransmission in the brain that may be involved in the pathogenesis of depression and that GABA may be an important target for the treatment of this disorder.

Depression has a high comorbidity with anxiety (Roy et al., 1995) and the symptoms of both of these disorders respond to similar treatments (Anderson et al., 2008; Baldwin et al., 2005; Nutt, 2000), which suggests possible common neurobiological dysfunctions in these disorders. SSRIs, tricyclic antidepressants (TCAs), and to a lesser extent, azapirone anxiolytics (e.g. buspirone) have been used to treat both anxiety and depression (Anderson et al., 2008; Baldwin et al., 2005; Nutt, 2000). Benzodiazepines, which act directly on $GABA_A$ signaling, are extremely effective in treating anxiety disorders (Baldwin et al., 2005) but less effective in the treatment of depression (Anderson et al., 2008). Although the benzodiazepine alprazolam has shown some promise in treating depression for short durations (Jonas and Cohon, 1993; Jonas and Hearron, Jr., 1996; Petty, 1995; Remick et al., 1988), many of the side effects associated with benzodiazepine use, such as dependence and sedation, make it a less than ideal choice for long-term treatment (Anderson et al., 2008).

Preclinical research has shown that chronic stress and glucocorticoid exposure can decrease GABA expression and alter $GABA_A$ receptor expression in the hippocampus and other structures (Drugan et al., 1989; Orchinik et al., 1995; Verkuyl et al., 2005). Moreover, homozygous $GABA_A$ α2 receptor global knockout mice show increased immobility in the forced swim test and latency to eat in the novelty suppression feeding test (Vollenweider et al., 2011). The authors suggest that this deficit in α2 may indicate a role of this receptor in depressive-like behavior and may be an important target for treatment of this disorder (Vollenweider et al., 2011). Consistent with this idea, research has recently shown that selective α2, α3 $GABA_A$ receptor modulators have potential antidepressant effects (Luscher et al., 2011; Mohler, 2012; Vollenweider et al., 2011). The above results suggest that GABA and its receptors are important in the development of depressive-like behavior and may be important in treating this debilitating disease.

Specific Aims and Goals

The general aim of this dissertation is to examine the potential role of the extracellular matrix protein reelin in the pathogenesis of depression. More specifically, I have examined how exposure to glucocorticoids affects reelin expression in the hippocampus, and how deficiencies in reelin impact the course of hippocampal neurogenesis and promote a depressive phenotype. In addition, I have done preliminary examination into the role of repeated stress on markers of GABAergic and glutamatergic signaling in the hippocampus and amygdala.

Question 1: Reelin has been shown to be decreased in post-mortem examination of hippocampal tissue from patients with depression (Fatemi et al., 2000; Knable et al., 2004). Reelin has also been shown to be involved in synaptic plasticity, neurogenesis, and dendritic morphology and spine density (Niu et al., 2008; Pujadas et al., 2010; Qiu et al., 2006a; Rogers et al., 2011; Won et al., 2006), which are factors also shown to be altered in depression (Duman, 2002; McEwen and Magarinos, 2001; Sapolsky, 2001). Because our laboratory has previously shown that repeated CORT increases depressive-like behavior in the forced swim test, while restraint stress does not (Gregus et al., 2005), I was interested in examining if changes in reelin cell numbers could be associated with these behavioral differences. To examine this question, the effect of administering CORT injections (40mg/kg) or repeated restraint stress (6hrs/day) for 21 consecutive days on the number of reelin-positive cells in several subregions of the hippocampus (e.g., CA1 stratum lacunosum, SGZ, hilus, CA1 stratum radiatum and stratum oriens, CA3 stratum radiatum and stratum oriens, and the inner, middle, and outer molecular layer) were investigated.

Hypothesis: Repeated CORT injections should decrease the number of reelin-positive cells in the hippocampus as this paradigm produces robust increases in depression-like behavior. In contrast, repeated restraint stress should produce few changes in the number of reelin-positive cells because previous work has shown that this paradigm does not reliably enhance depression-like behavior in rats (Chapter 2).

Question 2: The observation of decreased reelin-positive cells in the subgranular zone of the dentate gyrus in rats subjected to 21 days of high dose CORT injections led me to wonder what

the time course of the change in reelin expression might be with CORT administration. More specifically, does the time course of reelin loss in GABAergic basket cells in the subgranular zone coincide with the time course for decreased neurogenesis in the adjacent granule cell layer and the time course for the expression of depression-like behavior? To examine these questions rats were subjected to either 7, 14, or 21 days of CORT (40 mg/kg; s.c.) or vehicle injections and depressive-like behavior, reelin cell number and immature neuron number and maturation were measured.

Hypothesis: There should be a gradual decrease in hippocampal neurogenesis and reelin cell number within the subgranular zone over time as the rats receive additional injections of CORT. These neurobiological changes should parallel the increasing severity of depression-like behavior, as measured by the forced swim test. In addition, the losses in immature cell numbers will parallel decreases in dendritic morphology in this subpopulation of cells. (Chapter 3).

Question 3: Chapters 2 and 3 revealed decreases in reelin positive cells in the hippocampus as a result of repeated CORT exposure. This raises the question of whether levels of reelin represent a susceptibility factor for depression. Are animals with less reelin expression more susceptible to the depressogenic effects of repeated CORT? To tackle this question, heterozygous reeler mice (HRM) were utilized as they have approximately half of the reelin expression as wild-type mice (WTM), and exposed to different doses of CORT injections (5, 10, and 20 mg/kg; s.c.) over a 21 day period. After the period of CORT administration, mice

were tested in the forced swim test and assessed for alterations in reelin cell number and immature neuron number and maturation.

Hypothesis: The HRM should be more susceptible than the WTM to the depressogenic effects of CORT. This should manifest itself as dose-dependent increases in depressive-like behaviour and dose-dependent decreases in reelin cell number and neurogenesis within the hippocampus that are greater in magnitude in the HRM than in the WTM. (Chapter 4).

Question 4: Because repeated exposure to CORT was associated with a reduction in reelin cell numbers and that reelin is expressed in GABAergic interneurons within the adult hippocampus, the next question was: Does repeated stress produce alterations in GABAergic markers and receptors subtypes in the hippocampus and amygdala? To address this question, rats were subjected to CORT injections or restraint stress for 21 consecutive days and then protein levels of GAD65 and GAD67, as well as the α1, α2, α3, and β2-3 $GABA_A$ receptor subunits, and the glutamate transporter VGLUT2 were examined using Western blots.

Hypothesis: The CORT injections, but not restraint stress, should produce decreases in several markers of GABAergic activity, with associated increases in markers of glutamatergic activity. These changes should be prominent in both the hippocampus and amygdala, as both of these brain structures have been implicated in the depression (Chapter 5).

CHAPTER 2

Repeated Exposure to Corticosterone, but not Restraint, Decreases the Number of Reelin-Positive Cells in the Adult Rat Hippocampus

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1. **Introduction**

Stress is an important risk factor for the onset of major depression. The association between stress and depression is based primarily on a large body of correlational evidence, including observations that many patients with depression exhibit hypersecretion of cortisol and disrupted cortisol rhythmicity (Belmaker and Agam, 2008), that they often respond positively to antidepressant drugs that act on the neuroendocrine substrates that regulate cortisol secretion (O'Dwyer et al., 1995), and that they frequently experience traumatic or stressful life events prior to the onset of depression (Keller et al., 2007). These findings have spurred a sustained preclinical research effort to identify the neurobiological mechanisms by which stress might influence the development of depression. In general, these experiments have shown that exposure to various forms of repeated stress in laboratory animals has deleterious effects on the hippocampus, such as impaired neurogenesis (Galea et al., 1997; Gould et al., 1997; Galea et al., 1997; Gould et al., 1998) and long-term potentiation (Artola et al., 2006), decreased dendritic complexity within CA3 pyramidal cells and dentate granule cells (Bessa et al., 2009; Magarinos et al., 1996; Vyas et al., 2002), and altered gene and protein expression (Alfonso et al., 2005; Hunter et al., 2009; Joels et al., 2004).

There are striking similarities between the hippocampal processes affected by exposure to repeated stress and the biological activities of the extracellular matrix protein reelin. Reelin is synthesized and released by specific GABAergic interneurons in the adult hippocampus, where it enhances cell migration and integration, synaptogenesis, dendritic arborization and spine density, and synaptic plasticity (Forster et al., 2006; Gong et al., 2007; Niu et al., 2008; Pesold et al., 1998). It is also critically important for the induction and maintenance of long-term

potentiation (Weeber et al., 2002). Not surprisingly, deficits in reelin signaling have negative consequences for hippocampal functioning. For example, HRM with approximately 50% of normal levels of reelin show impaired hippocampal-dependent learning and hippocampal plasticity (Qiu et al., 2006a). Interestingly, analyses of samples from patients with depression have revealed general decreases in reelin in hippocampal sections from postmortem brain tissue (Knable et al., 2004; Fatemi et al., 2000). Therefore, we hypothesized that hippocampal reelin signaling could be down regulated by exposure to repeated stress and that this might be related to depressive symptomatology. To our knowledge, the relationship between stress, reelin and depression has not been studied in a preclinical model of depression in adult rats (although some recent work has been done on anxiety and impulsivity in reeler mice subjected to maternal separation (Laviola et al., 2009; Ognibene et al., 2007).

We tested this hypothesis using two different stress paradigms in rats: 21 days of corticosterone (CORT) injections and 21 days of repeated physical restraint (6 h/day).We chose these paradigms because we have previously shown that repeated CORT injections produce reliable and robust increases in depression-like behavior but repeated restraint does not (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Knable et al., 2004). If stress-induced alterations in hippocampal reelin plays a role in the expression of depressive symptomatology, we would expect to see decreased reelin in rats subjected to repeated CORT injections but not in rats subjected to repeated restraint.

2. Materials and Methods

The subjects were 26 male Long-Evans rats (purchased from Charles River Laboratories, Montreal, QC, Canada) that weighed approximately 225–250 g at the time of arrival. The subjects were housed individually in standard polypropylene cages with Purina rat chow and water available ad libitum. The colony room was maintained at a temperature of 21 ± 1 °C with a 12 h:12 h light/dark cycle (lights on at 8:00 a.m.). All experimental procedures were carried out during the light phase of the light/dark cycle in strict accordance with the Canadian Council on Animal Care guidelines and a protocol approved by the University of Saskatchewan Animal Care and Use committee. The rats were handled in the colony room for a short period of time once per day for 7 days before the stress procedures began. Rats were randomly assigned to weight-matched groups and received one of the following four treatments for 21 consecutive days: repeated CORT injections (CORT group, $n = 6$), repeated vehicle injections (vehicle group, $n = 6$), repeated physical restraint (restraint group, $n = 7$), or repeated handling (handled group, $n = 7$). All CORT and vehicle injections were delivered subcutaneously at a volume of 1 ml/kg, between 9:00 and 10:00 a.m. each day. CORT (MP Biomedicals, Solon, OH) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitanmonooleate (Tween-80; Sigma–Aldrich, St. Louis, MO) and given at a dose of 40 mg/kg because previous work has shown that this dose reliably increases depression-like behavior in the forced swim test (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Knable et al., 2004). All daily restraint sessions were 6 h in duration and conducted during the light phase of the light/dark cycle (Gregus et al., 2005). Rats were transported out of the colony room to an adjacent, brightly lit, quiet room, and were placed into restrainers. The restrainers were transparent Plexiglas tubes (VWR Canlab, Mississauga, ON, Canada) measuring 24 cm long × 9 cm wide × 6 cm high. The length of each restrainer was

adjusted to limit, but not completely restrict, head and limb movements. Following each restraint session, rats were transported back to the colony room. To control for handling, each rat from the handled group was briefly picked up once each day and then placed back into its home cage. The body weight of each rat in each group was recorded for each day of the 21-day treatment period.

On day 22, the day after the final stress treatment, all rats were anesthetized with an overdose of sodium pentobarbital (i.p.) and perfused transcardially with 0.9% (w/v) saline and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH7.4). The brains were removed and postfixed in 4% (w/v) paraformaldehyde for 48 h at 4 °C. They were then placed in a 0.1 M phosphate buffered saline (PBS)/0.1% (w/v) sodium azide solution at 4 °C until sectioning. The tissue was sectioned using a vibrating microtome at 50 μm and stored in cryoprotectant at −20 °C until it was used for immunohistochemistry.

Reelin immunohistochemistry was performed on free-floating sections according to the following parameters. The sections were washed (5×10 min) in 0.1 M PBS and incubated in 0.3% (v/v) H_2O_2 in PBS for 30 min to block endogenous peroxidase activity. After 3 washes for 5 min each in PBS, the sections were pre-incubated for 30 min in a blocking solution containing 0.3% (v/v) Triton X-100, 1.5% (v/v) normal horse serum, and 1% (v/v) bovine serum albumin dissolved in PBS in order to block non-specific antibody binding. Sections were incubated for 48 h at 4 °C with reelin antimouse monoclonal antibody (Chemicon International, Temecula, CA) at 1:2000 diluted in a blocking solution. After incubation, the sections were washed in PBS (3 × 10 min) and incubated with a biotinylated horse anti-mouse IgG (1:200, Sigma–Aldrich, St. Louis, MO) secondary antibody diluted in 0.3% (v/v) Triton

X-100 PBS for 2 h at room temperature. After another set of washes in PBS (3 × 10 min), the sections were incubated for 1 hr in avidin-biotin complex (ABC; 1:500, Vectastain Elite ABC reagent, Vector Laboratories, Burlingame, CA) at room temperature. After three 5 min washes in PBS, the sections were stained using 0.033% (w/v) 3'- diaminobenzidine (Sigma-Aldrich, St. Louis, MO) and 0.00786% (v/v) H_2O_2 dissolved in PBS. The reaction was stopped by washing the sections several times in PBS. The sections were then mounted onto slides and left to dry overnight and dehydrated using a graded alcohol and xylene series, then coverslipped using Entellan resin solution.

For each rat in each group, the number of reelin-positive cells was visualized using a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc, Williston, VT) that was attached to a Nikon Eclipse E800 microscope. The following regions were quantified: CA1 stratum oriens (CA1so), CA1 stratum radiatum (CA1sr), CA1 stratum lacunosum (CA1sl), CA3 stratum radiatum (CA3sr), CA3 stratum oriens (CA3so), outer molecular layer (OML), middle molecular layer (MML), inner molecular layer (IML), subgranular zone of the dentate gyrus (SGZ) and the hilus. The pyramidal cell layers and the dentate granule cell layer were not quantified because the cells in these areas do not express reelin (Pesold et al., 1998). An experimenter who was blind to the treatment conditions manually counted reelin-positive cells using a profile counting method, which involved using a meander scan setting to sample nonoverlapping fields from each brain region. Five sections per brain were analyzed, one every 300 µm (from −2.40 mm from bregma to approximately −3.94 mm from bregma, according to Paxinos and Watson 1998). The number of reelin-positive cells counted for each brain region was averaged across the number of sections. This profile counting method is thought to be comparable to stereological cell counting because the

sections were sampled randomly (i.e., one in every six sections was sampled), which avoided multiple counting of the same cell (each section was 300 µm apart). Recent studies using this cell counting method have provided results that are similar to those obtained with stereological cell counting methods (Nixon and Crews, 2004; Yang et al., 2008).

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, v 16.0, Chicago, IL, USA). The criterion for statistical significance was set at $p < 0.05$. All data are represented as means ± the standard error of the mean.

3. Results

Figure 2-1 shows the mean body weight for the rats in each group during the 21-day stress phase of the experiment. The rats in each group were weight-matched, so they started with very similar body weights. However, the CORT rats weighed significantly less than the vehicle rats on days 7, 14 and 21 of the injections ($t(10) = -2.68$, $p < 0.05$, $t(10) = -3.97$, $p < 0.05$, and $t(10) = -3.45$, $p < 0.05$, respectively, Student *t*-test). Similarly, the restraint rats weighed significantly less than the handled rats on days 14 and 21 ($t(12) = -2.65$, $p < 0.05$ and $t(12) = -3.00$, $p < 0.01$, respectively, Student *t*-test).

Figure 2-2 shows the distribution of reelin-positive cells across the hippocampus and dentate gyrus for the rats in each group. Reelin-positive cells were observed throughout the hippocampus, but mostly in the CA1 region and the SGZ of the dentate gyrus. Our quantification revealed decreases in the number of reelin-positive cells in the CORT rats but

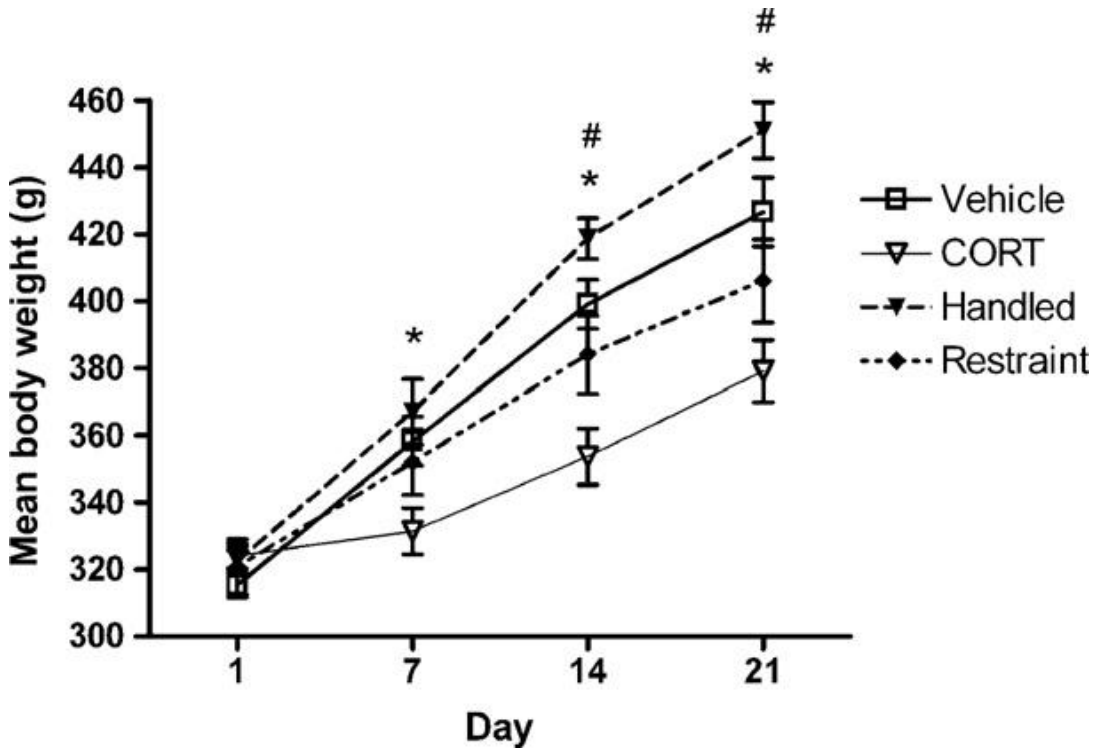

Figure 2-1. The effect of both stress paradigms on body weight during the 21-day stress phase of the experiment. Error bars represent mean ± standard error of the mean. Symbols (*) denotes a statistically significant difference between the vehicle and CORT rats ($p < 0.05$). Symbol (#) denotes a statistically significant difference between handled and restraint rats ($p < 0.05$).

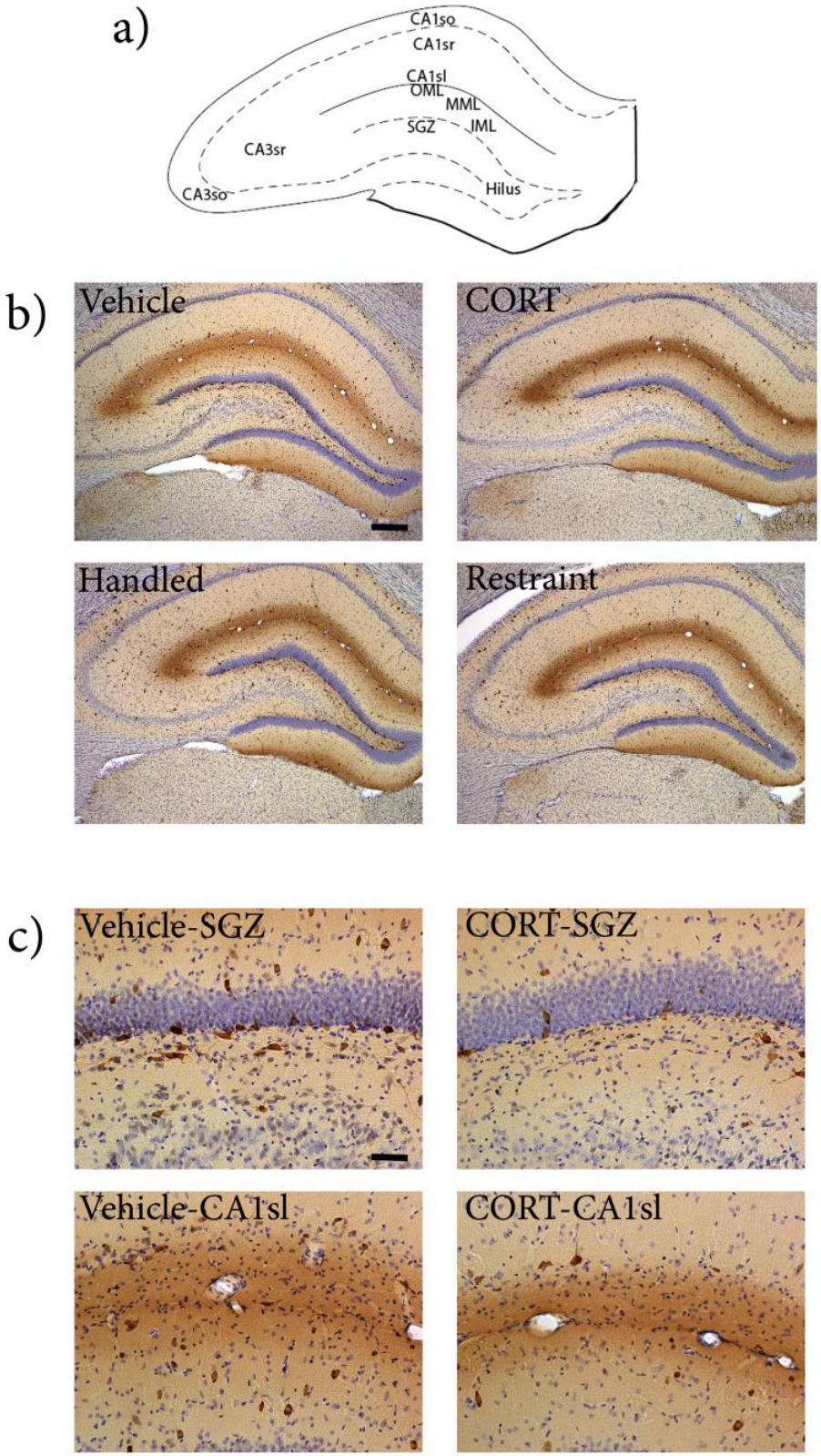
a)
CA1so
CA1sr
CA1sl
OML
MML
SGZ
IML
CA3sr
CA3so
Hilus
b)
Vehicle
CORT
Handled
Restraint
c)
Vehicle-SGZ
CORT-SGZ
Vehicle-CA1sl
CORT-CA1sl

Figure 2-2. (A) Diagram of the hippocampus showing regions in which reelin-positive cells were counted (adapted from Paxinos and Watson, 1998). (B) Photomicrographs showing examples of reelin immunolabeling across the hippocampus and dentate gyrus of rats that received repeated vehicle injections, repeated CORT injections, repeated handling, or repeated restraint stress. Scale bar: 300µm. (C) Higher power magnification of reelin immunolabeling in the CA1sl and SGZ of rats that received repeated CORT injections or repeated vehicle injections. Scale bar: 60 µm. *Abbreviations*: Stratum oriens (CA1so), CA1 stratum radiatum (CA1sr), CA1 stratum lacunosum (CA1sl), CA3 stratum radiatum (CA3sr), CA3 stratum oriens (CA3so), outer molecular layer (OML), middle molecular layer (MML), inner molecular layer (IML), and the subgranular zone of the dentate gyrus (SGZ).

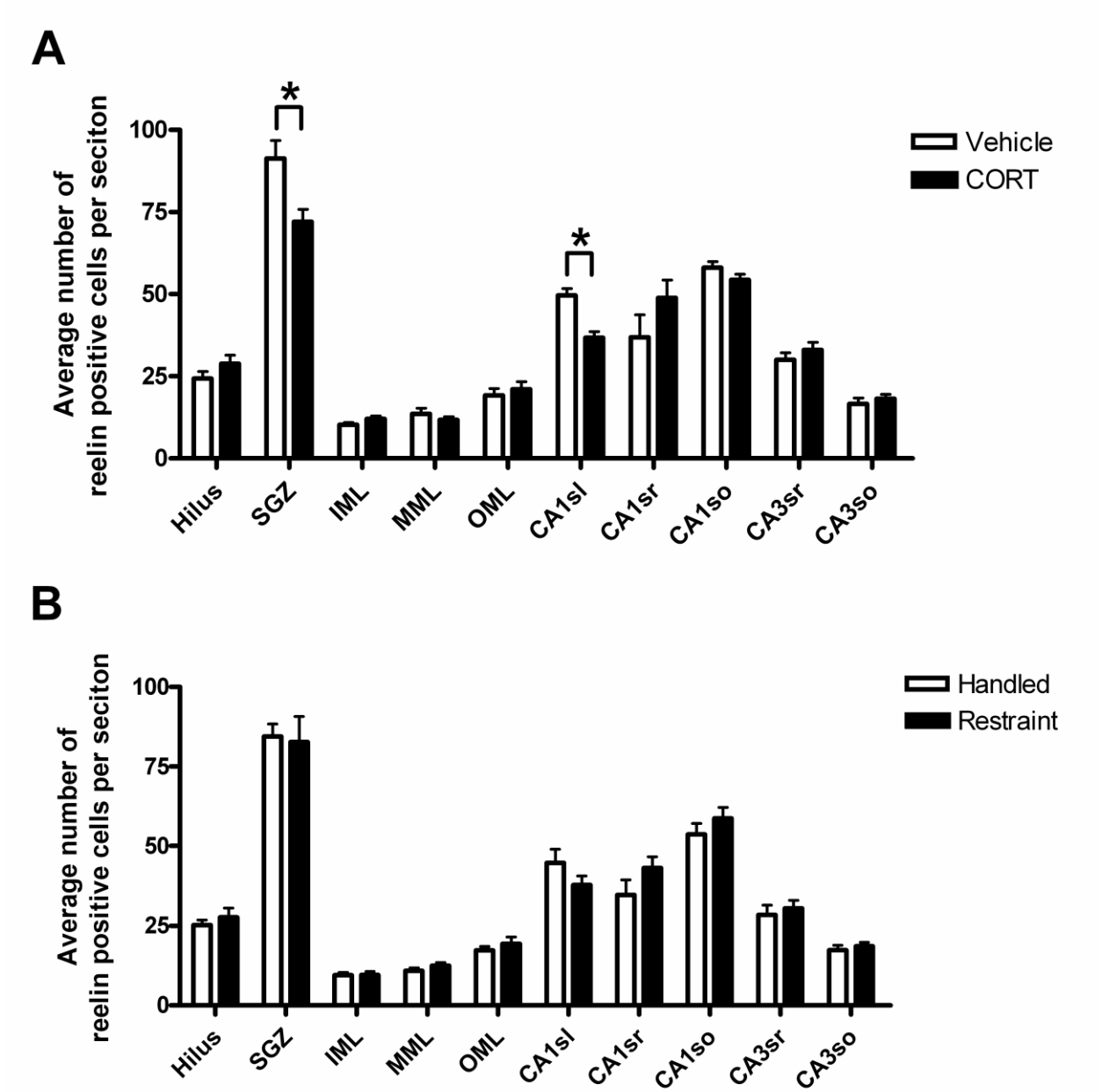

Figure 2-3. The mean number of reelin-positive cells in quantified regions of the hippocampus from rats exposed to repeated vehicle injections or repeated CORT injections (shown in panel A), and repeated handling or repeated restraint stress (shown in panel B). Error bars represent the mean ± standard error of the mean. Asterisk (*) denotes a statistically significant difference between the vehicle and CORT rats ($p < 0.05$). *Abbreviations*: Stratum oriens (CA1so), CA1 stratum radiatum (CA1sr), CA1 stratum lacunosum (CA1sl), CA3 stratum radiatum (CA3sr), CA3 stratum oriens (CA3so), outer molecular layer (OML), middle molecular layer (MML), inner molecular layer (IML), and the subgranular zone of the dentate gyrus (SGZ).

not the restraint rats. This is shown in Figure 2-3. The CORT rats had a 26% decrease in the number of reelin-positive cells in the CA1sl ($t(10) = -4.51$, $p < 0.001$, Student t-test) and a 21% decrease in the number of reelin-positive cells in the SGZ ($t(10) = -2.87$, $p < 0.05$, Student t-test) compared to the vehicle rats. In contrast, there were no significant differences between the restraint and handled rats in the number of reelin-positive cells in any quantified region of the hippocampus or dentate gyrus (all p values > 0.05).

4. Discussion

The present experiment provides the first evidence that stress can decrease reelin immunolabeling in a preclinical animal model of depression. We found a significant decrease in the number of reelin-positive cells in the CA1sl and the dentate SGZ in rats treated with repeated CORT injections but not in rats subjected to repeated physical restraint. This observation is quite important in lieu of the behavioral differences between these two stress paradigms. We have consistently shown that repeated CORT injections increase depression-like behavior in the forced swim test (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004). The depressogenic effects of CORT are dose and time specific, with higher doses and longer courses of treatment producing larger increases in depression-like behavior (Johnson et al,. 2006). However, we (Gregus et al., 2005) and others (Platt and Stone, 1982) have found that repeated exposure to physical restraint (2.5–6 h/day) for 11–21 consecutive days does not produce depressive-like behavior in the forced swim test. These behavioral differences likely reflect underlying neurobiological processes that are affected in a different way by repeated CORT and repeated restraint, and identifying these neurobiological processes could lead to a better understanding of the pathophysiology of depression. To that end, our

present results suggest that deficits in reelin in specific hippocampal regions could play a role in the depressive symptomatology produced by high levels of circulating glucocorticoids.

The site specificity of the changes in reelin seen in this experiment might help explain the mechanisms by which CORT-induced changes in reelin could enhance depressive symptomatology. Although reelin is expressed by GABAergic interneurons in all regions of the hippocampus and dentate gyrus, CORT selectively decreased the number of reelin-positive cells in the CA1sl (where there was a decrease of about 1/4 of reelin-positive cells) and the dentate SGZ (a decrease of about 1/5 of the number of reelin-positive cells). The stratum lacunosum has a high level of diffuse reelin immunoreactivity, which is released by stratum lacunosum GABAergic interneurons and perforant pathway terminals impinging onto the distal region of CA1 pyramidal cell dendrites (Alcantara et al., 1998; Pesold et al., 1998). Reelin release in the stratum lacunosum is thought to regulate synaptic spine development in this area and contribute to the induction of long-term potentiation (see Niu et al., 2008; Weeber et al., 2002). In contrast, reelin is expressed in the dentate SGZ primarily by GABAergic basket cells that make synaptic contacts with granule cell somata (Pesold et al., 1998). Reelin release by these cells could regulate the migration and maturation of newborn granule cells into the granule cell layer. Therefore, our results suggest that the CORT-induced decrease of reelin-positive cells could impair both synaptic plasticity in the CA1sl and the integration of newborn granule cells into the granule cell layer. Viewed this way, deficient reelin signaling could be a precursor to subsequent deficits in hippocampal plasticity, with reelin providing a link between the hypersecretion of cortisol and the numerous alterations in hippocampal plasticity that have been found in various models of depression.

Although reelin has not previously been studied in animal models of depression, there is evidence that reelin is decreased in postmortem hippocampal tissue from patients with depression (Knable et al., 2004; Fatemi et al., 2000). Interestingly, the link between reelin and psychiatric illness goes well beyond depression as reelin has also been implicated in the pathogenesis of schizophrenia and bipolar illness (Fatemi et al., 2000; Guidotti et al., 2000; Impagnatiello et al., 1998; Knable et al., 2004). However, the reelin deficits seen in postmortem brain tissue from patients with these disorders are more extreme and widespread than those reported for depression. This is not surprising given that schizophrenia and bipolar disorder are generally more debilitating and have an earlier life onset than depression, and a recent meta-analysis revealed that hippocampal reelin protein levels in postmortem brain tissue from psychiatric patients are negatively correlated with illness duration and severity (Knable et al., 2004). Our novel findings of decreased reelin-positive cells in specific hippocampal regions in the CORT model of depression appear to support the idea of circumscribed reelin deficits in depression, and indicate that the use of the repeated CORT injection paradigm – but not a repeated physical restraint paradigm – could be of primary interest to further explore how alterations in reelin are involved in the pathogenesis of depression.

CHAPTER 3

The progressive development of depression-like behavior in corticosterone-treated rats is paralleled by slowed granule cell maturation and decreased reelin expression in the subgranular zone of the dentate gyrus

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INTRODUCTION

Depression is a chronic disease with a huge impact on society. The World Health Organization has predicted that by the year 2020, depression will be the second leading global burden of illness. Therefore, it is imperative that rapid progress is made in understanding the pathophysiology of depression and identifying new neurobiological targets for novel antidepressants that work more quickly, with fewer side effects, and in a greater percentage of patients.

Much of the current research on depression is focused on the fact that stress is a major risk factor for the onset of symptoms. Correlational support for this theory includes the fact that the depressive symptoms are often preceded by stressful or traumatic life events (Keller et al., 2007) and observations of hypercortisolemia and disrupted cortisol rhythmicity in patients with depression (Belmaker and Agam, 2008; Burke et al., 2005). Interestingly, a longer course of depression (i.e., number and duration of episodes) is associated with more substantial neurobiological alterations, such as decreasing hippocampal volume (Bremner et al., 2000; Sheline et al., 1999; Sheline et al., 2003). This suggests that depression may be a progressive disorder, with increasingly larger neurobiological perturbations leading to increasingly problematic symptoms.

Given the link between stress and depression, most preclinical animal models of depression make use of repeated stress paradigms to study the neurobiological correlates of depression (Sterner and Kalynchuk, 2010). In general, these experiments have shown that exposure to various forms of repeated stress in laboratory animals has deleterious effects on the hippocampus, such as decreased dendritic complexity within CA3 pyramidal cells and dentate

granule cells (Bessa et al., 2009; Magarinos et al., 1996; Vyas et al., 2002), impaired long-term potentiation (Artola et al., 2006), altered gene and protein expression (Alfonso et al., 2005; Hunter et al., 2009; Joels et al., 2004), and impaired neurogenesis (Galea et al., 1997; Gould et al., 1998; Brummelte and Galea, 2010b). These results are encouraging although incomplete and what is needed now is a more systematic investigation of the molecular mechanisms that instigate a loss of hippocampal plasticity in the depressed brain.

We have been working on the idea that deficits in the extracellular matrix protein reelin may be involved in the pathogenesis of depression. In the adult brain, reelin is secreted by a subset of GABAergic interneurons in the cortex and hippocampus (Abraham and Meyer, 2003; Alcantara et al., 1998; Pesold et al., 1998; Pesold et al., 1999), where it plays important roles in learning and memory, cell migration and integration, synaptogenesis, and stabilizing synaptic contacts onto dendritic spines (Frotscher et al., 2003; Niu et al., 2008; Qiu et al., 2006b; Rodriguez et al., 2000; Rodriguez et al., 2002; Rogers et al., 2011; Weeber et al., 2002; Zhao et al., 2004). In the hippocampus, reelin also promotes adult neurogenesis (Pujadas et al., 2010). Importantly, reelin levels are decreased in a number of neuropsychiatric disorders, including schizophrenia, bipolar disorder, and depression (Knable et al., 2004; Fatemi et al., 2000; Guidotti et al., 2000; Impagnatiello et al., 1998). This prompted us to assess reelin might be altered in a preclinical model of depression. To that end, we studied the effect of either 21 days of restraint stress or 21 days of corticosterone injections on the number of reelin+ cells throughout the hippocampus. We found that repeated exposure to CORT, which increases depression-like behavior in the forced swim test (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009), significantly decreased the number of reelin+ cells in the CA1 stratum lacunosum and subgranular zone (SGZ) of the dentate gyrus (Lussier et al.,

2009), but restraint stress, which does not affect depressive behavior (Gregus et al., 2005), had no effect on reelin. We were intrigued by the selective effects of CORT on SGZ reelin, given that this is the proliferative zone for hippocampal neurogenesis, and reelin+ positive cells in this region are GABAergic pyramidal-basket cells that extend processes into the granule cell layer and likely modulate key aspects of the neurogenic cascade (Ge et al., 2006; Jagasia et al., 2009; Kim et al., 2012; Tozuka et al., 2005). Therefore, we went on to determine the effect of CORT on depression-like behavior and hippocampal neurogenesis in heterozygous reeler mice (HRM), which express about 50% of normal levels of reelin. We found that CORT had much greater effects in the HRM than their wildtype controls, as evidenced by increased immobility in the forced swim test, decreased reelin+ cells in the SGZ, and decreased immature granule cell number and maturation (Lussier et al., 2011). At the same time, we became aware of data from another group, who found that transgenic mice overexpressing reelin are protected from the depressogenic effects of CORT (Teixeira et al., 2011). Taken together, these results provide strong support for the idea that reelin plays an important role in the pathogenesis of depression and that this involvement may take the form of modulating the differentiation and maturation of newborn granule cells within the dentate gyrus.

The purpose of this experiment was to determine whether the progressive development of depression-like behavior in CORT-treated rats is paralleled by progressive changes in SGZ reelin and immature granule cell number and maturation. We mapped the effect of CORT on these parameters after 7, 14, and 21 days of treatment with CORT. Our results indicate that CORT does indeed produce parallel changes in all of these measures, lending more support to the idea that reelin plays an important role in the depressogenic effects of chronic glucocorticoids.

MATERIALS AND METHODS

Subjects

We used 63 male Long-Evans rats in this study (purchased from Charles River Laboratories, Montreal, QC, Canada). The rats weighed approximately 225–250 g upon arrival in our facility. The rats were individually housed in standard polypropylene cages with water and food available ad libitum (unless otherwise indicated). The temperature of the colony room was maintained at 21±1 °C and all experimental procedures were conducted during the light phase of the light/dark cycle (lights on at 8 a.m.). All experimental procedures were carried out in strict agreement with the Canadian Council on Animal Care guidelines and a protocol approved by the University of Saskatchewan Animal Care and Use committee.

CORT Administration

We handled the rats briefly once per day for 7 days prior to the start of the CORT injections. We then weight-matched the rats and randomly assigned them to one of the following four treatment groups: 21 days of CORT injections (CORT 21, n = 16), 7 days of vehicle injections followed by 14 days of CORT injections (CORT 14, n=16), 14 days of vehicle injections followed by 7 days of CORT injections (CORT 7, n=15), and 21 days of vehicle injections (vehicle, n = 16). This experimental design allowed us to compare the behavioral and neurobiological consequences of differing amounts of CORT exposure while ensuring that every rat received an equal amount of handling and an equal number of injections (see figure 3-1).

We injected each rat once per day between 9:00-10:00 a.m. CORT (MP Biomedicals) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) Tween-80 (Sigma–Aldrich) and injected subcutaneously at a dose of 40 mg/kg with a volume of 1 ml/kg. Previous work has shown that this dose reliably increases depression-like behavior in rats (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009). We also recorded the body weight of each rat for each day of the 21-day treatment period.

Behavioral Testing

We conducted the behavioral testing in a different room from those used for the CORT injections and housing. A subset of rats (n = 10-11) from each group was used for the behavioral testing. We videotaped all behaviors and scored them at a later date. We used the forced swim test to assess depression-like behavior and the novelty-suppressed feeding test to assess anxiety-like behavior (Gregus et al., 2005; Santarelli et al., 2003).

Forced Swim Test (FST). The FST was conducted on the day after the final injection. We used a modified version of the Porsolt test, as described previously (Marks et al., 2009). Briefly, each rat was placed individually into a Plexiglas swim tank (25 cm wide X 25 cm long X 60 cm high, 27 ± 2 ºC water, 30 cm deep) for 10 min. We measured the duration of time each rat spent immobile, swimming, and struggling during the 10 min test and we also recorded the latency to immobility (i.e., the amount of time before the first episode of immobility) (Cryan et al., 2005; Marks et al., 2009).

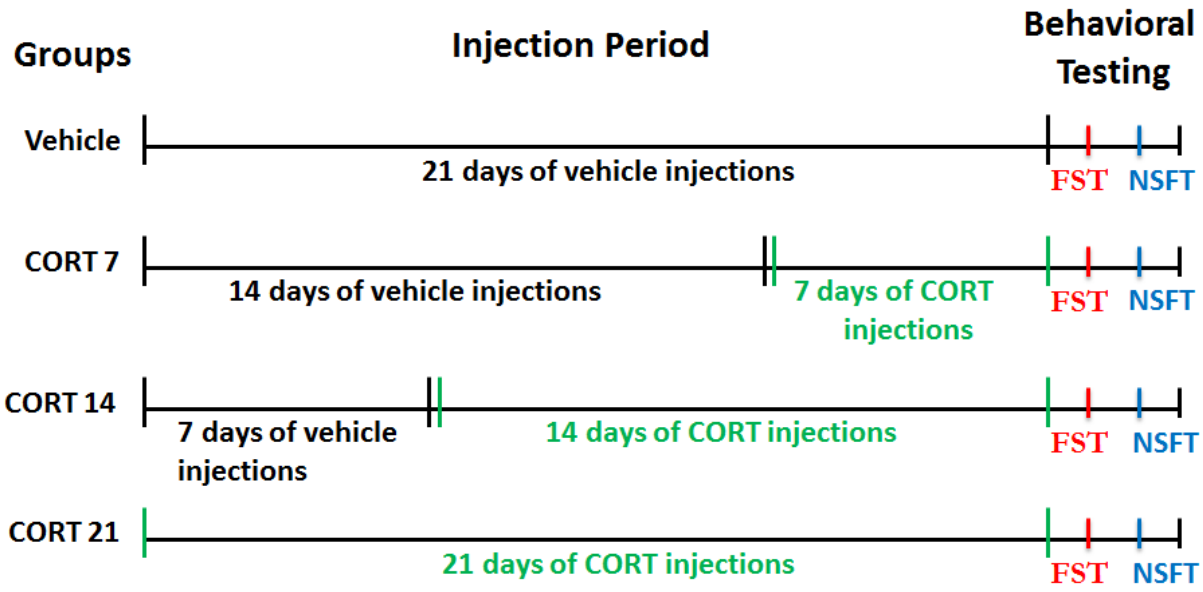


Figure 3-1. Diagram representing the duration and time points of the CORT injections and behavioral testing.

Novelty-Suppressed Feeding Test (NSFT). The NSFT was conducted on the day following the FST. We used the method previously described (Li et al., 2011). Briefly, the testing was done in a black wooden box (70 cm long X 70 cm wide X 60 cm high) with a Plexiglas floor. We food-deprived rats for 24 hr prior to the test. On the test day, we placed 5 standard food pellets in the center of the wooden box. Each rat was placed in a corner of the box and allowed to explore freely for 8 min. We recorded the latency to feed and immediately after the test, we put each rat back into the home cage and recorded the amount of food consumed over an 8 min period to assess general motivation to eat.

Perfusions and Tissue Preparation

On day 22, a subset of rats from each group (n = 6) that was not used for behavioral testing was anaesthetized with an overdose of sodium pentobarbital (i.p.) and perfused transcardially with 0.1 M phosphate buffer (PB, pH 7.4) and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and cut along the longitudinal fissure. One brain hemisphere was used for Golgi impregnation and the other hemisphere was used for immunohistochemistry.

Immunohistochemistry

Brain tissue processed for immunohistochemistry was postfixed in 4% (w/v) paraformaldehyde for 48 h at 4 ˚C and sectioned using a cryostat at 40µm. We used every sixth section for immunostaining and we quantified 5 sections per animal. Immunohistochemistry was performed on free-floating brain sections as described previously (Lussier et al., 2011). To visualize reelin+ cells, sections were incubated for 48 hr at 4°C with mouse anti-reelin primary

antibody (Chemicon, 1:2000) followed by incubation in biotinylated horse anti-mouse IgG secondary antibody (1:200, Sigma-Aldrich). To visualize immature neurons, sections were incubated for 24 hr at room temperature with rabbit anti-DCX primary antibody (1:1000, Cell Signaling), followed by incubation in biotinylated goat anti-rabbit IgG secondary antibody (1:500, Sigma-Aldrich). In both cases, this was followed by incubation in an avidin-biotin complex (1:500, Vecta Stain Elite ABC reagent, Vector Labs) for 1 hr at room temperature. The sections were then mounted onto slides and coverslipped using Entellan resin solution.

Analyses of Immunohistochemical Labeling

The total number of reelin+ cells and DCX+ cells in the dentate gyrus was estimated using the unbiased optical fractionator method as described in detail elsewhere (Lussier et al., 2011; West et al., 1991). We examined tissue sections at 400 × magnification using a NikonE800 microscope and a computerized stereology system (Stereo Investigator, MicroBrightField). We counted DCX+ cells in the SGZ and granule cell layer (GCL) throughout the dentate gyrus (from -2.40 mm to approximately -3.94 mm from bregma) and reelin+ cells in the SGZ. We estimated the total number of DCX+ and reelin+ cells using the following formula: $N_{\text{total}} : \Sigma Q^{-} \times 1/\text{ssf} \times A(x,y \text{ step})/ a(\text{frame}) \times t/h$; where ΣQ^{-} is the number of counted cells; ssf is the section sampling fraction (1/6); A(*x*,*y* step) is the area associated with each *x*,*y* movement (5,625 μm^2); *a*(frame) is the area of the counting frame (2,500 μm^2); *t* is the weighted average section thickness; and h is the height of the dissector (12 μm). We used a guard zone of 5 μm during cell counting to avoid sectioning artifacts.

To semi-quantify the dendritic morphology of immature granule cells, we categorized dendritic branching in a subset of DCX+ cells from each rat according to previously described methods (Lussier et al., 2011; Plumpe et al., 2006). A meander scan method was utilized to randomly select an average of 100 cells per rat for categorization. We calculated the percentage of cells falling into each of these categories for each animal. The six categories were defined by the presence and extent of apical dendrites (see Fig. 3-7A). Briefly, categories 1 and 2 represented the proliferative stage of development, categories 3 and 4 represented an intermediate development of the cell, and categories 5 and 6 represented more mature cell development.

Golgi Procedure and Analyses

We conducted Golgi impregnation of dentate granule cells using an FD Rapid Golgi Stain kit (FD NeuroTechnologies). Brain tissue used for the Golgi analyses was sectioned at 240 µm using a cryostat. We took Bright-field microscopy image stacks of dentate granule cells (an average of 5 cells per animal from -2.40 mm from bregma to approximately -3.94 mm from bregma) and traced them offline using Neurolucida software (MicroBrightField). We chose Golgi-impregnated neurons for analysis randomly based on the following criteria: (1) relative isolation from neighbouring impregnated cells in order to avoid interference in analysis; (2) dark and consistent impregnation throughout the cell body and dendrites; and (3) cell could not have truncated dendrites. We measured the total dendritic length for each Golgi-impregnated cell chosen for analysis and performed a Sholl's analysis on cells with concentric rings at 20 µm intervals to measure the number of intersections per radial distance from the cell body (Sholl, 1956).

Statistical Analyses

We used separate one-way ANOVA's to assess the statistical significance of group differences in each measure from this experiment (i.e., body weight, FST behavior, NSFT behavior, reelin, DCX, and Golgi analyses). Significant main effects were followed by Fisher's LSD post-hoc tests where appropriate. The criterion for statistical significance was $p < 0.05$. All data are represented as means ± the standard error of the mean.

RESULTS

The physiological effects of corticosterone were revealed through changes in body weight

Consistent with past research, CORT had significant effects on body weight in this experiment (see Fig 3-2). We began the experiment by weight-matching the rats into 4 separate groups prior to the onset of any CORT injections. However, we found significant differences among the groups in body weight on days 7 [F (3,62) = 19.756, $p < .001$], 14 [F (3,62) = 43.579, $p < .001$], and 21 [F (3,62) = 33.422, $p < .001$] of the CORT injections. Post-hoc analyses of these main effects revealed that on day 7, the CORT 21 rats weighed significantly less than all other groups and the CORT 14 rats weighed significantly more than the vehicle group [all p values <.05]. On day 14, the CORT 21 rats weighed significantly less than all other groups and the CORT 14 rats weighed significantly less than the CORT 7 and vehicle rats [all p values < .001]. On day 21, the CORT 21, CORT 14, and CORT 7 rats all weighed significantly less than the vehicle rats and the CORT 21 and CORT 14 rats weighed significantly less than the CORT 7 rats [all p values < .01].

Corticosterone increased depression-like behavior in the FST but did not alter anxiety in the NSFT

We used the FST and NSFT to assess progressive changes in depression- and anxiety-like behavior after CORT administration. CORT produced clear behavioral changes in the FST but not the NSFT (see Fig 3-2). More specifically, our statistical analyses revealed a significant effect of CORT on immobility time [F (3,37) = 6.13, p <0.01], swimming time [F (3,37) = 5.465, p <0.01], and the latency to immobility [F (3,37) = 4.002, p < 0.05] in the FST. Post-hoc analyses of these main effects revealed that the CORT 21 and CORT 14 rats showed significantly more immobility than the vehicle rats (both p values < 0.05) and the CORT 21 rats showed significantly more immobility than the CORT 7 rats (p < 0.01). In addition, the CORT 21, CORT 14, and CORT 7 rats all showed significantly less swimming than the vehicle rats (all p values < 0.01). Finally, the CORT 21 and CORT 14 rats had a significantly shorter latency to immobility than the vehicle rats (p < 0.01).

As mentioned above, there were no significant group differences in the latency to feed in the NSFT [F(3,37) = 0.96, p = 0.96] or the amount of food consumed in the home cage [F(3,37) = 0.62, p = 0.60].

Corticosterone decreased reelin expression in the SGZ and immature neuron number and maturation in the SGZ and granule cell layer

We used immunohistochemistry to assess whether the progressive effects of CORT on behavior in the FST are paralleled by progressive changes in reelin+ cells in the SGZ (see Fig 3-3). CORT had a significant effect on the number of reelin+ cells in this region [F (3,18) =

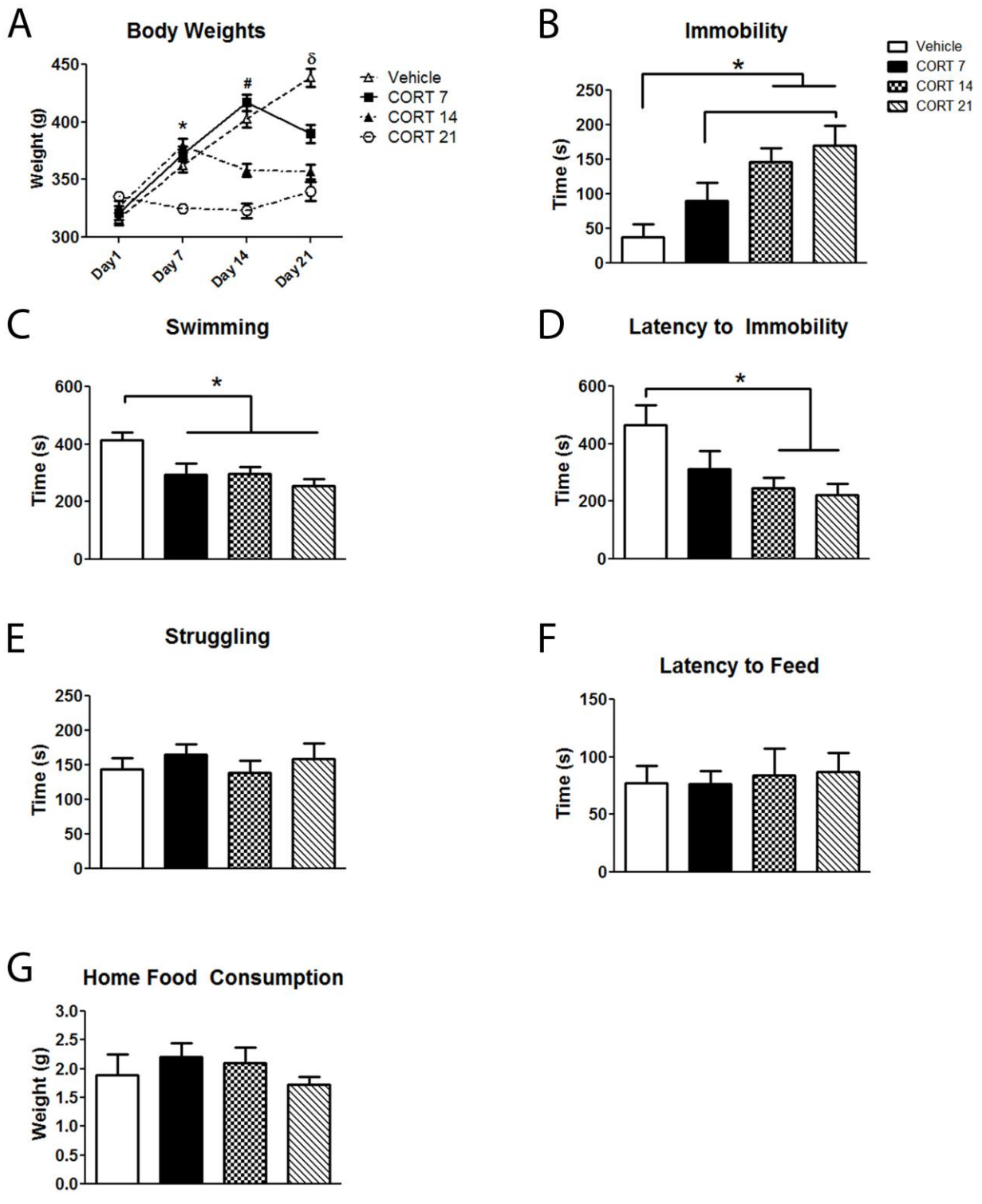

A
Body Weights
Weight (g)
450
400
350
300
Day1
Day 7
Day 14
Day 21
*
#
δ
Vehicle
CORT 7
CORT 14
CORT 21
B
Immobility
Time (s)
250
200
150
100
50
0
*
Vehicle
CORT 7
CORT 14
CORT 21
C
Swimming
Time (s)
600
400
200
0
*
D
Latency to Immobility
Time (s)
600
400
200
0
*
E
Struggling
Time (s)
250
200
150
100
50
0
F
Latency to Feed
Time (s)
150
100
50
0
G
Home Food Consumption
Weight (g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0

Figure 3-2. The effect of different numbers of repeated CORT exposure on body weight over the 21 days of injections (A). Symbols (*) denotes that the CORT 21 group weighs significant less than all of other groups and the CORT 14 group weighs more than the vehicle group ($p < 0.05$). Symbol (#) denotes that the CORT 14 and CORT 21 groups weigh less than the CORT 7 and vehicle groups and that the CORT 21 group weighs less than the CORT 14 group ($p < 0.001$). Symbol (δ) denotes that all of the CORT groups weigh less than the vehicle group and that the CORT 14 and CORT 21 groups weigh less than the CORT 7 group ($p < 0.01$). The effect of CORT on behavior in the forced swim test (B-E) and novelty suppression feeding test (F & G). Panel B shows the time spent immobile, panel C shows the time spent swimming, panel D shows the latency to immobility, and panel E shows struggling behavior. CORT decreased swimming and increased immobility behavior, as well as decreased latency to immobility. All rats injected with CORT showed a decrease in swimming behavior. Rats injected with CORT for 14 and 21 days show significantly more immobility behavior compared to the vehicle control groups and rats injected with CORT for 21 days show significantly more immobility behavior than rats injected with CORT for 7 days. In addition, the rats injected with CORT for 14 and 21 days show significantly less latency to immobility than the vehicle control group. Panel F shows the latency to feed behavior and panel G shows the home cage food consumption. Asterisks (*) denote a statistical significant difference of one-way ANOVAs ($p < 0.05$). Error bars represent the mean ± standard error of the mean.

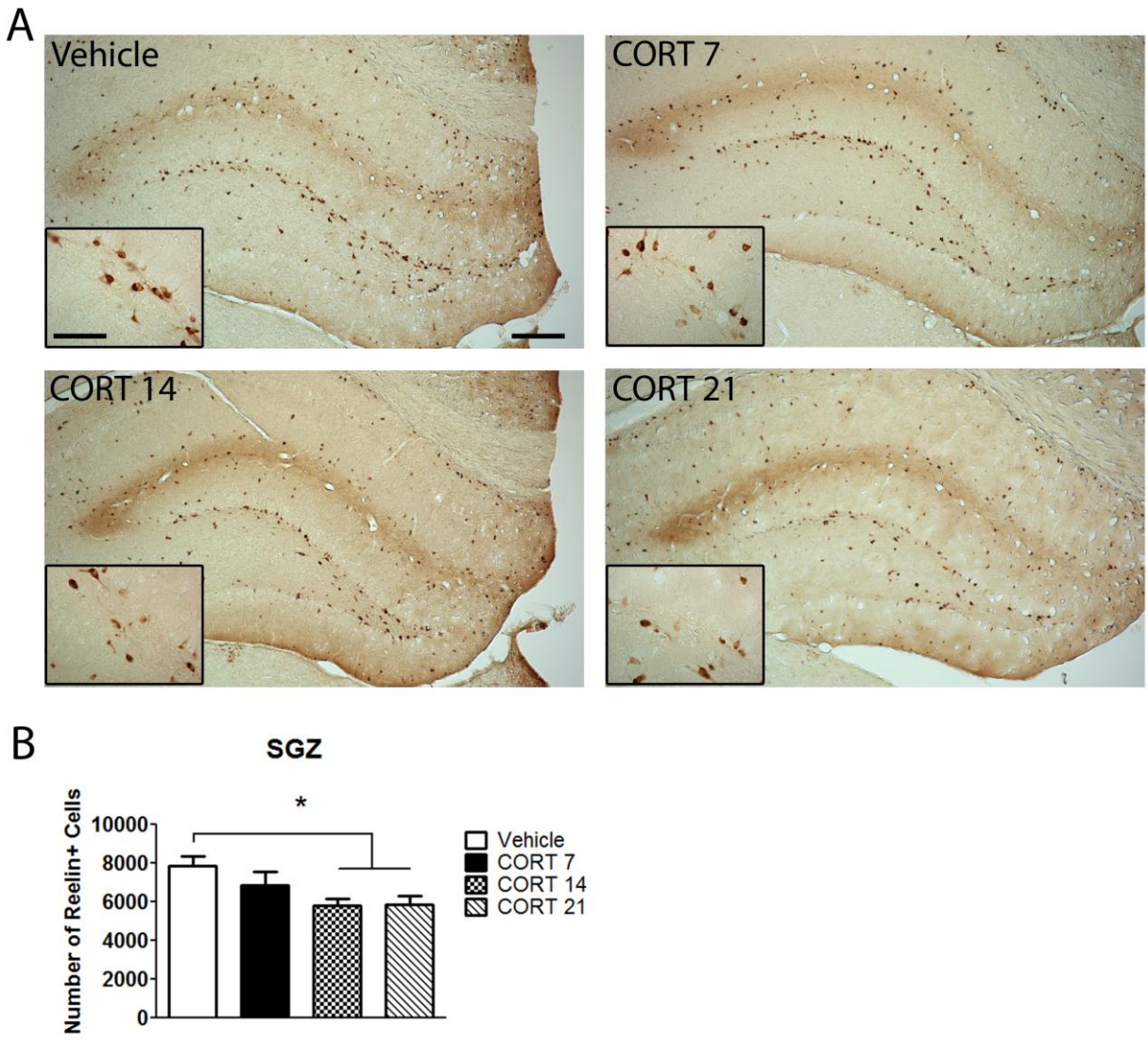


Figure 3-3. The effect of CORT on the number of reelin-positive cells in the SGZ: Panel A shows representative micrographs of reelin immunoreactivity. Panel B show the number of reelin positive-cells between the different time points of CORT exposure. Error bars represent the mean ± standard error of the mean. Rats that received CORT for 14 and 21 days had significantly fewer reelin-positive cells than the vehicle control group. Asterisk (*) denotes statistical significance ($p < 0.05$). Scale bar: 200 µm (lower power magnification) and 20 µm (higher power magnification insert).

3.84, $p < .05$]. Post hoc analyses of this main effect revealed that both the CORT 21 and CORT 14 rats had significantly fewer reelin+ cells in the SGZ than the vehicle rats [all p values < .01]. The CORT 7 rats did not differ statistically from the vehicle rats in this measure.

We then assessed the progressive effect of CORT on the number of immature granule cells in the SGZ and GCL and the maturation of these neurons using immunohistochemistry for DCX (Fig 3-4). As is typical, DCX+ cells were clearly visible in the SGZ and the inner inner one-third of the GCL. As such, the cell counts from both these regions were combined for the statistical analyses. CORT had a significant effect on the number of DCX+ cells [$F(3,17) = 4.325$, $p < .05$]. Post hoc analyses revealed that the CORT 21 and CORT 14 rats had significantly fewer DCX+ cells in the SGZ/GCL than the vehicle rats (p values < 0.01). To quantify the effect of CORT on the maturation rate of newborn neurons, we then examined dendritic branching in a subset of DCX+ cells. Each DCX+ cell was placed into a category based on the presence and extent of dendritic branching (according to Fig 3-4C). Consistent with previous work using this approach (Lussier et al., 2011; Plumpe et al., 2006), DCX+ cells showed a range of morphological complexity, from essentially no dendritic branching to well-defined and complex dendritic branching. In general, CORT had progressively greater effects on neuronal maturation, with longer exposures to CORT being associated with more category 2 cells and fewer category 4, 5, or 6 cells. These observations were confirmed statistically. CORT had significant effects on the percentage of category 2, 4, 5, and 6 cells [category 2: $F(3,17) = 5.235$, $p < 0.01$; category 4: $F(3,17) = 7.619$, $p < 0.01$; category 5: $F(3,17) = 6.374$, $p < 0.01$; category 6: $F(3,17) = 4.825$, $p < 0.05$]. Post-hoc analyses of these main effects revealed several group differences. First, the CORT 21 and CORT 14 rats had significantly more

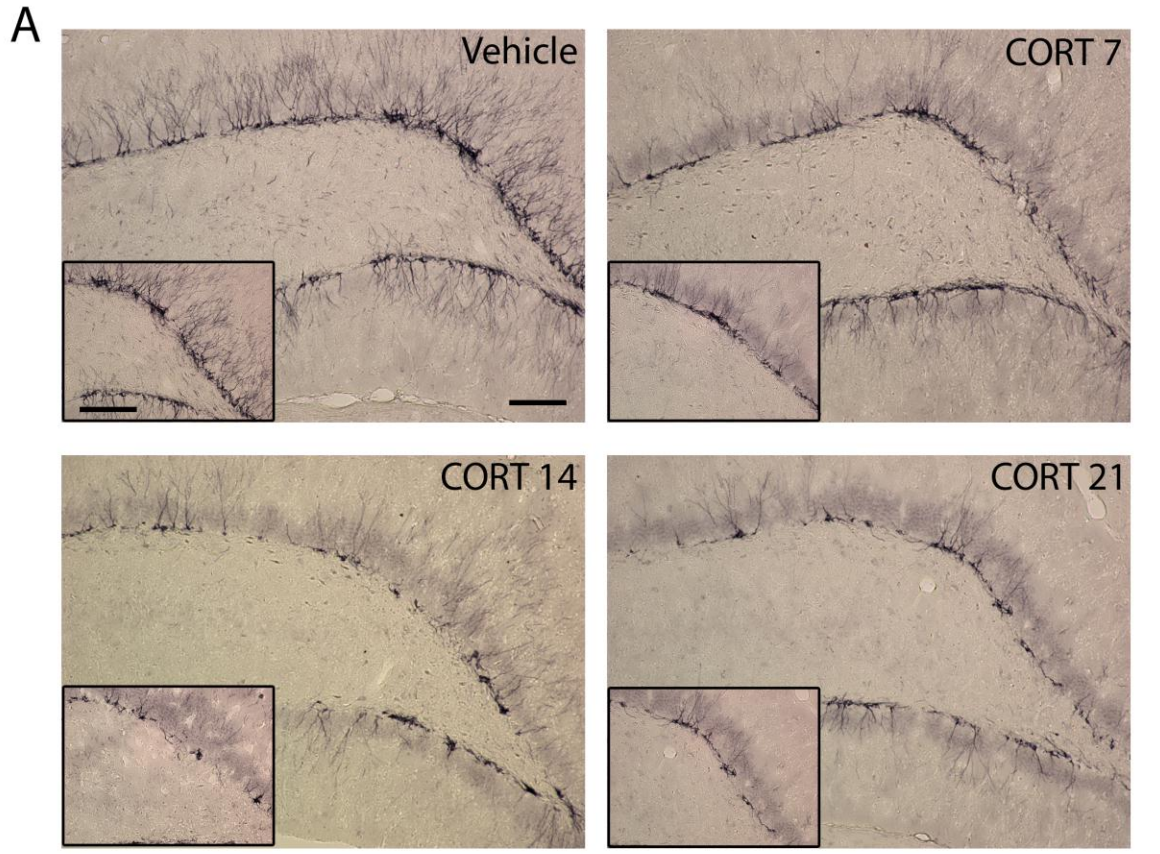

A
Vehicle
CORT 7
CORT 14
CORT 21
B
SGZ and GCL
Number of DCX+ Cells
25000
20000
15000
10000
5000
0
*
Vehicle
CORT 7
CORT 14
CORT 21
C
Categories
1
2
3
4
5
6
D
Category 1
Category 2
Category 3
Category 4
Category 5
Category 6
Percentage of Cells
40
30
20
10
0
*
Vehicle
CORT 7
CORT 14
CORT 21

Figure 3-4. The effect of CORT on the number of DCX-positive cells in the SGZ and GCL: Panel A shows representative micrographs of DCX immunoreactivity. Panel B show the number of DCX-positive cells between the different time points of CORT exposure. Rats that received CORT for 14 and 21 days had significantly fewer DCX-positive cells than the vehicle control group. Panel C provides an example of each of the six categories of dendritic complexity used in this analysis. Panel D shows the percentage of cells placed into each category for the rats in each group. CORT had progressive effects on the percentage of cells in categories 2, 4, 5, and 6, with an overall increase in the percentage of DCX-positive cells that fell into category 2 (i.e., more immature cells) and an overall decrease in the percentage of cells that fell into categories 4, 5, and 6 (more complex dendrites). In category 2 the rats that received CORT for 14 and 21 days had significantly higher percentage of cells than the vehicle control group. In category 4 the CORT groups that received 14 and 21 days of injections had a significantly fewer percentage of cells than the rats that received 7 days of CORT and the vehicle group. In category 5, the CORT 14 and CORT 21 groups had significantly fewer percentages of cells than the vehicle group. In category 6 the CORT 21 group has a significantly fewer percentage of cells than the vehicle group and the CORT 7 group. Error bars represent the mean ± standard error of the mean. Asterisk (*) denotes statistical significance ($p < 0.05$). Scale bars: 80 µm (lower power magnification) and 40 µm (higher power magnification insert).

category 2 DCX+ cells than the vehicle rats [all p values < 0.05]. Second, the CORT 21 and CORT 14 rats had significantly fewer category 4 DCX+ cells than the CORT 7 or vehicle rats [all p values < 0.02]. Third, the CORT 21 and CORT 14 rats had significantly fewer category 5 DCX+ cells than the vehicle rats [all p values < 0.01]. And finally, the CORT 21 rats had significantly fewer category 6 DCX+ cells than the CORT 7 or vehicle rats [all p values < 0.02].

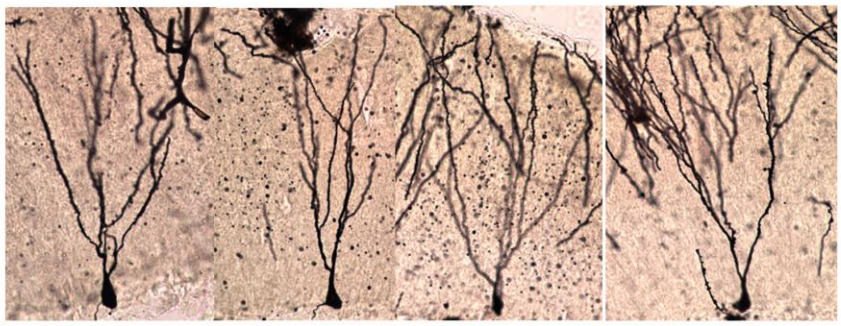
Corticosterone had no effect on dendritic complexity in mature granule cells

Finally, we used Golgi impregnation to assess the progressive effects of CORT on dendritic complexity in mature granule cells (Fig 3-5). In contrast to the significant effect of CORT on immature granule cells labelled with DCX, we found no significant differences in dendritic length [$F(3,16) = 0.176$ $p = 0.91$] or complexity of Golgi-stained granule cells (all F values < 0.986, all p values > 0.43).

DISCUSSION

In this study, we showed that repeated administration of varying durations of CORT produced progressive changes in depressive-like behavior, as well as decreases in reelin expression and neurogenesis within the dentate gyrus. In addition, we also found that there were progressive alterations in the dendritic complexity of cells within the dentate gyrus across the different CORT groups with the earliest changes noted after two weeks of treatment. Furthermore, we showed that the alterations in dendritic morphology were specific to newborn neurons as shown by DCX immunostaining and that there were no significant differences in the dendritic structure of mature granule cells within the dentate gyrus as shown by Golgi

A

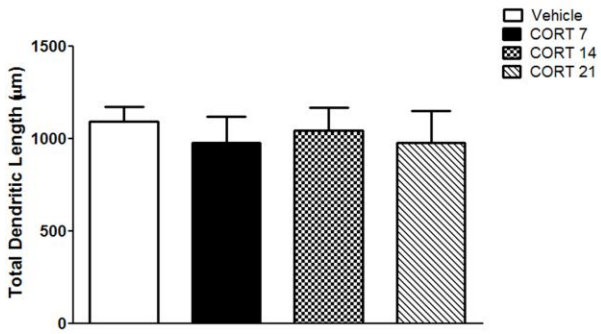


Vehicle CORT 7 CORT 14 CORT 21

B

Vehicle CORT 7 CORT 14 CORT 21

C



D

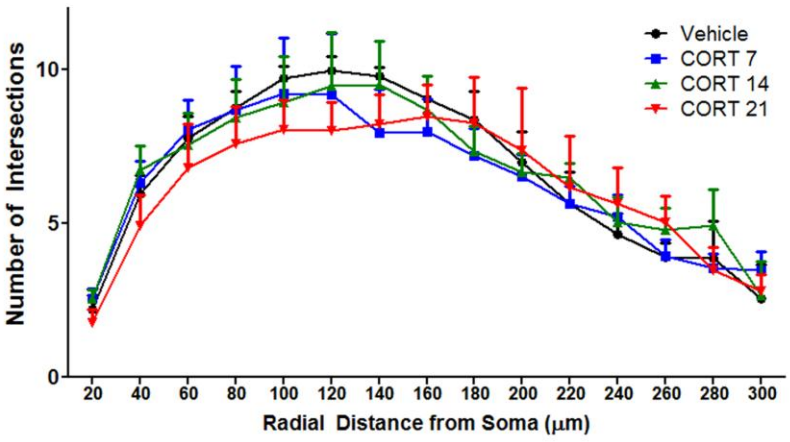


Figure 3-5. The effects of CORT on dendritic length and complexity within Golgi-impregnated cells. Panel A provides an example of the tracings from the Golgi-impregnated cells from each of the groups. Panel B shows representative photomicrographs of the Golgi-impregnated cells from each of these groups. Panel C shows the total dendritic length of each of the groups. Panel D shows the number of intersections in each of the groups. Error bars represent the mean ± standard error of the mean.

analyses. These results make two important points. First, they show that the progressive development of depression-like behavior with CORT administration is paralleled by progressive decreases in the number of reelin+ cells and the rate of hippocampal neurogenesis, which suggests these neurobiological processes may play a role in the depressive-like behavior produced by high levels of glucocorticoid exposure. Second, they show that CORT has greater effects on dendritic complexity in granule cells that are maturing during the period of CORT exposure than it does on mature granule cells with an existing dendritic architecture, which suggests that newborn neurons are particularly sensitive to the deleterious effects of glucocorticoids.

Our behavioral results demonstrate that the behavioral consequences of CORT develop in a progressive manner with repeated exposure. Immobility behavior in the FST clearly increased in a gradual manner with increasing exposure to CORT, and similarly, swimming behavior and the latency to immobility decreased in a gradual manner with increasing exposure to CORT. We have previously shown on several occasions that 21 days of high dose CORT administration increases depression-like behavior in the FST (see Sterner and Kalynchuk 2010 for a review). The results from this experiment add to this by showing that 14 days of high dose CORT treatment is sufficient to induce a depressive phenotype but 7 days of high dose CORT treatment is not. Previous research is consistent with these results. In rats, CORT given at 20 mg/kg for 20 days increases depression-like behavior but the same dose given for 10 days does not (Hill et al., 2003; Brotto et al., 2001). Furthermore, CORT given at 10 mg/kg for 21 consecutive days has no significant effect on depression-like behavior (Johnson et al., 2006). Consistent with the rat literature, mice given 20 mg/kg of CORT display depressive-like behavior after 21-36 days of exposure but not after 6 or 18 days of exposure (Zhao et al., 2008a;

Zhao et al., 2009; Lussier et al., 2011). Taken together, these findings strongly suggest that the deleterious effects of CORT on depression-like behavior accumulate with time and dose, which is consistent with clinical observations associating the onset of depression with exposure to stressful life events over a prolonged period of time (Keller et al., 2007; Kendler et al., 1995; Kendler et al., 1999).

Our behavioral analyses also reveal that high dose CORT administration has greater effects on depression-like behavior than it does on anxiety. The FST is typically viewed as a behavioral assay of despair (Porsolt et al., 1978). We found that both the CORT 21 and CORT 14 rats showed increased depression-like behavior in the FST, as measured by increased immobility, decreased swimming, and a decreased latency to immobility. In contrast, the NSFT is typically viewed as a behavioral assay of anxiety (Dulawa and Hen, 2005). In this case, treatment with CORT did not affect the latency to feed in the NSFT. This lack of effect could not be attributed to non-specific effects of CORT on feeding behavior because the rats in all the groups ate nearly the same amount of food in their home cages. These observations are generally consistent with previous results from studies using CORT-administration paradigms in rodents. For example, we and others have reported that 21 days of high dose CORT injections increases depression-like behavior in the FST (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009; Brummelte et al., 2006) with limited changes in anxiety-like behavior in an open field test, social interaction test, and predator odor test (Gregus et al., 2005; Kalynchuk et al., 2004; Lussier et al., 2011). This suggests that a CORT administration paradigm such as the one employed here may be more useful for studying the neurobiological mechanisms that lead to depression than it would be for studying the mechanisms involved in anxiety.

One purpose of the present experiment was to investigate whether the progressive effects of CORT on behavior might be paralleled by progressive changes in the glycoprotein reelin. We have previously reported that 21 days of high dose CORT injections significantly decreases the number of reelin+ cells in the SGZ and CA1 stratum lacunosum (Lussier et al., 2009), and that heterozygous reeler mice, which have about 50% of normal levels of reelin, are more susceptible to the depressogenic effect of CORT than wildtype mice (Lussier et al., 2011). These results suggested to us that reelin may be involved in the pathogenesis of depression. If this idea is true, then we would expect to see alterations in reelin+ cells in the hippocampus at the time that depression-like behavior emerges. The results of this experiment confirmed this idea. We found that CORT produced time-dependent decreases in the number of reelin+ cells within the SGZ, starting with moderate but nonsignificant decreases at 7 days of exposure and reaching significant decreases by 14 and 21 days of CORT exposure. Importantly, this timeline of changes in the expression of reelin in the SGZ matches the timeline of changes in depression-like behavior in the FST discussed above. This provides additional support for our hypothesis that a loss of reelin in the hippocampus, and specifically in the SGZ of the dentate gyrus, plays a role in the pathogenesis of depression.

Another way to approach the hypothesis that reelin is involved in depression is to assess the effects of overexpressing reelin in a preclinical model of depression. Teixeria and colleagues (2011) have done just that. They used a transgenic strain of mice with a threefold increase in hippocampal reelin expression. The mice received CORT in the drinking water for three weeks prior to behavioral testing. The transgenic mice did not show an increase in depressive-like behavior after repeated CORT exposure, whereas their wild-type mice did. When combined with our results described above, these result point to an emerging picture in

which the absolute amount of reelin in hippocampal regions may regulate vulnerability to depression: Low levels of reelin increase vulnerability whereas high levels of reelin decrease vulnerability. The next step would seem to be determining whether novel pharmaceutical agents that act on reelin and its receptors have therapeutic efficacy against depression.

A second purpose of the present experiment was to investigate whether the progressive effects of CORT on behavior might be paralleled by progressive changes in hippocampal neurogenesis. There is a large literature implicating hippocampal neurogenesis in depression (Barha et al., 2011; Brummelte and Galea, 2010b; Duman et al., 2001; Gould et al., 1997; Pham et al., 2003; Plumpe et al., 2006; Snyder et al., 2011) and we have recently reported that CORT produces dose-dependent decreases in DCX+ cells and morphology after 3 weeks of exposure (Lussier et al., 2011). Moreover, CORT-induced decreases in reelin expression are most pronounced in the SGZ of the dentate gyrus, which is the precise location of hippocampal cell proliferation. Indeed, we have previously hypothesized that a loss of reelin after chronic stress could dampen the maturation rate of newborn granule cells (Lussier et al., 2009; Lussier et al., 2011). In this experiment we found that CORT exposure affected immature dentate granule neurons in a time-dependent manner. More specifically, we found moderate but nonsignificant decreases in the number of DCX+ cells in the CORT 7 rats, and significant decreases in the number of DCX+ cells in the CORT 14 and CORT 21 rats. In addition, we also found that DCX+ cells in the CORT 14 and CORT 21 rats were characterized by smaller and less complex dendritic processes. Similar to the effect we saw with our reelin analyses, the timeline of CORT-induced changes in DCX+ cell number and morphology fits with the timeline for the onset of depression-like behavior, suggesting that there could be an important

relationship between the effects of prolonged glucocorticoid exposure on hippocampal reelin expression and neurogenesis and depression-like behavior.

In contrast to the effects of CORT on immature granule neurons, our Golgi analyses revealed no significant effect of CORT on dendritic length or branching in mature granule neurons. This suggests that immature neurons may be more susceptible to the deleterious effects of CORT than mature neurons with established synaptic connections. One possible caveat with this conclusion is that we cannot be sure that all of the Golgi impregnated neurons in this experiment were mature neurons. It is possible that some of the Golgi stained neurons were immature neurons. However, we believe that this is unlikely, because our data clearly show that DCX+ cells in CORT 14 and CORT 21 rats have less complex dendrites, and if cells at this level of maturity were capture by the Golgi and quantified in our analyses, this would have increased the probability of us finding a decrease in dendritic length or branching in the Golgi-impregnated cells. Therefore it follows that the majority of the Golgi cells in this experiment were mature neurons. This interpretation is also consistent with the results of other researchers showing no difference in dendritic morphology in neurons within the GCL in after a period of CORT exposure (Woolley et al., 1990). One question to consider then is why CORT seems to preferentially affect immature neurons over their more mature cousins?

One answer to this question may come from a consideration of the specific population of cells that DCX identifies. DCX is expressed by both type-2b and type-3 progenitor cells but not type-1 or type-2a cells (Kronenberg et al., 2003). In adult neurogenesis, the type-2 stage is a transition stage from a glia-like precursor to a neuronal fate choice, which is thought to occur when the cells are type-1 or type-2a. Type-3 cells are characterized by an intense period of

morphological development and significant radial migration into the GCL. Type-3 cells therefore represent a transition from a proliferative stage to an immature neuronal stage. Importantly, DCX+ cells with complex dendritic morphology show increased synaptic plasticity compared to older cells in the GCL (Ge et al., 2007b; Schmidt-Hieber et al., 2004; Wang et al., 2000). Immature neurons appear to be favorably recruited over mature neurons during dentate activation because of this greater plasticity (Kee et al., 2007). This propensity for activation and recruitment seems to occur when neurons are between 2 and 6 weeks of age (Ge et al., 2007b; Kee et al., 2007; Schmidt-Hieber et al., 2004; Wang et al., 2000; Zhao et al., 2006a). In this experiment, we found decreases in DCX+ cell number and morphology after 2 or 3 weeks of CORT administration. These DCX+ cells would have just be entering the critical period of synaptic plasticity mentioned above, and this may have made these cells particularly vulnerable to changing environmental conditions, such as a sudden increase in circulating glucocorticoids. In fact, research has previously shown that the survival of newborn neurons can be influenced by external factors such as environmental enrichment (Brown et al., 2003; Kempermann et al., 1997b), spatial learning (Kee et al., 2007), exercise (Kronenberg et al., 2003) and stress (Brummelte and Galea, 2010b; Gould et al., 1997; Plumpe et al., 2006).

Another interesting idea regarding the effects of CORT on these immature neurons is that the cell subtypes differentially express the glucocorticoid receptor types. GR have a relatively low affinity for CORT, and they typically become occupied when CORT levels are very high, such as during periods of prolonged stress. On the other hand, MR have a high affinity for CORT, and they are occupied when CORT is at basal levels (de Kloet et al., 1998; Plumpe et al., 2006; Sapolsky, 2000). MR seem to be only expressed on mature granule cells, whereas GR are found in newborn cells that are at progenitor stages (Garcia et al., 2004a;

Garcia et al., 2004b). For example, Garcia and colleagues (2004a) have shown that 50% of type-1, type-2a, and type-3 cells express GR, but not MR. Type-2b cells do not express either MR or GR (Garcia et al., 2004a). Given that the DCX cells with more mature morphology (categories 5-6) are type-3 cells (Plumpe et al., 2006) and the DCX cells with no or short processes (categories 1 & 2) are likely type-2b cells (Garcia et al. 2004b), then the GR expression in type-3 neurons would make these cells more sensitive to high levels of circulating glucocorticoids than the type 2 cells. This makes sense with our results showing decreases seen in the immature neurons with more complex dendritic processes (likely Type-3 cells) and the increase in the number of immature neurons with less complex morphology (likely type-2b cells), as CORT should have direct effects on the Type-3 population while not having a direct influence on the type-2b cells. As GR provide the conduit by which stress or glucocorticoids can influence gene expression and cell metabolism (Calfa et al., 2003; Pariante and Miller, 2001), constant occupation of these receptors in newborn neurons could dramatically affect their course of development. This coincides quite nicely with the observation that 4 days of treatment with mifepristone, a GR antagonist, can normalize DCX expression in rats that were previously subjected to 21 days of chronic unpredictable stress (Oomen et al., 2007). In addition, mifepristone reduces symptoms of depression in 30-50% of patients with psychotic major depression (Belanoff et al., 2002). It would be interesting to investigate whether mifepristone had similar beneficial effects on DCX cell number and morphology in CORT-treated rats and whether it might also normalize the effect of CORT on reelin and FST behavior.

In conclusion, our results reveal that the progressive development of a depressive phenotype in CORT-treated rats is paralleled by decreases in reelin in the SGZ and the number

and maturity rate of newborn neurons in the granule cell layer. These results add to the growing literature implicating reelin in the pathogenesis of major depression and in the regulation of hippocampal neurogenesis. Future work should be directed at determining whether novel pharmaceuticals that work on reelin signal transduction pathways have antidepressant efficacy.

CHAPTER 4

Reelin as a Putative Vulnerability Factor for Depression: Examining the Depressogenic Effects of Repeated Corticosterone in Heterozygous Reeler Mice

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1. Introduction

Depression is a debilitating syndrome characterized by sadness, lack of motivation, anhedonia, helpless behaviors, cognitive deficiencies, and sleep and appetite disturbances. The vast majority of basic research on depression has focused on a putative link between exposure to stressful life events and the onset of depressive symptoms (reviewed in Sterner and Kalynchuk, 2010). This link is evidenced by a number of correlational observations, including the fact that many patients with depression show hypercortisolemia and disrupted cortisol rhythmicity (Belmaker and Agam, 2008; Burke et al., 2005), elevated corticotrophin-releasing hormone (CRH) levels in cerebrospinal fluid, and increased levels of CRH mRNA and protein expression in limbic brain regions (Merali et al., 2004). Not surprisingly, most animal models of depression make use of repeated stress paradigms to study the neurobiological correlates of depression. However, depression is not caused solely by environmental conditions. The etiology of depression also has a significant genetic component, with heritability estimates ranging from 33% to 50% (Fava and Kendler, 2000; Levinson, 2006). This suggests that like schizophrenia and bipolar disorder, depression may be best conceptualized using a two-hit hypothesis (Maynard et al., 2001), where genetic alterations in early development confer a biological vulnerability that can lead to the disorder in individuals who are subsequently exposed to precipitating environmental events. Indeed, depressive symptomatology is not restricted to patients with major depression: Depressive symptoms are a key component of bipolar disorder and they also frequently occur in patients with schizophrenia (Hafner et al., 2005b; Hafner et al., 2005a). The manifestation of depressive symptoms in any one of these disorders could involve an interaction between genetic abnormalities during development and exposure to chronic stress later in life.

In this experiment, we asked whether a developmental genetic deficit in reelin signaling could make mice more vulnerable to the depressogenic effects of repeated exposure to glucocorticoids in adulthood. Reelin is a large extracellular matrix protein that is active in both the developing and mature brain. In early development, reelin is expressed by cortical and hippocampal Cajale Retzius cells, olfactory bulb mitral cells, and cerebellar granule cells, where it controls radial neuronal migration and cell layer formation (reviewed in (Tissir and Goffinet, 2003). In the adult brain, reelin is secreted by a subset of GABAergic interneurons in the cortex and hippocampus (Abraham and Meyer, 2003; Alcantara et al., 1998; Pesold et al., 1998; Pesold et al., 1999), where it plays a prominent role in regulating neural plasticity by enhancing cell migration and integration, facilitating synaptogenesis, and stabilizing synaptic contacts onto dendritic spines (Drakew et al., 2002; Forster et al., 2006; Frotscher et al., 2003; Gong et al., 2007; Herz and Chen, 2006; Liu et al., 2001; MacLaurin et al., 2007; Niu et al., 2004; Niu et al., 2008; Pujadas et al., 2010; Rodriguez et al., 2000; Rodriguez et al., 2002; Weiss et al., 2003; Zhao et al., 2004; Zhao et al., 2006b).

Given that many of the functional properties of reelin mirror those that are disrupted or impaired in schizophrenia, bipolar disorder, and depression, it is logical to wonder whether reelin might be involved in the pathogenesis of these disorders. This issue was first addressed by the pioneering studies carried out under the direction of Dr. Erminio Costa at the University of Illinois at Chicago, which clearly demonstrated a widespread downregulation of reelin expression levels in post-mortem brain samples from patients with schizophrenia and bipolar disorder (Guidotti et al., 2000; Impagnatiello et al., 1998). These results were subsequently replicated and extended by other researchers (Eastwood and Harrison, 2006; Fatemi et al., 2000; Fatemi et al., 2001; Fatemi et al., 2005b; Knable et al., 2004). Of particular importance is

the observation by Fatemi et al. (2000) that reelin levels are downregulated in post-mortem hippocampal tissue not just in schizophrenia and bipolar disorder, but also in major depressive disorder (albeit the decrease in depression is more subtle than in schizophrenia and bipolar disorder). Taken together, these findings suggested that a deficit in reelin signaling could be important for the development of several psychiatric disorders, including depression, and that this was an issue that should be investigated in preclinical animal models of these disorders.

To study whether a loss of reelin could be a vulnerability factor for depression, we used a strain of haploinsufficient heterozygous reeler mice (HRM), which typically have about 50% of normal brain levels of reelin. Behaviorally, these mice are not obviously different from wild-type mice (WTM), but they do show subtle deficits in prepulse inhibition and impairments on some measures of cognition and executive function (Ammassari-Teule et al., 2009; Barr et al., 2008; Brigman et al., 2006; Krueger et al., 2006; Qiu et al., 2006a; Tueting et al., 1999; Tueting et al., 2006). To our knowledge, depression-like behavior has not been assessed in these mice. Interestingly, HRM do show neuroanatomical and neurochemical alterations in brain regions that have been implicated in depression (i.e., hippocampus, cortex, prefrontal cortex, see Tueting et al., 2006 for a review), which may make them more vulnerable than WTM to the deleterious effects of stress or glucocorticoids. Therefore, we challenged HRM and WTM mice with repeated exposure to different doses of corticosterone (CORT), and measured changes in depression-like behavior, the number of reelin-positive cells in the dentate gyrus, and hippocampal neurogenesis. We chose to use CORT for this experiment because we have previously shown that repeated daily injections of CORT reliably increase depression-like behavior in a dose-dependent manner in normal rodents (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009). In a preliminary study,

we also found that repeated CORT injections decrease the number of reelin-positive cells selectively in the CA1 stratum lacunosum-moleculare and the subgranular zone (SGZ) of the dentate gyrus (Lussier et al., 2009). This latter change was particularly thought-provoking, because a lack of reelin within pyramidal-basket cells in the SGZ could have direct effects on hippocampal neurogenesis, and one prominent hypothesis about the neurobiology of depression suggests that a deficit in neurogenesis could play a causal role in depression (e.g., (Dranovsky and Hen, 2006; Jacobs, 2002; Koo et al., 2010; Kempermann and Kronenberg, 2003). Importantly, we also found that rats exposed to a repeated restraint stress procedure that does not increase depression-like behavior show no changes in the number of reelin-positive cell anywhere in the hippocampus (Gregus et al., 2005; Lussier et al., 2009). Therefore, exposure to stress or glucocorticoids does not seem to produce a deficit in hippocampal reelin expression unless the stressor also produces a behavioral phenotype of depression.

Based on the findings from post-mortem tissue and animal models described above, our primary hypothesis was that the HRM mice would be more susceptible to the depressogenic effects of repeated CORT than WTM. We expected to see greater increases in depression-like behavior in a dose-dependent manner in the HRM, as well as fewer reelin-positive cells and more deficits in hippocampal neurogenesis and neuronal maturation. The present report evaluates this hypothesis by employing several behavioral and immunohistochemical paradigms.

2. Materials and Methods

2.1. Animals

We used 45 male wild-type mice (WTM) and 45 male reelin haploinsufficient heterozygous reeler mice (HRM) in this experiment. HRM and their wild-type littermates were generated from heterozygous (B6C3Fe-a/a-Reln$^{rl/+}$) breeding pairs (Jackson Laboratory, Bar Harbor, ME) maintained in our colony at the University of Santiago de Compostela, Spain. The genotype of each mouse was confirmed using standard polymerase chain reaction (PCR) techniques on tail samples as described previously (D'Arcangelo et al., 1995). The following oligonucleotide primers were used: 50-TAA TCT GTC CTC ACT CTG CC-30, 30-ACA GTT GAC ATA CCT TAA TC-50, 30-TGC ATT AAT GTG CAG TGT TGT-50. The PCR products were analyzed in a 2% agarose gel: The product from wild type mice DNA is 266 bp long and the product from heterozygous reeler mice is 363 bp long. All mice used in this experiment were randomly chosen from a number of litters that were born at about the same time.

The mice were individually housed under standard conditions with food and water available ad libitum. They were approximately 3 months of age at the start of experiment and weighed an average of 30.59 g. There was a 12 h:12 h light/dark cycle with the lights turned on at 8:00 am.

2.2. Experimental Procedures

All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care, the University of Saskatchewan Committee on Animal

Care, the European Communities Council directive of 24 November 1986 (86/609/EEC), the Spanish royal decree 1201/2005, and the Bioethics Committee of the University of Santiago de Compostela. Both HRM and WTM were randomly assigned to groups that received one of the following four treatments by subcutaneous injections of CORT or vehicle: CORT injections at a dose of 20 mg/kg (n =12 HRM; n = 12 WTM), CORT injections at a dose of 10 mg/kg (n = 11 HRM; n = 11 WTM), CORT injections at a dose of 5 mg/kg (n = 11 HRM; n = 11 WTM) or vehicle injections (n = 11 HRM; n = 11 WTM). These doses were chosen on the basis of previous work in our laboratory showing that repeated CORT injections have dose dependent depressogenic effects in rats (Johnson et al., 2006) and the recent observation that 21 days of 20 mg/kg of CORT injections increase depression-like behavior in the forced swim test in c57 mice (Zhao et al., 2008a). We included doses below 20 mg/kg because we hypothesized that the HRM might be more vulnerable to the deleterious effects of CORT and we wanted to include doses that might be below threshold for the WTM but not the HRM. All CORT and vehicle injections were delivered subcutaneously at a volume of 5 ml/kg, between 9:00 and 11:00 am each day, for 21 consecutive days. CORT (Sigma-Aldrich, St. Louis, MO) was suspended in .9% physiological saline with 2% polyoxyethylene glycol sorbitan monooleate (Tween-80; SigmaeAldrich, St. Louis, MO).

2.3. Behavioral Testing

All behavioral testing was conducted in a different room from those used for the injections and housing. Mice were tested between 11 am and 7 pm. All behaviors were videotaped and scored at a later date by a trained observer who was blind to the experimental conditions.

2.3.1. Forced Swim Test

The forced swim test was conducted on the day after the final injection. Each mouse was placed individually into a 3 L beaker containing 25 ± 1°C water that was 15 cm deep. The mouse was left in the beaker for 6 min. Immobility, swimming and struggling behaviors were timed (in sec) over the last 4 min of the 6 min trial (Porsolt et al., 1977). Immobility was defined as the absence of head movement or body propulsion (Cryan et al., 2005; Stranahan et al., 2007; Strekalova et al., 2004), swimming was defined as the directed movement of the animal's head and body, and struggling was defined as more forceful movements than swimming, usually against the walls of the beaker. The water in the beaker was changed after each mouse.

2.3.2. Open Field Test

The open field test was conducted on the day after the forced swim test. Each mouse was placed individually into the center of a 40 cm × 40 cm wooden box, which was divided into 16 equal squares. The mouse was left in the box for 10 min. The inner 4 squares were defined as the center and the outer 12 squares were defined as the periphery. Four behavioral measures were quantified for each mouse: the latency to leave the center start area (in sec), the amount of time spent in the center (in sec), the amount of time spent in the periphery (in sec), and the total distance traveled (in cm). All behaviors were coded using Ethovision software (version 3.0; Wageningen, The Netherlands).

2.4. Perfusions and Tissue Preparation

On day 24, the day after the open field test, all mice were anesthetised with 15% chloral hydrate (i.p.) and perfused transcardially with .9% saline and 4% paraformaldehyde in .1 M phosphate buffer (PB, pH 7.4). The brains were removed and postfixed in 4% paraformaldehyde for 72 h at 4 °C, and then they were placed in a .1 M phosphate buffered saline (PBS)/.1% (w/v) sodium azide solution at 4 °C until sectioning. The brains were sectioned using a vibrating microtome (Vibratome 3000, Vibratome Company, St. Louis, MO) at 40 µm and the resulting sections were stored in cryoprotectant at -20 °C until used for immunohistochemistry.

2.5. Immunohistochemistry

Every sixth section (1 in 6 series) was utilized for immunostaining and approximately 5 sections per animal were quantified. Immunohistochemistry was performed on free-floating brain sections as follows:

2.5.1. Reelin Immunohistochemistry

The sections were washed in .1 M PBS and incubated in .3% (v/v) H_2O_2 in PBS for 30 min to block endogenous peroxidase activity. The sections were pre-incubated for 30 min in a blocking solution containing .3% (v/v) Triton X-100, 1.5% (v/v) normal horse serum (NHS), and 1% (v/v) bovine serum albumin (BSA) dissolved in PBS in order to block nonspecific antibody binding. The sections were then incubated for 48 h at 4 ºC with mouse anti-reelin primary antibody (Chemicon International, Temecula, CA) at 1:2000 diluted in a blocking

solution. After incubation and PBS washes, the sections were incubated with biotinylated horse anti-mouse IgG (1:200, SigmaeAldrich, St. Louis, MO) secondary antibody diluted in .3% (v/v) Triton X-100 PBS at room temperature for 2 h. After another set of PBS washes, the sections were incubated for 1 h in an avidinebiotin complex (1:500, Vecta Stain Elite ABC reagent, Vector Labs, Burlingame, CA) at room temperature. The sections were stained using .033% (w/v), 30-diaminobenzidine (DAB, SigmaeAldrich, St. Louis, MO) dissolved in .00786% (v/v) H_2O_2 and PBS. The reaction was stopped using PBS as a rinse. The sections were then mounted onto slides and left to dry overnight and then counterstained with cresyl violet and coverslipped using Entellan resin solution.

2.5.2. Doublecortin (DCX) Immunohistochemistry

The sections were washed in PBS and incubated in sodium citrate (pH 6.0; 95 °C) for 30 min. After 3 PBS washes, the sections were incubated for 24 h at room temperature with rabbit anti-DCX primary antibody (Cell Signaling, Danvers, MA) at 1:1000 diluted in a solution containing Triton X-100 (.5%, v/v), normal goat serum (NGS; 5%; v/v), and BSA (1%; w/v) dissolved in PBS. After incubation in the primary antibody, the sections were washed in PBS then incubated in 5% (v/v) H_2O_2 in PBS for 30 min to block endogenous peroxidase activity. The tissue was then incubated with biotinylated goat anti-rabbit IgG (1:500, Sigma Aldrich, St. Louis, MO) secondary antibody diluted in .5% (v/v) Triton X-100, 5% (v/v) NGS, and 1% (w/v) BSA in PBS for 1 h at room temperature. This was followed by incubation in an avidin biotin complex (1:500, Vecta Stain Elite ABC reagent, Vector Labs) for 1 h at room temperature. After washes in PBS and 2 washes in .175 M sodium acetate, the sections were stained using .025% DAB and 4.167% NiSO4 dissolved in .002% H_2O_2 and .175 M sodium

acetate. The reaction was stopped using .175 M sodium acetate as a rinse. The sections were then mounted onto slides and coverslipped using Entellan resin solution.

2.6. Analyses of Immunohistochemical Labeling

All analyses were conducted by individuals who were blind to the experimental conditions. The total number of reelin-positive cells and DCX-positive cells in the dentate gyrus was estimated using the unbiased optical fractionator method (West et al., 1991). Sections were examined at 400 × and 1000 × (oil immersion) magnification using a Nikon E800 microscope equipped with a motorized stage and a computerized stereology system (Stereo Investigator, version 9.0, MicroBrightField Inc, Williston, VT). DCX-positive cells were counted in the subgranular zone (SGZ) and granule cell layer (GCL) throughout the dorsal dentate gyrus (from -2.40 mm from bregma to approximately -3.94 mm from bregma, according to Paxinos and Watson, 1998). Reelin-positive cells were counted in the SGZ and hilus of the dentate gyrus from the same A-P plane as described above. The granule cell layer was not counted because there are typically very few reelin-positive cells located in this region. The total number of DCX-positive and reelin-positive cells was estimated using the following formula: $N\text{total} : \Sigma Q^- \times 1/\text{ssf} \times A(x,y\ \text{step})/a(\text{frame}) \times t/h$; where ΣQ^- is the number of counted cells; ssf is the section sampling fraction (1/6); $A(x,y\ \text{step})$ is the area associated with each x,y movement (5625 μm^2); a(frame) is the area of the counting frame (2500 μm^2); t is the weighted average section thickness; and h is the height of the dissector (12 µm). A guard zone of 5 µm was used during cell counting to avoid sectioning artifacts. Both hemispheres were quantified and combined for the statistical analyses.

To semi-quantify the morphology of the DCX-positive cells, dendritic branching in a subset of DCX-positive cells from each mouse was categorized according to the method described by Plumpe et al. (2006). Between 80 and 120 cells per animal were randomly selected using a meander scan method. Each cell was assigned to one of six categories based on the presence and extent of its apical dendrites (see Figure 4-5). Briefly, categories 1 and 2 represented the proliferative stage of development and cells placed into these categories had either no processes (category 1) or one short process (category 2). Categories 3 and 4 represented an intermediate stage of cell development. Category 3 cells showed a medium process that reached the granular cell layer but did not extend into the molecular layer. Category 4 cells were similar except that they had a process that reached the molecular layer. Categories 5 and 6 represented more mature cell development. Category 5 cells had at least one major dendrite branching into the molecular layer. Category 6 cells had a defined dendritic tree with delicate branching within the granule cell layer. The percentage of cells falling into each of these categories was calculated for each animal.

2.7. Statistical Analyses

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS v 18.0, Chicago, IL).We examined the statistical significance of group differences in two different ways. First, we examined the effect of genotype and CORT dose using two-way ANOVAs for each measure (i.e., each behavior in the forced swim test and open field, reelin cell number, DCX cell number, and DCX cell category). Second, because we had hypothesized a priori that the HRM would be more affected by CORT than the WTM, we examined the effect of CORT on each measure using separate one-way ANOVAs for the HRM and WTM.

This provided more statistical power for us to detect CORT effects within each strain of mouse. For all analyses, significant main effects were followed by Fisher's least significant difference post-hoc tests or post-hoc t-tests when appropriate. All results are represented as mean ± standard error of the mean. The criterion for statistical significance was set at $p < .05$.

3. Results

3.1. Forced Swim Test Behavior

Figure 4-1 shows the effect of CORT on behavior in the forced swim test. A two-way ANOVA on immobility behavior revealed a significant main effect of CORT dose [F (3,77) = 3.832, $p < .02$] but no significant effect of genotype [F (1,77) = .439, $p > .50$] and no significant CORT dose by genotype interaction [F (3,77) = 1.027, $p > .38$]. Post-hoc analyses confirmed that the mice that received 20 mg/kg of CORT showed significantly more immobility behavior than the mice that received 10 mg/kg of CORT, 5 mg/kg of CORT or vehicle injections (all p values <.05). Similarly, a two-way ANOVA on swimming behavior revealed a significant main effect of CORT dose [F (3,77) = 3.425, $p < .03$] but no significant effect of genotype [F (1,77) = .749, $p > .38$] and no significant CORT dose by genotype interaction [F (3,77) = .743, $p > .53$]. Post-hoc analyses confirmed that the mice that received 20 mg/kg of CORT showed significantly less swimming behavior than the mice that received 10 mg/kg of CORT, 5 mg/kg of CORT or vehicle injections (all p values <.02). Finally, a two-way ANOVA on struggling behavior revealed no significant main effects or interaction effects [all p values >.089], suggesting that none of the groups differed in the amount of struggling behavior.

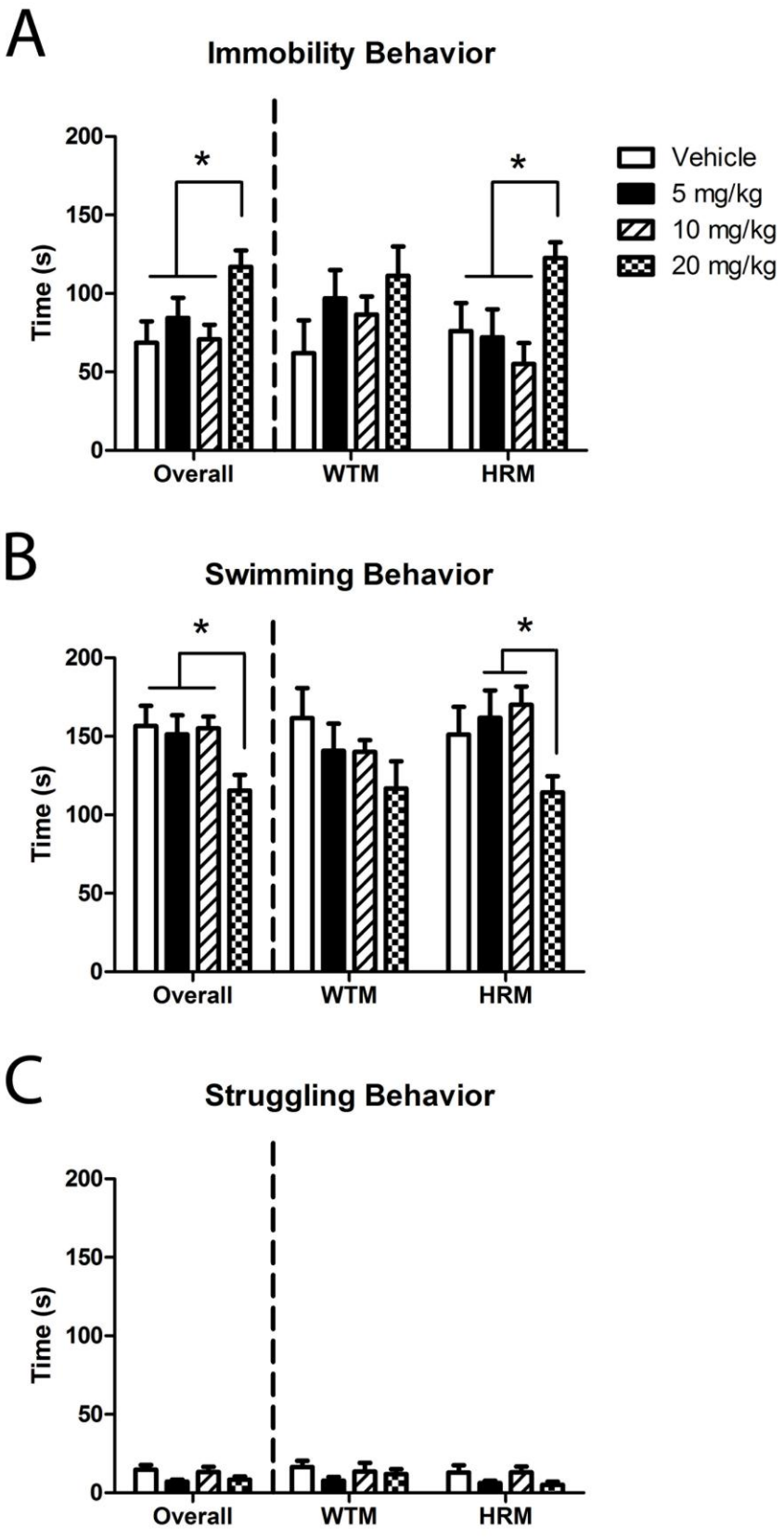

A
Immobility Behavior
Time (s)
200
150
100
50
0
*
*
Overall
WTM
HRM
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg
B
Swimming Behavior
Time (s)
200
150
100
50
0
*
*
Overall
WTM
HRM
C
Struggling Behavior
Time (s)
200
150
100
50
0
Overall
WTM
HRM

Figure 4-1. The effect of CORT on behavior in the forced swim test: Panel A shows the time spent immobile, panel B shows the time spent swimming, and panel C shows the time spent struggling. Error bars represent the mean ± standard error of the mean. Each panel shows the overall time spent by the WTM and HRM in each behavior and asterisks in this area represent the results of the two-way ANOVAs. The data are also shown with the WTM and HRM plotted separately, and in these cases, asterisks represent the results of the one-way ANOVAs within each genotype. CORT had dose dependent effects on immobility and swimming behavior. The mice injected with 20 mg/kg CORT spent significantly more time immobile than the mice in the three other groups. The same pattern of results was seen within the HRM alone. Similarly, the mice injected with 20 mg/kg CORT spent significantly less time swimming than the mice in the three other groups. Within the HRM alone, the mice injected with 20 mg/kg CORT spent significantly less time swimming than the mice injected with 10 mg/kg CORT or 5 mg/kg CORT.

Within the HRM only, one-way ANOVAs of each forced swim test behavior revealed significant main effects of CORT dose on immobility [F (2,38) = 4.08, p < .02] and swimming [F (2,38) = 3.16, p < .04]. Post-hoc analyses of these main effects further revealed that the HRM mice injected with 20 mg/kg CORT group showed significantly more immobility than the mice injected with 10 mg/kg CORT, 5 mg/kg CORT, or vehicle [all p values <.04]. In addition, the HRM mice injected with 20 mg/kg CORT showed significantly less swimming than the mice injected with 10 mg/kg CORT or 5 mg/kg CORT [both p values <.02].

Within the WTM only, one-way ANOVAs of each forced swim test behavior revealed no significant effects [all p values >.268].

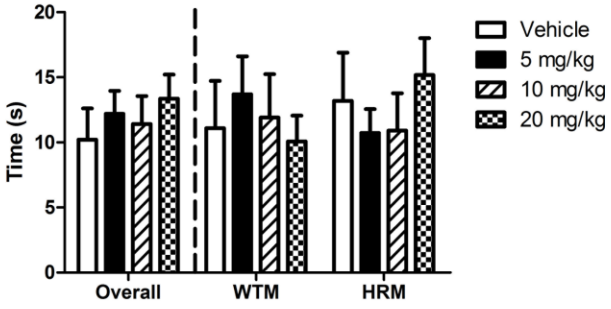
3.2. Open Field Behavior

Figure 4-2 shows the effect of CORT on behavior in the open field test. Two-way ANOVAs on each measure in the open field revealed no significant main effects of genotype or CORT dose and no significant interactions between genotype and CORT dose [all p values >.23]. Similarly, one-way ANOVAs on each measure in the open field within the HRM only or WTM only revealed no significant main effects of CORT [all p values >.246].

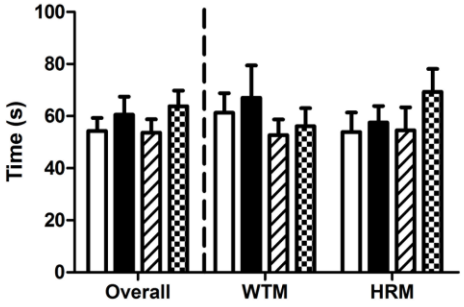
3.3. Reelin Cell Counts in the Hilus and SGZ

Figure 4-3 shows the effect of CORT on the number of reelin-positive cells in the SGZ and hilus. A two-way ANOVA of the number of reelin-positive cells in the SGZ revealed a significant main effect of genotype [F (1,65) = 9.569, p < .003] but no significant main effect of CORT dose [F (3,65) = 1.557, p > .21] and no significant CORT dose by genotype

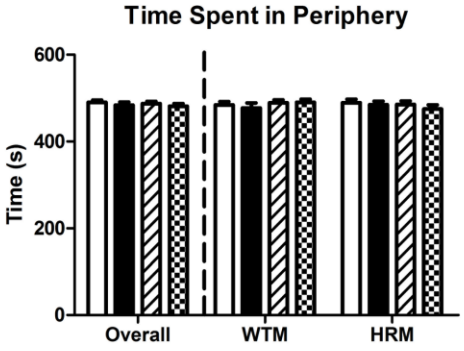
A


Latency to Leave Center Area
Time (s)
20
15
10
5
0
Overall
WTM
HRM
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg

B


Time Spent in Center
Time (s)
100
80
60
40
20
0
Overall
WTM
HRM

C


Time Spent in Periphery
Time (s)
600
400
200
0
Overall
WTM
HRM

D

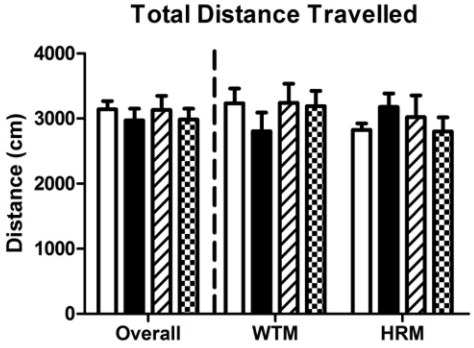

Total Distance Travelled
Distance (cm)
4000
3000
2000
1000
0
Overall
WTM
HRM

Figure 4-2. The effect of CORT on behavior in the open field: Panel A shows the latency to leave the center area, panel B shows the time spent in the center, panel C shows the time spent in the periphery, and panel D shows the total distance traveled. Error bars represent the mean ± standard error of the mean. Each panel shows the overall time spent by the WTM and HRM in each behavior, followed by the data for the WTM and HRM plotted separately. There were no significant genotype or dose differences in open field behavior.

interaction [F (3,65) = 1.299, p > .28]. The main effect of genotype confirmed that in general, the HRM had significantly fewer reelin-positive cells in the SGZ than did the WTM. The same pattern of results was also seen in the hilus. A two-way ANOVA of the number of reelin-positive cells in the hilus revealed a significant main effect of genotype [F (1,65) = 6.735, p < .02] but no significant main effect of CORT dose [F (3,65) = 2.116, p > .10] and no significant CORT dose by genotype interaction [F (3,65) = .942, p > .42].

From looking at Figure 4-3, it is apparent that the significant genotype main effect described above is probably driven primarily by the sharp decrease in the number of reelin-positive cells in the HRM injected with 10 mg/kg CORT and 20 mg/kg CORT. To examine this possibility statistically, we compared the number of reelin-positive cells across genotype for each dose of CORT. We found that the HRM injected with 20 mg/kg CORT had significantly fewer reelin-positive cells in both the SGZ and hilus than the WTM injected with 20 mg/kg CORT [SGZ: t (13) = 2.188, p < .03; hilus: t (13) = 2.684, p < .01]. The HRM had 43% and 33% fewer reelin-positive cells than the WTM in the SGZ and hilus, respectively.

Similarly, the HRM injected with 10 mg/kg CORT had significantly fewer reelin-positive cells in both the SGZ and hilus than the WTM injected with 10 mg/kg CORT [SGZ: t (15) = 2.557, p < .02; hilus: t (15) = 1.791, p < .05]. The HRM had 24% and 15% fewer reelin-positive cells than the WTM in the SGZ and hilus, respectively.

Within the HRM only, one-way ANOVAs revealed significant main effects of CORT dose on the number of reelin-positive cells in the SGZ and hilus [SGZ: F (3,29) = 4.20, p < .02; hilus: F (3,29) = 4.42, p < .02]. Post-hoc analyses of these main effects showed that the HRM mice injected with 20 mg/kg CORT group had significantly fewer reelin-positive cells in the

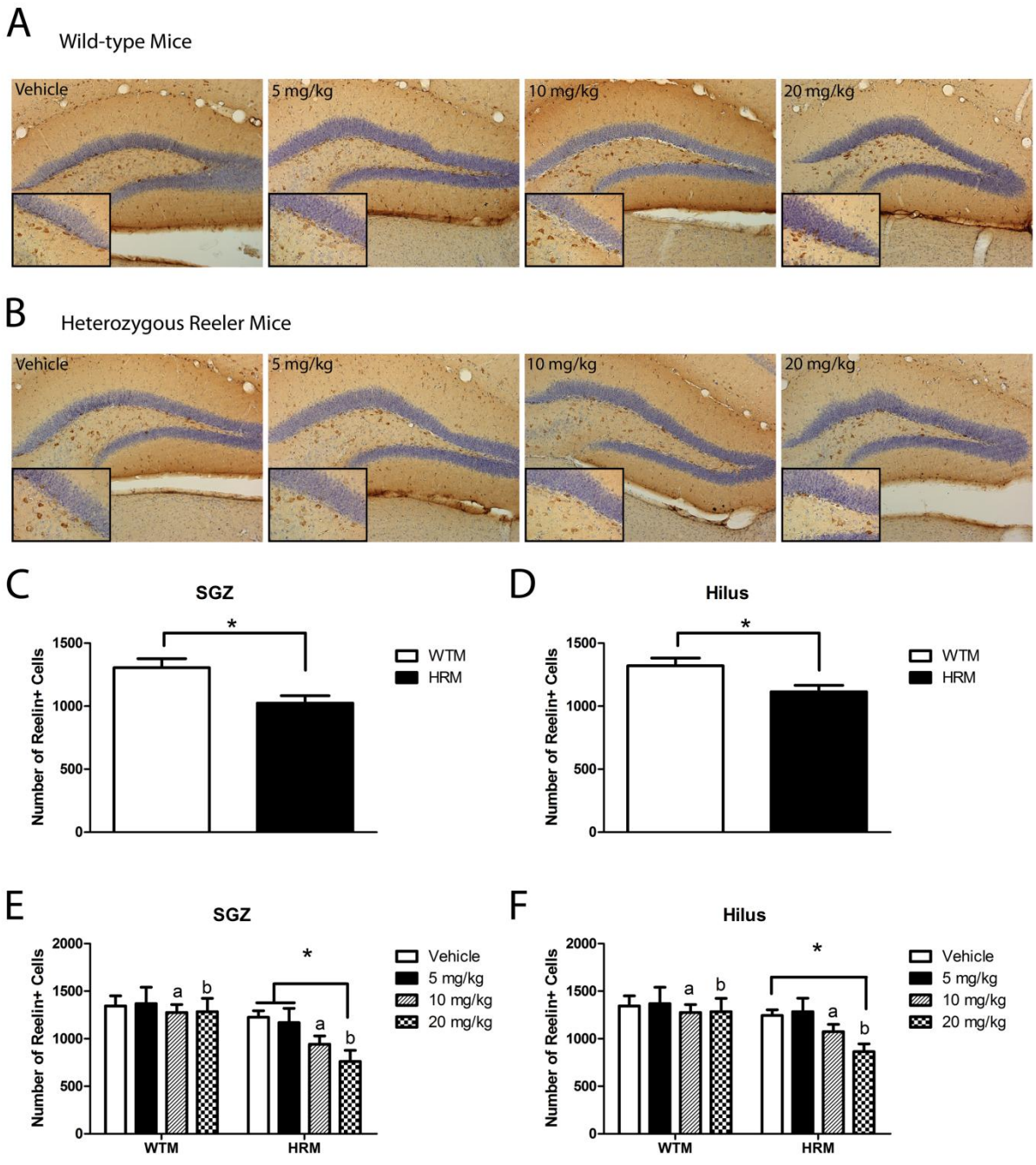

A
Wild-type Mice
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg
B
Heterozygous Reeler Mice
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg
C
SGZ
Number of Reelin+ Cells
1500
1000
500
0
*
WTM
HRM
D
Hilus
Number of Reelin+ Cells
1500
1000
500
0
*
WTM
HRM
E
SGZ
Number of Reelin+ Cells
2000
1500
1000
500
0
a
b
a
b
*
WTM
HRM
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg
F
Hilus
Number of Reelin+ Cells
2000
1500
1000
500
0
a
b
a
b
*
WTM
HRM
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg

Figure 4-3. The effect of CORT on the number of reelin-positive cells in the SGZ and hilus: Panel A shows representative micrographs of reelin immunoreactivity in the WTM and panel B shows representative micrographs of reelin immunoreactivity in the HRM. Panels C and D show the number of reelin-positive cells for all the WTM and HRM in both the SGZ and hilus. Panels E and F show the same data separated by dose of CORT. Error bars represent the mean ± standard error of the mean. The HRM had significantly fewer reelin-positive cells in the SGZ and the hilus, showing a clear effect of genotype on this parameter. This effect was driven by the fact that the HRM injected with 10 mg/kg CORT or 20 mg/kg CORT had significantly fewer reelin-positive cells in the SGZ and hilus than the WTM injected with 10 mg/kg CORT or 20 mg/kg CORT (shown by a, b in the figure). Further analyses within each genotype separately revealed that the HRM injected with 20 mg/kg CORT had significantly fewer reelin-positive cells in the SGZ than the HRM injected with 5 mg/kg CORT or vehicle, and they had significantly fewer reelin-positive cells in the hilus than HRM injected with vehicle.

SGZ and hilus than did the HRM mice injected with 5 mg/kg CORT or vehicle [all *p* values < .02]. In practical terms, the differences among these groups were quite large. In the SGZ, the HRM mice injected with 20 mg/kg CORT had 38% fewer reelin-positive cells than the vehicle-injected mice and 35% fewer reelin-positive cells than the mice injected with 5 mg/kg CORT. In the hilus, the HRM mice injected with 20 mg/kg CORT had 31% fewer reelin-positive cells than the vehicle-injected mice and 32.7% fewer reelin-positive cells than the mice injected with 5 mg/kg CORT.

Within the WTM only, one-way ANOVAs revealed no significant effects of any CORT dose on the number of reelin-positive cells in the SGZ or hilus [all *p* values > .945].

3.4. DCX Cell Counts in the SGZ and Granule Cell Layer

Figure 4-4 shows the effects of CORT on the number of DCX-positive cells. As is typical, these cells were primarily located in the subgranular zone and the inner one-third of the granule cell layer. The cells counts from both these regions were combined for the statistical analyses. A two-way ANOVA revealed a significant main effect of CORT dose [F (3,48) = 7.711, $p < .001$] but no significant effect of genotype [F (1,48) = .211, $p > .64$] and no significant CORT dose by genotype interaction [F (3,48) = .750, $p > .52$]. Post-hoc analyses showed a number of differences among the groups. First, the mice that received 20 mg/kg of CORT had significantly fewer DCX-positive cells than did the mice that received 5 mg/kg of CORT [$p < .001$] or vehicle [$p < .02$]. The difference between the 20 mg/kg mice and the 10 mg/kg mice narrowly missed statistical significance [$p < .06$]. Second, the mice that received 10 mg/kg CORT had significantly fewer DCX-positive cells than did the mice that received

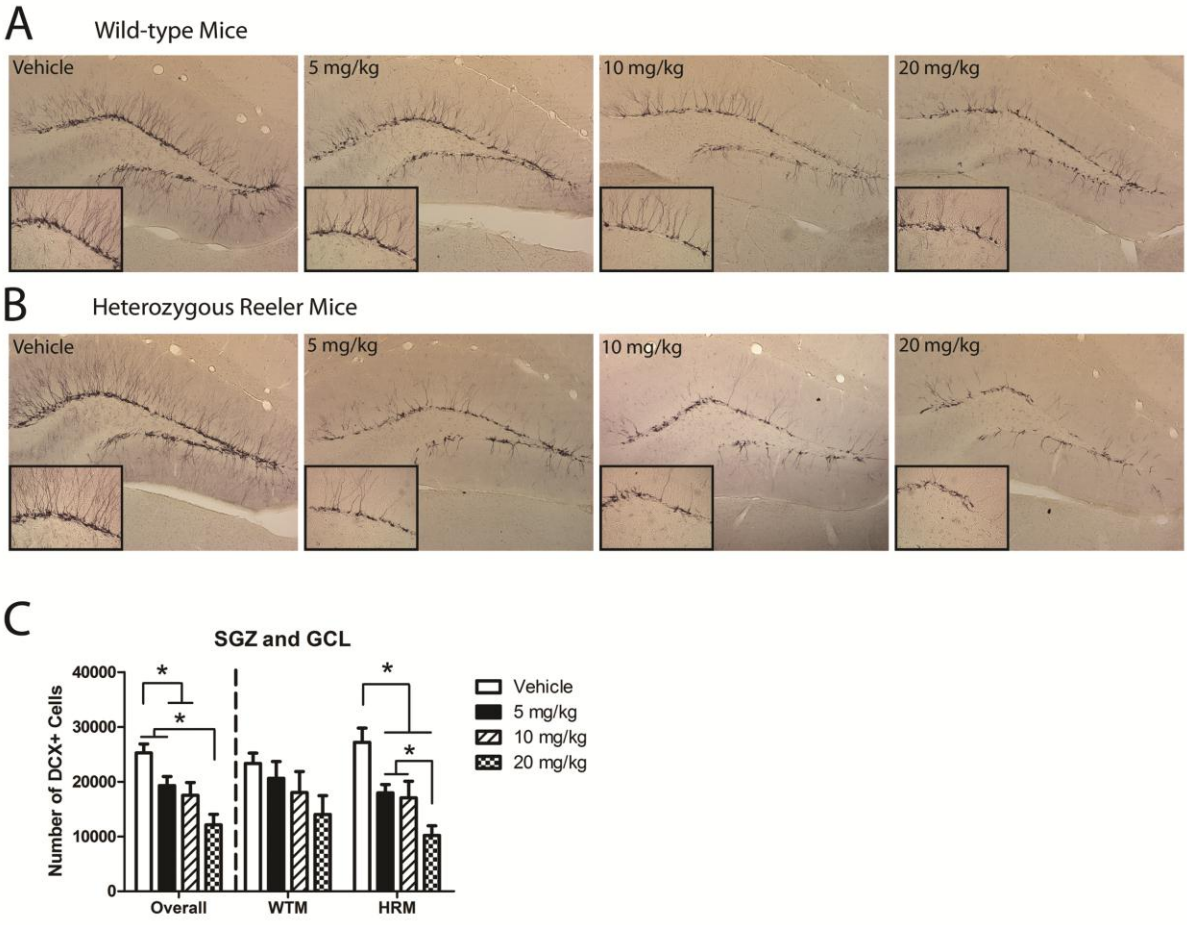


Figure 4-4. The effect of CORT on the number of DCX-positive cells in the SGZ and GCL: Panel A shows representative micrographs of DCX immunoreactivity in the WTM and panel B shows representative micrographs of DCX immunoreactivity in the HRM. Panel C shows the quantified results of the DCX cell counts. Error bars represent the mean ± standard error of the mean. As in the previous figures, the data are plotted first with all mice from both genotypes combined and asterisks in this area represent the results of the two-way ANOVA. The data are also shown with the WTM and HRM plotted separately, and asterisks in these areas represent the results of the one-way ANOVAs within each genotype. CORT had dose-dependent effects on the number of DCX-positive cells. The mice injected with 20 mg/kg CORT had fewer DCX-positive cells than the mice injected with 5 mg/kg CORT or vehicle. In addition, the mice injected with 5 mg/kg CORT or 10 mg/kg CORT had fewer DCX-positive cells than the mice injected with vehicle. Within the HRM alone, all doses of CORT significantly decreased the number of DCX-positive cells. Furthermore, the HRM injected with 20 mg/kg CORT had significantly fewer DCX-positive cells than the HRM injected with 10 mg/kg or 5 mg/kg CORT.

vehicle [$p < .008$]. And finally, the mice that received 5 mg/kg CORT had significantly fewer DCX-positive cells than did the mice that received vehicle [$p < .04$].

Within the HRM only, a one-way ANOVA revealed a significant main effect of CORT dose on DCX cell number [F (3,23) = 9.133, $p < .001$]. Post-hoc analyses of this main effect revealed that the HRM mice injected with each dose of CORT had fewer DCX-positive cells than the mice injected with vehicle [all p values <.012]. There was a 34%, 37%, and 63% decrease in the number of DCX-positive cells seen in the HRM mice injected with 5 mg/kg CORT, 10 mg/kg CORT, and 20 mg/kg CORT, respectively. In addition, the HRM mice injected with 20 mg/kg CORT had significantly fewer DCX-positive cells than the HRM mice injected with 10 mg/kg CORT or 5 mg/kg CORT [both p values <.05]. The mice injected with 20 mg/kg CORT had 40.21% and 43.23% fewer DCX-positive cells than the mice injected with 10 mg/kg CORT and 5 mg/kg CORT, respectively.

Within the WTM only, a one-way ANOVA revealed no significant effect of CORT dose on the number of DCX-positive cells [F (3,23) = 1.587, $p > .22$].

3.5. DCX Dendritic Morphology

Figure 4-5A shows the categorization of DCX-positive cells across all groups. Consistent with Plumpe et al. (2006), the DCX-positive cells showed a range of morphological complexity, from essentially no dendritic branching to well defined and complex branches. Each panel of Figure 4-5B shows the results for one of the six categories. In general, CORT appeared to slow DCX cell maturation, with more category 1 cells and fewer category 4, 5, and 6 cells in the CORT injected rats. A two-way ANOVA on the percentage of category 1 cells

revealed a significant main effect of CORT dose [F (3,45) = 11.166, $p < .001$] but no significant effect of genotype [F(1,45) = .294, $p > .59$] and no significant CORT dose by genotype interaction [F (3,45) = .659, $p > .58$]. Post-hoc analyses of this effect revealed that the mice injected with 20 mg/kg CORT had significantly more category 1 cells than the mice injected with 10 mg/kg CORT, 5 mg/kg CORT, or vehicle [all p's < .009]. In addition, the mice injected with 10 mg/kg CORT had significantly more category 1 cells than did the vehicle-injected mice [$p < .007$].

There were no significant main effects or interaction effects for the category 2 or 3 cells [all p values >.13]. However, a two-way ANOVA on the percentage of category 4 cells revealed a significant main effect of CORT dose [F (3,45) = 4.727, $p < .007$] but no significant effect of genotype [F (1,45) = 1.651, $p > .207$] and no significant CORT dose by genotype interaction [F (3,45) = .468, $p > .707$]. Post-hoc analyses revealed that the mice injected with 20 mg/kg CORT had significantly fewer category 4 cells than the mice injected with 5 mg/kg CORT or vehicle [all p's < .006]. Similar effects were seen for the category 5 and 6 cells. In both cases, two-way ANOVAs revealed significant main effects of CORT dose [category 5: F (3,45) = 4.962, $p < .005$; category 6: F (3,45) = 7.077, $p < .001$] but no significant effects of genotype [category 5: F (1,45) = .001, $p > .987$; category 6: F (1,45) = .001, $p > .987$] and no significant CORT dose by genotype interactions [category 5: F (3,45) = .06, $p > .981$; category 6: F (1,45) = .06, $p > .981$]. Post-hoc tests revealed that the mice injected with all doses of CORT had significantly fewer category 5 cells than the mice injected with vehicle [all p's < .05], and that the mice injected with 20 mg/kg CORT had significantly fewer category 6 cells than the mice in all other groups [all p's < .003].

Within the HRM only, a one-way ANOVA revealed a significant main effect of CORT dose on the percentage of category 1, 4, 5, and 6 cells [category 1: F (3,23) = 4.471, p < .02; category 4: F (3,23) = 3.122, p < .05; category 5: F (3,23) = 4.321, p < .02; category 6: F (3,23) = 4.466, p < .02]. Post-hoc analyses of these main effects revealed several group differences. First, the HRM injected with 20 mg/kg CORT had significantly more category 1 cells than the mice in all other groups [all p values <.03]. Second, the HRM injected with 20 mg/kg had significantly fewer category 4 cells than the mice injected with 5 mg/kg CORT or vehicle [both p values <.02]. Third, the HRM injected with 20 mg/kg CORT had significantly fewer category 5 cells than the vehicle-injected mice [p < .02]. The difference in category 5 cells between the mice injected with 20 mg/kg CORT and those injected with 5 mg/kg narrowly missed statistical significance [p < .061]. Fourth, the HRM injected with 10 mg/kg CORT had significantly fewer category 5 cells than did the mice injected with 5 mg/kg CORT or vehicle [both p values <.04]. And finally, the HRM injected with 20 mg/kg had significantly fewer category 6 cells than the HRM mice injected with 10 mg/kg CORT, 5 mg/kg CORT, or vehicle [all p values <.02]. There was also a significant main effect of CORT dose on the percentage of category 1 cells in the WTM [F (3,20) = 9.073, p < .001]. Post-hoc analyses of this effect revealed that the WTM injected with 20 mg/kg CORT or 10 mg/kg CORT had significantly more category 1 cells than the WTM injected with 5 mg/kg CORT or vehicle [all p values <.05]. There were no other significant differences in DCX cell category for the WTM, but the differences in category 5 cells and category 6 cells narrowly missed statistical significance [p > .065 for category 5 and p > .069 for category 6].

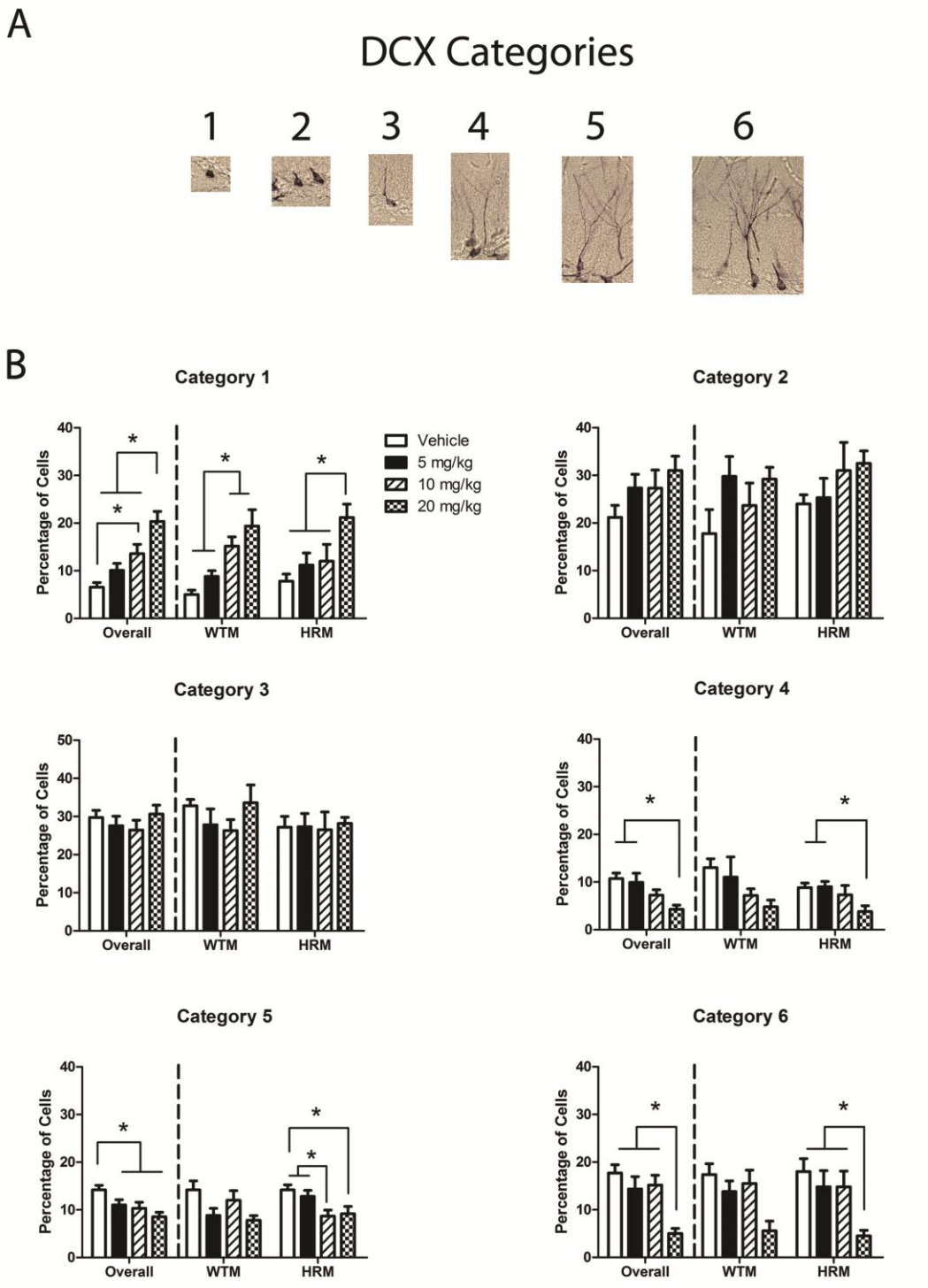

A
DCX Categories
1
2
3
4
5
6
B
Category 1
Category 2
Category 3
Category 4
Category 5
Category 6
Vehicle
5 mg/kg
10 mg/kg
20 mg/kg
Percentage of Cells
Overall
WTM
HRM

Figure 4-5. The effect of CORT on dendritic complexity within DCX-positive cells: Panel A provides an example of each of the 6 categories of dendritic complexity used in this analysis. Panel B shows the percentage of cells placed into each category for the mice in each group. Error bars represent the mean ± standard error of the mean. As in the previous figures, the data are plotted first with all mice from both genotypes combined and asterisks in this area represent the results of the two-way ANOVAs. The data are also shown with the WTM and HRM plotted separately, and asterisks in these areas represent the results of the one-way ANOVAs within each genotype. CORT had dose-dependent effects on the percentage of cells in categories 1, 4, 5, and 6, with an overall increase in the percentage of DCX-positive cells that fell into category 1 (i.e., more immature cells) and an overall decrease in the percentage of cells that fell into categories 4, 5, and 6 (more complex dendrites). Within the HRM alone, the mice injected with 20 mg/kg CORT had significantly more category 1 cells and significantly fewer category 6 cells than the HRM in all other groups. They also had significantly fewer category 4 cells than the HRM injected with 5 mg/kg CORT or vehicle, and significantly fewer category 5 cells than the HRM injected with vehicle. In addition, the HRM injected with 10 mg/kg CORT had significantly fewer DCX-positive cells than the HRM injected with 5 mg/kg CORT or vehicle. Finally, the WTM injected with 10 mg/kg or 20 mg/kg CORT had significantly more category 1 cells than the WTM injected with 5 mg/kg CORT or vehicle.

4. Discussion

The results of this experiment can be summarized as follows. We found that 21 days of repeated CORT administration produced dose-dependent increases in depression-like behavior and decreases in neurogenesis that are consistent with the results of previous experiments (for a review see Sterner and Kalynchuk, 2010). CORT also decreased dendritic complexity in newly born dentate granule cells, suggesting that the rate of cell maturation is compromised in mice exposed to high levels of glucocorticoids. Interestingly, there were no differences between the vehicle injected HRM and WTM in depression-like behavior, the number of reelin-positive cells in the SGZ and hilus, or hippocampal neurogenesis and neuronal maturation. However, the effects of CORT on behavior, reelin cell number, and neurogenesis were more pronounced in the HRM than in the WTM, providing support for the idea that mice with impaired reelin signaling may be more vulnerable to the deleterious effects of glucocorticoids. These findings are discussed in more detail in the following paragraphs.

4.1. Effect of CORT on Depression-like Behavior

CORT had significant effects on depression-like behavior in this experiment. The highest dose of 20 mg/kg increased the time spent immobile and decreased the time spent swimming in the forced swim test, which are measures typically used to infer a depressive phenotype in rats and mice (Cryan et al., 2005; Gregus et al., 2005; Porsolt et al., 1977). Importantly, these effects occurred independently of nonspecific changes in motoric behavior, as the CORT injected rats showed a similar amount of exploration in the open field compared to vehicle-injected rats. These results are consistent with previous findings (Marks et al., 2009), and reinforce the idea that the depressogenic effects of exogenous CORT administration are

time- and dose-dependent. We have worked extensively with a rat paradigm of exogenous CORT administration and our studies have revealed that 21 days, but not 1 day, of 40 mg/kg CORT injections produce large and reliable increases in depression-like behavior, whereas 21 days of 20 mg/kg injections produce smaller and less reliable increases and 21 days of 10 mg/kg injections produce no increases in depression-like behavior (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009). Results reported by other researchers largely confirm these observations. Brummelte et al. (2006) found that 26 days of 40 mg/kg CORT increased forced swim test immobility and Hill et al. (2003) found the same result with 20 days of 20 mg/kg CORT injections. However, a 20 mg/kg dose given for at least 20 days appears to be the minimum amount required for eliciting a depressive phenotype because (Brotto et al., 2001) found no behavioral effects with only 10 days of 20 mg/kg CORT injections. Work in mice has confirmed this observation, as Zhao et al. (2008, 2009) reported that c57 mice injected with 20 mg/kg CORT for 21, 35, or 36 days show increased immobility behavior but mice injected with 20 mg/kg CORT for either 6 or 18 days do not. Taken together, our findings and those of previous studies confirm that medium to high dose CORT administration can produce depressogenic effects in both rats and mice, and further validate the value of this approach for understanding the relationship between glucocorticoids and depressive behavior.

4.2. Effect of CORT on Hippocampal Neurogenesis

CORT also had significant effects on hippocampal neurogenesis in this experiment. This is not a novel finding: Numerous previous reports have shown that exposure to chronic stress or glucocorticoids causes rapid and robust decreases in hippocampal cell proliferation

and survival in the adult rodent brain (e.g., Brummelte and Galea, 2010b; Pham et al., 2003; Sheline, 2000; Wong and Herbert, 2006). However, we believe that our results are the first to show that a dose as low as 5 mg/kg can decrease neurogenesis in mice. We found that all doses of CORT significantly decreased the number of DCX-positive cells in the subgranular zone and granule cell layer. Our results also showed that CORT decreases dendritic complexity in surviving newly born neurons. In general, a greater percentage of DCX-positive cells from CORT-injected mice had no or little dendritic branching compared to the vehicle injected mice. Similarly, a smaller percentage of DCX-positive cells from CORT-injected mice had extensive dendritic branching. Both of these effects increased with the dose of CORT given. Interestingly, Wong and Herbert (2004, 2006) recently showed that dentate granule cells born under conditions of high glucocorticoid levels are less likely to survive and less likely to take on a neuronal phenotype if they do survive. Our results take this one step further, and suggest that even if cells do survive, they may be slow to mature and integrate into existing circuitry. An important implication of this finding is that glucocorticoid-induced decreases in neurogenesis may not be the only issue to consider in the context of mood disorders. The cells that do survive may do so abnormally, contributing to disrupted hippocampal plasticity and function. If these cells are involved in the expression of depressive symptoms, then strategies that can repair them may have antidepressant properties. This idea is speculative, but it may be a fruitful topic for future investigation.

One other interesting observation from the present results is that all doses of CORT significantly decreased the number of DCX-positive cells, but only the highest dose of CORT increased immobility behavior in the forced swim test. On the surface, this suggests that the

effects of CORT on cell proliferation and survival and depression-like behavior may not be completely in sync. This is relevant to the prominent hypothesis that deficient hippocampal neurogenesis may play a causal role in depression (Dranovsky and Hen, 2006; Duman and Charney, 1999; Jacobs et al., 2000). This hypothesis has been quite controversial, with support gathered from reports that antidepressants increase neurogenesis while stress decreases it (reviewed by Kempermann and Kronenberg, 2003), and resistance building from observations that neurogenesis can be dissociated from depression-like behavior in some preclinical animal models and that it may not be decreased in post-mortem tissue from patients with depression (e.g., Reif et al., 2006; Vollmayr et al., 2003). Nevertheless, the vast majority of current data support the idea that deficient neurogenesis plays a role in the pathogenesis of depression. One possible interpretation of our results is that neurogenesis must dip below a certain level before functional consequences emerge. Our data show that CORT had graded effects on the number of DCX-positive cells, with cell number becoming increasingly smaller as the dose of CORT increased. Overall, the 20 mg/kg dose produced about a 50% decrease in the number of DCX-positive cells. In addition, the 20 mg/kg dose was the only one to significantly increase the percentage of DCX-positive cells with no dendritic arborization and decrease the percentage of DCX-positive cells with extensive dendritic branching and this is consistent with the fact that the 20 mg/kg dose was the only one to significantly increase depression- like behavior in the forced swim test. This suggests to us that the combined effect of a substantial loss of proliferating and surviving cells along with a deficit in dendritic maturation in surviving cells could be necessary for depressive symptoms to become apparent.

4.3. Differential Effects of CORT in the WTM and HRM

An important aspect of the present study was to determine whether HRM are more sensitive to the depressogenic effects of CORT than WTM. Our results appear to generally support this idea. The vehicle-injected HRM and WTM showed very similar patterns of behavior in the forced swim test and open field and they also did not differ in terms of the number of reelin-positive and DCX positive cells in regions of the dentate gyrus that were examined in this experiment. Although there are numerous subtle behavioral, neuroanatomical, and neurochemical differences between HRM and WTM (reviewed in Tueting et al., 2006), our results suggest that the effect of genotype per se is not sufficient to produce differences in depressive behavior, exploratory behavior, or the number of reelin-positive and DCX-positive cells. However, when we examined the effects of CORT within each genotype, we found dose dependent effects of CORT in the HRM that were not observed in the WTM, such as increased immobility and decreased swimming in the forced swim test, decreased reelin-positive cells in the SGZ and hilus, decreased DCX-positive cells in the SGZ and GCL, and decreased dendritic complexity within DCX-positive cells. The fact that the HRM express less than normal levels of reelin may have contributed to their vulnerability to CORT: A direct comparison between the HRM and WTM in the number of reelin-positive cells located in the SGZ showed that the number of reelin-positive cells was significantly reduced in the HRM with respect to WTM at CORT doses of 10 mg/kg and 20 mg/kg. This type of genotype effect was not apparent for the number of DCX-positive cells, which suggests a bigger effect of CORT on reelin-positive cells that are already intrinsically altered in HRM (i.e., because they express only about one-half of reelin levels than WTM littermates, see Tueting et al., 1999). The effect of CORT on the number of reelin-positive cells in the SGZ, the site of adult neurogenesis, may have led to a

secondary decrease in the number of DCX-positive cells. In a previous paper, we have shown that CORT treatment specifically affects the number of reelin-positive pyramidal-basket cells located in the dentate subgranular zone, and we have hypothesized that these cells may influence the integration of newborn granule cells by secreting reelin into the dentate granule cell layer (Lussier et al., 2009). We have also recently demonstrated that a subpopulation of reelin-positive pyramidal-basket cells in the dentate gyrus co-express neuronal nitric oxide synthase, and that this co-expression is reduced in HRM, and we have proposed that the production of nitric oxide by these cells may make them more vulnerable to the deleterious effects of glucocorticoids (Romay-Tallon et al., 2010). A loss of reelin-positive pyramidal-basket cells under conditions of high glucocorticoids would accordingly decrease the integration of newborn granule cells. This idea is consistent with the very recent report of Pujadas et al. (2010), who found that transgenic mice overexpressing reelin show increased hippocampal neurogenesis, hypertrophy of dendritic spines, and enhanced long-term potentiation. This implies that the higher vulnerability of reelin-positive dentate pyramidal-basket cells to CORT treatment in the HRM could be the trigger for subsequent deficits in DCX-positive cell number and integration, and secondarily, the expression of depression-like behavior.

An important question that needs to be addressed in any preclinical animal study of psychiatric illness is how well the results mirror the human condition. A specific deficit in the number of hippocampal CA4 reelin-positive cells has been shown albeit at a non-significant level- in post-mortem samples of patients with depression (Fatemi et al., 2000). It is possible that a more detailed count of specific types of reelin-positive cells (i.e., identifying SGZ pyramidal-basket cells and other hilar cells) could reveal significant deficits in reelin-positive

cells in post-mortem tissue from depressed human subjects. A deficit in reelin-positive pyramidal basket cells associated with human depression would strengthen our idea discussed above about expanding experimental examinations of deficient neurogenesis in depression to include studies on newborn cell maturation and integration in the granule cell layer.

One should also point out that hippocampal reelin appears to have a prominent role in regulating working memory and hippocampal long-term potentiation (Krueger et al., 2006; Qiu et al., 2006a; Wedenoja et al., 2010; Weeber et al., 2002; Levenson et al., 2008), and given the cognitive deficits associated with psychiatric disorders showing decreased hippocampal reelin (e.g., schizophrenia, bipolar disorder, depression, and autism), it would be of interest in future studies to analyze cognitive performance in HRM treated with CORT. This could help enhance our understanding of the potential interaction between hippocampal reelin levels and cognitive alterations in these disorders.

As discussed above, the results of this experiment suggest that a deficit in reelin could increase vulnerability to the depressogenic effects of CORT, and as such, a general reelin decrease of possible genetic and/or epigenetic origin could be operative as a primary event in a two-hit model of depression. It is difficult to reconcile this idea with the current human literature, and more studies are needed from both the preclinical and clinical literature on this topic. On one hand, it should be noted that post-mortem brain studies to date have not revealed widespread alterations in reelin expression associated with depression, whereas these kinds of alterations are found in schizophrenia, and to a lesser degree in bipolar disorder (Fatemi et al., 2000; Fatemi et al., 2005b; Guidotti et al., 2000). On the other hand, recent data have confirmed that chronic psychotropic drug treatment with antidepressants such as fluoxetine, or

atypical antipsychotics such as olanzapine, increase the level of reelin expression, whereas typical antipsychotics like haloperidol, or mood stabilizers like lithium, showed the opposite effect (Fatemi et al., 2009). Perhaps the best way to conceptualize our data given what is currently known about depression and reelin from human studies is to consider them with a broader perspective. Depressive symptoms are observed in early stage and prodromal schizophrenia (Hafner et al., 2005b; Hafner et al., 2005a), and post-mortem brain studies by Reif et al. (2006) have suggested that an alteration in hippocampal neurogenesis may be more characteristic of schizophrenia than of major depression. These data suggest that a developmental deficit in reelin could underlie a susceptibility to stress-related depressive symptoms within a number of psychiatric illnesses that have been associated with a deficit in reelin. For example, this could help explain the onset of depressive symptoms during the prodromal stage of schizophrenia, and therefore, the HRM CORT treated mouse could provide an interesting two-hit model of the early stages schizophrenia before the onset of psychotic symptoms. Additional studies addressing behavioral, neurochemical and neuroanatomical alterations in the HRM CORT model are needed to evaluate the validity of this idea in correlation with behavioral and/or imaging studies in the early course of schizophrenia and other psychiatric illnesses in human patients.

5. Conclusions

Our results confirm the depressogenic effects of repeated CORT treatment in mice, and show that HRM have a higher level of vulnerability to the depressogenic effects of CORT than WTM, both in terms depression-like behavior and in terms of hippocampal neurogenesis-cell maturation. This suggests that depression and/or depressive symptoms may arise from a similar

kind of two-hit scenario that has previously been suggested to occur in schizophrenia. Additional studies are necessary to evaluate the degree to which this HRM CORT model approximates the behavioral and neurobiological correlates of depression or of depressive symptoms in other psychiatric illnesses such as prodromal stage schizophrenia.

CHAPTER 5

Chronic Corticosterone Exposure alters GABAergic and Glutamatergic Activity within the Rat Hippocampus and Amygdala: Relevance to Depression

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1. **Introduction**

GABA ($_{\Upsilon}$-aminobutyric acid) is the primary inhibitory neurotransmitter in the brain, acting in concert with glutamate to control the balance of excitation and inhibition in many brain circuits. Of the classical neurotransmitters, serotonin and norepinephrine have been exhaustively studied in the context of major depression, with relatively little attention paid to the potential role of GABA. However, there is intriguing evidence from studies of clinical populations that GABA could be involved in the pathogenesis of depression. For example, GABA is synthesized from glutamate by the enzyme glutamate decarboxylase (GAD), which exists in two isoforms: GAD67 (thought to be involved in synthesis of GABA for general metabolic activity) and GAD65 (thought to be involved in synaptic transmission). Patients with depression show decreased levels of both GAD65 and GAD67 in the cerebellum and prefrontal cortex (Fatemi et al., 2005b; Karolewicz et al., 2010). In addition, patients with depression have lower levels of GABA in blood plasma, cerebrospinal fluid, and brain regions such as the prefrontal and occipital cortices (Gerner and Hare, 1981; Kasa et al., 1982; Petty et al., 1992; Sanacora et al., 1999; Sanacora et al., 2000) and electroconvulsive therapy and antidepressant treatment can both normalize GABA levels in the occipital cortex (Sanacora et al., 2002; Sanacora et al., 2003a). Moreover, patients with depression also express fewer $GABA_A$ receptors within temporal and parahippocampal regions (Klumpers et al., 2010) and fewer GABAergic neurons in the orbitofrontal cortex and prefrontal cortex (Rajkowska et al., 1999; Rajkowska et al., 2007). Collectively, these observations reveal a dysfunction in GABAergic neurotransmission that could play a role in the manifestation of a depressive phenotype.

Stress is a risk factor for the onset of depressive symptoms (see Sterner and Kalynchuk 2010 for a review) and there appears to be a reciprocal relationship between the stress hormone corticosterone (CORT) and GABAergic signalling in brain regions that have been implicated in depression, such as the hippocampus and amygdala. Under normal conditions, the hypothalamic-pituitary-adrenal axis response to a stressor is regulated by negative feedback and a significant component of that negative feedback occurs through GABAergic signalling from the ventral hippocampus to the paraventricular nucleus of the hypothalamus (Cullinan et al., 1993). However, GABAergic control of the HPA axis appears to be diminished under conditions of chronic stress, when glucocorticoid levels are high (Verkuyl et al., 2005). Indeed, under some conditions, CORT can alter the uptake and release of GABA and it can also decrease $GABA_A$ receptor binding and benzodiazepine receptor binding in the hippocampus and amygdala (Drugan et al., 1989; Goeders et al., 1986; Miller et al., 1988; Orchinik et al., 1995; Weizman et al., 1990; Wilson and Biscardi, 1994). These observations prompted us to consider the effect of elevated glucocorticoids on protein levels of specific $GABA_A$ receptor subunits. $GABA_A$ receptors are ionotropic heteropentameric GABA-gated chloride channels that are assembled from 19 different subunits (α1-6, β1-3, ϒ1-3, δ, ε, π, θ, and ρ1-3). Many $GABA_A$ receptor subunits appear to preferentially congregate together (e.g., a common configuration is two α and β subunits and one γ subunit (Angelotti et al., 1993), but in theory, there are a vast number of potential subunit combinations. The structural heterogeneity of $GABA_A$ receptors confers differences in their pharmacological and functional characteristics (Burt and Kamatchi, 1991; Sanger et al., 1994). For example, the $GABA_A$ α1 receptor subunit has been associated with anxiety, whereas the α2 receptor subunit has been linked to both anxiety and depressive-like behavior (see Smith and Rudolph, 2011 for review). Therefore, we

hypothesized that depression may be associated with decreases in some $GABA_A$ receptor subunits, such as the α2 subunit, but not others. Examining this association could provide important information to guide the development of novel therapeutics for treating mood disorders.

One way to study the relationship between depression and GABAergic neurotransmission is to use animal models that capitalize on the association between chronic stress and depression (Sterner and Kalynchuk, 2010). In the experiment described here, we investigated the effect of two well-characterized preclinical models of depression--the repeated CORT injection paradigm and repeated restraint stress—on markers of GABAergic and glutamatergic activity in the hippocampus and amygdala. We chose these two models because 21 days of repeated CORT injections (40 mg/kg) reliably increase depression-like behavior in rats (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009; Brummelte et al., 2006), whereas 21 days of repeated restraint stress with plastic restrainers does not (Gregus et al. 2005). Therefore, we would expect neurobiological changes that are associated with a depressive phenotype to be present in CORT-treated rats but not in rats subjected to restraint stress. Given the relationship between stress, depression, and GABA, we sought to examine the effect of repeated stress or glucocorticoid administration on protein levels of GAD65, GAD67, and the $GABA_A$ receptor subunits α1-3 and β2-3 in the hippocampus and amygdala. As a preliminary examination of corresponding changes in the glutamatergic system, we also assessed the effect of repeated stress or glucocorticoid administration on protein levels of the vesicular glutamate transporter VGLUT2.

2. Materials and Methods

2.1 Animals

The subjects were 25 male Long-Evans rats (purchased from Charles River Laboratories, Montreal, QC, Canada) that weighed approximately 225–250 g at the time of arrival. The subjects were housed individually in standard polypropylene cages with Purina rat chow and water available ad libitum. The colony room was maintained at a temperature of 21±1 °C with a 12 h:12 h light/dark cycle (lights on at 8:00 a.m.). All experimental procedures were carried out during the light phase of the light/dark cycle in strict accordance with the Canadian Council on Animal Care guidelines and a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply. All efforts were made to minimize animal suffering and to use the fewest number of animals possible.

2.2 Stress Procedures

The rats were handled in the colony room for a few minutes once per day for a week before the stress procedures began. Rats were randomly assigned to weight-matched groups and received one of the following four treatments for 21 consecutive days: single daily CORT injections (CORT group, n = 7), single daily vehicle injections (vehicle group, n = 6), daily physical restraint (restraint group, n = 6), or daily handling (handled group, n = 6). All CORT and vehicle injections were delivered subcutaneously at a volume of 1 ml/kg between 9:00 and 10:00 a.m. each day. CORT (MP Biomedicals, Solon, OH) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitanmonooleate (Tween-80; Sigma–Aldrich, St. Louis, MO) and given at a dose of 40 mg/kg because previous work has

shown that this dose reliably increases depression-like behavior (Brummelte et al., 2006; Gregus et al., 2005; Johnson et al., 2006; Zhao et al., 2008b). All daily restraint sessions were 6 hr in duration and conducted during the light phase of the light/dark cycle (Gregus et al. 2005). Rats were transported out of the colony room to an adjacent, brightly lit, quiet room, and placed into individual restrainers. The restrainers were transparent Plexiglas tubes (VWR Canlab, Mississauga, ON, Canada) measuring 24 cm long × 9 cm wide × 6 cm high. The length of each restrainer was adjusted to limit, but not completely restrict, head and limb movements. Following each restraint session, the rats were transported back to the colony room. To control for handling, each rat from the handled group was briefly picked up once per day and then placed back into its home cage. The body weight of each rat in each group was recorded for each day of the 21-day treatment period.

2.3 Tissue Preparation

On day 22, all rats were anesthetised with an overdose of sodium pentobarbital (i.p.) and decapitated with a standard rodent guillotine. Each brain was rapidly excised from the cranial cavity and the hippocampus and amygdala were dissected out. Hippocampal tissue was removed by gross dissection and a final protein concentration of 60 µg/ml was used for analysis. Amygdalar tissue was removed using bilateral punches with a disposable pipette tip from approximately -1.8 mm to -3.6 mm relative to bregma (according to Paxinos and Watson, 1998), and a final protein concentration of 60 µg/ml was used for blotting. All tissue was immediately flash frozen using liquid nitrogen and stored at -80 ˚C until it was utilized for protein extraction.

2.4 Protein Extraction and Western Blot Analyses

Total protein was extracted from hippocampal and amygdalar tissue samples in 0.3 M Sucrose Tris-EDTA solution and the concentration was measured using a BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) supplemented with protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche, Laval, QC) after microcentrifugation. Protein levels were determined using the BSA method. A final protein concentration of 60 µg/ml in Laemmli Sample Buffer (Bio Rad) and 5% 2-mercaptoethanol was separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) running gels and transferred to nitrocellulose blotting membranes (Pall Corporation, Pensacola, FL). The membranes were blocked in PBS containing 0.1% Tween-20 and 5% milk to prevent nonspecific binding. The membranes were then probed with primary antibody (see Table 5-1 for a list of the primary antibodies that were used) diluted in a blocking buffer overnight. The next day, the membranes were washed and then incubated in secondary antibody (see Table 5-1 for a list of the secondary antibodies that were used) diluted in PBS containing 0.1% Tween-20 and 5% milk. The blots were reprobed with β-actin (see Table 5-1) and then rinsed and incubated with secondary and normalized to verify equivalent loading of protein. Primary antibody binding was detected using ECL Plus reagent (GE Healthcare) for chemiluminescence on autoradiography X-ray film (Amersham Hyperfilm ECL, GE Healthcare).

The films were digitized and optical densities were determined using a computerized image analysis system with a high powered scanner and the software program Image J (v1.43u, National Institutes of Health). Single autoradiographic signal bands of appropriate molecular weights (see Table 5-1) were identified and quantified. Protein samples from 4 animals per

group were analyzed in all Western blots. An experimenter who was blind to the treatment conditions measured the optical densitometry of the bands.

2.5 Statistical Analyses

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, v 16.0, Chicago, IL, USA). We used independent samples t-tests (2-tailed) to examine the statistical significance of differences between the CORT and vehicle groups and the restraint and handled groups for all variables of interest (i.e., body weights, protein levels). The criterion for statistical significance was set at $p < 0.05$. All data are represented as means ± the standard error of the mean.

Primary	Concentration	Supplier	Molecular Weight (kDa)	Secondary	Concentration	Supplier
GAD65	1:5000	Millipore	65	Goat anti-rabbit	1:5000	Santa Cruz Biotechnology
GAD67	1:5000	Millipore	67	Goat anti-mouse	1:2000	Santa Cruz Biotechnology
$GABA_A$ B2-3	1:1000	Millipore	55	Goat anti-mouse	1:2000	Santa Cruz Biotechnology
$GABA_A$ α1	1:2000	Millipore	~51	Goat anti-rabbit	1:5000	Santa Cruz Biotechnology
$GABA_A$ α2	1:1000	Millipore	53	Goat anti-rabbit	1:5000	Santa Cruz Biotechnology
$GABA_A$ α3	1:1000	Abcam	55	Goat anti-rabbit	1:5000	Santa Cruz Biotechnology
VGLUT2	1:2000	Millipore	~56	Goat anti-mouse	1:2000	Santa Cruz Biotechnology
β Actin	1:10000	Millipore	~43	Goat anti-mouse	1:10000	Santa Cruz Biotechnology

Table 5-1. List of primary and secondary antibodies used in Western blotting.

3. Results

3.1 Body Weight

Figure 5-1 shows the mean body weight for the rats in each group during the 21-day stress phase of the experiment. Because the rats in each group were weight-matched, they started the stress procedures with very similar body weights. However, the CORT rats weighed significantly less than the vehicle rats on days 7, 14 and 21 of the injections (Panel A: $t(11) = 7.02$, $p < 0.001$, $t(11) = 9.82$, $p < 0.001$, and $t(11) = 10.885$, $p<0.001$, respectively). Similarly, the restraint rats weighed significantly less than the handled rats on days 14 and 21 of the restraint sessions (Panel B: $t(10) = 2.932$, $p = 0.015$ and $t(10) = 2.879$, $p < 0.016$, respectively).

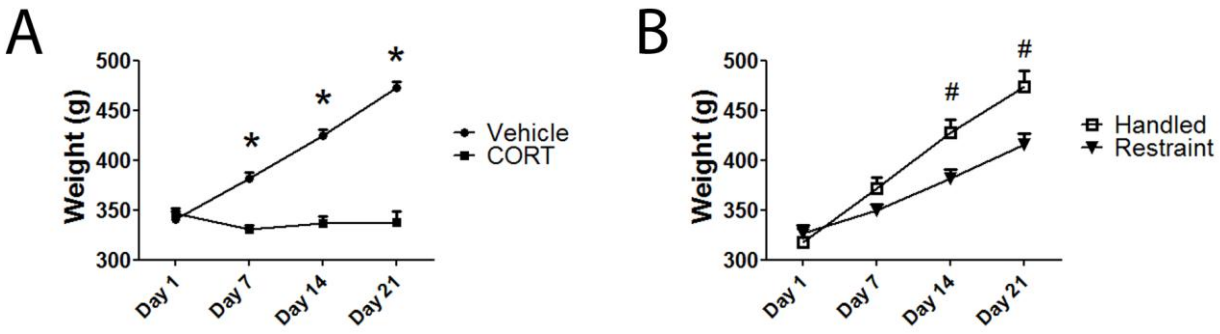

Figure 5-1. The body weight of rats in each group during the stress phase of the experiment. Panel A shows the effects of 21 days of CORT (40 mg/kg, s.c.) and vehicle injections on body weight. Panel B shows the effects of 21 days of restraint stress (6 hrs/day) and handling on body weight. Error bars represent mean ± the standard error of the mean. * denotes a statistically significant difference between vehicle and CORT groups ($p < 0.001$). # denotes a statistically significant difference between the handled and restraint groups ($p < 0.05$).

3.2 Protein Analyses

We used Western blotting to determine the effect of either repeated CORT administration or repeated physical restraint on protein levels of GAD65, GAD67, and the $GABA_A$ receptor subunits α1, α2, α3, and β2-3 in the hippocampus and amygdala. Although the primary focus of this experiment was on the GABAergic system, we also assessed the effect of CORT administration and repeated physical restraint on VGLUT2 expression as a preliminary examination of parallel changes in the glutamatergic system and as a positive control.

Figure 5-2 shows the effect of CORT administration on protein levels in the hippocampus. CORT significantly decreased expression of GAD65 [$t(6) = -3.979$, $p < 0.05$] and the α2 [$t(6) = -2.595$, $p < 0.05$] subunit of the $GABA_A$ receptor. At the same time, CORT significantly increased VGLUT2 levels in this region [$t(6) = 3.612$, $p < 0.05$]. No other group differences in the hippocampus were statistically significant (all p values > 0.05).

Figure 5-3 shows the effect of CORT administration on protein levels in the amygdala. In this region, CORT significantly decreased expression of GAD67 [$t(6) = -3.964$, $p < 0.05$] and the α2 subunit of the $GABA_A$ receptor [$t(6) = -2.512$, $p < 0.05$]. No other group differences in the amygdala were statistically significant (all p values >0.05).

Figures 5-4 and 5-5 show the effect of repeated physical restraint on protein levels in the hippocampus and amygdala respectively. There were no statistically significant differences in either brain region for any of the measured proteins (all p values >0.05).

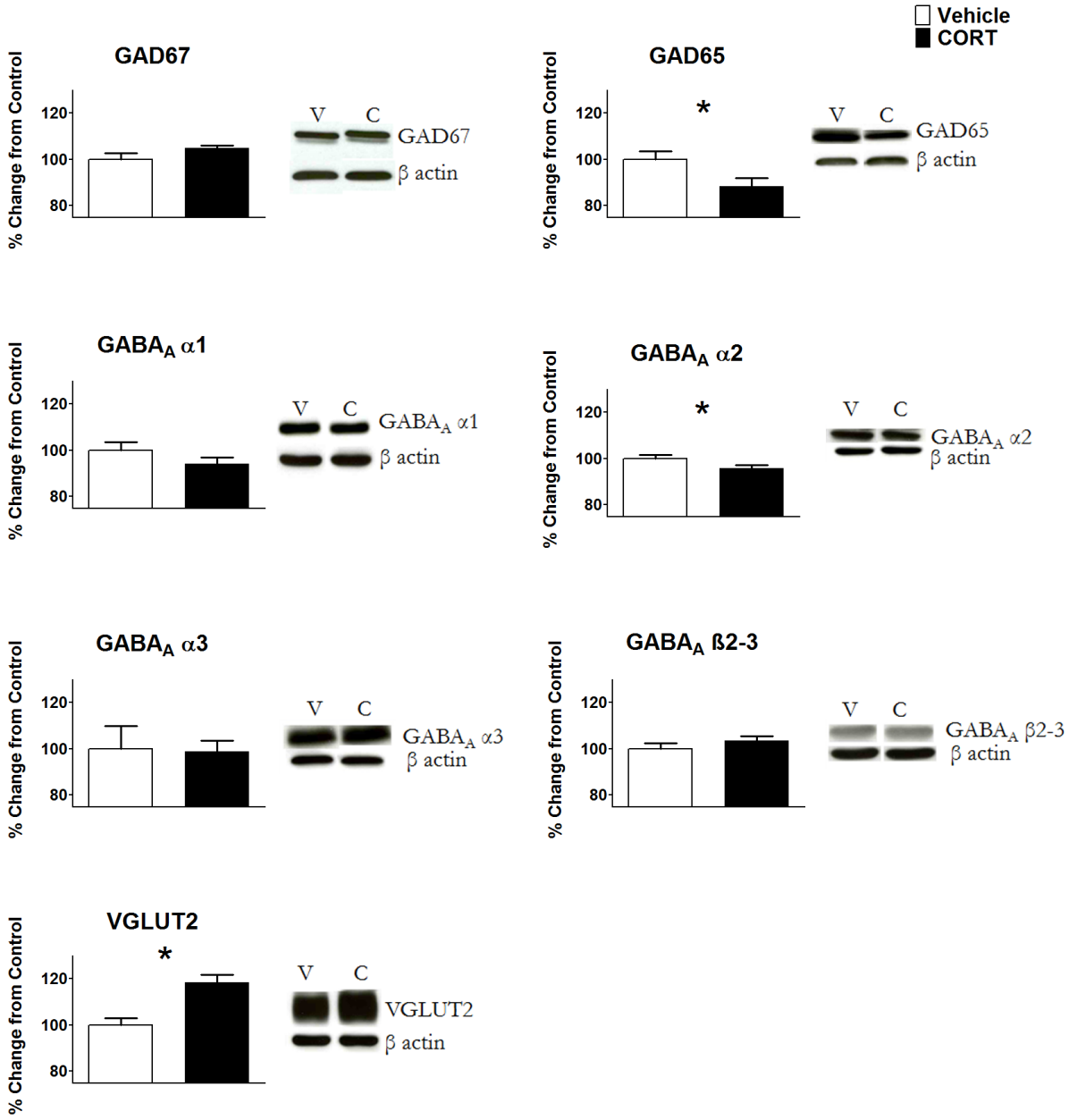

Figure 5-2. The effect of 21 days of CORT injections on protein levels of GAD67, GAD65, the GABAA α1, α2, α3, and β2-3 receptor subunits, and VGLUT2 in the hippocampus. The values are shown as a percentage change in optical density scores from the control group ± the standard error of the mean. * denotes a statistically significant difference between the CORT and vehicle groups ($p < 0.05$). Representative scanned western blots are shown to the right of each histogram.

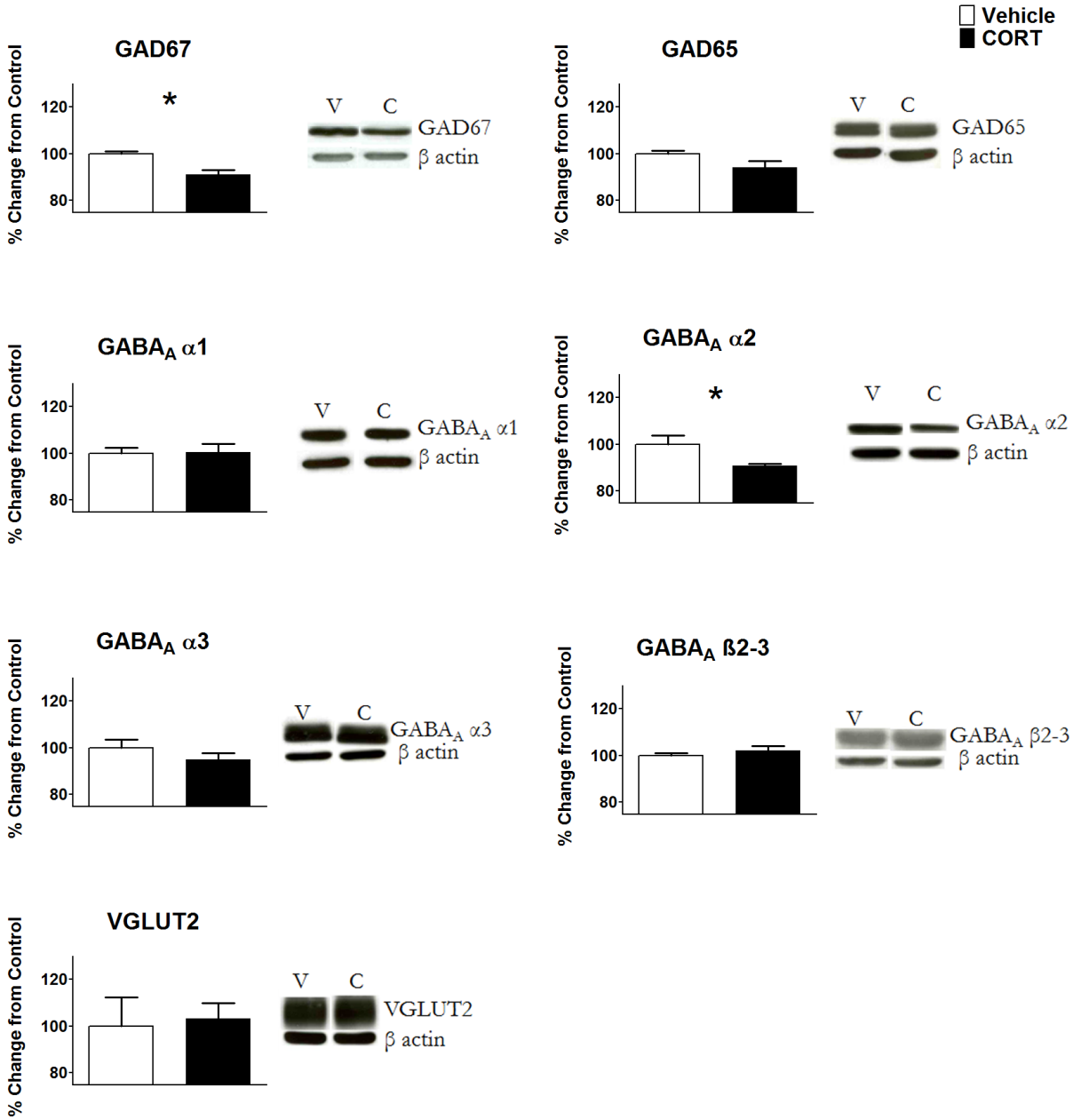

Figure 5-3. The effect of 21 days of CORT injections on protein levels of GAD67, GAD65, the GABAA α1, α2, α3, and β2-3 receptor subunits, and VGLUT2 in the amygdala. The values are shown as a percentage change in optical density scores from the control group ± the standard error of the mean. * denotes a statistically significant difference between the CORT and vehicle groups ($p < 0.05$). Representative scanned western blots are shown to the right of each histogram.

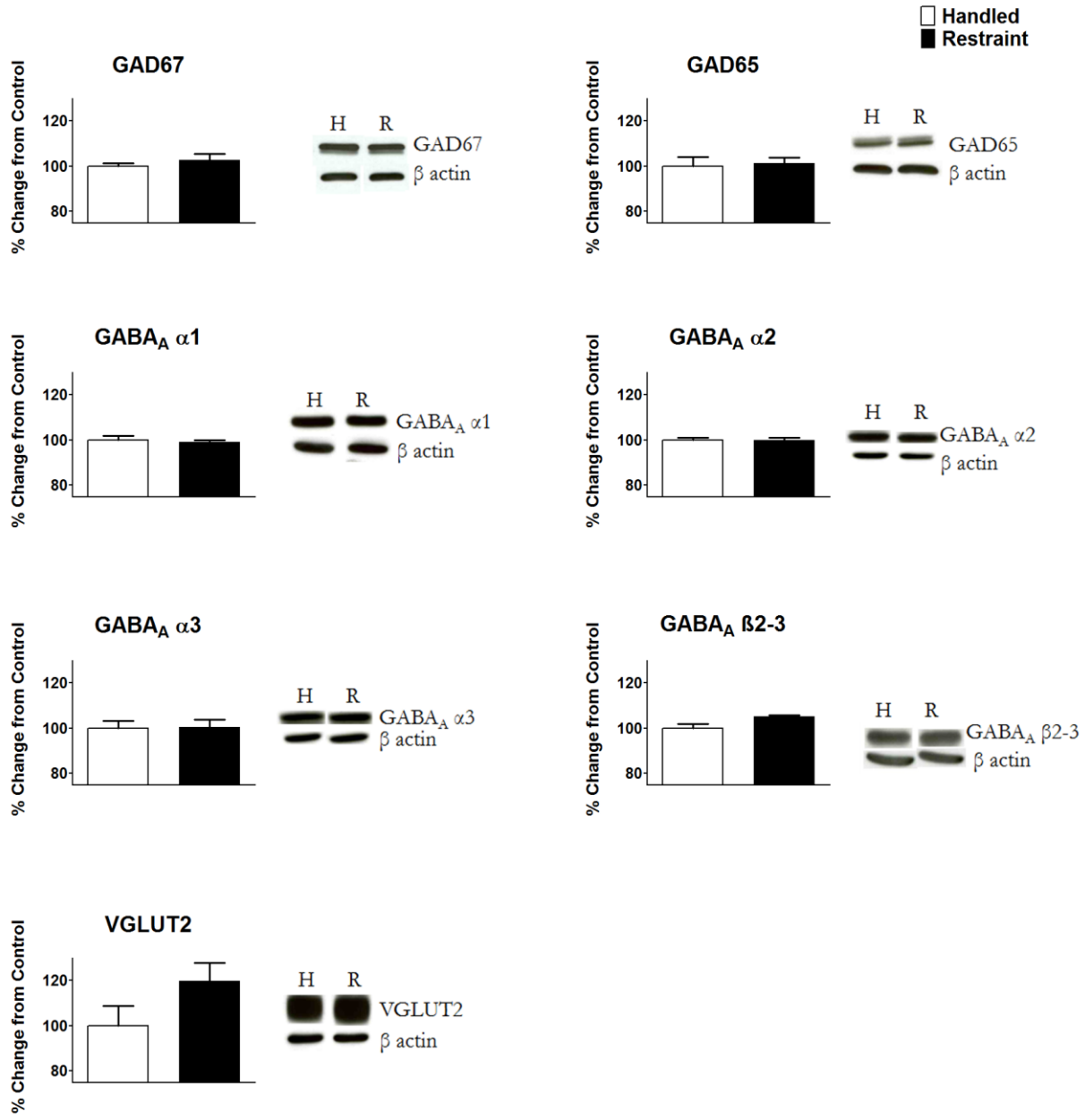

Figure 5-4. The effect of 21 days of restraint stress on protein levels of GAD67, GAD65, the GABAA α1, α2, α3, and β2-3 receptor subunits, and VGLUT2 in the hippocampus. The values are shown as a percentage change in optical density scores from the control group ± the standard error of the mean. There were no significant differences between the groups. Representative scanned western blots are shown to the right of each histogram.

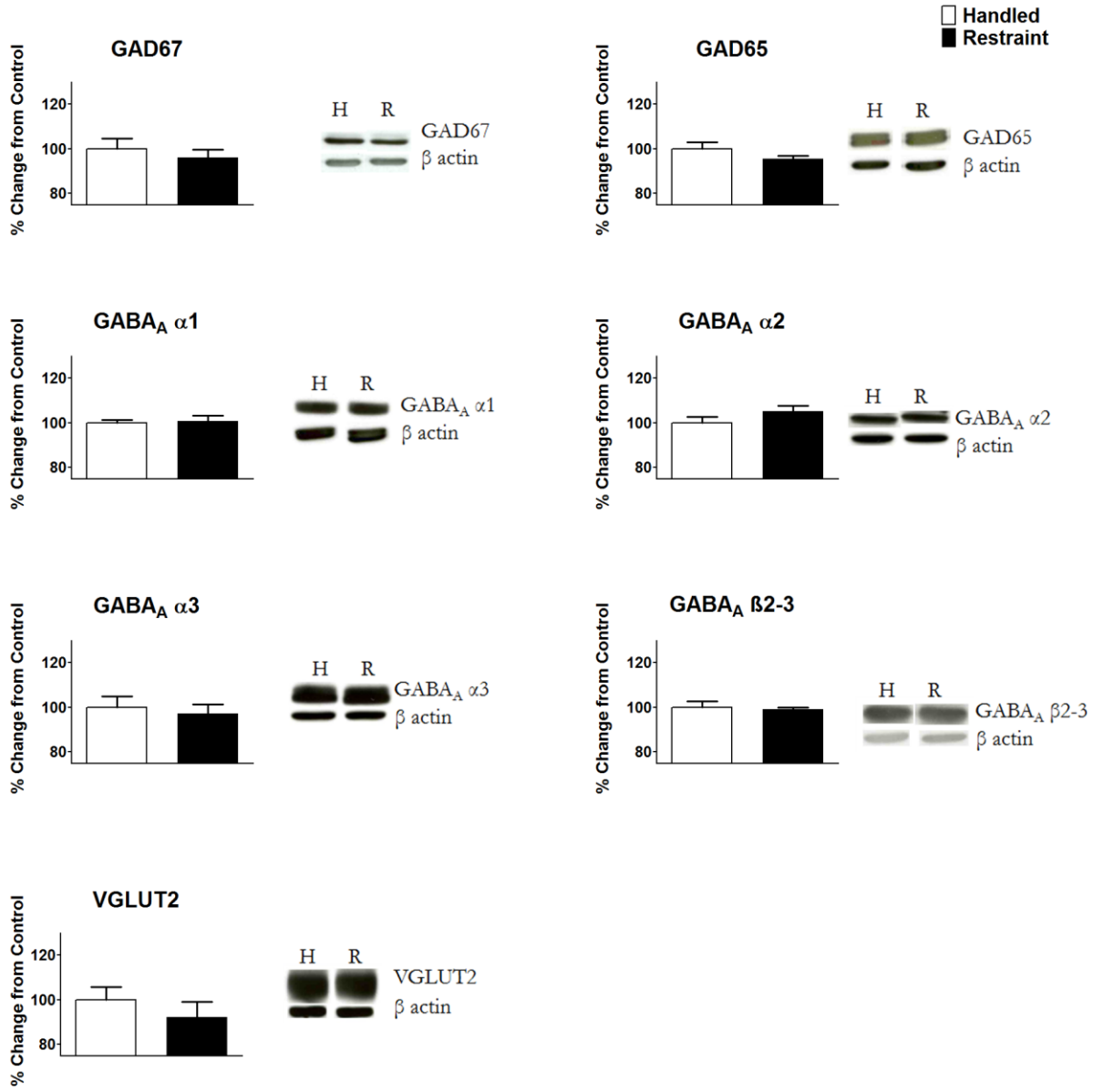

Figure 5-5. The effect of 21 days of restraint stress on protein levels of GAD67, GAD65, the GABAA α1, α2, α3, and β2-3 receptor subunits, and VGLUT2 in the amygdala. The values are shown as a percentage change in optical density scores from the control group ± the standard error of the mean. There were no significant differences between the groups. Representative scanned western blots are shown to the right of each histogram.

4. Discussion

The results of this experiment reveal that repeated exposure to the primary stress hormone CORT has significant effects on GABAergic and glutamatergic signalling within the hippocampus and amygdala. More specifically, we found that repeated CORT administration decreases protein levels of GAD65 and the α2 subunit of the $GABA_A$ receptor in the hippocampus and decreases protein levels of GAD67 and the α2 subunit of the $GABA_A$ receptor in the amygdala. This was accompanied by an increase in VGLUT2 protein levels in the hippocampus only. However, there were no significant effects of repeated restraint stress on any of our markers of GABAergic or glutamatergic activity in either the hippocampus or amygdala.

The distinction between the effects of repeated CORT and repeated restraint is an important one in the context of depression, because we have previously shown that CORT significantly increases depression-like behavior as measured by the forced swim test whereas restraint stress does not (Gregus et al., 2005). Other researchers have reported similar results, with CORT administration producing a depressive phenotype characterized by forced swim test immobility, decreased sucrose preference, decreased sexual behavior, decreased reward behavior, and impaired spatial working memory and executive function (Coburn-Litvak et al., 2003; Gorzalka et al., 2003; Gorzalka and Hanson, 1998; Gourley et al., 2008a; Hill et al., 2003; Sousa et al., 2000). The differential effects of CORT and restraint stress on behavioral and neurobiological induces of depression likely stems from the fact that the amount of CORT delivered with each daily injection remains constant, whereas rats tend to habituate to the aversive effects of restraint over time, with smaller and smaller HPA axis responses occurring

with each subsequent exposure to the restrainer (Galea et al., 1997; Grissom et al., 2007). Indeed, the effects of CORT on depression-like behavior are time and dose dependent, with higher doses and durations of treatment producing more robust depressive phenotypes (Johnson et al., 2006; Brummelte et al., 2006; Zhao et al., 2008a). The fact that repeated CORT administration increases depression-like behavior but repeated restraint stress does not gives traction to the idea that the alterations in GABAegic and glutamatergic markers seen in this experiment could be involved in the neurobiological milieu that underlies depression.

Our data reveal differential effects of CORT on GAD65 and GAD67 in the hippocampus and amygdala, with CORT inducing a decrease in GAD65 but not GAD67 in the hippocampus and a decrease in GAD67 but not GAD65 in the amygdala. Although GAD is generally involved in the synthesis of GABA from glutamate, the GAD65 and GAD67 isoforms show distinct subcellular expression and functionality in brain GABAergic neurons. For example, GAD65 is primarily expressed in synaptic terminals and is associated with vesicle release, whereas GAD67 is mostly expressed in neuronal cell bodies and proximal dendrites (Erlander et al., 1991; Esclapez et al., 1994). Accordingly, GAD65 is thought to participate in the fast conversion of glutamate to GABA in axon terminals, providing a pool of vesicular GABA for release following an action potential (Kaufman et al., 1991; Martin et al., 1991a; Martin and Rimvall, 1993). In contrast, GAD67 likely participates in the synthesis of GABA that is released through GABA transporters to act as trophic factors on extra-synaptic $GABA_A$ receptors (Kaufman et al., 1991; Martin et al., 1991a; Martin and Rimvall, 1993). In addition, it has been estimated that 50% of all GAD is present in the brain in apoenzyme (apoGAD) form, which provides a pool of inactive GAD that can be drawn upon when additional GABA is needed (Itoh and Uchimura, 1981). The majority of apoGAD appears to be

in the form of GAD65 (Martin et al., 1991b; Martin et al., 1991a). Taken together, these observations suggest that the CORT-induced decrease in GAD65 in the hippocampus reflects dampened hippocampal GABAergic neurotransmission, which could have negative implications for hippocampal neurogenesis, negative feedback of the HPA axis, and hippocampal-dependent behavior, including cognition and mood regulation, whereas the CORT-induced decrease in GAD67 in the amygdala may reflect secondary deficits in GABAergic neurotransmission related to extrasynaptic $GABA_A$ receptors. These ideas are consistent with the results of clinical studies of patients with depression, which have revealed reduced GABA levels in brain regions such as the prefrontal cortex, occipital cortex, and cerebellum and also peripherally in blood plasma and cerebrospinal fluid (Brambilla et al., 2003; Karolewicz et al., 2010; Kasa et al., 1982; Petty et al., 1992; Sanacora et al., 1999; Fatemi et al., 2005b). Further examination of the effects of glucocorticoids and/or chronic stress on specific aspects of GABA synthesis and release in the hippocampus and amygdala are clearly needed.

Our data also reveal a selective effect of CORT on the α2 subunit of the $GABA_A$ receptor, with decreased protein levels of this subunit seen in both the hippocampus and amygdala of CORT-treated rats. No other $GABA_A$ receptor subunits that we examined (α1, α3, or β2-3) were altered by protracted exposure to CORT. $GABA_A$ receptors containing the α2 subunit are highly expressed in the hippocampus, amygdala, and nucleus accumbens (Fritschy and Mohler, 1995; Kaufmann et al., 2003; Pirker et al., 2000). Previous findings from gene knockout and pharmacological studies support the idea that the α2 subunit is involved in the pathogenesis of depression (for a review see Smith and Rudolph, 2012). For example, heterozygous and homozygous α2 subunit knockout mice show increased immobility in the

forced swim test and tail suspension test compared that is independent of nonspecific changes in locomotor activity (Vollenweider et al., 2011). These mice also show increased aversion to novelty (Vollenweider et al., 2011). Furthermore, the addition of eszopiclone (a preferential $GABA_A$ α2, α3, and α1 subunit modulator) to serotonin selective reuptake inhibitors (SSRIs) in the treatment of depressed patients increases the effectiveness of the antidepressant with respect to SSRIs alone (Fava et al., 2006; Fava et al., 2011; Krystal et al., 2007). Our results showing decreases in the α2 receptor subunit in both the hippocampus and amygdala, in combination with our previous report of increased depressive-like behavior in the forced swim test in CORT-treated rats compared to vehicle controls (Gregus et al., 2005), provide further support for the importance of the α2 subunit of the $GABA_A$ receptor in the pathogenesis of depression, and suggest that augmentation of the activity of $GABA_A$ receptors containing the α2 subunit should be investigated as a putative novel antidepressant mechanism.

It is important consider how alterations in GABAergic activity might affect hippocampal functioning and subsequently, the development of a depressive phenotype. A significant number of GABAergic interneurons within the hippocampus release the extracellular matrix protein reelin (Pesold et al., 1998), and in the adult brain, reelin regulates hippocampal plasticity by enhancing cell migration and integration, facilitating synaptogenesis, and stabilizing synaptic contacts onto dendritic spines (Frotscher et al., 2003; Gong et al., 2007; Herz and Chen, 2006; Niu et al., 2008; Pujadas et al., 2010; Qiu et al., 2006a). Importantly, we have previously shown that repeated CORT administration, but not restraint stress, decreases the number of reelin-positive GABAergic interneurons in the CA1 stratum lacunosum and subgranular zone of the dentate gyrus (Lussier et al., 2009). We have also found that heterozygous reeler mice, which express about 50% of the normal levels of reelin, are more

susceptible to the depressogenic effects of CORT than wildtype mice (Lussier et al., 2011). The demise of a subpopulation of reelin-positive hippocampal GABAergic cells after prolonged exposure to CORT could account for the decrease in hippocampal GAD65 observed in the present experiment. More generally, a loss of GABA with repeated CORT administration likely affects the course of hippocampal neurogenesis, especially given that CORT-induced loss of reelin-positive GABAergic cells occurs selectively in the proliferative subgranular zone of the dentate gyrus (Lussier et al., 2009; Lussier et al., 2011). There are compelling data that GABA is essential for survival and dendritic outgrowth of newborn granule cells that are less than two weeks old (Ge et al., 2006; Jagasia et al., 2009; Tozuka et al., 2005). This occurs through GABA-mediated excitation (Sernagor et al., 2010). Indeed, our laboratory and others have shown that repeated CORT administration decreases hippocampal cell proliferation (Brummelte and Galea, 2010b; Wong and Herbert, 2006) and slows the maturation rate of newborn neurons that do survive (Lussier et al., 2011). These findings suggest that the CORT-induced dampening of GABAergic neurotransmission seen in this experiment could be a mechanism by which hippocampal cell proliferation is decreased and the integration of surviving newborn neurons is impaired. Certainly it is of interest to study if a concurrent decrease of GAD and reelin in the subgranular zone and hilus have synergistic effects on the differentiation and migration of newborn granule cells and how this could relate to the depressive phenotype observed in CORT-treated animals.

A final point that should be mentioned is the fact that in addition to the decreases in GABA discussed above, we also found that repeated CORT administration increased VGLUT2 protein levels in the hippocampus. Vesicular glutamate transporters exist in three isoforms—VGLUT1, 2, and 3—and they are essential for synaptic vesicle loading of glutamate (Takamori,

2006). The VGLUT2 isoform in particular appears to participate in quantal release of glutamate at the synapse (Moechars et al., 2006) and as such, alterations in VGLUT2 levels can provide a snapshot of the relative amount of glutamatergic neurotransmission that is present at a given point in time. Our finding of increased VGLUT2 protein in the hippocampus of CORT-treated rats suggests that prolonged exposure to CORT increases glutamatergic activity in hippocampal circuits. This interpretation is consistent with recent research showing that early life stress and glucocorticoids can enhance other markers of glutamatergic neurotransmission in the hippocampus (Martisova et al., 2012). It also dovetails nicely with observations of enhanced glutamate expression in patients with depression (Altamura et al., 1993; Levine et al., 2000; Mitani et al., 2006). An important question to consider is how increased glutamate neurotransmission might affect hippocampal function and the manifestation of a depressive phenotype when combined with the decreased GABAergic activity that is also evident in our findings. Krystal and colleagues (2002) discussed this idea in view of reports that antidepressant drugs reduce NMDA receptor function while enhancing GABA levels in patients with depression. They pointed out that structural deficits in cortico-limbic circuits in depressed patients may be linked to dysfunction in amino acid neurotransmitters and that correcting this dysfunction could represent a new treatment strategy for mood disorders. Ten years later, their ideas have proven to be correct as the NMDA receptor antagonist ketamine seems to be the most promising new therapeutic for patients with treatment-resistant depression (Connolly and Thase, 2012; Mathew et al., 2012). This leaves us with an important question about the mechanisms by which ketamine might exert its antidepressant effects: Could ketamine work by restoring the balance between glutamate and GABA in hippocampal and other limbic circuits?

5. Conclusions

Our results show that repeated CORT administration, but not repeated restraint stress, alters the balance of GABAergic and glutamatergic activity in the hippocampus and decreases GABAergic activity in the amygdala. These results are consistent with observations of reduced levels of GABA and increased levels of glutamate in brain and peripheral samples from human patients with depression. Combined with our past research showing that repeated CORT administration, but not restraint stress, increases depression-like behavior and decreases hippocampal reelin expression and hippocampal neurogenesis (Gregus et al., 2005; Lussier et al., 2009), these results hint at an emerging picture of dysfunctional GABAergic and glutamatergic neurotransmission that may be at play in the pathogenesis of depression and that could be exploited in the development of novel antidepressant treatments.

CHAPTER 6

General Discussion

1. **A Summary of the Main Findings**

The purpose of this dissertation was to gain further understanding of neurobiological alterations associated with repeated stress and the behavioral consequences associated with these changes. More specifically, I examined the role of reelin and neural plasticity in the hippocampus and amygdala following repeated stress. In order to study this, I examined the effects of different types, duration, and intensities of stress paradigms on a number of different measures including behavior, reelin cell numbers, dendritic morphology, and neurogenesis within the hippocampus. In the last experiment I examined the effects of repeated stress on GABAergic and glutamatergic markers in the hippocampus and amygdala.

In **Chapter 2**, I examined the effects of two different stress paradigms (CORT exposure and restraint) on the number of reelin-positive cells within several regions of the hippocampus. I found a significant decrease in the number of reelin-positive cells in the CA1sl as well as in the SGZ of the CORT-treated group compared to the vehicle control group. There were no significant differences in the number of reelin-positive cells in any of the areas within the hippocampus when comparing to the handled-only and restraint groups. Importantly, our laboratory has previously shown that the chronic treatment with corticosterone increases depressive-like behavior in the forced swim test, whereas chronic restraint stress does not seem to change depressive behavior (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al.,

2004; Marks et al., 2009). Together, these results suggest that alterations in reelin cell number in specific regions of the hippocampus are associated with increases in depression-like behavior.

This experiment was an important starting point for all subsequent experiments in this thesis because it was the first preclinical study to examine the effects of repeated stress on reelin cell numbers in the hippocampus. After characterizing the effects of repeated stress throughout the various hippocampal subfield regions, the decrease found in the SGZ further engaged my interest because it is one of two areas in the adult brain where neurogenesis occurs. When considering that depressed patients show decreases in hippocampal reelin expression (Fatemi et al., 2000; Knable et al., 2004), that the hippocampus has been shown to be involved in learning and memory, which is impaired in some depressed patients (Erickson et al., 2003; Rubinow et al., 1984; Sternberg and Jarvik, 1976), that reelin has been shown to be involved in hippocampal synaptic plasticity (Niu et al., 2008; Weeber et al., 2002), and that reelin has been shown to influence the proliferation and migration of adult born granule cells (Courtes et al., 2011; Drakew et al., 2002; Hack et al., 2002; Hoe et al., 2009; Kim et al., 2002; Pujadas et al., 2010; Won et al., 2006), it was clear that investigating the relationship between depression-like behavior, reelin, hippocampal neurogenesis and hippocampal plasticity could be a fruitful avenue for further research.

In **Chapter 3**, I set out to examine the effects of different durations of CORT exposure on depression-like behavior, reelin cell number, dendritic morphology, and neurogenesis in the dentate gyrus. I was interested in characterizing the progression of the effects of CORT exposure on behavior and neurobiological alterations in order to further understand the

development of these consequences. Understanding the progression of repeated stress in regard to neurobiological and behavioral changes is important given that there is clinical evidence that supports a relationship between the duration of HPA axis overactivity following stress and the magnitude and severity of clinical depression (Belmaker and Agam, 2008; Burke et al., 2005; Checkley, 1996; de Kloet et al., 1998; Sapolsky et al., 1986). Our laboratory has previously described a dose-dependent effect of CORT on HPA axis activation and depressive-like behavior (Johnson et al., 2006), therefore, I was interested in examining the effects of the different durations of CORT administration on behavior and neurobiology. I found that there was an increase in depressive-like behavior and decreases in reelin cell number, and number and maturation of immature neurons following repeated CORT exposure. There were intermediate effects after one week of CORT exposure and significant effects by 2 and 3 weeks of CORT exposure. Importantly, there were no differences in anxiety-like behavior or dendritic morphology of mature granule cells within the dentate gyrus. These results show that CORT exposure causes progressive alterations in the neurobiology of the hippocampus and depressive-like behavior. Consistent with the previous research in Chapter 2, only the durations of CORT (2 and 3 weeks) that produced increased depressive-like behavior showed decreased reelin cell number. In addition, the number of immature neurons, as labeled with DCX, was also significantly less in the groups exposed to CORT for 2 and 3 weeks. Moreover, the percentage of DCX-positive cells with mature dendritic morphology was decreased, while DCX cells with immature morphology were increased in the groups exposed to CORT for 2 and 3 weeks. This implies that the CORT 14 and CORT 21 groups had fewer immature neurons and that the population of DCX cells expressed in these groups were also less likely to show mature morphology and therefore would be less likely to be functionally incorporated

into existing circuitry. The corresponding decreases in maturation and number of immature cells and decreased reelin expression, a protein involved in migration and maturation of newborn neurons (Courtes et al., 2011; Won et al., 2006), suggests a relationship between these factors.

In **Chapter 4**, I further examined the role of CORT on depressive-like behavior, reelin cell number, and neurogenesis by using HRM, as these mice express approximately half of the reelin as their wild-type counterparts. My hypothesis was that if a decrease in reelin was associated with increased depressive-like behavior, as we showed in Chapter 2 and Chapter 3, then the HRM should be more susceptible to the effects of repeated CORT because they start the experiment with less reelin than their wild-type counterparts. As this was the first experiment looking at the effects of repeated CORT exposure on HRM, I decided to use 3 different doses of CORT to systematically characterize the neurobiological and behavioral effects of chronic stress in these animals. I found that the HRM were more susceptible to the effects of repeated CORT than the WTM when examining depressive-like behavior, reelin cell numbers, and number and maturation rate of immature granule cells. The HRM showed an increase in immobility in the forced swim test at the 20 mg/kg dose, whereas the WTM did not show any increase in depressive-like behavior at any of the doses. No alterations in anxiety or locomotor behavior, as measured by the open field test, were seen in any of the groups. Importantly, there were no differences in the behavioral or neurobiological measures between these two genotypes when injected with vehicle, indicating the effects shown are in fact due to the CORT exposure. In addition, the HRM showed decreases in reelin cell number in both the SGZ and hilus when compared to WTM. This decrease was driven by the HRM having significantly fewer cells in the 10 mg/kg and 20 mg/kg conditions than the WTM at these two

doses. When examining the effect of CORT doses in each mouse strain separately, the HRM showed significant decreases in reelin-positive cells in the 20 mg/kg dose compared to the vehicle and 5 mg/kg dose in the SGZ and a similar decrease was found in the hilus. This finding is consistent with our previous research demonstrating that the group that shows increases in depression-like behavior is the only group that shows the significant decrease in reelin cell numbers (Chapter 3). When examining the DCX cells, we found a significant decrease in cell numbers in the 20mg/kg CORT dose group compared to the vehicle and 5 mg/kg groups. This effect was driven by the HRM; no significant effects were seen in the WTM. When examining the morphology of these immature neurons the HRM showed the alterations, which were consistent with the results in Chapter 3, with the largest dose that showed increased depressive-like behavior showing greatest decreases in complex morphology. Again, the HRM were driving the effects seen in this experiment. The WTM did show an increase in the number of cells with immature morphology but no other significant alterations were shown in the DCX cell morphology. The above results suggest that the HRM are more susceptible to the effects of CORT exposure.

Finally, in **Chapter 5**, because reelin is expressed in specific subpopulations of GABAergic cells within the adult hippocampus (Abraham and Meyer, 2003; Campo et al., 2009; Pesold et al., 1998) and we have consistently shown decreases in reelin expression in animals with a depressive phenotype, I wanted to examine if there were alterations in expression of various inhibitory markers and $GABA_A$ receptor subtypes after CORT treatment. To maintain consistency with my previous experiment, I used both the repeated CORT and restraint stress paradigms. As the hippocampus and amygdala are two brain regions that are sensitive to the effects of elevated glucocorticoids and neuroplastic alterations in these regions

have been linked to the onset of depressive symptoms, I examined the consequences of restraint or CORT treatment on inhibitory markers and GABA receptor subtypes in these structures. I found decreases in GABAergic markers and receptor subtypes in the hippocampus and amygdala following CORT exposure but an increase in a glutamatergic marker in the hippocampus. No alterations were found when comparing the handled and restraint groups. Although these preliminary results require further examination, they suggest that imbalances in excitation and inhibition might play an important role in development and/or susceptibility of depression after chronic exposure to stress hormones. Taken together, the overall findings of this dissertation provide compelling evidence that repeated CORT exposure can affect depression-like behavior, neurogenesis and reelin expression within the hippocampus through a mechanism that may involve imbalances in inhibitory/excitatory neurotransmission particularly in the hippocampus.

2. The Role of Reelin in Depression

Reelin has been shown to be altered in psychiatric disorders such as schizophrenia, bipolar disorder, autism, epilepsy, and depression (Fatemi et al., 2000; Fatemi et al., 2001; Frotscher et al., 2003; Guidotti et al., 2000; Haas et al., 2002; Impagnatiello et al., 1998; Knable et al., 2004; Fatemi, 2001; Fatemi et al., 2005a). Thus, the findings from my dissertation are in accordance with these clinical studies, as only animal models that produced increased depressive-like behavior showed decreases in reelin expression (Chapters 2-4). Consistent with the effects of repeated stress on reelin expression, others have shown that two weeks of maternal separation also decreases reelin expression in the hippocampus one week following stress exposure (Qin et al., 2011). Interestingly, double-labeling of reelin and GR

within the stratum oriens, stratum lacunosum molecular and GCL revealed that virtually all reelin-positive cells are GR positive (Gross et al., 2010), which suggests that reelin-positive cells can be directly affected by circulating levels of central glucocorticoids. These results indicate that different types of repeated stress can decrease reelin expression and suggest that these effects are robust and potentially enduring.

Research examining reelin levels in mice has begun to further our understanding of this protein in affective behavior. For example, HRM exposed to maternal separation show increased anxiety behavior compared to their wild-type counterparts (Laviola et al., 2009; Ognibene et al., 2007). Interestingly, when examining the other side of this relationship Teixeria et al. (2011) found that mice overexpressing reelin (reelin-OE) were more resistant to the effects of repeated CORT exposure. Under basal conditions, there were no behavioral alterations between these reelin-OE mice and wild-type control mice. Importantly, with the exposure to CORT the reelin-OE mice did not show an increase in immobility behavior on the forced swim test, whereas the wild-type mice exposed to CORT had significantly increased time spent immobile. The authors suggest that the reelin-OE mice were resilient to the effects of CORT exposure and suggest that these mice may be interesting to use to examine the protective effects of reelin against environmental influences (Teixeira et al., 2011). Taken together, these results suggest that decreased reelin can be detrimental and increased reelin can be protective when exposed to repeated stress. As these are novel findings, further investigation is needed to elucidate a potential of reelin as a novel therapeutic target for treating depression.

It is known that antidepressants can reverse or protect against the negative behavioral and neurobiological consequences of repeated stress. However, the effects of antidepressants on reelin following repeated stress are not well understood. A recent study examining psychotropic medications, such as fluoxetine, haloperidol and olanzapine, have shown that these drugs alter reelin expression in the frontal cortex of naïve rats (Fatemi et al., 2009). Moreover, citalopram, a selective serotonin reuptake inhibitor, has been shown to prevent the decrease in reelin expression following kainic acid exposure (Jaako et al., 2011), a model of epilepsy previously shown to decrease reelin cell numbers (Gong et al., 2007; Heinrich et al., 2006). Infusion of exogenous reelin directly into the hippocampus has also been show to prevent granule cell dispersion in a kainic acid model of epilepsy (Muller et al., 2009), which suggests that directly increasing reelin levels can have protective effects in the brain. Another study examining intraventricular reelin infusion in freely moving animals showed an increase in contextual fear conditioning and spatial learning and memory in the Morris water maze, both hippocampal dependent tasks, 5 to 12 days following reelin infusions (Rogers et al., 2011). This research provides direct evidence for reelin to influence behavior. Further research on how antidepressants influence reelin expression should be done to further elucidate the mechanisms involved with this relationship.

The above results support the potential of reelin as a therapeutic target for treating and possibly preventing neuropsychiatric disorders. This area of research is still in its infancy and considerably more investigation is needed to understand the relationship between reelin and affective behavior. Although there is support for the role of reelin in depression-like behavior that is consistent with studies of depressed patients (Fatemi et al., 2000; Knable et al., 2004),

there is an abundance of potential research to further elucidate the relationship between reelin and depression: a few key experiments will be mentioned in the section below.

3. Relationship Between Reelin and Neurogenesis

Neurogenesis has been hypothesized to be involved in the development of depression for many years. Support for this relationship comes from numerous studies showing that repeated stress decreases neurogenesis, whereas antidepressants increase neurogenesis (Gould and Tanapat, 1999; Gould and Gross, 2002). Although there has been strong support for the role of repeated stress in decreases in proliferation, conflicting results have been reported (Vollmayr et al., 2003). Indeed, recent studies have shown that some antidepressant-induced changes can occur independently of changes in neurogenesis (David et al., 2009), whereas other studies have shown that neurogenesis is pivotal in antidepressant efficacy (Santarelli et al., 2003). Moreover, neurogenesis suppressed conditional knockout mice showed increased stress responses following a novel stressor compared to wild-type mice (Schloesser et al., 2009; Snyder et al., 2011). To further complicate things, there has been support for decreases in proliferation following repeated stress (Brummelte and Galea, 2010b; Duman et al., 2001; Gould et al., 1997; Pham et al., 2003) but others suggest that it is the differentiation and maturation phases, and not proliferation, that are most adversely affected with stress (Lee et al., 2006; Jayatissa et al., 2008; Jayatissa et al., 2009). Overall, these results propose a complex interaction between neurogenesis, repeated stress, and antidepressant treatments and further suggest that more than one time point may be necessary to extensively examine the effects of repeated stress on neurogenesis.

Examination into the effects of reelin on neurogenesis has interesting implications. For example, decreased adult hippocampal proliferation and immature neurons have been shown in reeler mice when compared to wild-type mice (Won et al., 2006; Zhao et al., 2007). Moreover, the number of BrdU-NeuN co-labeled cells are also decreased, whereas the number of double-labeled BrdU-GFAP cells are increased in reeler mice, showing that in the absence of reelin the proliferating cells are more likely to differentiate into astrocytes (Zhao et al., 2007). Interestingly, examination of neurobiological alterations in reelin-OE mice revealed that they not only had an increase in reelin-expressing cells, but they also reported that the rates of neurogenesis increased in the reelin-OE mice compared to wild-type control mice (Pujadas et al. 2010). Taken together, these results indicate that the level of reelin expression may alter the stages of proliferation, differentiation and maturation of newborn granule cells. Direct examination of this relationship would be an interesting area of research that could be important in furthering our understanding of neurogenesis and may potentially lead to novel treatments of neurodegenerative disorders, such as Alzheimer's disease.

The role of reelin in cellular migration was first recognized in reeler mice as they have abnormal hippocampal, cortical, and cerebellar lamination (Curran and D'Arcangelo, 1998; D'Arcangelo et al., 1995; Stanfield and Cowan, 1979). Further support for the role of reelin in newly generated cell migration comes from animal models of epilepsy. For example, a number of animal models of epilepsy have shown decreases in reelin expression, which is associated with aberrant distribution of the GCL (Fournier et al., 2010; Gong et al., 2007; Heinrich et al., 2006; Muller et al., 2009), can be prevented with recombinant reelin infusions (Muller et al., 2009). Importantly, aberrant migration of the newly generated cells was also shown in the reelin-OE mice in both the RMS and in the dentate gyrus (Pujadas et al. 2010). Overall, these

data suggest that excess or deficient reelin may be detrimental to normal cellular migration, and suggests that there may be an optimal level of reelin expression necessary to instigate proper cellular migration.

4. Role of GABA in Depression

As decreased GABA levels have been shown in depressed patients (Gerner and Hare, 1981; Gold et al., 1980; Kasa et al., 1982; Petty et al., 1992), while glutamate plasma levels and its metabolites have been shown to be increased (Altamura et al., 1993; Levine et al., 2000), an interest in these neurotransmitters in the pathogenesis of depression has grown. Furthermore, specific GABA receptor subtypes have begun to be implicated as novel targets for the treatment of depression (Cryan and Slattery, 2010; Ghose et al., 2011; Luscher et al., 2011; Vollenweider et al., 2011). For example, the mice with deficits in $GABA_A$ α1 receptor subunit have shown increases in anxiety; while the α2 subunit is involved in both depression- and anxiety-like behavior (see Smith and Rudolph 2011 for review).

One potential implication for the alterations in GABA and glutamate in the brain is related to altered neurogenesis. For example, research has shown that an increase in GABA influences not only the differentiation of newly generated cells (Tozuka et al., 2005) but also the integration of these cells into the surrounding network (Ge et al., 2006). As GABA is excitatory in developing neurons (Ben-Ari et al., 2007; Ge et al., 2006; Ge et al., 2007a; Liu et al., 2005; Loturco et al., 1995; Sernagor et al., 2010) and regulates the differentiation and integration of these new born neurons, the decrease in GABA markers we found after CORT exposure has important implications for neurogenesis within our CORT model (Chapter 5). In

fact, our laboratory and others have shown that repeated CORT exposure decreases neurogenesis (Chapters 2 and 3; Brummelte and Galea 2010; Sterner and Kalynchuk 2010). In addition to the decreased number of immature neurons, those existing have fewer and less complex dendritic morphology and are therefore less likely to be functionally integrated into the surrounding network (Chapters 3 and 4). These findings suggest that the decrease in GABA expression found within the CORT model may be a mechanism by which CORT decreases neurogenesis and potentially the integration of these newborn neurons into the circuitry. Although the findings in Chapter 5 are preliminary, they do offer support for this theory and gives a foundation for further research into the relationship of GABA and neurogenesis within the CORT model.

5. Limitations

Clinical Relevance of CORT Injections

There were a few limitations in these studies. The first limitation is that exogenous CORT administration may not be a realistic stimulation of daily life stressors. Given the difficulty in reproducing the human experience, the unrealistic nature of the stressors used in animal models is a common criticism. For example, repeated restraint stress is not a realistic simulation of the human experience because it utilizes a physical stressor and habituation that can develop quickly in this model (Galea et al., 1997; Gregus et al., 2005; Grissom et al., 2007). It has been argued that the CMS paradigm is the most representative model of the human experience with stress because it has altering milder stressors used throughout the paradigm. However, there is a lot of variability in the types of stressors used and results found across

different laboratories using this model (Willner et al., 1992; Willner, 2005). In this regard, it is interesting that the CORT model produces reliable behavioral, cellular, and morphological alterations that are typically more robust than those produced by other repeated stress models, such as the repeated restraint stress model (see Sterner and Kalynchuk, 2010). For example, repeated CORT exposure can produce increased immobility in the forced swim test, increased anhedonia, and learning and memory deficits (Sterner and Kalynchuk, 2010). Moreover, the CORT paradigm allows researchers greater control over glucocorticoid levels and to directly examine the effects of CORT on behavior and neurobiological factors, without the influence of psychosocial factors. It would be important to examine other repeated stress paradigms to see if the models that produced depressive-like behavior could also show decreases in reelin expression.

Sex Differences in Depressive-like Behavior and Neurobiology

Although the prevalence of depression is higher in females than males (Weissman et al., 1993), no females were used in these studies. Our laboratory has previously examined sex differences in the CORT model (40mg/kg for 21 days) and found increases in immobility in the forced swim test in both female and male rats, albeit to a lesser extent in females (Kalynchuk et al., 2004). In addition, 20 days of CORT (20 mg/kg) exposure did not increase immobility in the forced swim test in female rats, but did so in male rats (Hill et al., 2003). Moreover, 40 mg/kg of CORT has been shown to decrease neurogenesis in both male and female ventral hippocampus, but only in the male dorsal hippocampus as seen by decreased Ki67 and DCX expressing cells, which was not seen at the lower CORT dose (10mg/kg) (Brummelte and Galea, 2010b). Neurogenesis has been shown to be altered through the

different phases of the female estrus cycle (Galea et al., 2006) and elevated levels of CORT exposure has been shown to decrease number of estrous cycles (Brummelte and Galea, 2010b). In addition, exposure to estradiol shows dose-dependent effects on proliferation in female but not male rodents, with lower doses (10 µg) enhancing but supraphysiological doses (50 µg) not showing significant results (see Brummelte and Galea 2010 for review). Research examining sex differences have also found differential effects depending on the stressors being used and factors being measured (Brummelte and Galea, 2010b; Barha et al., 2011; Hill et al., 2003; Perrot-Sinal et al., 2004; Galea et al., 1997; Brotto et al., 2001), suggesting complicated relationships between sex differences in stress experience, behavioral consequences, and neurobiology.

There have been some conflicting results about sex differences in reelin and GABA expression. For example, some studies have shown that the reelin gene is associated with an increased risk for the development of schizophrenia and bipolar disorder in females and not in males (Goes et al., 2010; Shifman et al., 2008). Other researchers have found no significant differences between genders in regard to levels of reelin and GAD67 levels in neuropsychiatric disorders, including depression (Guidotti et al., 2000; Fatemi et al., 2000). Interestingly, female HRM showed protective effects in cerebellar cell loss when compared to male HRM (Hadj-Sahraoui et al., 1996). Again, these results suggest a complicated relationship between sex differences, GABA and reelin. Further investigation into these differences is needed to further understand this relationship.

Other Markers that May be Involved in the Pathogenesis of Depression

Although the research discussed in this dissertation provides support for the role of reelin in depression-like behavior (Chapters 2, 3, and 4), other molecular markers may also be involved. For example, neurotropic factors have been implicated in a number of animal models of depression and in depressed patients (Duman and Monteggia, 2006). Specifically, post-mortem and serum analysis of depressed patients have shown decreases in BDNF levels in patients not receiving treatment (Chen et al., 2001; Karege et al., 2002; Karege et al., 2005) and increases seen in patients treated with antidepressants (Chen et al., 2001; Aydemir et al., 2005). Similar findings have been shown in animal models, as BDNF is decreased following repeated stress paradigms (Barbany and Persson, 1992; Schaaf et al., 1998; Roceri et al., 2004; Nibuya et al., 1999) and increased as a result of antidepressant administration (Nibuya et al., 1995). Similar changes are also found for VEGF in repeated stress animal models of depression and following antidepressant treatment (Duman and Monteggia, 2006; Fournier and Duman, 2012). Importantly, these neurotropic factors are directly associated with neurogenesis (Duman and Monteggia, 2006; Fournier and Duman, 2012). None of these mechanisms are mutually exclusive. In fact, recent evidence points to interactions between these growth factors and reelin signalling (Lindhorst et al., 2012; Ringstedt et al., 1998). Therefore, it is possible that there are interactions between these and a number of other mechanisms which results in the changes seen after repeated stress.

6. Future Studies

What is the Role of Reelin in the Effects of Antidepressants?

Further examination into the role of reelin in the development of depression is important to elucidate the potential therapeutic implication of these findings. Examination into the effects of antidepressants following CORT exposure would be important to relate to alterations in depressive-like behavior. Research has already shown that antidepressants can alter reelin expression and other markers involved in the reelin signalling pathway, as well as GABA markers in naïve rodents (Fatemi et al., 2009). In addition, our laboratory has examined the effects of 10 and 15 mg/kg of imipramine given in parallel with 40mg/kg of CORT for 21 consecutive days which showed a protective effect of the larger dose of imipramine in both depressive-like behavior and reelin cell numbers (Kalynchuk et al. in progress). In further support of the role of reelin in the treatment of neuropsychiatric disorders, citalopram, a selective serotonin reuptake inhibitor, has been shown to reverse the kainic acid-induced reelin cell loss (Jaako et al., 2011). Interestingly, mice with reduced reelin expression show more robust behavioral alterations following maternal separation than their wild-type counterparts (Laviola et al., 2009; Ognibene et al., 2007). Moreover, adult HRM are more susceptible to the effects of repeated stress than the wild-type controls, both behaviorally and neurobiologically (Chapter 4). Furthermore, Teixeira and colleagues (2011) have shown that reelin-overexpression has a protective effect against CORT exposure, which shows strong therapeutic implications for reelin. Further research with different types of antidepressants following repeated stress is needed in order to understand the importance of reelin in the therapeutic effects of antidepressants.

In addition, directly examining the effects of reelin infusions into the ventricles following repeated stress would be another important step in understanding the function of this protein on depressive-like behavior. As previously mentioned, reelin infusions into the

ventricles of naïve mice has been shown to increase hippocampal dependent learning and memory and increased synaptic plasticity (Rogers et al., 2011). In addition, there are protective effects of increased reelin levels in reelin-OE mice against CORT exposure compared to wild-type mice (Teixeira et al. 2011). These studies indicate that there are potential therapeutic effects of reelin and provide a starting point to begin addressing whether reelin could alter affective behavior.

Are the Decreases in Reelin-Positive Cells Following Repeated CORT Exposure Actually Reflecting a Loss of Reelin-Expressing Neurons?

In Chapters 2-4, I showed that there were decreases in the number of reelin-positive cells in the hippocampus following repeated CORT exposure. As reelin is expressed in GABAergic interneurons in the dentate gyrus of the hippocampus (Pesold et al., 1998), my final experiment (Chapter 5) was a preliminary step into examining the effects of repeated stress on GABA and its related receptors. The decreases in GABAergic markers and an increase in the glutamatergic marker in the CORT model are consistent with an enhanced excitatory-inhibitory ratio seen in depressed patients (Sanacora et al., 1999; Sanacora et al., 2003b; Sanacora et al., 2004; Bhagwagar et al., 2007) and in an animal model of depression (Martisova et al., 2012). However, there are many subtypes of GABAergic neurons, and reelin is heterogeneously expressed in them. For example, reelin has a less than 6% frequency of co-expression with parvalbumin-, vasoactive intestinal polypeptide-, and cholechytokinin-immunoreactive neurons, while reelin is co-expressed with calretinin, somatostatin, calbindin, and neuropeptide Y approximately 58%, 75%, 14%, and 30%, respectively (Alcantara et al., 1998). Given the heterogeneity of reelin expression in GABAergic neurons, it would be

important to further examine the effects of CORT on the expression of the GABAergic neuronal subpopulation. Is it that all GABAergic cells are decreased equally? Or are the reelin-GABA co-expressing subtypes more vulnerable to the effects of CORT than the others?

There is some evidence of alterations in the different cell types that co-express reelin following repeated stress. For example, maternal separation was shown to decrease hippocampal neuropeptide Y expression (Husum et al., 2002; Jimenez-Vasquez et al., 2001). However, maternal separation stress does not significant affect the expression of calretinin and calbindin in the dentate gyrus and even increased density of these proteins in the CA1 area of the rat hippocampus (Giachino et al., 2007). Further examination into the changes in cells double-labelled for reelin and these proteins following repeated stress is necessary to understand whether any of these cell types are more susceptible to the effects of repeated stress.

Changes in Reelin Expression in Other Animal Models of Depression?

Repeated CORT administration consistently decreased reelin expression in the adult hippocampus (Chapters 2-4), while repeated restraint stress did not alter reelin-positive cell numbers (Chapter 2). Some inconsistent findings in reelin cell counts have been found with maternal separation. For example, maternal separation for 3 hrs per day for 14 days did not show changes in reelin-positive cell numbers in the hippocampus immediately following or 2 months after the stress (Gross et al., 2010). However, another study examining maternal separation revealed a decrease in reelin mRNA in the hippocampus (Qin et al., 2011). These results are similar to other studies of maternal separation showing inconsistencies in neurobiology (Macri et al., 2011; Newport et al., 2002). Although not directly examined in these studies, it would be interesting to see if these animals that showed decreases in reelin

expression also show alterations in depressive-like behavior. Interestingly, HRM have shown a susceptibility to anxiety behavior following repeated maternal separation (Laviola et al., 2009; Ognibene et al., 2007). Future research should utilize other repeated stress models, such as CMS, to ensure that the decreases in reelin-positive cells can be replicated across repeated stress models. Of course, depressive-like behaviors should also be measured in these models to further elucidate the importance of reelin expression in depression-like behavior, specifically. In addition, the use of HRM or reelin-OE mice in different types of repeated stress would also be an interesting angle to examine the relationship of reelin and depressive-like behavior.

Are Specific Subtypes of Neuronal Progenitors More Susceptible to Stress?

In Chapters 3 and 4, DCX cell numbers were decreased following different durations and doses of CORT exposure, respectively. In addition, DCX morphology was significantly decreased following repeated CORT exposure. DCX cells in the SGZ and GCL are type-2b and type-3 progenitor cells (Plumpe et al., 2006). Type-2b cells show co-expression with nestin which reflects its precursor cell lineage. It is during the type-2 phase where cell fate is determined and in type-3 phase where migration and integration occurs (Plumpe et al., 2006). When examining DCX cells in the dentate gyrus it was found that the cells with the most complex morphology (categories 5 and 6) were co-expressed with calretinin which suggests that these cells were in the postmitotic phase of cell development (Plumpe et al., 2006). The results from Chapters 3 and 4 suggest that the percentages of cells that have more complex morphology are decreased following CORT exposure while cells with immature morphology are increased. This suggests that not only does CORT decrease the number of DCX expressing cells, but also potentially halts the development of the remaining cells. This could imply that

there is a potential difference in the population of cells with regards to the sensitivity to CORT exposure. Future research is necessary to examine the specific subtypes of precursor cells in regard to this sensitivity. For example, is a specific subtype of precursor cells (i.e. type-2a, 2b, or 3) more vulnerable to the effects of CORT? Double-labeling studies could be performed in this context to elucidate the vulnerability of these cell types to the effects of repeated CORT exposure.

Another way to examine this effect would be to co-label GR expression with the DCX cells and compare them in regard to the difference in morphological development to reveal any susceptibilities of the stages in development to changes in receptor availability. Perhaps the reason that we see the decreases in the DCX cells with more complex morphology compared to the increase in immature morphology is that the more complex cells have increased GRs and therefore CORT could have a direct effect of this cell population. In fact, Garcia and colleagues (2004) have shown that GR were expressed in 50% of type-1, type-2a, and type-3 cells, while type-2b did not show any GR expression. Given that DCX labels type-2b (no GR expression) and type-3 (50% GR expression) cells and that the type-3 cells have more mature morphology (Plumpe et al., 2006), it suggests that the mature DCX cells can be directly influenced by increased CORT levels.

Are there Changes in Reelin Expression in Other Brain Structures Following Repeated Stress?

Although the hippocampus has been implicated in depression for a number of reasons previously mentioned, other structures have also been implicated in the pathogenesis of depression. For example, PFC volume has been shown to be decreased in depressed patients (Hastings et al., 2004) and in animal models of depression (Akana et al., 2001; Cerqueira et al.,

2005; Radley et al., 2005), which has been associated with changes in PFC-dependent behaviors (Cerqueira et al., 2005). In addition, research has supported a role of the PFC in the negative feedback control of the HPA axis (Diorio et al., 1993). Interestingly, decreases in reelin expression have been shown in the PFC of patients with schizophrenia and bipolar disorder; however, no alterations were seen in PFC of depressed patients (Guidotti et al., 2000). To my knowledge, no studies have examined the levels of reelin expression in the PFC in an animal model of depression. However, alterations of reelin expression in the frontal cortex have been shown following psychotropic drug exposure (Fatemi et al., 2009), which suggests that the regulation of reelin expression in this area could be important in antidepressant efficacy. A more direct examination into the changes of reelin expression in the PFC after repeated stress and antidepressant treatment is needed.

Another structure potentially involved in the pathogenesis of depression is the amygdala. Alterations in amygdala volume have been shown in patients with depression (Hastings et al., 2004; Sheline et al., 1998). In addition, dendritic complexity and length, and spine density within the amygdala has been shown to be increased after repeated stress (Mitra et al., 2005a; Mitra and Sapolsky, 2008; Vyas et al., 2002; Vyas et al., 2006). Interestingly, decreases in GAD67 and the $GABA_A$ α2 receptor subunit were shown in the amygdala after repeated CORT exposure (Chapter 5). In addition, there does seem to be some decreases in reelin expression in the Me and cortical amygdala (Lussier and Kalynchuk unpublished observation, 2010). Further investigation is needed to understand the role of reelin in the amygdala on behavior. One way to directly examine the role of reelin in the amygdala would be to infuse CR-50 into the amygdala as it is known to disrupt reelin signaling (D'Arcangelo et

al., 1997; Nakajima et al., 1997; Matsuzaki et al., 2007) and examine amygdala dependent behavior such as fear conditioning.

7. Conclusion

The goal of this dissertation was to conduct research that provides a neurobiological and behavioral examination of the effects of repeated stress to further understand the role of stress hormones in the pathogenesis of depression. Preclinical research utilizing different types, duration, and intensities of stress were examined to further elucidate the neuropathological and behavioral alterations in rodents. The decreases in hippocampal reelin cell number following repeated stress are consistent with human studies showing decreases reelin levels in neuropsychiatric disorders (Fatemi et al., 2000; Fatemi et al., 2001; Fatemi, 2005; Knable et al., 2004). In addition, the decreases in reelin cell number were associated with increased depressive-like behavior and decreases in neurogenesis and maturation of newly born neurons following repeated stress. Moreover, HRM were more susceptible to the effects of repeated CORT than the WTM which further support the role of reelin in the pathology of depression. Lastly, repeated CORT exposure, but not repeated restraint, decreases GABAergic activity in the hippocampus and amygdala and increases glutamatergic activity in the hippocampus. Combined, these results provide a novel examination of preclinical models of depression on reelin cell number, maturation and number of newly born hippocampal neurons, behavior, and may provide avenues for future research that could lead to novel therapeutic targets for the treatment of depression.

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