Examining Hippocampal Reelin Expression and Neural Plasticity in an Animal Model of Depression

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By
Erin Y. Fenton

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ABSTRACT

Stress is an important risk factor for the development of clinical depression, yet little is known about the neurobiological mechanisms by which stress might promote depressive symptomatology. The brain is particularly susceptible to the negative effects of stress, as high levels of stress hormones result in decreased hippocampal neurogenesis, slowed cell maturation, and decreased cell complexity. Although we already know that these neurobiological changes are associated with significant impairments in important psychological functions such as learning, memory and motivation, we know little about the molecular details of this stress-induced remodeling and how it contributes to the development of depression. Currently, one candidate molecule of particular interest is reelin, an extracellular matrix protein responsible for regulating neuronal maturation and synaptic plasticity in the adult brain. Interestingly, recent post- mortem analyses indicate that reelin expression is decreased in depressed patients. Similarly, preclinical research has shown that repeated glucocorticoid administration significantly reduces reelin expression in the adult hippocampus. Combined, these results suggest that reelin may be an important protein to examine in regards to the pathogenesis of depression as well as a potential therapeutic target for the treatment of this disorder.

The goal of this dissertation is to provide a comprehensive examination of the influence repeated glucocorticoid administration has on reelin expression in the rat hippocampus, and how this relates to the pathogenesis of depression. In chapter 2 we examined how co-treatment with the stress hormone corticosterone (CORT), and the antidepressant imipramine, influence reelin expression in the proliferative region of the hippocampus. In addition we determined whether changes in reelin expression are associated with alterations in neurogenesis and behavioral measures of depression. Results revealed that imipramine prevents CORT-induced downregulation of reelin in the hippocampus, and that these changes parallel improvements in FST behavior, increased neurogenesis and enhanced maturation of immature granule cells. Importantly, these data provide further evidence of reelin's role in depression and establish this protein as a target of antidepressant treatment. In chapter 3 we examined the effect of CORT on a number of interneuron markers that co-localize with reelin throughout the hippocampus to determine whether the populations of neurons that express reelin are lost or are no longer expressing this protein. Results of this study indicate that CORT influences a number of interneuron markers in a region-specific manner in the hippocampus, but does not cause these

cell populations to die, suggesting that CORT exploits an intracellular mechanism to regulate reelin expression in the hippocampus. Finally, in chapter 4, the influence of CORT on MeCP2 and DNMT1, two markers associated with DNA methylation, was examined in the hippocampus to elucidate a potential intracellular mechanism for CORT-induced reelin deficits. Results of this study indicate that CORT has no influence on global protein levels of these markers, but significantly increases the number of MeCP2-expressing cells in the proliferative subgranular zone of the hippocampus, suggesting that there is an increase in the number of methylated cells in this region. While it cannot be conclude from this study that increased methylation causes reelin deficits, the fact that an increase in MeCP2 is seen in the exact region where reelin deficits are most pronounced suggest it is possible. Moreover, these findings are novel, and suggest a role for MeCP2, and more generally, DNA methylation, in the neurobiology of depression. Collectively, the results of this dissertation enhance our understanding of the functional consequences of altered hippocampal neuroplasticity on the development of depressive symptomatology, and the role that reelin may play in this process. They also provide further support for reelin as a novel therapeutic target for the treatment of major depression.

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DEDICATION

I would like to dedicate this dissertation to all of my friends and family who were there with me when I started this journey, those I have been fortunate enough to meet along the way, and those who will be there with me when I graduate. Nothing is more powerful than the encouragement and support of loved ones – they are the compass that guides us, the inspiration to reach great heights, and our comfort when we falter.

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LIST OF ABBREVIATIONS

3’UTR three prime untranslated region

ABC avidin-biotin complex

ACTH adrenocorticotropin hormone

ApoER2 apolipoprotein E receptor 2

BDNF brain-derived neurotrophic factor

BSA bovine serum albumin

CA Cornu Ammonis

CA1P pyramidal cell layer of CA1

CA1SO stratum oriens of CA1

CA1SR stratum radiatum of CA1

CA3P pyramidal cell layer of CA3

CA3SL stratum lucidum of CA3

CA3SO stratum oriens of CA3

CA3SR stratum radiatum of CA3

$Ca2^{+}$ calcium

cAMP cyclic adenosine monophosphate

CB calbindin

CCK colechystokinin

ChIP chromatin immunoprecipitation

CI-10 CORT plus 10mg/kg of imipramine

CI-15 CORT plus 15mg/kg of imipramine

CMS chronic mild stress

CNS central nervous system

CORT corticosterone

CpG cytosine dinucleotides

CR calretinin

CREB cyclic AMP-response element binding protein

CRH corticotropin-releasing hormone

CUS chronic unpredictable stress

DAB 3,3’-diaminobenzidine

Dab1 disabled-1

DCX doublecortin

DG Dentate gyrus

DNA deoxyribonucleic acid

DNMTs DNA methylstransferases

DNMT1 DNA methyltransferase 1

EC Entorhinal cortex

ECL enhanced chemiluminescence

ECT electroconvulsive therapy

FST forced swim test

GABA γ-amminobutyric acid

GAD glutamate decarboxylase

GCL granule cell layer

GR glucocorticoid receptor

Gsk3β glycogen synthase kinase 3β

HAT histone acetyltransferase

HDAC histone deacetylase

H3K9 histone 3 lysine 9

H_2O_2 hydrogen peroxide

HPA hypothalamic pituitary adrenal

HRM heterozygous reeler mice

Lis1 lissencephaly 1

lMol Stratum lacunosum-moleculare

LTP long-term potentiation

MBP methyl-CpG-binding protein

MeCP2 methyl-CpG-binding protein 2

miRNA micro ribonucleic acid

Mol D Molecular layer of the dentate gyrus

mPFC medial prefrontal cortex

MR mineralocorticoid receptor

MRI magnetic resonance imaging

mRNA messenger ribonucleic acid

MWM Morris water maze

NeuN neuron-specific nuclear protein

NGS normal goat serum

NHS normal horse serum

NMDA N-methyl-D-aspartate

NMDAR N-methyl-D-aspartate receptor

NR2 NMDAR subunit 2

NPY neuropeptide-y

PBS phosphate buffered saline

PBSx phosphate buffered saline with triton X-100

PFC prefrontal cortex

PI3K phosphatidylinositol-3-kinase

PKB protein kinase B

PPI prepulse inhibition

PRS prenatal restraint stress

PSD-95 postsynaptic density protein 95

PTSD posttraumatic stress disorder

PVB parvalbumin

PVN paraventricular nucleus

REM rapid eye movement

RRS repeated restraint stress

RTT Rett's Syndrome

SAM S-adenosyl methionine

SFKs src family tyrosine kinases

SOM somatostatin

SSRI selective serotonin reuptake inhibitor

TBS tris buffered saline

TBST tris buffered saline with tween-20

TBSx tris buffered saline with triton X-100

RELN reelin gene

SGZ subgranular zone

VEGF vascular endothelial growth factor

VIP vasoactive intestinal peptide

VPA valproic acid

VLDLR very low density lipoprotein receptor

WT wild-type mice

CHAPTER 1
General Introduction[1]

1. Thesis Outline

A fundamental aspect of our nervous system is its ability to adapt to ever-changing stimuli in our environment. These adaptations are a response to the need for our body to maintain a stable internal environment (e.g. temperature, pH, glucose levels, etc), otherwise known as homeostasis. Homeostatic regulation is imperative for survival – harnessing physiological mediators such as adrenalin, glucocorticoids, and cytokines, to act upon receptors in various tissues and organs to produce effects that are acutely adaptive. However, prolonged activation of these systems is maladaptive and may have detrimental consequences to our mental and physical well-being. For example, prolonged or even irregular elevation of stress hormones such as cortisol and epinephrine can weaken the immune system and lead to the development of hypertension, heart disease, and poor mental health. This cost of adaptation is often referred to as "allostatic load" (McEwen, 1998b; McEwen & Stellar, 1993).

Neuropsychiatric disorders provide a good example of the extent to which homeostatic imbalance can disrupt functioning in the central and peripheral nervous systems. In particular, many of these disorders are characterized by alterations in various neurotransmitters and neuroendocrine hormones in the brain and periphery, which are important for the regulation of mood, arousal and cognition (Laryea, Arnett, & Muglia, 2012; Syvalahti, 1987; Urban & Abi-Dargham, 2010). Major depression is among the most common of neuropsychiatric disorders, affecting up to 16% of the population (Kessler et al., 1994; Kessler, 2003). In fact, according to the World Health Organization, depression is the fourth leading cause of overall disease burden and the leading cause of years lost to disability worldwide in individuals 15-44 years of age. Although core symptoms of depression include low mood and anhedonia (i.e., lack of interest in pleasurable acts), the disorder is characterized by a complex cluster of clinical symptoms that may include psychomotor agitation and/or retardation, decreased energy, altered appetite and weight,

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nervousness, irritability, sleep disturbances, and cognitive deficits including an impaired ability to think, concentrate, or make decisions (Buchwald & Rudick-Davis, 1993). Moreover, individuals suffering from depression have increased physical illness, decreased social functioning, and a high mortality rate (Nemeroff, 1998). The complexity of this disorder is further compounded by the fact that it often co-occurs with other neuropsychiatric conditions. For instance, it is reported that 51% of those with depression also suffer from anxiety disorders (Kessler et al., 1996), which can have a profound impact on the course of depressive illness, with delayed recovery, increased risk of relapse, greater disability and increased suicide attempts (Hirschfeld, 2001).

Generally it is thought that a combination of genes, psychosocial factors, and alterations in neurotransmission and hormone levels influence the development of depression (Kalia, 2005; Nestler et al., 2002a). Recent hypotheses about the etiology of depression involve alterations in proteins and growth factors that regulate plasticity in the adult brain. These hypotheses are supported by a wealth of post-mortem evidence indicating volumetric reductions in brain regions thought to regulate cognitive and emotional processes (Sheline, Gado, & Kraemer, 2003), as well as decreased cell proliferation, complexity and survival in these same regions in rodent models of depression, and recovery of these changes after antidepressant treatment (Bambico & Belzung, 2012; Sterner & Kalynchuk, 2010). These data have fuelled intense research efforts aimed at characterizing the cellular and molecular mechanisms underlying the development of depressive symptomatology, as well as the long-term adaptations required for the therapeutic action of antidepressants. Although a wealth of clinical and preclinical evidence indicates that many of the cellular changes associated with depression significantly impair important psychological functions (Conrad, 2010; McEwen & Magarinos, 2001), our understanding of the molecular details of this atrophy and how it contributes to the development of clinical depression is still rudimentary.

This dissertation includes a collection of experiments that attempt to further our understanding of the relationship between glucocorticoids, alterations in hippocampal plasticity (a structure known to influence the stress response and be negatively affected in depressed patients), and depressive symptomatology. More specifically, my research is focused on how glucocorticoids regulate reelin, a glycoprotein important for neural development and synaptic plasticity, in the rodent hippocampus, and how this relates to depression. To begin addressing these issues, I examined whether antidepressant treatment could rescue corticosterone (CORT)-induced

downregulation of reelin-positive (+) cells in the hippocampus, and whether these changes are associated with improvements on behavioral measures of depression and alterations in neurogenesis and neuronal matruation. My second experiment was designed to address how different populations of γ-aminobutyric acid (GABA) interneurons in the hippocampus are affected by repeated CORT treatment. This study allowed me to determine how the populations of cells that express reelin are affected by this model of depression, and assess the extent to which repeated gluccorticoid exposure influences GABAergic interneuron markers in the hippocampus. Finally, my last experiment examines how CORT influences methylation markers globally and regionally in the hippocampus to determine how methylation may be altered by repeated glucocorticoid exposure, and to elucidate a potential mechanism for altered reelin expression in this model of depression.

The following sections of this chapter will detail what is currently known about the link between stress and depression, associated neurobiological changes and how reelin might be involved. First, I will provide an overview of the hypothalamic-pituitary-adrenal (HPA) axis and its relationship to depression. I will then go on to discuss the structural and functional abnormalities associated with the disorder and how stress may play a role, followed up by an in depth discussion of the anatomy of the hippocampus. To substantiate the use of exogenous CORT administration as a model of depression, an overview of the model will be provided, with a focus on cognitive, behavioral, and neurobiological function, as well as considerations when choosing this model. I will then discuss the link between GABA, major depression, glucocorticoids, and reelin, with a focus on inhibitory interneurons in the hippocampus. Finally, I will provide an introduction to epigenetics, focusing on mechanisms of methylation and acetylation, and discuss how these mechanisms may influence reelin expression in the brain. I will conclude this chapter with a discussion of specific questions that remain unanswered in the context of stress and depression that will be directly addressed in this dissertation.

Chapters 2, 3 and 4 provide experimental data that address the specific aims outlined in this chapter. Chapter 5 provides a general overview of my experimental research, implications of the results, a discussion of the limitations of the work presented in this dissertation, and specific areas in which further research is necessary.

2. HPA Axis Under Normal Circumstances

Although individual vulnerability to developing depression appears to be heritable, a large body of evidence suggests that prolonged exposure to stress may be a precipitating factor in developing the disorder (Checkley, 1996). In fact, there are data to suggest that up to 85% of patients experience significant stress prior to the onset of their depressive symptoms (Parker, Schatzberg, & Lyons, 2003). A key question then becomes, can stress precipitate depression by altering brain structure and function, and if so, by what mechanisms? The normal physiological and neurobiological responses to stress are well characterized. Exposure to an acute stressor activates the HPA axis (See Figure 1-1; Herman and Cullinan, 1997), resulting in a cascade of endocrine events. This cascade includes the release and transport of corticotropin-releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN) of the hypothalamus to the anterior pituitary, where it stimulates the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary into the circulatory system. ACTH then travels to the adrenal glands, where it stimulates the production and release of glucocorticoids (cortisol in humans and CORT in rodents) from the adrenal cortex into the circulatory system. Once released, glucocorticoids act on bodily tissues to limit non-essential functions and mobilize energy to deal with the stressor. Glucocorticoids also travel to the brain where they exert an inhibitory influence on the PVN to halt HPA axis activity through a negative feedback process (Herman & Cullinan, 1997).

Negative feedback of the HPA axis appears to be heavily dependent on the integrity of the hippocampus. The hippocampus expresses both mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), which are the main sites of glucocorticoid action. Mineralocorticoid receptors have a high affinity for CORT and thus, they are activated even when circulating CORT levels are low. In contrast, glucocorticoid receptors have a low affinity for CORT, and only become activated when CORT levels are relatively high (de Kloet, Vreugdenhil, Oitzl, & Joels, 1998). Hippocampal glucocorticoid receptors are critically involved in negative feedback control of the HPA axis. When hippocampal glucocorticoid receptor levels are high, feedback inhibition of the PVN is enhanced and HPA axis activity is tightly controlled. However, when hippocampal glucocorticoid receptor levels are low, feedback inhibition is inefficient, and stimuli that evoke an HPA axis response lead to higher than normal levels of CORT (de Kloet et al., 1998). Not surprisingly, the hippocampus is particularly susceptible to the damaging effects of prolonged elevations of CORT, evidenced by decreased dendritic branching of CA3 pyramidal neurons, decreased hippocampal neurogenesis, and decreased hippocampal glucocorticoid receptor

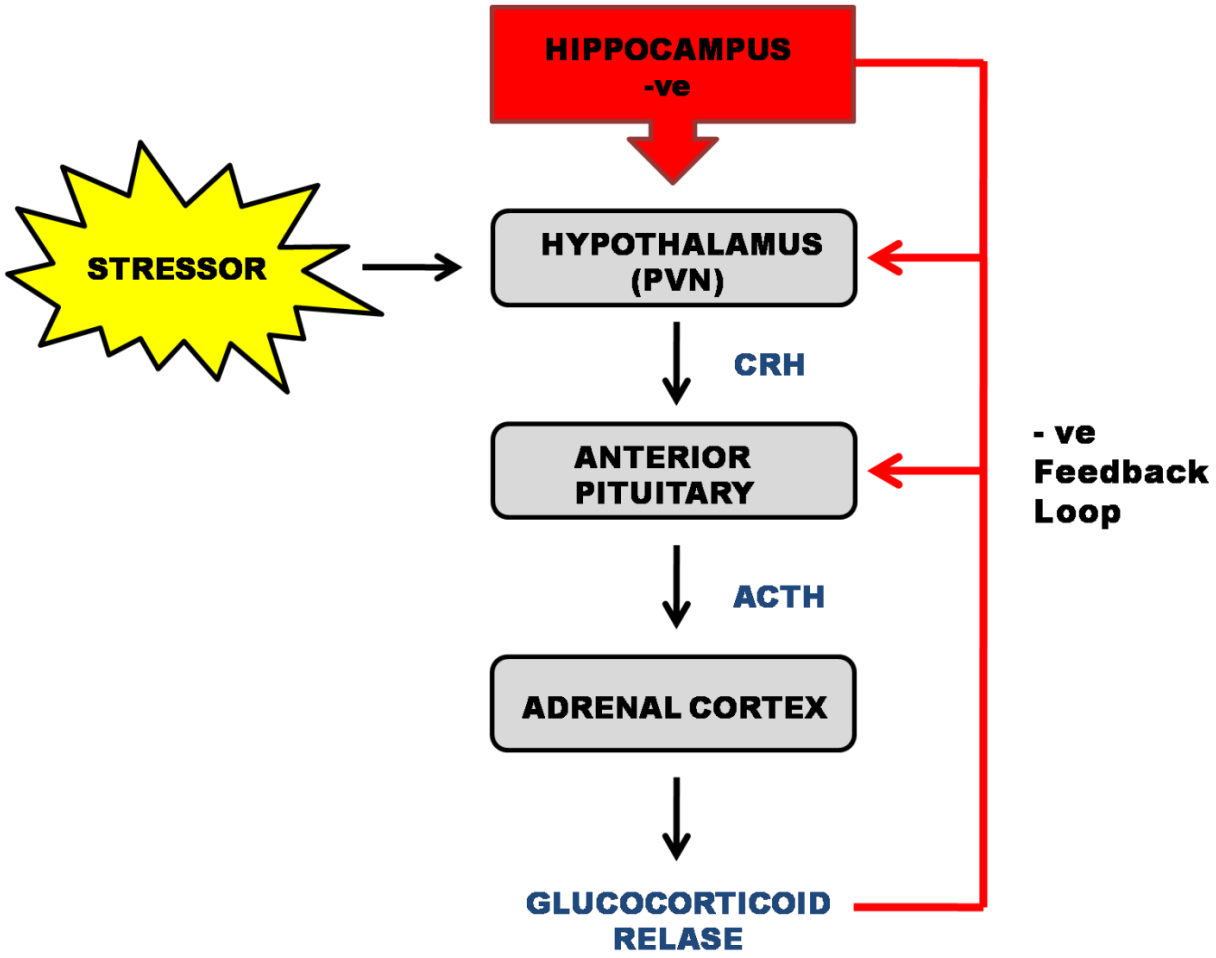

Figure 1-1. Schematic of Hypothalamic-Pituitary-Adrenal Axis Regulation. At the onset of an acute stressor, parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus are stimulated to release the peptide corticotropin releasing hormone (CRH) into hypophysial portal vessels that access the pituitary gland. CRH then 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 (i.e., CORT in rodents, cortisol in humans). 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). Hippocampal glucocorticoid receptors area also critically involved in negative feedback control of the HPA axis (red arrows). When hippocampal glucocorticoid receptor levels are high, feedback inhibition of the PVN is enhanced and HPA axis activity is tightly controlled. However, when hippocampal glucocorticoid receptor levels are low, feedback inhibition is inefficient, and stimuli that evoke an HPA axis response lead to higher than normal levels of CORT (de Kloet et al., 1998). At elevated levels, glucocorticoids impair and/or damage the neurons, or alter neuronal function. The hippocampus is rich in MR and GR and so this region is particularly susceptible to the deleterious effects of stress. For example, prolonged exposure to high levels of corticosteroids results in dendritic atrohphy and loss of pyramidal neurons in the hippocampus (McEwen & Magarinos, 2001).

messenger ribonucleic acid (mRNA; Fuchs & Gould, 2000; McEwen, 2000; Pham, Nacher, Hof, & McEwen, 2003). The functional implications of this damage are not clear, but presumably it reduces the feedback control exerted by the hippocampus over the HPA axis, further increasing CORT levels and creating even more hippocampal damage (de Kloet et al., 1998; Nestler et al., 2002a,b).

Fundamentally, activation of the HPA axis in response to a stressor is essential for survival. Its primary purpose is to prepare the body to deal with an acute stressor, and then to restore bodily homeostasis once the stressor has diminished (de Kloet et al., 1998). However, prolonged activation of the HPA axis can present a serious health risk, leading to immunosuppression, growth inhibition, sleep disorders, impaired memory, decreased sexual behavior, and chronic dysphoria (Conrad, Galea, Kuroda, & McEwen, 1996; D'Amato, Rizzi, & Moles, 2001; McEwen, 1998a,b). Thus, the same stress hormones that are vital for an organism's survival during acute stress can also predispose an organism to illness if the period of stress is prolonged. The challenge is to identify the mechanisms by which prolonged stress might impair brain structure and function and eventually lead to the manifestation of depressive symptomatology.

3. Evidence of HPA Axis Deregulation in Depression

Several lines of research in human patients reveal an important connection between excessive HPA axis activation and depression. For example, about half of all depressed patients display hypercortisolemia and disrupted cortisol rhythmicity (Sachar & Baron, 1979), which can be reversed by antidepressant treatment (Holsboer, 2001). Additionally, many depressed patients show an exaggerated cortisol response to supraphysiological doses of exogenous ACTH (Amsterdam, Lucki, & Winokur, 1985; Amsterdam, Maislin, Abelman, Berwish, & Winokur, 1986; Amsterdam, Maislin, Berwish, Phillips, & Winokur, 1989; Amsterdam, Maislin, Droba, & Winokur, 1987; Jaeckle, Kathol, Lopez, Meller, & Krummel, 1987). Many hypercortisolemic depressed patients also show an attenuated ACTH response to exogenously administered CRH (Amsterdam et al., 1988; Amsterdam, Maislin, Winokur, Kling, & Gold, 1987; Gerken & Holsboer, 1986; Gold et al., 1986; Holsboer, von, Gerken, Stalla, & Muller, 1984; Rubin, Phillips, Sadow, & McCracken, 1995), and ACTH responses tend to be most attenuated in depressed patients with the most severe hypercortisolism (Gold et al., 1986).There is also evidence of increased levels of CRH in cerebrospinal fluid, increased urinary free cortisol, and a

decreased tendency for the synthetic glucocorticoid dexamethasone to suppress plasma cortisol (Nestler et al., 2002a; Southwick, Vythilingam, & Charney, 2005). In healthy subjects, dexamethasone peripherally suppresses ACTH and cortisol release by binding to GRs, which inhibit the synthesis, and secretion of ACTH and, consequently, the secretion of cortisol. In depressed patients, however, if the dexamethasone suppression test result is initially abnormal (i.e., showing inadequate cortisol non-suppression after dexamethasone treatment), a normalization usually occurs during successful antidepressant treatment, and in a majority of studies, failure to normalize has been associated with poor outcome and early relapse (Greden et al., 1983; Holsboer, Liebl, & Hofschuster, 1982). More evidence for a link between excessive HPA axis activation and depression comes from observations that the onset of depressive illness is correlated with major stressful life events, such as divorce, unemployment, or the death of a family member (Checkley, 1992; Kessing, Agerbo, & Mortensen, 2003). Furthermore, patients with Cushing's disease, a disorder marked by chronically high levels of cortisol, show unusually high rates of depression (Sonino & Fava, 2002), creating a strong argument for the influence of a dysregulated stress system in the development of a depressed state. Interestingly, in patients with Cushing's disease, hippocampal volume and function can also undergo recovery when glucocorticoid levels return to normal (Bourdeau et al., 2002; Heinz, Martinez, & Haenggeli, 1977; Starkman et al., 1999), and some of the depressive symptoms manifest in this disorder can be relieved following successful treatment for hypercortisolemia (Starkman, Giordani, Gebarski, & Schteingart, 2007; Starkman, Schteingart, & Schork, 1986). Finally, several existing classes of effective antidepressant drugs act on the neuroendocrine substrates that regulate cortisol secretion (Pariante et al., 2003; Pariante et al., 2001; Pariante & Miller, 2001) and novel antidepressant therapies that inhibit cortisol secretion have shown promise in clinical trials (Laakmann, Hennig, Baghai, & Schule, 2004; O'Dwyer, Lightman, Marks, & Checkley, 1995; Schule, Baghai, Eser, & Rupprecht, 2009). Although all the evidence listed above is correlational, it is nonetheless substantial and it provides a strong indication that some aspects of depression may arise from a dysregulated HPA axis.

4. Structural and Functional Abnormalities in Depression

Although for many years depression has been primarily linked to abnormalities in monoaminergic neurotransmission, it is now well accepted that this condition is characterized by

profound alterations in brain structure, function, and responsiveness (Berton & Nestler, 2006; Pittenger & Duman, 2008). Consequently depressed patients display an inability to cope or adapt to the environment and may be more vulnerable to challenging and stressful experiences. Generally, the pattern of metabolic changes during major depressive episodes suggests that brain structures thought to play a key role in mediating emotional and stress responses (e.g., the amygdala, hippocampus) are pathologically activated. Brain areas thought to modulate or inhibit emotional expression are also activated (e.g., subgenual prefrontal cortex). However, areas implicated in attention and sensory processing are deactivated (Drevets, 2001). Incidentally, this pathological activation is accompanied by structural abnormalities. Typically, neuroimaging and postmortem analyses of patients with depression identify structural changes in limbic and forebrain regions including the hippocampus, amygdala, and prefrontal cortex (PFC; Drevets, 2000; Drevets, 2001; Jaracz, 2008). The hippocampus is the most extensively studied region in depression, and the resulting findings, albeit not homogeneous, suggest that reductions in hippocampal volume are associated with depression. Interestingly, smaller hippocampal volumes have been more commonly found in patients suffering multiple depressive episodes rather than patients in remission or those experiencing their first episode (Caetano et al., 2004; Frodl et al., 2004; Frodl et al., 2006; Jaracz, 2008; Lorenzetti, Allen, Fornito, & Yucel, 2009). This suggests that the reduction in hippocampal volume may be associated with the severity of the disorder (MacQueen et al., 2003). Moreover, there seems to be an association between volume loss in this region and time spent with untreated depression; however, no such association is found while depression is being treated (Sheline et al., 2003). Similarly, there are consistent reports of reduced PFC volumes in patients with depression, specifically in sub-regions including the dorsolateral PFC, orbitofrontal cortex, and subgenual PFC (Konarski et al., 2008). In the amygdala, however, volumetric changes appear to be dynamic throughout the course of depressive illness, with an initial enlargement, followed by a volume reduction as the illness progresses (Lorenzetti et al., 2009). Importantly, these regions are part of the limbic-cortico-thalamic circuit which plays an integral role in cognition and emotional processing (Soares & Mann, 1997). The fact that all of these regions, to some degree, function pathologically in depression supports a neural model of depression in which dysfunction involving regions that modulate or inhibit emotional behavior may result in the emotional, motivational, cognitive, and behavioral manifestations of mood disorders.

Stress has also been implicated in some of the volumetric changes observed in patients with depression. More specifically, it has been suggested that dysregulation of the HPA system and subsequent changes in glucocorticoid secretion could result in both reversible remodelling and irreversible cell death in limbic and forebrain regions, which could account for the volumetric changes and subsequent pathological functioning, seen in patients with depression (Drevets, 2001; McEwen, 2007; Sapolsky, 2000). Importantly, the hippocampus, amygdala and PFC express GR and MR, making them likely targets for glucocorticoid action and thus particularly susceptible to stress-induced neuronal atrophy or hypertrophy (Herman et al., 2003; Herman, Ostrander, Mueller, & Figueiredo, 2005). Typically, the hippocampus and PFC inhibit HPA axis activity, whereas the amygdala enhances it (Feldman, Conforti, & Weidenfeld, 1995; Herman & Cullinan, 1997; Jacobson & Sapolsky, 1991). As patients with depression show decreased hippocampal and PFC volumes and increased amygdala volumes, this could indicate that prolonged stress intereferes with the ability of the hippocampus and PFC to inhibit HPA axis activity, while facilitating amygdalar enhancement of the HPA axis. Interestingly, there is evidence supporting an association between hypercortisolemia and hippocampal volume in depressed patients (Lorenzetti et al., 2009; Pariante, 2003; Pariante & Miller, 2001; Sheline, 2000). Similar results have also been reported in the PFC, medial orbitofrontal cortex and the amygdala (Lorenzetti et al., 2009). Preclinical evidence corroborates this further, as a prolonged elevation of glucocorticoids is known to induce dendritic remodeling, and in some cases, neuronal death in hippocampal and PFC regions, and dendritic hypertrophy in amygdalar regions (McEwen, 2007; Mitra & Sapolsky, 2008). It is therefore likely that the connection between limbic structures, mood, and HPA axis dysfunction is associated with impaired integration of hippocampal, amygdalar and/or prefrontal cortical information.

5. Anatomy of the Hippocampal Formation

As discussed in the previous section, some of the most consistent findings in the brains of depressed patients are abnormalities in the structure and function of the hippocampus (Caetano et al., 2004; Frodl et al., 2004; Frodl et al., 2006; Jaracz, 2008; Lorenzetti et al., 2009). Given that this brain region is highly involved in the stress response (de Kloet et al., 1998), as well as memory and emotional processing (Conrad, 2010; Soares & Mann, 1997), a discussion of hippocampal circuitry will aid in understanding how damage to various hippocampal sub-regions

might influence the overall function of this brain structure, and lead to the development of depressive symptomatology. Accordingly, the following section will provide a general overview of how information is processed in the hippocampal formation, as well as a closer examination of the cellular organization in the hippocampus proper and dentate gyrus (DG).

Trisynaptic Circuit

Located in the temporal lobe of the mammalian brain, the hippocampus is one of several structures that together form what is known as the hippocampal formation. The hippocampus proper, or Cornu Ammonis (CA), has three major sub-divisions: CA1, CA2 and CA3 (See Figure 1-2). The remaining regions of the hippocampal formation include the DG, the subicular cortex (including the subiculum, presubiculum, and parasubiculum) and the entorhinal cortex (Amaral, Dolorfo, & Alvarez-Royo, 1991; Amaral & Witter, 1989; Lopes da Silva, Witter, Boeijinga, & Lohman, 1990). Much of the cortical input reaching the hippocampal formation does so via the EC which is why this structure is considered to be the first step in the hippocampal circuit. In particular, neurons in the EC give rise to axons that project to the DG, forming the major hippocampal input pathway called the perforant path. The principal cells (i.e., excitatory granule cells) of the DG then give rise to axons called mossy fibers which synapse onto the somata of pyramidal cells in CA3; the axons of these pyramidal cells (called Shaeffer collateral axons) in turn provide the major input to the CA1 subfield of the hippocampus, making synaptic connections with proximal dendrites of pyramidal cells in this region. The axons of CA1 pyramidal cells then project to the subiculum and EC providing the major excitatory output for the hippocampus (Amaral, Ishizuka, & Claiborne, 1990; Amaral & Witter, 1989; Lopes da Silva et al., 1990). Collectively, the synaptic connections formed by the perforant path, mossy fibers and Shaeffer collaterals in this hippocampal network form what is known as the trisynaptic circuit. Unlike the reciprocal connections made between most regions of the cortex (Felleman & Van Essen, 1991), the passage of information through the intrahippocampal circuit is largely unidirectional (i.e, the DG does not project back to the EC, CA3 does not project back to the DG, CA1 does not project back to CA3 and the subiculum does not project back to CA1), making the neuroanatomy of this structure unique (Amaral et al., 1990; Amaral & Witter, 1989; Lopes da Silva et al., 1990).

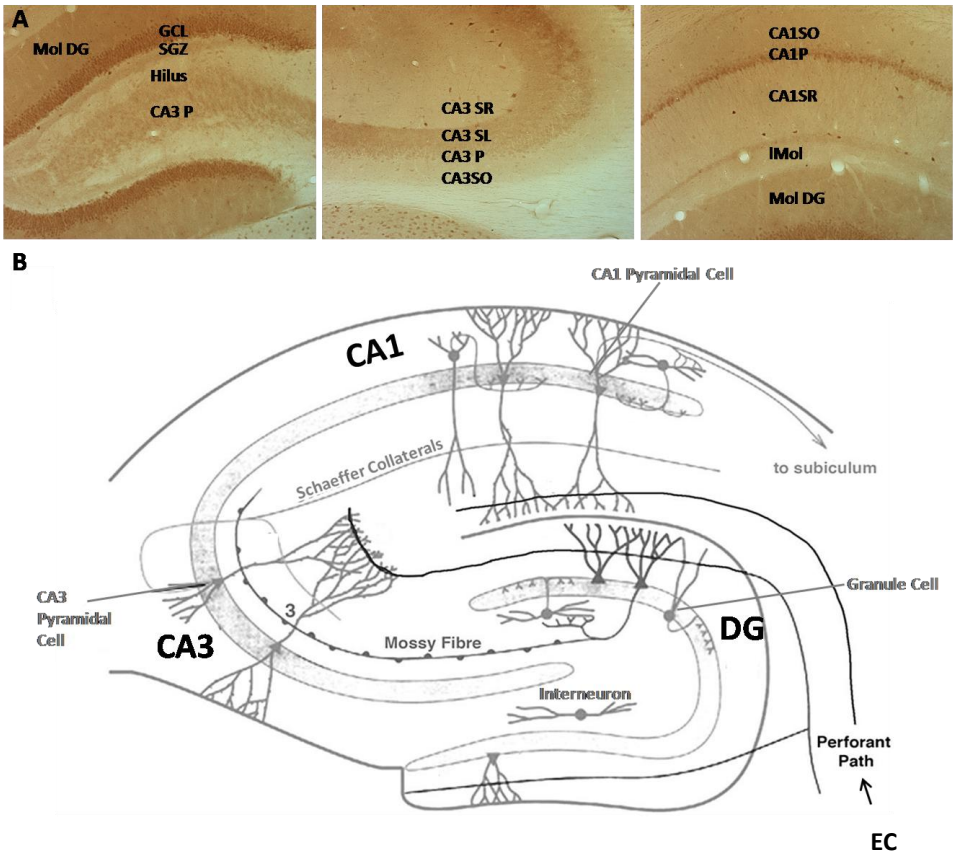

Figure 1-2. Anatomy of the rat hippocampus and related circuitry. **A)** Schematic representation of the rat hippocampus depicting the laminar organization of the DG, CA1 and CA3. The DG is divided into three layers: the stratum molecular layer (Mol DG), dentate granule cell layer (GCL), and the polymorphic layer or hilus. The molecular layer consists of granule cell dendrites. The dentate granule cells constitute the next layer. The polymorphic layer lies between the upper and lower blades of the granule cell layer and comprises a number of cell types. The principle cell type in the hippocampus is the pyramidal cell and the cell bodies of these neurons form the stratum pyramidale in CA1 (CA1P), CA2 (not shown) and CA3 (CA3P). Just below the pyramidal cell layer is the stratum oriens. In CA3, there is an additional stratum referred to as the stratum lucidum (CA3SL), located between the pyramidal cell layer and the stratum radiatum (CA3SR). Adjacent to the stratum lucidum is the stratum radiatum (CA1SR and CA3SR) and stratum lacunosum-moleculare (lMol). The stratum radiatum contains Shaeffer collateral fibers which are the main projection forward from CA3 to CA1.The stratum lacunosum-moleculare is the region where perforant path fibers form synapses onto the apical dendrites of pyramidal cells (for review see Amaral et al., 2006 and Lopes da Silva et al., 1990). **B)** Depicts the intrahippocampal circuitry and classic tri-synaptic circuit (for review see Amaral et al., 2006). Neurons in the entorhinal cortex (not shown) give rise to axons that project to the DG, forming the major hippocampal input pathway called the perforant path. Granule cells of the DG give rise to mossy fiber axons that synapse onto the somata of pyramidal cells in CA3. The Shaeffer collateral axons of CA3 pyramidal cells in turn provide the major input to CA1. The axons of CA1 pyramidal cells then project to the subiculum and EC providing the major excitatory output for the hippocampus. Figure adapted from (Daumas, Ceccom, Halley, Frances, & Lassalle, 2009).

Hippocampus Proper

The principal cell layer in the CA subfields of the hippocampus proper is the stratum pyramidale, housing the cell bodies of pyramidal neurons (i.e., the principal excitatory neurons in the hippocampus). Pyramidal cells typically have large apical dendrites, along with several shorter basal dendrites and a single axon. These basal and apical dendrites terminate in different strata on either side of the pyramidal cell layer, with basal dendrites extending into the stratum oriens and apical dendrites branching extensively throughout the remaining strata. The stratum pyramidale also contains cell bodies of many interneurons sub-types that project to surrounding pyramidal cells and interneurons in the CA sub-fields. Just below the pyramidal cell layer is the stratum oriens, which contains the cell bodies of inhibitory interneurons innervating all lamina of the CA sub-field, along with the basal dendrites of pyramidal cells. The CA3 sub-field also contains one of the thinnest strata in the hippocampus known as the stratum lucidum, which serves as the major site for mossy fiber innervation from the DG. Adjacent to the stratum lucidum, and above the pyramidal layers of CA2 and CA1, are the stratum radiatum and stratum lacunosum-moleculare. The stratum radiatum contains Shaeffer collateral fibers which are the main projection forward from CA3 to CA1. This stratum also contains a number of interneuron sub-types that project to adjacent excitatory and inhibitory neurons. The stratum lacunosum-moleculare is the most superficial stratum in the hippocampus. It is in this stratum where perforant path fibers form synapses onto distal regions of the apical dendrites on pyramidal cells (Amaral & Witter, 1989; Lopes da Silva et al., 1990). Inhibitory GABAergic interneurons are mainly distributed basal and apical to the pyramidal cell layer in stratum oriens, stratum radiatum and stratum lacunosum-moleculare. These local inhibitory interneurons function to shape the activity of excitatory neurons through a complex array of feedback and feedforward inhibitory mechanisms (for review see Freund & Buzsaki, 1996).

Dentate Gyrus

The DG is the primary target of cortical input to the hippocampal formation, and has three layers: the polymorphic layer (or hilus), the stratum granulosum (or granule cell layer), and the stratum moleculare (Lopes da Silva et al., 1990). A small region of the hilus, approximately two cell widths below the granule cell layer (GCL), can be further differentiated as the subgranular zone (SGZ), one of two sites in the mamalian brain where neurogenesis takes place. The principal cell layer of the DG is the GCL. This layer is comprised of densely packed

excitatory granule cells, whose apical dendritic processes extend into the molecular layer and axons enter the hilar region. The hilus comprises a variety of neurons that include excitatory mossy cells and inhibitory interneurons. The most extensively studied interneurons in the hilus are the pyramidal basket cells, located along the deep surface of the granule cell layer (or the SGZ). These basket cells have a single apical dendrite that extends into the molecular layer, several principal basal dendrites that ramify in the hilus, and axons that synapse with cell bodies of granule cells. The hilus also contains several other inhibitory interneurons whose processes innervate distinctive regions of both excitatory and inhibitory cells in the DG, depending on the subtype (Freund and Buzsaki, 1996). The molecular layer of the DG is primarily occupied by dendritic processes of granule, basket, and hilar cells as well as axons from the EC and other sources; the cell bodies of inhibitory interneurons innervating granule cells and other inhibitory cells can also be found in this region. Similar to the hippocampus proper, the function of excitatory granule and mossy cells in the DG is modified by a network of local interneurons that shape their activity and functioning of the DG (Amaral & Witter, 1989; Freund & Buzsaki, 1996; Lopes da Silva et al., 1990). Given that the DG is situated in a critical position to modulate incoming information to the hippocampus, dysfunction of the DG can significantly impact the entire hippocampal network.

6. Assessing the Validity of Animal Models of Depression

Although a large body of clinical evidence suggests a link between chronic stress and depressive illness, these data are correlational and subject to variability. Furthermore, it has been difficult to determine whether the high cortisol levels seen in depressed patients are causal to, or are a by-product of depression. Therefore, an important question that remains unanswered is whether or not high levels of CORT can actually cause depression and if so, by what mechanisms. Preclinical animal models provide an efficient way to study the etiology of human disorders such as depression by allowing for strict control over stressor manipulation and subject homogeneity. However, it can be difficult to study "depression" in rodents. One challenge is selecting qualitative measures in rats that are indicative of human depression. Indeed, symptoms of human depression are often subjective and variable, and can also be contradictory (i.e., one patient shows psychomotor agitation whereas another shows psychomotor retardation). Moreover, some symptoms simply cannot be measured in laboratory animals (i.e., suicidal

ideation). However, if animal models are to be useful there must be a means of evaluating the degree of commonality they possess with the clinical disorder, that is, the model must comprise a set of established criteria for validity. Therefore, specific criteria have been developed to include the following considerations: 1) that the precipitating factors for developing the disorder are present, there are behavioral and motivational similarities with the clinical disorder, and there are common neurobiological changes (i.e., they have good face validity), and 2) the model shows a response to therapeutic intervention similar to that seen in patient populations (i.e., they have good predictive validity; (McKinney, Jr. & Bunney, Jr., 1969). Willner (2005) further suggests that animal models should be developed so that a rational theory can be constructed to explain and test theories regarding the etiology of the disorder (i.e., they have good construct validity).

Numerous animal models have been developed for the purpose of studying depressive symptomatology; however, the decision on which to choose is sometimes difficult, and can depend on what aspect of the disorder you are trying to examine. For example, animal assays (e.g., olfactory bulbectomy, forced swim test) have good predictive value and are often used in the pharmaceutical industry to screen potential drug treatments for depression. However, these are not really "models" of depression because they have little face or construct validity and are not well suited for understanding the etiology of depression (Newport, Stowe, & Nemeroff, 2002). Conversely, more homologous models, predicated on the belief that certain aspects of animal behavior and physiology emulate that of humans, have predictive, face, and construct validity, and thus may be better suited for examining antecedents of depressive symptomatology (Newport et al., 2002). Therefore, it is important to remember that models of depression can serve many different purposes and their inherent value lies in the specific aim of the individual model.

7. Experimenter-Applied Stress Models of Depression

A number of experimenter-applied animal models have been developed in an attempt to determine the influence of stress on the development of depression, of which repeated restraint stress and chronic mild stress (CMS) are two of the most widely used (Kim & Han, 2006). In general, repeated restraint stress has been very helpful for understanding stress-induced remodeling of specific brain regions, as well as some of the cognitive deficits associated with depression (Beck & Luine, 2002; Bowman, Beck, & Luine, 2003; Conrad, Grote, Hobbs, &

Ferayorni, 2003; Luine, Martinez, Villegas, Magarinos, & McEwen, 1996; Luine, Villegas, Martinez, & McEwen, 1994; Pham, Nacher, Hof, & McEwen, 2003; Vyas, Bernal, & Chattarji, 2003; Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Watanabe, Gould, & McEwen, 1992); however, its ability to reliably produce behaviors associated with the disorder has been questioned. For example, although there is some evidence to suggest that repeated restraint stress produces changes in emotionality related to increased anxiety and aggression (Beck & Luine, 2002; Conrad, LeDoux, Magarinos, & McEwen, 1999; Sandi, Merino, Cordero, Touyarot, & Venero, 2001; Wood, Young, Reagan, & McEwen, 2003), there are also reports suggesting no effect of repeated restraint on anxiety (Gregus, Wintink, Davis, & Kalynchuk, 2005; Perrot-Sinal, Gregus, Boudreau, & Kalynchuk, 2004). There are also mixed findings with regard to the effect of restraint stress on depression-like behavior, ranging from increased immobility in the forced swim test (FST) and decreased sucrose preference (Bravo et al., 2009; Joo et al., 2009; Regenthal, Koch, Kohler, Preiss, & Krugel, 2009; Veena, Srikumar, Raju, & Shankaranarayana Rao, 2009), to no effect on FST behavior (Gregus et al., 2005; Lussier, Caruncho, & Kalynchuk, 2009; Perrot-Sinal et al., 2004), to even decreased depressive behaviors (Dunn & Swiergiel, 2008; Platt & Stone, 1982). Similarly, conflicting behavioral results have been reported after CMS, although the difficulty in implementing the model may be partially responsible for these findings, as the ability of CMS to produce depressive-like behaviors is better established than that of repeated restraint stress (Willner, 1997; Willner, 2005). Nevertheless, the CMS procedure tends to be quite labor intensive, demanding of space, and is often long in duration (Kim & Han, 2006; Willner, 1997; Willner, 2005). The procedure itself can also be quite variable, which may further account for inconsistencies across laboratories (Willner, 2005).

8. Exogenous CORT Administration as a Preclinical Model of Depression

Essentially, one of the biggest problems with experimenter-applied stress models such as repeated restraint stress and CMS is a lack of control over individual differences in responsivity to physical and psychological stressors. Stressful stimuli can differ in their physical qualities (i.e., actual qualities) and in terms of their psychological qualities (i.e., perceived qualities). This may result in differing CORT levels between different rats exposed to the same stressor, which in turn could lead to increased experimental variability. Furthermore, many animals habituate to the aversive effects of these stressors when they are repeatedly exposed to them (Galea et al.,

1997; Gregus et al., 2005; Grissom, Iyer, Vining, & Bhatnagar, 2007). This might explain why these paradigms have produced conflicting behavioral results. Depending on the question being asked, these issues can be advantageous in the sense that they may provide excellent paradigms for studying issues related to individual differences in habituation, resiliency, and susceptibility. However, in situations where the goal is to produce more robust and reliable behavioral symptomatology, it is desirable to avoid these individual differences. To that end, an animal model of depression has been developed whereby exogenous CORT is administered over a period of weeks to months either by subcutaneous injection, pellet implantation, osmotic pump, or passively in drinking water or food, allowing for more stringent control over hormone levels. An advantage of this model is that it allows experimenters to examine the direct influence of glucocorticoids on the development of depressive symptomatology.

8.1 Influence of Repeated CORT Administration on Depression and Anxiety-Like Behaviors

Although it is impossible to examine all symptoms of depression manifest in patient populations, a wide range of behavioral measures have been used in an attempt to capture "depression" in rodents (e.g., weight loss, memory impairments, sleep disturbances, open field exploration, anhedonia, and helpless behaviors), of which anhedonia and learned helplessness are the most frequently used. Anhedonia, or the lack of interest in pleasurable acts, is typically inferred by measuring an animal's preference for a sucrose solution over a plain water solution. Normal rats prefer the sucrose solution, so a decrease in sucrose preference is thought to be indicative of depression. Importantly, antidepressant drugs increase preference for sucrose (Willner, 1997). Learned helplessness can be assessed in rats in several ways, but the most common method involves the use of the FST. In this test, rats are forced to swim in a tank of water with no possibility of escape. Increases in passive behavior such as immobility and decreases in active behaviors such as swimming or climbing are indicative of depression-like behavior (Lucki, 1997). The FST is thought to be a valid test for depression because all forms of antidepressant treatment, including typical and atypical antidepressants, rapid eye movement (REM) sleep deprivation, and electroconvulsive therapy (ECT) all decrease immobility in the FST, whereas compounds that increase depression in humans increase immobility in the forced swim test (Armario, Gavalda, & Marti, 1988; Porsolt, Anton, Blavet, & Jalfre, 1978). In fact, there is a significant correlation

between the clinical potency of antidepressants in humans and their potency in the FST that is not found with any other measure of depression (Borsini & Meli, 1988; Porsolt, Le, & Jalfre, 1977).

An extensive body of literature indicates that exogenous CORT administration produces reliable and robust changes in a variety of behaviors that could be considered symptomatic of depression (See Table 1-1). Specifically, rodents administered CORT via injection, pellet implantation, or drinking water (Brummelte, Pawluski, & Galea, 2006; David et al., 2009; Gourley, Kiraly, Howell, Olausson, & Taylor, 2008; Gregus et al., 2005; Hill, Brotto, Lee, & Gorzalka, 2003; Johnson, Fournier, & Kalynchuk, 2006; Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004; Marks, Fournier, & Kalynchuk, 2009; Murray, Smith, & Hutson, 2008; Zhao et al., 2008b,c), show a decrease in latency to immobility, an increase in time spent immobile, and a decrease in time spent swimming in the FST - all measures typically used to infer a depressive phenotype (Cryan, Valentino, & Lucki, 2005). Importantly, our laboratory (Johnson et al., 2006), as well as others (Zhao et al., 2008b,c), have shown that the effect of CORT on depression-like behavior is both dose and time dependent. For example, we have demonstrated that a daily injection of 10, 20 or 40 mg/kg CORT for 21 consecutive days produces a graded increase in depression-like behavior on the FST. Specifically, rats injected with 40 mg/kg CORT spend significantly more time immobile and less time climbing and have a shorter latency to immobility compared to vehicle rats. Those receiving 20mg/kg also have a shorter latency to immobility; however, rats receiving 10 mg/kg of CORT show no significant increases in depression-like behavior on any measures in the FST. Moreover, a single injection of CORT has no effect on FST behavior (Gregus et al., 2005), and in some cases, smaller doses given over a short period of time have been shown to decrease depression-like behavior in the FST (Stone & Lin, 2008). Collectively, these findings confirm that the amount of CORT administered and the duration of CORT treatment are critical determinants for the expression of a depressive phenotype.

Importantly, the depressogenic effects of CORT go beyond behavior on the FST. For example, rats subjected to various repeated CORT administration paradigms often show inhibited sexual behavior (Gorzalka, Brotto, & Hong, 1999; Gorzalka & Hanson, 1998; Gorzalka, Hanson, & Hong, 2001; Hanson & Gorzalka, 1999), decreased sucrose intake (David et al., 2009; Gorzalka, Hanson, Harrington, Killam, & Campbell-Meiklejohn, 2003; Gourley et al., 2008a), decreased responding for food reinforcement (Gourley et al., 2008 a,b) and decreased grooming behavior (David et al., 2009), all of which are indicative of anhedonia. There is also evidence to suggest that

Table 1-1. Similarities Between the Consequences of Exogenous CORT Administration and the Human Manifestation of Depression

Symptom Category	Human Manifestation	Reference	Animal Manifestation	CORT dose/duration	Admin. Method	Species/Sex	Reference
Depression	Depressed mood, decreased energy, feelings of worthlessness and helplessness	Buchwald & Rudick-Davis (1993) Nemeroff (1998) Nestler et al. (2002a)	Increased Immobility	40mg/kg; 26 days 40mg/kg; 21 days 20mg/kg; 20 days 10-40mg/kg 21 days 40mg/kg; 21 days 40mg/kg; 21 days 20mg/kg; 3 and 5 weeks 35µg/ml; 4 weeks 25 or 100µ/ml; 14 days 40mg/kg; 7-28 days	s.c. injection s.c. injection s.c. injection s.c. injection s.c. injection s.c. injection s.c. injection drinking water drinking water s.c. pellet implant	SD rat; F dams LE rat; M LE rat; M&F LE rat; M LE rat; M&F LE rat; M C57BL/6 N mice; M C57BL/6Ntac mice; M C57BL/6 mice; M CD1 mice; M	Brummelte et al. (2006) Gregus et al. (2005) Hill et al. (2003) Johnson et al. (2006) Kalynchuk et al. (2004) Marks et al. (2009) Zhao et al. (2008b,c) David et al. (2009) Gourley et al. (2008a) Murray et al. (2008)
	Anhedonia (i.e., lack of interest in pleasurable things)	Buchwald & Rudick-Davis (1993) Nemeroff (1998) Nestler et al. (2002a)	Decreased Sucrose Preference Decreased Reward Responding Decreased Sexual Behavior Decreased Grooming	50mg/kg; 14 days 35µg/ml; 4 weeks 25µg/ml; 14 days 25µg/ml; 14 days 20mg/kg; 14 days 20mg/kg; 10 days 50mg/kg; 10 days 35µg/ml; 4 weeks	s.c. injection drinking water drinking water drinking water s.c. injection s.c. injection s.c. injection drinking water	LE rat; M C57BL/6Ntac mice; M C57BL/6 mice; M C57BL/6 mice; M LE rat; M LE rat; M LE rat; M C57BL/6Ntac mice; M	Gorzalka et al. (2003) David et al. (2009) Gourley et al. (2008a) Gourley et al. (2008a,b) Gorzalka et al. (1999) Gorzalka et al. (2001) Gorzalka & Hanson (1998) David et al. (2009)
	Alterations in Weight	Buchwald & Rudick-Davis (1993) Nestler et al. (2002a)	Decreased Weight Gain	40mg/kg; 26 days 26.8mg/kg; 21 or 56 days 40mg/kg; 21 days 40mg/kg; 21 days 25mg/kg; 4 weeks 35µg/ml; 4 weeks 100mg/65.2mg; 7 days 35% pellet implant	s.c. injection s.c. injection s.c. injection s.c. injection s.c. injection drinking water s.c. pellet implant s.c. pellet implant	SD rat; F dams SD rat; M LE rat; M LE rat; M W rat; M C57BL/6Ntac mice; M LH rat; M W rat; M	Brummelte et al. (2006) Coburn-Litvak et al. (2003) Gregus et al. (2005) Johnson et al. (2006) Pego et al. (2008) David et al. (2009) Bush et al. (2003) Meijer et al. (1997)
Anxiety	Co-morbid Symptoms of Anxiety	Hirschfeld (2001)	Increased Defensive Behavior Decreased Rime in Open Arms of EPM Increased Escape	40mg/kg; 26 days 40mg/kg; 19 days 25mg/kg; 4 weeks 15 or 30µg; 5 days 30µg; 7 days 4-35mg/kg; 3 days	s.c. injection s.c. injection s.c. injection s.c. pellet implant s.c. pellet implant N/A	LE rat; M SD rat; M W rat; M Fisher 344 rat; M W rat; M N/A	Kalynchuk et al. (2004) Lee et al. (2009) Pego et al. (2008) Myers et al. (2007) Shepard et al. (2000) Stone et al. (1988)

			Behavior				
			Increased Freezing in CFC task	5 or 20mg/kg; 25 days 5mg/kg; 5.5 days 400µg/ml; 21 days	s.c. injection s.c. injection drinking water	W rat; M SD rat; M SD rat; M	Skorzewska et al. (2006) Thompson et al. (2004) Conrad et al. (2004)
			Decreased Time in Center of OFT	5 or 20mg/kg; 25 days 35µg/ml; 4 weeks	s.c. injection drinking water	W rat; M C57BL/6Ntac mice; M	Skorzewska et al. (2006) David et al. (2009)
			Increased Latency to Feed in the NSFT	35µg/ml; 4 weeks	drinking water	C57BL/6Ntac mice; M	David et al. (2009)
			Decreased Time in Light Side of LDB	40mg/kg; 21-28 days	s.c. pellet implant	CD1 mice; M	Murray et al. (2008)
Cognition & Memory	Impaired Spatial Working Memory and Executive Function	Austin et al. (2001) Castaneda et al. (2008) Fleming et al. (2004) Taylor Tavares et al. (2007) Veiel (1997) Zakzanis et al. (1998)	Decreased Time in Novel Arm of Y-Maze	26.8mg/kg; 56 days	s.c. injection	SD rat; M	Coburn-Litvak et al. (2003)
			Increased Errors on the Barnes Maze	26.8mg/kg; 21 days 5mg/kg; 21 days 5mg/kg; 21 days	s.c. injection s.c. injection s.c. injection	SD rat; M W rat; M N/A	Coburn-Litvak et al. (2003) Tromofiuk & Braszko (2008) Walesiuk & Braszko (2009)
			Decreased Place Learing/Increased Latency to find platform on MWM	40mg/kg; 4 weeks 5mg/kg; 21 days 27-43µl/dl; 3 months 150mg; 80 days	s.c. injection s.c. injection s.c. pellet implant s.c. pellet implant	W rat; M W rat; M LE rat; M Fisher 344xBN rat; M	Sousa et al. (2000) Walesiuk & Braszko (2009) Bodnoff et al. (1995) McLay et al. (1998)
			Decreased Reversal Learning in MWM	25mg/kg; 4 weeks	s.c. injection	W rat; M	Cerqueira et al. (2005)
			Impaired Acquisition/Increased Errors on the RAM	1mg/0.2ml; 40days 3,300 mg; 21 day release 600mg; 9 weeks	s.c. injection s.c. pellet implant s.c. pellet implant	C57BL/6CrSlc mice; M Fisher 344 rat; M Fisher 344 rat; M	He et al. (2008) Arbel et al. (1994) Dachir et al. (1993, 1997)
			Impaired Object Recognition	300mg; 12 weeks 5mh/kg; 21 days	s.c. pellet implant s.c. injection	WI rat; M W rat; M	Endo et al. (1996) Walesiuk et al. (2005)
Structural Abnormalities	Decreased Hippocampal Volume	Caetano et al. (2004) Drevets (2000, 2001) Frodl et al. (2004, 2006) Jaracz (2008) Konarski et al. (2008) Lorenzetti et al. (2009) MacQueen et al.	Decreased Neurogenesis/Cell Proliferation	40mg/kg; 21 days 40mg/kg; 5 days 10mg/kg; 21days 35µg/ml; 4 weeks 75mg implant 30% implant 5mg; 14 days	s.c. injection s.c. injection s.c. injection drinking water s.c. pellet implant s.c. pellet implant s.c. pellet implant	W rat; M LH rat; M W rat; M C57BL/6Ntac mice; M LH & SD rat; M LH rat; M CD1 mice; M	Hellesten et al. (2002) Karishma & Herbert (2002) Mayer et al. (2006) David et al. (2009) Alahmed & Herbert (2008) Huang & Herbert (2006) Murray et al. (2008)
			Cell Death	10mg/kg; 12 weeks 5mg/kg; 3 months 10-40mg/kg; 30-180 days	s.c. injection s.c. injection s.c. injection	LE rat; M Fisher 344 rat; M W rat; M	Clark et al. (1995) Sapolsky et al. (1985) Sousa et al. (1998)

		(2003) Sheline et al. (2003)	Dendritic Remodeling Decreased Mossy Fiber Synapses	40mg/kg; 4 weeks 40mg/kg; 21 days 10mg/kg; 21 days 400µg/ml; 21 days 400µg/ml; 5 weeks 40mg/kg; 4 weeks 40mg/kg; 60 days	s.c. injection s.c. injection s.c. injection drinking water drinking water s.c. injection s.c. injection	W rat; M SD rat; M SD rat; M SD rat; M SD rat; M W rat; M SD rat; M	Sousa et al. (2000) Watanabe et al. (1992a) Woolley et al. (1990) Magarinos et al. (1998) Magarinos et al. (1999) Sousa et al. (2000) Tata et al. (2006)
	Decreased PFC Volume	Drevets (2000, 2001) Jaracz (2008) Konarski et al. (2008) Lorenzetti et al. (2009)	Dendritic Remodeling Decreased Glial Cells	25mg/kg; 4 weeks 40mg/kg 3 weeks 40mg/kg; 3 weeks 10mg/kg; 15 days	s.c. injection s.c. injection s.c. injection s.c. injection	W rat; M SD rat; M SD rat; M SD rat; M	Cerqueira et al. (2005, 2007) Seib & Wellman (2003) Wellman (2001) Alonso (2000)
	Increased Amygdala Volume	Konarski et al. (2008)	Dendritic Hypertrophy	10mg/kg; 1 or 10 days	s.c. injection	W rat; M	Mitra & Sapolsky (2008)
Molecular Abnormalities	Decreased BDNF	Dwivedi et al. (2003) Karege et al. (2002, 2005) Sen et al. (2008) Shimzu et al. (2003)	Decreased BDNF	32mg/kg; 21 days 40mg/2ml/kg; 10 days 100mg; 21 days	s.c. injection s.c. injection pellet implant	W rat; M W rat; M SD rat; M	Jacobsen & Mork (2006) Nitta et al. (1997) Dwivedi et al. (2006)
	Decreased CREB	Dowlatshahi et al. (1998)	Decreased CREB	25 or 100 µg/ml; 14 days	drinking water	C57BL/6Ntac mice; M	Gourley et al. (2008a)
	Decreased Reelin	Fatemi et al. (2000) Knable et al. (2004)	Decreased Reelin	40mg/kg; 21 days	s.c. injection	LE rat; M	Lussier et al. (2009)

NOTE: CFC: contextual fear conditioning; EPM: elevated plus maze; FST: forced swim test; LDB: light/dark box; MWM: Morris Water Maze; NSFT: novelty suppressed feeding test; OFT: open field test; RAM: radial arm maze; BN: Brown Norway; LE: Long Evans; LH: Lister Hooded; SD: Sprague Dawley; W: Wistar; WI: Wistar-Imamichi; M: male; F: female; s.c.: subcutaneous

repeated administration of exogenous CORT produces anxiety-like behaviors in a variety of tasks including the elevated plus maze (Lee, Shim, Lee, Yang, & Hahm, 2009; Myers & Greenwood-Van, 2007; Pego et al., 2008; Shepard, Barron, & Myers, 2000), the light/dark box (Murray et al., 2008), the predator odor test (Kalynchuk et al., 2004), tests of escape behavior (Stone, Egawa, & McEwen, 1988), the novelty suppressed feeding test, and the open field test (David et al., 2009; Skorzewska et al., 2006). The manifestation of pure depression without any symptoms of anxiety occurs very infrequently in patient populations (Breslau, Schultz, & Peterson, 1995; Mineka, Watson, & Clark, 1998), so the development of anxiety-like behaviors in this model is highly plausible, and attests to the face validity of the model. Physiologically CORT administration also produces a variety of changes indicative of depression, including decreased weight gain (Barr, Brotto, & Phillips, 2000; Brummelte et al., 2006; Bush, Middlemiss, Marsden, & Fone, 2003; Coburn-Litvak, Pothakos, Tata, McCloskey, & Anderson, 2003; Gregus et al., 2005; Johnson et al., 2006; Magarinos, Orchinik, & McEwen, 1998; Meijer, Van Oosten, & de Kloet, 1997; Pego et al., 2008), dysregulated HPA axis function (Johnson et al., 2006), and decreased adrenal weight (Bush et al., 2003; Meijer et al., 1997; Murray et al., 2008). Importantly, many of the behavioral changes associated with repeated CORT administration can be reversed with antidepressant treatment (David et al., 2009), supporting the predictive validity of CORT administration as a preclinical model of human depression.

8.2 Influence of Repeated CORT Administration on Cognition

As discussed previously, depression is often associated with impaired cognition (Buchwald & Rudick-Davis, 1993), with the largest deficits seen in verbal and spatial working memory and executive function. These are typically forms of memory associated with hippocampal, amygdalar and/or PFC functioning (Austin, Mitchell, & Goodwin, 2001; Castaneda, Tuulio-Henriksson, Marttunen, Suvisaari, & Lonnqvist, 2008; Fleming, Blasey, & Schatzberg, 2004; Taylor Tavares et al., 2007; Veiel, 1997; Zakzanis, Leach, & Kaplan, 1998). Interestingly, several lines of evidence also indicate that exposure to stress or stress hormones impair hippocampal-dependent forms of memory in humans (Lupien & McEwen, 1997). For example, Vietnam combat veterans diagnosed with post-traumatic stress disorder (PTSD) exhibit marked deficits in verbal recall tasks when compared with other military enlistees not diagnosed with PTSD (Bremner et al., 1993; Vasterling, Brailey, Constans, & Sutker, 1998). In healthy

subjects, administration of high doses of cortisol selectively impairs verbal declarative memory, without affecting nonverbal (non-hippocampal) memory (Newcomer et al., 1999). Moreover, certain depressed patients with chronic hypercortisolaemia, or individuals with Cushing's disease exhibit impairments in declarative memory (Sapolsky, 2000; Starkman, Gebarski, Berent, & Schteingart, 1992).

Consistent with human studies, rodents exposed to stress or repeatedly administered CORT exhibit deficits in various tasks thought to reflect spatial abilities and executive function (Lupien & McEwen, 1997; Sapolsky, 2000). For instance, rats repeatedly exposed to CORT show decreased spontaneous alternation on a T-maze (Bardgett, Taylor, Csernansky, Newcomer, & Nock, 1994), impaired object recognition (Walesiuk, Trofimiuk, & Braszko, 2005) and impaired acquisition of a radial arm maze task (Arbel, Kadar, Silbermann, & Levy, 1994; Dachir, Kadar, Robinzon, & Levy, 1993; Dachir, Schmidt, & Levy, 1997; Endo, Nishimura, & Kimura, 1996; He et al., 2008; Walesiuk et al., 2005). Moreover, using the Y-maze, deficits in spatial working memory have been found after 56 days but not after 21 days of CORT (26.8mg/kg) administration (Coburn-Litvak et al., 2003). Conversely, deficits in spatial reference memory can be seen in the Barnes maze after only 21 days of CORT administration at the same dose (Coburn-Litvak et al., 2003; Trofimiuk & Braszko, 2008; Walesiuk & Braszko, 2009). Using the Morris Water Maze (MWM), Sousa et al. (2000) found similar results, whereby rats treated with CORT (40mg/kg) for one month showed deficits in place learning but not repeated acquisition in the MWM, indicating deficits in spatial and reference memory but not working memory. Importantly, these results suggest that, similar to depression-like behavior, stress-induced cognitive impairments may be dose and time dependent. Corroborating this assumption, previous reports of spatial learning deficits have been found after CORT treatment for 3 months (Bodnoff et al., 1995), and 80 days (McLay, Freeman, & Zadina, 1998), but not after 15 days (Hebda-Bauer, Morano, & Therrien, 1999). Repeated CORT administration is also known to decrease behavioral flexibility in reversal learning tasks, indicative of impaired PFC-mediated executive function (Cerqueira et al., 2005). However, it is important to note that CORT does not appear to impair all hippocampal-dependent memory tasks. For example, recent studies have showed that repeated CORT administration enhances hippocampal-dependent aversive tasks such as contextual fear conditioning (Conrad et al., 2004; Gourley, Kedves, Olausson, & Taylor, 2009; Skorzewska et al., 2006; Thompson, Erickson, Schulkin, & Rosen, 2004). Similarly,

cortisol treatment in humans can also enhance long-term recall of emotionally arousing (but not neutral) pictures (Buchanan & Lovallo, 2001), suggesting that stress produces differential effects on different memory systems. It may be that memories for emotional stimuli are enhanced by stress, partly due to enhanced amygdala function and increased anxiety, whereas memories for contextual information or executive function are impaired, due to impaired hippocampal and PFC functioning after stress. This is speculative at this point and requires more study, particularly to better illuminate the facilitative effect of CORT on fear conditioning. What is clear from reviewing these findings is that many of the CORT-induced cognitive changes described here have been found in other preclinical models of depression (Alonso, 2000; Henningsen et al., 2009; Lupien, McEwen, Gunnar, & Heim, 2009; Mitra & Sapolsky, 2008; Murray et al., 2008; Rodrigues, LeDoux, & Sapolsky, 2009; Wood, Norris, Waters, Stoldt, & McEwen, 2008; Woolley, Gould, & McEwen, 1990), and are similar to what is seen in patient populations (Austin et al., 2001; Castaneda et al., 2008; Fleming et al., 2004; Taylor Tavares et al., 2007; Veiel, 1997; Zakzanis et al., 1998), providing empirical support for the utility of this model for studying depressive symptomatology.

8.3 Neurobiological Changes Associated with CORT Administration and Human Depression

Effects of CORT on Structural Plasticity

Prolonged exposure to stress induces a considerable degree of structural plasticity in the adult brain, especially in the limbic system, where these changes are accompanied by impairments in learning and memory (McEwen, 2000; McEwen, 2007; Southwick et al., 2005). Importantly, these neurobiological changes are thought to underlie depressive symptomatology, and have been reliably characterized using exogenous CORT administration. For example, several groups have identified CORT-induced dendritic remodeling in the hippocampus (Magarinos, Deslandes, & McEwen, 1999; Magarinos et al., 1998; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000; Southwick et al., 2005; Watanabe, Gould, Daniels, Cameron, & McEwen, 1992; Woolley et al., 1990), amygdala (Mitra & Sapolsky, 2008), and medial PFC (Cerqueira et al., 2005; Cerqueira, Taipa, Uylings, Almeida, & Sousa, 2007; Seib & Wellman, 2003; Wellman, 2001), reminiscent of what has been documented in post-mortem tissue of patients with depression (Konarski et al., 2008). Specifically, it is well known that prolonged

administration of CORT induces extensive dendritic remodeling in hippocampal pyramidal cells, with significant volume loss of CA3 and CA1 apical dendrites (Magarinos et al., 1999; Magarinos et al., 1998; Sousa et al., 2000; Sousa, Madeira, & Paula-Barbosa, 1998; Woolley et al., 1990) and a loss of mossy fiber CA3 synapses (Sousa et al., 2000; Tata, Marciano, & Anderson, 2006). Although these effects are often reversible upon cessation of the stressor (Pittenger & Duman, 2008; Sousa et al., 2000; Sousa et al., 1998), a high-dose and/or prolonged administration can result in cell death (Sapolsky, Krey, & McEwen, 1985; Sousa et al., 1998). Similarly, atrophic changes in the mPFC occur after repeated CORT administration, evidenced by extensive reorganization of apical arbors of layer II-III neurons (Cerqueira et al., 2005; Cerqueira et al., 2007; Seib & Wellman, 2003; Wellman, 2001), and reduced proliferation of glia and endothelial cells (Alonso, 2000). However, in contrast to what is seen in the hippocampus and PFC, repeated CORT treatment causes hypertrophy of amygdalar neurons (Mitra & Sapolsky, 2008). As mentioned above, prolonged CORT treatment impairs hippocampal-dependent function (Bardgett et al., 1994; Cerqueira et al., 2005; Coburn Litvak et al., 2003; Dachir et al., 1993; McLay et al., 1998; Sousa et al., 2000) and enhances amygdala-dependent unlearned fear and fear conditioning (Gourley et al., 2009; Skorzewska et al., 2006), which are consistent with the opposite effects of stress on hippocampal and amygdala cell morphology. This suggests that stress-induced alterations in morphology and function may be region and circuit dependent (Pittenger & Duman, 2008).

Another form of structural plasticity associated with depression is that of reduced cell proliferation and neurogenesis in the hippocampus. Neurogenesis can be described as the process by which new neurons are generated from neural stem cells and progenitor cells in the brain. Historically it was believed that we were born with all of the neurons we would have for the rest of our lives, and that new neurons could not be generated in the adult brain. However, in the 1960's it was discovered that new neurons are in fact born in the olfactory bulb and dentate gyrus in young and adult rodents (Altman, 1962; Altman, 1969: Altman & Das, 1965). A more compelling argument for this came roughly 30 years later when Eriksson and colleagues showed that neurogenesis also occurs in the adult human brain (Eriksson et al., 1998). Since this time, a wealth of research has focused on the functional role of these newborn neurons in learning, memory, and pathology. One of the primary catalysts for focusing on neurogenesis in depression comes from the observation that all known antidepressants, including antidepressant medication,

electroconvulsive shock, and physical exercise seem to increase hippocampal cell proliferation (Malberg, Eisch, Nestler, & Duman, 2000; Scott, Wojtowicz, & Burnham, 2000; van, Kempermann, & Gage, 1999). Magnetic resonance imaging (MRI) studies also indicate that in depression and Cushing's disease, the duration of illness and not the age of onset, predicts a progressive reduction in hippocampal volume (Sheline, Sanghavi, Mintun, & Gado, 1999; Starkman, Gebarski, Berent, & Schteingart, 1992). Importantly, many preclinical models of depression, including repeated CORT administration, have a profound effect on hippocampal neurogenesis, causing rapid and robust reductions in the proliferation and survival of newborn neurons in adult brain (Alahmed & Herbert, 2008; David et al., 2009; Fuchs & Gould, 2000; Hellsten et al., 2002; Huang & Herbert, 2006; Karishma & Herbert, 2002; Mayer et al., 2006; Murray et al., 2008; Pham et al., 2003; Sapolsky, 2004; Sheline et al., 1999; Starkman et al., 1992). These findings are particularly important as one of the most prominent current hypotheses regarding the etiology of depression is that chronic stress evokes pathological plasticity within the hippocampus, leading to decreased neurogenesis and eventually depressive symptomatology (Duman, Malberg, Nakagawa, & D'Sa, 2000; Jacobs, Praag, & Gage, 2000). However, opponents of the neurogenesis hypothesis correctly point out that there is no direct evidence that decreased neurogenesis causes human depression. In fact, recent post-mortem evidence has challenged this hypothesis, showing no significant decreases in hippocampal cell proliferation and no cell loss in post-mortem tissue from human patients with depression (Muller et al., 2001; Reif et al., 2006; Stockmeier et al., 2004). Additionally, in rodents, there is recent evidence that ablation of neurogenesis, through either irradiation (Santarelli et al., 2003; Surget et al., 2008) or genetic techniques (Zhao, Deng, & Gage, 2008), is not always accompanied by a depressive phenotype. Based on these results, it is very unlikely that a deficit in hippocampal neurogenesis is the singular cause of depression. More study is needed to determine whether neurogenesis might be related to selected symptoms of depression or whether it may only be critical for the behavioral effects of antidepressant treatment (Sahay and Hen, 2007). Importantly, in the exhaustive quest to determine whether hippocampal neurogenesis plays a causal role in depression, most researchers have paid little attention to the neurons that do survive under conditions of stress. It may be that the etiological problem lies not with an absolute loss of cell proliferation and survival, but rather, with abnormal characteristics within new dentate granule cells that do survive and integrate themselves into existing circuitry. Do they locate themselves

properly and form functional connections or is this process dysfunctional? There has been a suggestion that surviving cells may show abnormal migration or morphological and molecular characteristics that subsequently promote depressive symptomatology (Lussier et al., 2009), but this is a topic that requires more investigation.

Effects of CORT on Molecular and Cellular Mechanisms of Plasticity

Over the past 50 years the interaction between chronic stress and the molecular and cellular changes that influence the development of depression have become increasingly clear (Pittenger & Duman, 2008). For example, both CORT administration in animals and depression in humans have been associated with reductions in the transcription factor cyclic-adenosine monophosphate (cAMP) response element binding protein (CREB; Pittinger & Duman, 2008). Interestingly, CREB activation promotes neurogenesis (Nakagawa et al., 2002a) and CREB blockade decreases it (Nakagawa et al., 2002a; Nakagawa et al., 2002b), making this transcription factor a likely contributor to the alterations in neuronal stability associated with stress and depression. Furthermore, CREB and other transcription factors induce effector genes that contribute to the stabilization of synaptic plasticity (Kandel, 2001). Prominent among these is brain-derived neurotrophic factor (BDNF), which has a critical role in stabilizing neurons during development (Pittenger & Duman, 2008), and is important for the survival and function of mature neurons (Duman, 2002). Importantly, several forms of chronic stress, and glucocorticoid exposure, lead to impaired CREB and BDNF-mediated signaling in limbic and forebrain regions (Dwivedi, Rizavi, & Pandey, 2006; Focking, Holker, & Trapp, 2003; Gourley et al., 2008a; Jacobsen & Mork, 2006; Nitta, Fukumitsu, Kataoka, Nomoto, & Furukawa, 1997; Pittenger & Duman, 2008; Xu et al., 2006) similar to that seen in patients with depression (Dowlatshahi, MacQueen, Wang, & Young, 1998; Dwivedi et al., 2003; Karege et al., 2005; Karege et al., 2002; Sen, Duman, & Sanacora, 2008; Shimizu et al., 2003). These changes are also accompanied by depression-like behavior in rodents (Gourley et al., 2008a,b). Repeated CORT treatment is also known to reduce the expression of cell adhesion proteins (Nacher, Pham, Gil-Fernandez, & McEwen, 2004) and cytoskeletal proteins that play an important role in structural plasticity within the central nervous system (Cereseto et al., 2006). A recent gene profiling experiment in tree shrews exposed to cortisol has also identified other genes that are differentially regulated by glucocorticoids (Alfonso et al., 2004), many of which have roles in neuroplasticity.

Given the heterogeneity of depressive symptoms and illness course, it is clear that no single underlying mechanism could be the primary culprit for developing the disorder. Instead, the emerging picuture suggests that depression probably arises from a complex cascade of molecular and cellular events that influence gene transcription and protein expression and subsequently, cell morphology and connectivity. However, one must be cautious when interpreting these findings, as not all neural changes associated with stress are pertinent to depression (Gregus et al., 2005). For example, more subtle effects of CORT can be detected relatively quickly, as increases in the surface densities of rough endoplasmic reticulum and Golgi apparatus in pyramidal cells of the CA3 and CA1 regions have been observed in as little as three days of CORT treatment (Miller, Antecka, & Sapolsky, 1989), which is a time course that typically does not alter depression-like behavior. Moreover, smaller doses (e.g. 5-10mg/kg) of CORT, which do not result in a depressive phenotype (Johnson et al., 2006), produce significant neurobiological alterations in some areas of the hippocampus, PFC and amygdala (Alonso, 2000; Mayer et al., 2006; Mitra & Sapolsky, 2008; Murray et al., 2008; Woolley et al., 1990). Given that these neurobiological changes are not linked to behavioral changes, they likely do not relate to depression. Therefore, one strength of a preclinical model like exogenous CORT administration is that it can produce both behavioral and neurobiological changes associated with depression in the same animal, allowing for systematic investigation of neurobiological changes that may underlie specific symptoms and therapeutic strategies that may be most helpful in reversing specific manifestations of the disease.

8.4 Considerations When Using Exogenous CORT to Model and Study Human Depression

Although the validity of administering exogenous CORT to emulate a depressive phenotype is well established, there are a few considerations to be made when choosing this model. First, administration of exogenous CORT is not a realistic simulation of stressors encountered in daily life, and the amount of CORT needed to produce a depressive phenotype is often supraphysiological (Johnson et al., 2006). However, this criticism is probably not unique to the CORT model, as the human experience is difficult to reproduce. Repeated restraint, for example, is also not a realistic simulation of the human experience because of its use of a physical stressor and the quick development of habituation in many animals (Galea et al., 1997; Gregus et al, 2005). It is also important to understand that the CORT model induces very reliable behavioral, morphological, and cellular changes that are more robust than those produced by the

CMS model. The chronic mild stress model probably provides the most realistic simulation of the human experience of all the preclinical stress models currently in use (Willner, 1997), but it can be quite difficult to implement. In addition, the CORT model allows one to investigate the direct effects of CORT itself on depressive behavior and neurobiological factors, without the influence of habituation and psychosocial factors. Thus, the CORT method offers some advantages over other models but it also has some disadvantages and one should carefully consider which model is best suited for the experimental question being asked before deciding which model to use.

Second, there are a number of ways to administer CORT that need to be considered when implementing this model. The benefit of passive CORT administration via chow or drinking water is that there is little to no stress associated with the actual administration of the hormone. However, animals do not consume water or food continuously or consistently, which may cause excessive fluctuation of circulating drug concentrations within and between animals, potentially leading to individual variability. Another simple approach is the repeated subcutaneous injection of CORT. A potential advantage of this method is that it allows for control over increases in circulating glucocorticoids, which may not be achieved with other methods of administration. That is, administering CORT in drinking water and through pellet implantation may yield a more flattened variable hormone level throughout the day (Cereseto et al., 2006; Herrmann et al., 2009), whereas injections allow for strict control over the timing of glucocorticoid circulation. This can however, be a disadvantage if a more constant hormone level is desired. The injection process itself can also be a stressor. For instance, saline injections have been shown to induce dendritic remodeling similar to that of CORT, albeit the degree to which these changes occur is much less (Seib & Wellman, 2003). Therefore, the stress of injections could lead to uncontrolled release of endogenous corticosteroids, which may mask any intentional changes in plasma glucocorticoid levels. The use of micro-osmotic pumps and slow-release pellets for subcutaneous implantation are alternative options that are thought to provide constant delivery rates, as manufacturers claim a constant delivery rate up to 28 days for pumps, and up to 90 days for pellets (Herrmann et al., 2009). However, a recent study found that the implantation of micro-osmotic slow-release pumps for 21 days did not result in elevated CORT plasma levels. In animals treated with CORT at a dose of 5mg/kg, mean CORT plasma concentrations were nearly identical to controls at all time points. At day 7, only three animals had higher CORT plasma levels when compared to baseline, with

individual concentrations ranging between 140 and 290 μg/L, and after 14 days, only one of these animals maintained slightly elevated CORT plasma concentrations. In light of these findings, authors examined residual pump volumes to determine whether they were delivering their content as expected. Surprisingly, pump residual volumes varied greatly between animals, with volumes containing active drug ranging from 50 to 180 μl, indicative of major differences in the flow rate of individual pumps. In this same study, implantation of 10mg CORT pellets induced elevated plasma CS levels within 24 h of insertion, with individual values ranging between 1200 and 1400 μg/L. However, although these pellets were meant to last for 21 days as per the manufacturer's instructions, plasma levels of CORT returned to baseline within 7 days, and thus, further implantation was necessary to attain increased levels of circulating hormone. Similarly, a single injection of CORT 10mg/kg induced significant hypercortisolemia with peak values of 1000 to 2600 μg/L at 1 h post-injection, yet circulating plasma CORT levels returned to baseline at 4 h after the injection (Herrmann et al., 2009). Therefore, method of hormone delivery is an important consideration when using exogenous CORT to model depression. As discussed previously, the development of depression-like behaviors may be dose and time dependent, and thus, each method may vary in the amount of drug and time needed to induce such behaviors, especially if the delivery is not consistent. Nonetheless, once these methodological issues are addressed, the behavioral and morphological changes associated with stress are quite consistent across drug delivery methods, reinforcing the idea that repeated CORT administration is a valid and useful animal model for studying the interaction between stress, glucocorticoids, and depression.

9. Association Between Reelin, Hippocampal Neuroplasticity and Depression

Reelin is a large secretory protein that plays an important regulatory role in cell migration during embryonic development, and neural maturation and synaptic function in the adult brain (Herz & Chen, 2006; Teixeira et al., 2012). During embryonic development, reelin is expressed by a population of Cajal-Retzius cells in the marginal zone of the hippocampus and cortex, and in the cerebellum by cells in the external granule cell layer (Del Rio et al., 1997; Frotscher, Seress, Abraham, & Heimrich, 2001; Honda, Kobayashi, Mikoshiba, & Nakajima, 2011); at this stage, reelin plays an important role in guiding the lamination of these structures. During early postnatal stages of development, however, the majority of Cajal-Retzius cells disappear and reelin is synthesized and secreted by a sub-set of GABAergic interneurons in the cortex and

hippocampus (Guidotti, Pesold, & Costa, 2000; Pesold et al., 1998). The first evidence to suggest an important role for reelin in neural development came from the discovery of the "reeler" mouse in 1951 (Fatemi, 2001). These mice have a spontaneous deletion of the reelin (RELN) gene, which results in severe disruptions in cortical lamination, including abnormal positioning and orientation of neurons and fibers in the brain. Behaviorally, reeler mice display symptoms of severe ataxia, tremor, uncoordinated movement, and a reeling gait (D'Arcangelo & Curran, 1998; Lambert de & Goffinet, 1998). Nearly 40 years later, the RELN gene was mapped, cloned (D'Arcangelo et al., 1995), and antibodies raised against mouse extracts of reeler mice were found to be specific for reelin protein (Ogawa et al., 1995). Since this time, the role reelin plays in the regulation of cellular function in the adult brain has become increasingly clear.

Reelin is constitutively secreted into the extracellular matrix by GABAergic interneurons in the adult cortex and hippocampus, where it surrounds dendrites, spines, and neurite arborizations (Lacor et al., 2000). Reelin signaling occurs when it binds to the very-low density lipoprotein (VLDLR) and the apolipoprotein E receptor 2 (ApoER2) in target cells (D'Arcangelo et al., 1999; Trommsdorff et al., 1999). The binding of reelin to VLDLR and ApoER2 then results in the clustering of these receptors and intracellular activation of the cytoplasmic adaptor protein disabled-1 (Dab1). The clustering of VLDLR and ApoER2 is important for the activation of the Src family of tyrosine kinases (SFKs)/Fyn kinase, which in turn phosphorylates Dab1 (Hiesberger et al., 1999). In the phosphorylated state, Dab1 can bind members of the soluble tyrosine kinase family and translocate them to various cellular compartments including the nucleus, where these kinases initiate the transcription of specific genes important for synaptic plasticity and the promotion of cytoskeletal changes including dendritic spine maturation (See Figure 1-3; Guidotti et al., 2000b).

Specific to the hippocampus, proper functioning of the reelin signaling pathway is required for normal development and maintenance of dendritic structures in this brain region. For example, hippocampal neurons of reeler mice are characterized by stunted dendritic growth, decreased dendritic branching, and reduced spine density (Niu, Renfro, Quattrocchi, Sheldon, & D'Arcangelo, 2004; Niu, Yabut, & D'Arcangelo, 2008). Heterozygous reeler mice (HRM), which have a 50% reduction in reelin expression in the brain (Tueting et al., 1999), also display subtle neuroanatomical changes, including decreased spine density in CA1 pyramidal neurons in the hippocampus (Liu et al., 2001; Niu et al., 2008). Deletion of Dab1 in dentate neuroprogenitor

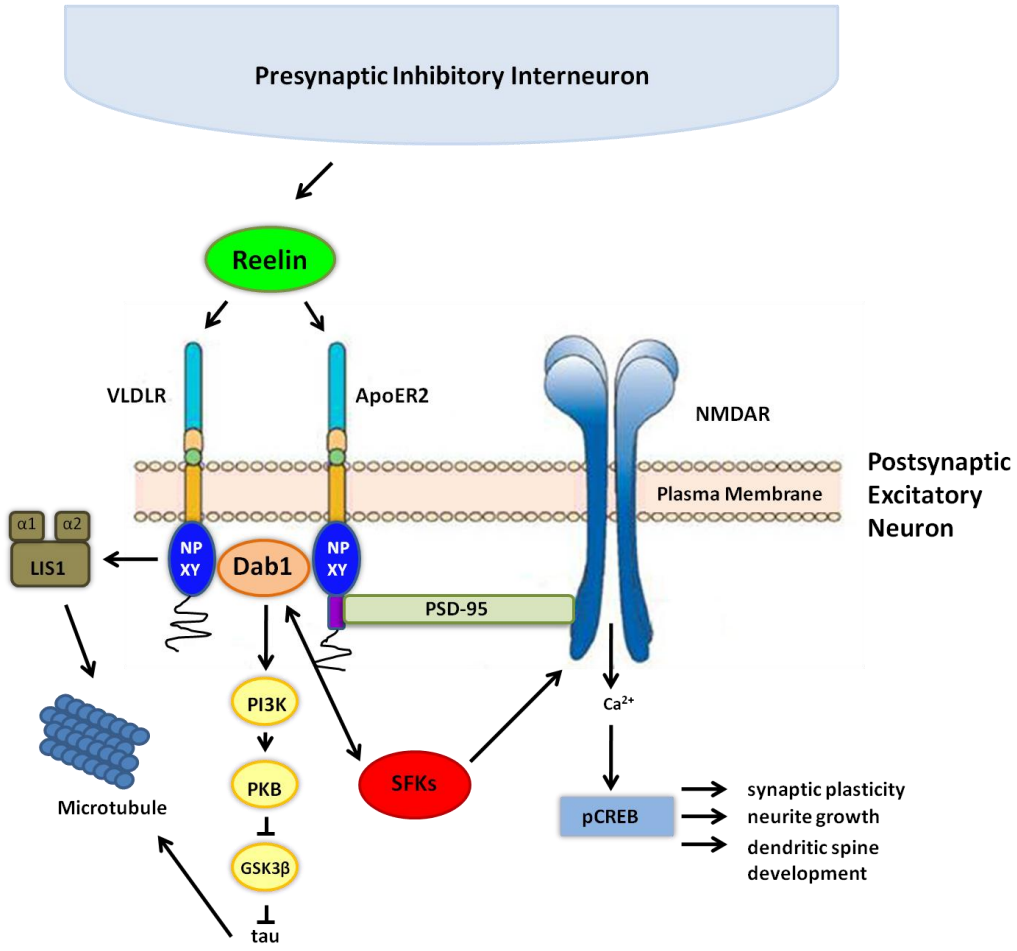

Figure 1-3. Schematic Representation of the Reelin Signaling Pathway through ApoER2 and Vldlr (Adapted from Herz, Chen, Masiulis, & Zhou, 2009). Reelin binds to Vldlr and Apoer2 and induces phosphorylation of Dab1, an adaptor protein that interacts with NPxY motifs in both receptor tails. Clustering of Dab1 activates SFKs, which potentiates tyrosine phosphorylation of Dab1. Phosphorylated Dab1 activates phosphatidylinositol 3-kinase (PI3K) and subsequently protein kinase B (PKB), which inhibits glycogen synthase kinase-3β (GSK3β) and reduces phosphorylation of tau, promoting microtubule stability (Beffert et al., 2002). Lis1 can also bind tyrosine-phosphorylated Dab1. It participates in the formation of a Pafah1b complex, which regulates microtubule functions (Assadi et al., 2003; Zhang et al., 2007). ApoER2 associates with the postsynaptic scaffolding protein PSD95 through an alternatively spliced exon. This interaction is critical for the coupling of the Reelin signaling complex to the NMDAR. Reelin-activated SFKs phosphorylate the NMDA receptor on tyrosines in the NR2 subunits, thereby potentiating NMDAR-mediated calcium ($Ca2^{+}$) influx. Elevated intracellular $Ca2^{+}$ that has entered the cell through NMDAR activates the transcription factor CREB (Beffert et al., 2002; Chen et al., 2005). This phosphorylation event initiates the expression of genes that are important for synaptic plasticity, neurite growth, and dendritic spine development.

cells also leads to abnormal orientation and ectopic migration of these cells into the hilus, and a marked reduction in dendritic length and complexity (Teixeira et al., 2012). Importantly, exogenous reelin application can reverse the reduction in dendritic growth exhibited in neuronal cultures lacking one or both reelin genes (Niu et al., 2004; Niu et al., 2008), and overexpression of this protein in the forebrain results in enhanced dendritic development (Teixeira et al., 2011).

Given its role in regulating dendritic development, it is not surprising that reelin plays an important role in synaptic plasticity and cognitive processing. In particular, reelin enhances N-methyl-D-aspartate receptor (NMDAR) mediated calcium ion influx in neurons by SFK tyrosine phosphorylation. This increased calcium influx then activates the transcription factor CREB, which initiates gene expression important for dendritic growth, spine formation, and synaptic plasticity (Chen et al., 2005). Recent research has also shown that exogenous application of reelin in cultured hippocampal neurons or slices dramatically elevates the magnitude of long-term potentiation (LTP) induction (Weeber et al., 2002). Similarly, intraventricular infusions of recombinant reelin have been shown to enhance spatial learning and memory in the MWM, increase LTP in the CA1 region of the hippocampus, and increase CA1 dendritic spine density (Rogers et al., 2011). However, blocking reelin signaling on any level (e.g. disrupting binding of reelin to its receptors, genetic inactivation of Dab1, or pharmacological inhibition of SFKs), prevents reelin-enhanced NMDAR-mediated calcium entry into the cell (Chen et al., 2005), which results in reduced synaptic efficacy and cognitive deficits. In particular, HRM and mice lacking receptors for reelin have impaired LTP in the CA1 region of the hippocampus (Beffert et al., 2006; Qiu et al., 2006; Valastro, Ghribi, Poirier, Krzywkowski, & Massicotte, 2001), and show impairments in contextual fear responding, acquisition of instrumental responding, and reversal learning (Brigman, Padukiewicz, Sutherland, & Rothblat, 2006; Krueger et al., 2006; Qiu et al., 2006). Similarly, reeler mice are severely impaired on tasks of spatial working memory, emotional learning, executive function, and response inhibition (Salinger, Ladrow, & Wheeler, 2003). Dab1 deficient mice also show severe behavioral impairments identical to that of the reeler mouse (Ware et al., 1997), suggesting that reelin and its signaling machinery are directly involved in the development and maintenance of normal synaptic function and stability.

Although the cellular functions of reelin in the adult brain are not entirely known, recent evidence implicates disrupted reelin signaling in the pathophysiology of a number of neuropsychiatric disorders, including schizophrenia, autism, bipolar disorder, epilepsy, and

major depression (Eastwood & Harrison, 2006; Fatemi, Earle, & McMenomy, 2000; Fatemi, Kroll, & Stary, 2001; Haas & Frotscher, 2010; Impagnatiello et al., 1998; Saez-Valero et al., 2003). The involvement of disrupted reelin signaling in depression is a relatively new concept, stemming from a few post-mortem studies showing a reduction in reelin in the hippocampus of depressed patients (Fatemi et al., 2000; Knable, Barci, Webster, Meador-Woodruff, & Torrey, 2004; Torrey et al., 2005). Subsequent preclinical research has supported these results. Specifically, our laboratory was the first to show that repeated CORT injections significantly downregulate reelin+ cells in the hippocampal DG of rodents (Lussier et al., 2009). Interestingly, repeated restraint stress, a model which does not produce reliable increases in depressive-like behaviors in the FST (Sterner and Kalynchuk, 2010), also does not decrease reelin expression in the hippocampus (Lussier et al., 2009), suggesting a link between disrupted reelin signaling and depressive symptomatology. In addition, we have found that HRM present with increased depressive-like behaviors in the FST after CORT treatment compared to controls (Lussier, Romay-Tallon, Kalynchuk, & Caruncho, 2011), suggesting that impaired reelin signaling may increase vulnerability to the deleterious effects of glucocorticoids. Conversely, after CORT treatment, mice that overexpress reelin in the forebrain spend less time immobile in the FST compared to their wild-type counterparts (Teixeira et al., 2011), pointing to the potential therapeutic capabilities of this protein.

Investigations into the downstream molecular events of medications used to treat neuropsychiatric illness have generated new theories about the etiology of these disorders, and the long-term adaptations required for treatment efficacy (Tanis & Duman, 2007; Tanis, Newton, & Duman, 2007). Specific to reelin, a great deal of overlap exists between the molecular and cellular changes induced by mood stabilizing medications and the biological activities of this protein (Fatemi, 2011; Folsom & Fatemi, 2012; Herz & Chen, 2006; Liu et al., 2001; Pujadas et al., 2010; Teixeira et al., 2012; Teixeira et al., 2011), suggesting that it may have an important role in the efficacy of these drugs. However, little is known about how medications currently used to treat neuropsychiatric illness influence reelin expression in the mammalian brain. Previous reports have shown that chronic treatment with the selective serotonin reuptake inhibitor (SSRI), fluoxetine, or the atypical antipsychotic olanzapine, enhances mRNA of reelin, Dab1, and various isoforms of the VLDLR receptor in the frontal cortex of rats (Fatemi, Reutiman, & Folsom, 2009). Olanzapine also increases total protein levels of reelin, whereas the

anticonvulsant valproic acid (VPA), enhances Dab1 mRNA, but not total protein levels of Dab1 or reelin (Fatemi et al., 2009). Chronic treatment with the SSRI citalopram was also shown to increase reelin mRNA in the hippocampus when compared to controls, and counteract kainic acid-induced decreases in reelin+ cells and mRNA in this region (Jaako et al., 2011). Collectively these data indicate that chronic treatment with psychotropic medication may influence the synthesis and overall protein levels of reelin and its signaling machinery in areas of the brain known be affected in neuropsychiatric disorders.

10. Association Between GABA, Reelin and Depression

Gamma aminobutyric acid (GABA) is the primary inhibitory neurotransmitter working alongside glutamate to control the balance of excitation and inhibition in the brain. It is this homeostatic regulation that helps control proper neural network function. Therefore, disruptions in either can have profound consequences on behavior and cognition. In particular, disrupted GABA function has been associated with a number of neuropsychiatric disorders, including schizophrenia, bipolar disorder, epilepsy, and major depression (Brambilla, Perez, Barale, Schettini, & Soares, 2003; Rowley, Madsen, Schousboe, & Steve, 2012; Stan & Lewis, 2012). In depressed patients, reduced levels of GABA in the plasma, cerebrospinal fluid, and areas of the prefrontal and occipital cortex have been documented (Gerner & Hare, 1981; Kasa et al., 1982; Petty, Kramer, Gullion, & Rush, 1992; Sanacora, Mason, & Krystal, 2000; Sanacora et al., 1999). Deficits in a number of neurochemical markers of GABAergic cells in the brain, including reelin, have also been documented in post-mortem tissues from depressed patients (Rajkowska, O'Dwyer, Teleki, Stockmeier, & Miguel-Hidalgo, 2007). Furthermore, the enzyme involved in converting glutamate to GABA, glutamate decarboxylase (GAD), is reduced in the brains of depressed individuals (Fatemi, Stary, Earle, Araghi-Niknam, & Eagan, 2005; Karolewicz et al., 2010). Similarly, preclinical research has shown that exposure to chronic stress and glucocortoicoids can lead to a reduction in GABA, its receptors, and neurochemical interneuron markers in a number of brain regions, including the hippocampus (Cullinan & Wolfe, 2000; Gronli et al., 2007; Orchinik, Weiland, & McEwen, 1995). Importantly, the decreased GABA concentrations in depressed patients can be reversed by treatment with SSRIs (Sanacora, Mason, Rothman, & Krystal, 2002) and ECT (Sanacora et al., 2003). Modulation of the α2 subunit of the $GABA_A$ receptor has also shown potential as an antidepressant in

preclinical models (Vollenweider, Smith, Keist, & Rudolph, 2011). Taken together, these data suggest that targeting the GABAergic system may be important for alleviating symptoms of depression.

Growing evidence suggests that GABA also plays an important role in adult hippocampal neurogenesis (Dieni, Chancey, & Overstreet-Wadiche, 2012; Masiulis, Yun, & Eisch, 2011; Vicini, 2008). For example, mice lacking the α4 subunit of the $GABA_A$ receptor have a reduction in the number of proliferating cells in the SGZ of the DG (Duveau et al., 2011). Treating cultured cells with GABA also causes proliferating cells to differentiate and take on a neuronal phenotype (Tozuka, Fukuda, Namba, Seki, & Hisatsune, 2005). The survival of newly generated neurons in the DG also seems to depend partially on GABA signaling. In particular, GABA receptor-mediated depolarization promotes CREB activation, which in turn promotes cell survival (Obrietan, Gao, & Van Den Pol, 2002). Conversely, cell autonomous loss of GABA depolarization leads to cell death in 1 to 2 week old granule cell neurons, which is rescued by enhanced CREB signaling (Jagasia et al., 2009). Deletion of the α4 subunit of the $GABA_A$ receptor also impairs dendritic development in 2 week old newborn granule cells (Duveau et al., 2011), suggesting that GABA signaling is important for the maturation of newborn neurons in the hippocampus. Interestingly, both chronic stress and prolonged glucocorticoid treatment lead to a reduction in cell proliferation and maturation of dentate granule cells (Alahmed & Herbert, 2008; David et al., 2009; Fuchs & Gould, 2000; Hellsten et al., 2002; Huang & Herbert, 2006; Karishma & Herbert, 2002; Mayer et al., 2006; Murray et al., 2008; Pham et al., 2003; Sapolsky, 2004; Sheline et al., 1999; Starkman et al., 1992). Given that these stressors also decrease GABA levels in the hippocampus (Cullinan & Wolfe, 2000; Gronli et al., 2007; Orchinik et al., 1995), dampened GABAergic neurotransmission may partially account for disrupted neurogenesis in these models of depression. Human patients with depression also have lower levels of GABA in brain and peripheral tissues, which could similarly contribute to the volumetric reductions in the hippocampus that is often found in post-mortem brain samples. Although the above data do not represent an exhaustive review of the literature on this topic, they do provide evidence that GABA plays at least some role in various stages of hippocampal neurogenesis in the adult brain. In addition, recent attention has been placed on understanding how cell-signaling molecules synthesized by inhibitory interneurons (e.g. reelin) influence adult neurogenesis (Masiulis et al., 2011). Given the proximity of GABAergic interneurons to adult neural stem cells and their

progenitors (Abrous, Koehl, & Le, 2005; Seri, Garcia-Verdugo, McEwen, & Alvarez-Buylla, 2001), these molecules are in a prime location to regulate all stages of neurogenesis and could tell us a great deal about how the inhibitory network influences hippocampal function. The ability to manipulate these cell-signaling molecules is also valuable in understanding disease states and may create new avenues for treating disorders characterized by abnormalities in neurogenesis and hippocampal dysfunction.

GABA is released by inhibitory interneurons in the brain. Unlike their excitatory neuron counterparts, whose morphological and physiological properties are relatively homogenous (DeFelipe & Farinas, 1992; Mendez & Bacci, 2011; Spruston, 2008), interneurons encompass a vast number of different cell types (Freund & Buzsaki, 1996; Mendez & Bacci, 2011). The classification of interneurons is based on the expression of certain calcium binding proteins and/or neuropeptides, their specific electrophysiological characteristics, functional characteristics of synapses that they form and receive, as well as specific anatomical and morphological properties (Freund & Buzsaki, 1996; Mendez & Bacci, 2011). Although inhibitory cells make up only 15 to 20% of the total neurons in our brain, they play an integral role in neural function. Overall, these cells provide inhibition to neuronal networks and dictate the temporal pattern of activity of principal pyramidal and other inhibitory neurons. By keeping the membrane potential below the threshold of spike generation, interneurons can also dynamically control the configuration of circuits (Klausberger & Somogyi, 2008). Ultimately, this control over activity is important for the integration of synaptic inputs and memory formation (Buzsaki & Chrobak, 1995; Buzsaki & Draguhn, 2004; Freund & Buzsaki, 1996; Paulsen & Moser, 1998; Somogyi & Klausberger, 2005).

As discussed previously, reelin is synthesized and secreted by GABAergic interneurons in the cortex and hippocampus in the adult brain (Pesold et al., 1998). In the adult hippocampus, reelin is highly expressed in interneurons contained in the stratum oriens, stratum-lacunosum moleculare and stratum radiatum regions of CA1-3. The pyramidal layers of CA1-3, however, are always devoid of reelin labeling. In the DG, reelin-containing interneurons are primarily located at the base of the granule cell layer (i.e., SGZ) and hilus, whereas the molecular layer contains very few reelin+ cells. However, the stratum-lacunosum moleculare and upper portion of the molecular layer of the DG has strong diffuse labeling of reelin protein. Although virtually all reelin+ cells co-localize with GAD67 and are therefore considered GABAergic, the number

of GAD67 cells expressing reelin varies by hippocampal region. In the stratum oriens of CA1-3, the percentage of GAD67+ cells expressing reelin is between 85 and 88%, whereas in the stratum lacunosum moleculare and stratum radiatum they make up between 40 to 50% of GABAergic cells. In the DG, reelin+ cells make up 70 to 85% of GABAergic cells in the SGZ and hilus, whereas they only account for about 24% of the interneuron population in the molecular layers (Pesold et al., 1998). The types of interneurons that express reelin are also varied. In particular, reelin is most highly co-localized with interneurons expressing the neuropeptide somatostatin (SOM; 75%), the calcium binding protein calretinin (CR; 58%), neuropeptide-y (NPY; 30%) and the calcium binding protein calbindin (CB; 14%) (Alcantara et al., 1998). Although these cells make up a heterogeneous population of intereneurons, most of them form inhibitory contacts on the dendrites of principal cells, and thus, exert control over their firing (Freund & Buzsaki, 1996). Calretinin+ interneurons also form contacts with other inhibitory neurons, and so they have the ability to exert control over hippocampal activity by modulating inhibitory output (Freund & Buzsaki, 1996; Gulyas, Hajos, & Freund, 1996). On the other hand, reelin co-localizes very little (1 to 6%) with interneurons expressing the calcium binding protein parvalbumin (PVB), the neuropeptide cholecystokinin (CCK) and vasoactive intestinal peptide (Alcantara et al., 1998), indicating that reelin is not highly expressed in chandelier or basket cells. Given that reelin is known to be downregulated in depressed patients, identifying the types of reelin-expressing interneurons that are affected by the disorder and how this relates to inhibitory function in the hippocampus is important for understanding how these alterations might relate to depressive symptomatology.

11. Introduction to Epigenetics

The term epigenetics was coined by Waddington in 1942, litereally meaning “above the level of the genome” (Jamniczky et al., 2010; Ng & Bird, 1999). At a basic level, epigenetics is the study of the regulation of genomic function that occurs through mechanisms other than changes in underlying DNA sequence (Callinan & Feinberg, 2006; Jamniczky et al., 2010; Rivera & Bennett, 2010). Essentially, epigenetic modifications function to establish and maintain different gene expression programs within cells, leading to phenotypic differences despite each cell sharing the same genetic information (Ng & Bird, 1999; Ng & Gurdon, 2008). Importantly, these changes can remain through cell divisions and may also last for multiple generations

(Rutten & Mill, 2009). In the past decade there has been an exponential increase in the number of studies demonstrating the dynamic nature of epigenetic processes and their influence on the regulation of genes and behavior. Importantly, much of this research has underscored the importance of epigenetic events in the control of both normal cellular processes and abnormal events associated with disease as well as the long-term regulation of gene expression (Callinan & Feinberg, 2006; Feng, Fouse, & Fan, 2007; Henikoff & Matzke, 1997; Mehler, 2008a,b).

In order to understand how epigenetic modifications take place, it is first important to understand some basic concepts of how DNA is packaged within the nucleus, and how this packaging is important for regulating gene expression. The primary epigenetic modifications that regulate gene expression patterns are DNA methylation and chromatin remodelling (Feng et al., 2007). That is, epigenetic modifications can either occur on the DNA itself, or to the proteins with which it associates. In all eukaryotes, DNA exists as a complex with specialized proteins, which together form chromatin. Histone proteins serve to package DNA, acting like spools around which DNA is coiled, allowing it to be contained inside the nucleus (Felsenfeld & Groudine, 2003). Importantly, chromatin not only serves as a way to condense DNA, but also as a way to control how DNA is used. In particular, specific genes are not expressed unless they can be accessed by RNA polymerase and proteins known as transcription factors. A tightly condensed chromatin structure limits the access of these substances to DNA. Therefore, a cell's chromatin must open in order for gene expression to take place. This process of relaxing the chromatin is called chromatin remodeling, and it is of vital importance to the proper functioning of eukaryotic cells (Berger, 2007; Downs, Nussenzweig, & Nussenzweig, 2007; Ito, 2007; Mehler, 2008a,b). DNA can also directly be modified through a process called methylation. This type of modification involves the addition of methyl groups to gene promoter regions ultimately resulting in gene silencing. The degree of promoter methylation is often correlated with the extent of gene activation, and can be reversed by the process of demethylation [i.e., removal of the methyl groups (Bird, 2002; Miranda & Jones, 2007; Ng & Bird, 1999)]. Other epigenetic processes also exist, such as non-coding RNAs and RNA and DNA editing (Mehler, 2008a,b), however, they will not be discussed at length in this chapter. Nonetheless, it is important to understand that none of these processes work alone; rather, it is the coordination of epigenetic modifications that maintain cellular function within the nervous system (Mehler, 2008a,b). In fact, many of these mechanisms have been implicated in the regulation of most cellular and

molecular processes that are essential for higher nervous system function (Graff & Mansuy, 2008; Mehler, 2008a,b; Miller, Campbell, & Sweatt, 2008), giving them great potential as targets of pharmacological intervention.

Methylation and acetylation are posttranslational modifications to proteins and/or DNA that play key roles in regulating gene expression (See Figure 1-4). Methylation was the first epigenetic mechanism identified in the mammalian genome, and it is present to varying degrees in all eukaryotes except yeast (Bredy, Sun, & Kobor, 2010). Methylation contributing to epigenetic inheritance can occur via alterations to either DNA or histone proteins. DNA methylation, the best understood epigenetic mechanism, is a stable heritable covalent modification that alters the DNA without changing its sequence. This modification consists of the addition of a methyl group from S-adenosyl methionine (SAM) to cytosine residues of the DNA template, and is catalyzed by a group of enzymes known as DNA methyltransferases (Bredy et al., 2010; Klose & Bird, 2006; Mehler, 2008a,b; Rivera & Bennett, 2010; Suzuki & Bird, 2008). DNA methylation specifically occurs on cytosines located at the 5th carbon position of the pyridine ring, within cytosine dinucleotides (CpG). These CpG dinucleotide sequences are usually found in and around gene regulatory regions in clusters known as CpG islands. Although CpG sequences throughout the genome are usually heavily methylated, CpG islands, found at the 5-prime regulatory regions of more than 50% of human genes, are ususally methylated to a much lesser degree in normal cells. Methylation in these regions usually represses gene transcription, with the amount of DNA methylation at a promoter correlating with the extent of gene inactivation (Bird, 2002; Mehler, 2008a,b; Miranda & Jones, 2007; Ng & Bird, 1999; Rivera & Bennett, 2010; Suzuki & Bird, 2008; Tucker, 2001). Essentially, DNA methylation is involved in silencing parasitic DNA sequences and the inactive X-chromosome, genomic imprinting, and developmental specific silencing and/or activation of gene transcription (Rivera & Bennett, 2010). In addition, DNMTs are expressed during development and in the adult brain in mature stem cell generative zones mediating neurogenesis (Feng et al., 2007), they are regulated by physiological and pathological states, and they promote neural survival, plasticity and stress responses (Klose & Bird, 2006; Ooi et al., 2007). Collectively, this evidence points to the integral role this epigenetic process plays in cellular function not only throughout development, but also in the adult CNS.

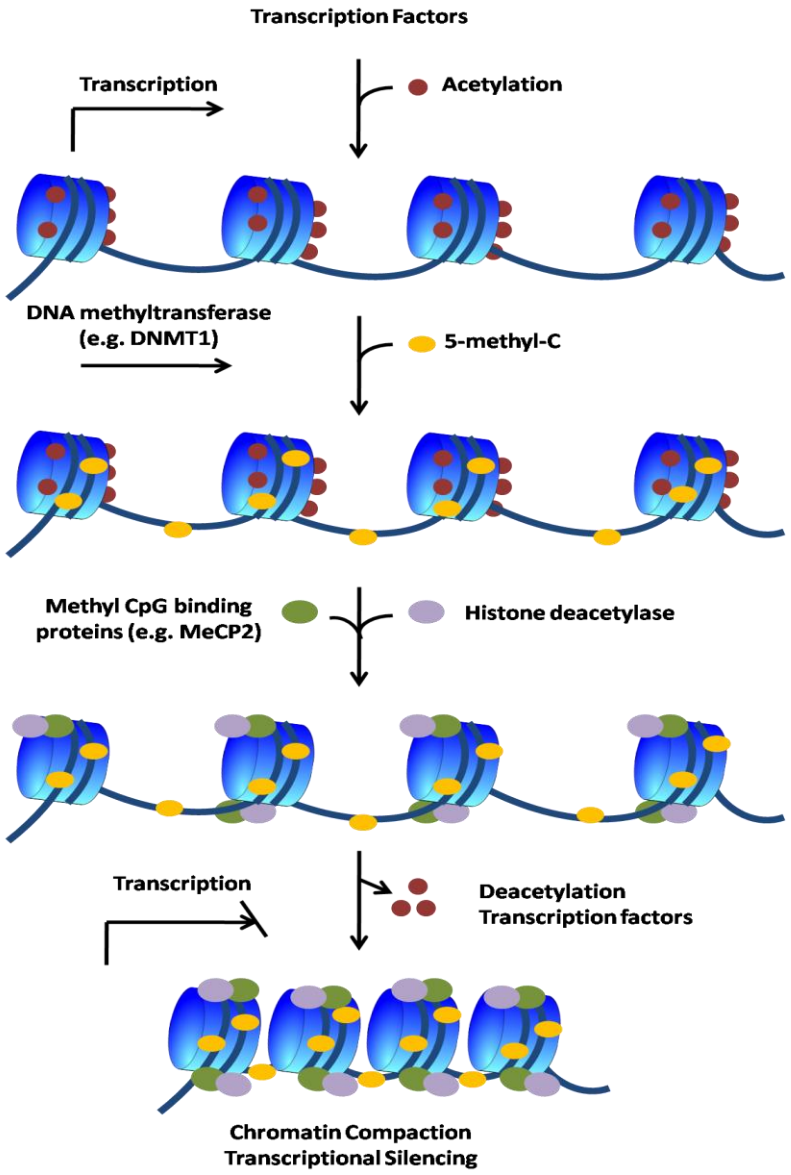

Figure 1-4. Schematic Representation of Transcriptional Repression via DNA Methylation and Histone Modifications. DNA methylation and histone acetylation and methylation are involved in gene activation and silencing. Acetylation of histone tails by histone acetyl transferases (HAT) leads to opening of the nucleosome chain which allows transcription factors (TF) to bind gene promoter regions on DNA and activate gene expression (Hong et al., 1993). In contrast, DNA methylation by DNA methylases causes gene silencing. A transcriptionally active region targeted for silencing becomes methylated via DNMT regulated transfer of methyl groups to cytosine residues of the DNA template (Suzuki & Bird, 2008). DNA methylation is also associated with histone modifications through methyl-DNA binding proteins (e.g. MeCP2). These binding proteins help recruit histone modification enzymes such as histone deacetylases (HDACs) that remove acetyl groups from histone proteins (Nightingale et al., 2008). This removal of acetyl groups leads to the compaction of chromatin, limiting the accessibility of transcriptional machinery, ultimately suppressing gene transcription. Adapted from Robertson & Wolffe (2000).

DNA methylation also occurs within the context of nucleosome and chromatin remodelling providing a mechanism for both short and long-term gene silencing (Metivier et al., 2008; Nightingale, O'Neill, & Turner, 2006). Specifically, histone proteins can be methylated on either lysine or arginine residues which can induce changes in chromatin structure. For example, the transfer of methyl groups from SAM to histones is catalyzed by enzymes known as histone methyltransferases. Histones that are methylated on certain residues can act epigenetically to either repress or activate gene expression depending on the residue acted upon (Grewal & Rice, 2004; Nakayama, Rice, Strahl, Allis, & Grewal, 2001; Tsankova, Renthal, Kumar, & Nestler, 2007). DNA methylation is also associated with histone modifications through methyl-DNA binding proteins (e.g. MeCP2). These binding proteins help recruit histone modification enzymes such as histone deacetylases (HDACs) that remove acetyl groups from histone proteins (Lachner & Jenuwein, 2002; Tsankova et al., 2007). This removal of acetyl groups leads to the compaction of chromatin, limiting the accessibility of transcriptional machinery, ultimately suppressing gene transcription.

In contrast to the primarily repressive actions of methylation, acetylation serves to enhance gene transcription. This epigenetic modification occurs on histones, with the unstructured N-termini (i.e., histone tail) being particularly susceptible to modifications. Specifically, histones can be acetylated and deacetylated on lysine residues in the N-terminal tail and on the surface of the nucleosome core as part of gene regulation. Typically, these reactions are catalyzed by enzymes known as histone acetyltransferases (HATs) or HDACs. Acetylation of the lysine residues at the N terminus of histone proteins brings in a negative charge which neutralizes the positive charge on the histones, thereby reducing the affinity between histones and DNA (Hong, Schroth, Matthews, Yau, & Bradbury, 1993). As a consequence, the condensed chromatin (i.e., heterochromatin) is transformed into a more relaxed structure (i.e., euchromatin) which allows RNA polymerase and transcription factors to more easily access the promotor region. Therefore, in most cases, histone acetylation enhances transcription whereas histone deacetylation represses it (Mizzen & Allis, 1998; Tsankova et al., 2007; Vidali, Ferrari, & Pfeffer, 1988).

Although epigenetic modifications to DNA and histones are often investigated separately, these modifications work in concert in a number of ways to regulate gene expression. Specifically, the complex interplay between DNA and histone modifications promotes both

transient and long-term profiles of synaptic and neural network modifications, of which both methylation and acetylation play integral roles (Levenson & Sweatt, 2005; Miller et al., 2008). Adult neurons, for example, respond to various environmental signals via dynamic changes in DNA methylation and histone modifications; many of these processes are important to mechanisms of memory formation and cognition via modulation of genes involved in synaptic plasticity (Bredy et al., 2010; Miller et al., 2008; Riccio, 2010; Roth, Lubin, Sodhi, & Kleinman, 2009). Collectively, this evidence suggests that a coordinated functioning of epigenetic modifications is essential for neural development, cellular differentiation and communication, as well as synaptic plasticity in general, which are all fundamental processes for normal brain activity.

12. Epigenetics Regulation of Reelin in Neuropsychiatric Illness

Emerging evidence in the field of epigenetics suggests that disturbances in epigenetic processes both during development and in the postnatal brain may influence the development of a number of neurological and neuropsychiatric disorders (Bredy et al., 2010; Cho, Elizondo, & Boerkoel, 2004; Kubota, Miyake, Hirasawa, Nagai, & Koide, 2010; Zhao, Pak, Smrt, & Jin, 2007). This is probably not surprising given the evidence suggesting that many epigenetic mechanisms are implicated in the regulation of most cellular and molecular processes that are essential for higher nervous system functioning. In particular, it has been suggested that transcriptional deregulation may underlie the behavioral manifestation of a number of neuropsychiatric illnesses, including major depression. For example, increased expression of HDAC and HDAC5 in the hippocampus has been associated with increased depression-like behavior in rodents (Tsankova et al., 2006). Conversely, inhibiting histone deacetylation in the hippocampus has been shown to reduce depression-like behaviors in rodents (Covington, III, Vialou, LaPlant, Ohnishi, & Nestler, 2011). HDAC inhibition can also promote recovery of associative and spatial learning after neuronal atrophy in transgenic mouse models (Fischer, Sananbenesi, Wang, Dobbin, & Tsai, 2007), and chromatin remodelling in general is known to regulate the expression of genes important for neural stem cell maintenance and fate, neuronal subtype specification and differentiation, and synaptic activity in adult neurons (Akhtar et al., 2009; Feng et al., 2007; Klose & Bird, 2006; Mehler, 2008a,b; Tsankova et al., 2007). DNMTs are also expressed during development and in the adult brain and are known to regulate

neurogenesis and promote cell survival (Feng et al., 2007). These methylation enzymes also act at genomic sites to promote differential expression of synaptic plasticity and memory suppressor genes (Miller & Sweatt, 2007). Although neuropsychiatric disorders are quite heterogenous in terms of both etiology and clinical manifestations, many of them share common underlying disturbances in neuronal morphology, synaptic connectivity and brain plasticity. In particular, alterations in dendritic branching and spine morphology play a central role in the pathophysiology of most neurological disorders (Dierssen & Ramakers, 2006; Goto, Yang, & Otani, 2010; Tamminga, Stan, & Wagner, 2010; van & Hoogenraad, 2010) suggesting that common pathways regulating neuronal function are affected. Given the intimate relationship that exists between epigenetic processes and neuroplasticity, much attention has been focused on what epigenetics can tell us about the etiology and treatment of disorders characterized by extensive neuronal remodeling.

As discussed previously in this chapter, reelin signalling plays an important role in governing cell migration, neuronal maturation and synaptic plasticity in the adult brain (Herz & Chen, 2006; Honda et al., 2011; Lakoma, Garcia-Alonso, & Luque, 2011; Niu et al., 2008; Teixeira et al., 2012). An interesting link also exists between numerous neuropsychiatric conditions and disrupted reelin signalling in brain regions that regulate mood and cognition (Fatemi et al., 2000; Guidotti et al., 2000a,b; Impagnatiello et al., 1998). Specific to major depression, a growing body of preclinical research indicates a relationship between dampened reelin signalling and depressive symptomatology (Lussier et al., 2009; Lussier et al., 2011; Teixeira et al., 2011). However, the mechanism by which reelin is downregulated in depression and other neuropsychiatric disorders is not entirely understood. One possibility is that increased methylation occurs at the reelin gene promoter, resulting in decreased transcription and translation of this protein. Although this has not been documented in depressed patients, a great deal of evidence from schizophrenia research suggests it may be possible. Specifically, in the 1960's, it was discovered that chronic administration of the methyl donor, L-methionine, exacerbated symptoms in schizoprehnic patients (Pollin, Cardon Jr., & Kety, 1961). It was believed that this worsening of symptoms was due to the conversion of methionine into SAM, leading to increased DNA methylation, and decreased transcription of reelin (Costa, Davis, Pesold, Tueting, & Guidotti, 2002). This hypothesis was confirmed with the discovery that the promoter region for reelin was hypermethylated in schizophrenic patients who had reduced reelin

expression (Grayson et al., 2005). Moreover, DNMT1 mRNA and protein levels were shown to be significantly increased in the cortex of schizophrenic patients (Veldic et al., 2004; Veldic, Guidotti, Maloku, Davis, & Costa, 2005) and these increases paralleled deficits in reelin (Ruzicka et al., 2007; Veldic et al., 2005). In further support of this methylation hypothesis of schizophrenia, a mouse model of the disorder was developed using protracted Lmethionine treatment in both wild-type (WT) and HRM (Tremolizzo et al., 2002). This treatment leads to a significant reduction in reelin mRNA expression in both WT and HRM and is associated with increased DNA promoter methylation and impaired prepulse inhibition (PPI), which is also impaired in schizophrenic patients (Tremolizzo et al., 2002). Interestingly, by increasing histone acetylation with VPA, which is an HDAC inhibitor, the impairment in PPI, promoter methylation, and downregulation of reelin expression can be reversed (Tremolizzo et al., 2002). Moreover, a direct role for DNMT1 in reelin promoter methylation was recently confirmed using an antisense-induced knockdown of DNMT1 to block methionine-induced downregulation of reelin (Noh et al., 2005). Dong and colleagues furthered these findings by demonstrating that L-methionine treatment leads to increased recruitment of the methyl-CpG binding proteins MeCP2 and MBD2 to the reelin promoter (Dong et al., 2005). Conversely, in vitro activation of reelin results in reduced DNMT1 protein levels, a dissociation of DNMT1 and MeCP2 from their promoters, and this is enhanced with DNMT inhibitors (Kundakovic, Chen, Costa, & Grayson, 2007; Kundakovic, Chen, Guidotti, & Grayson, 2009; Noh et al., 2005). It has also been demonstrated that treatment with the antipsychotics clozapine and sulpiride results in demethylation of the reelin gene promoter (Dong, Nelson, Grayson, Costa, & Guidotti, 2008), lending further support for this hypothesis. Collectively, these findings suggest that a complex interplay of DNA methylation and histone acetylation governs reelin expression, and that perturbation of these epigenetic processes can have profound effects on neuronal function. Moreover, they point to the potential for targeting epigenetic processes in the treatment of disorders characterized by decreased reelin expression.

13. Specific Aims and Goals

The specific aim of this dissertation is to further characterize the role of reelin in depression by examining how the expression of this protein is altered in an animal model of the disorder. To begin addressing this issue, a few key questions and hypotheses were developed:

Question 1: Can antidepressant treatment rescue CORT-induced downregulation of reelin+ cells in the hippocampus? And, if so, will these changes correlate with improvements on behavioral measures of depression and increased neurogenesis and neuronal maturation in the dentate gyrus? To examine these questions, I evaluated the effects of repeated injections of CORT (40mg/kg), CORT (40mg/kg) plus the tricyclic antidepressant imipramine (10mg/kg or 15mg/kg), or vehicle, on the number of reelin+ cells in the proliferative SGZ and hilus of the hippocampus in relation to dentate granule cell maturation, and depression-like behaviors in the FST.

Hypothesis: I expect to find a significant decrease in the number of reelin+ cells in the SGZ and hilus of the hippocampus after repeated CORT administration. Importantly, I expect that co-treatment with imipramine will prevent this effect. Because reelin plays an integral role in neural migration and development, and because antidepressants have the ability to block and/or rescue the deleterious effects of stress on neurogenesis and dendritic remodeling, I also expect that in the CORT plus imipramine treated groups, increased neurogenesis and maturation of immature granule cells will be associated with increased reelin expression in the SGZ and hilus. Previous reports from our laboratory have also found a link between decreased hippocampal reelin expression and depressive-like behaviors (Lussier et al., 2009; Lussier et al., 2011). Therefore, I expect that increased reelin expression in CORT plus imipramine groups will parallel improved behavior in the FST (Chapter 2).

Question 2: After identifying that CORT significantly decreases reelin+ cells in the hippocampus, and that imipramine can prevent this effect, it was important to determine whether glucocorticoids provoke degeneration of reelin-secreting GABAergic interneurons or whether these interneurons survive the period of glucocorticoid exposure but stop expressing reelin. Specifically, is the decrease in reelin+ cells reflective of an actual loss of GABAergic interneurons? To begin addressing this question, I examined the influence of repeated CORT (40mg/kg) injections on the expression of 7 known interneuron markers (i.e., GAD67, PVB, CR, CB, SOM, NPY, and CCK) in the hippocampus and DG (i.e., granule cell layer, SGZ, hilus, CA3 pyramidal, oriens, lucidum, and radiatum, CA1 stratum pyramidal, oriens, and radiatum).

These markers were chosen because they are all known to co-localize with reelin (Alcantara et al., 1998).

Hypothesis: I predict that repeated CORT treatment will lead to a significant reduction in some, but not all, interneuron markers in specific sub-regions of the hippocampus. Overall, the number of GAD67+ cells is unlikely to change, as previous reports from our laboratory indicate no influence of CORT on levels of this protein in the hippocampus of stressed rats (Lussier, Romay-Tallon, Caruncho, & Kalynchuk, 2013). Likewise, CCK+ cells have not been found to be altered by chronic stress, and so no change in these cells is expected (Hu, Zhang, Czeh, Flugge, & Zhang, 2010). However, previous research has shown that interneurons expressing PVB, NPY, SOM, CR, and CB are all susceptible to the deleterious effects of chronic stress, and so it is possible that CORT will cause a decrease in these markers in a number of hippocampal sub-regions (Hu et al., 2010; Lussier et al., 2013; Yu, An, & Lian, 2010). On the other hand, recent evidence suggests that in schizophrenia, the downregulation of reelin is not accompanied by interneuron loss (Guidotti et al., 2000a). Therefore, I hypothesize that I will not find a significant reduction in interneuron markers that highly co-localize with reelin (i.e., SOM and CR) in the SGZ or hilus of the DG (Chapter 3).

Question 3: Upon the discovery that a loss in GABAergic cells could not fully account for the decrease in reelin expression evident after CORT treatment, I was interested in examining the potential involvement of epigenetic processes in this deficit. Specifically, could DNA methylation play a role in the dowregulation of reelin expression in the hippocampus? As a preliminary examination into this possibility, I examined the influence of repeated CORT (40mg/kg) injections on global levels of DNMT1 and MeCP2 expression in the hippocampus. I also examined how CORT influences the number of MeCP2+ cells in the SGZ and hilus, the two regions where reelin is downregulated by stress.

Hypothesis: To date, levels of DNMT1 and MeCP2 have not been evaluated in the hippocampus of adult rats subjected to chronic stress. However, recent preclinical evidence does suggest that deregulation of gene expression via epigenetic processes are linked to depressive symptomatology (Sun, Kennedy, & Nestler, 2013). Although I do not expect to see a significant

change in global patterns of DNA methylation (as evidenced by changes in DNMT1 and MeCP2 expression), I believe that the number of methylated cells in SGZ and hilus will be increased. Although I cannot conclude from this work that the expected increase is occuring in reelin-expressing interneurons, a positive correlation between reelin downregulation and increased methylation markers in these hippocampal regions would suggest it is possible (Chapter 4).

CHAPTER 2

Downregulation of reelin in an animal model of depression is prevented by imipramine and correlates with deficient neuronal maturation in the adult dentate gyrus

Erin Y. Fenton[a], Neil M. Fournier[b], April L. Lussier[c], Hector J. Caruncho[d], Lisa E. Kalynchuk[e]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[b] Department of Psychiatry, Yale University School of Medicine, New Haven, CT, USA

[c] Department of Molecular Pharmacology and Physiology, University of South Florida, Tampa Bay, FL, USA

[d] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[e] College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Abstract

Prolonged exposure to stress or glucocorticoids is an important risk factor for the development of depression, as revealed in both clinical studies of patient populations and preclinical studies with animal models. However, the mechanisn by which repeated stress might lead to depression is not clear. Several prominent hypotheses have suggested that depression might arise from stress-induced decreases in adult neurogenesis. There is also recent evidence that glucocorticoids decrease expression of the glycoprotein reelin selectively in regions of the hippocampus that are involved in adult neurogenesis. The purpose of this experiment was to determine whether the deleterious effects of repeated glucocorticoid administration on hippocampal neurogenesis and reelin expression could be prevented by co-administration with the antidepressant imipramine. Rats were subjected to corticosterone injections, or corticosterone injections plus either 10 or 15mg/kg of imipramine injections, or vehicle injections, once per day for 21 consecutive days. They were then exposed to the forced swim test to assess depressive symptoms, and then sacrificed for immunohistochemical examination of immature neuron number and dendritic complexity and the presence of reelin+ interneurons. Our results show that corticosterone increases depression-like behavior, decreases the number of reelin+ cells, and decreases the number and complexity of immature neurons in the dentate gyrus. Importantly, all of these behavioral and cellular phenotypes were prevented by imipramine, suggesting that reelin may be a key mechanism involved in the long-term adaptations necessary for antidepressant efficacy.

1. Introduction

Depression is a severe and devastating neuropsychiatric illness that affects roughly 16% of the population (Kessler et al., 2003). In the quest to better understand the neurobiological basis of depression, growing evidence has suggested that dysfunction of the hippocampus may underlie, at least in part, the etiology and treatment of this disorder (Nestler et al., 2002a; Nestler et al., 2002b). Indeed, both neuroimaging and post-mortem studies have consistently reported decreased hippocampal volume and altered cell morphology in depressed patients (MacQueen et al., 2003; Sheline et al., 2003; Sheline et al., 1999; Sheline et al., 1996). Although a number of different mechanisms could play a role in the hippocampal pathology observed in major depressive disorder, one of the most compelling hypotheses suggests that stress-related illnesses, including depression, might arise from abberant hippocampal neurogenesis (Fournier & Duman, 2012; Sahay & Hen, 2007). In particular, preclinical work with animal models of depression has shown that various forms of chronic stress or glucocorticoid exposure decrease the proliferation, differentiation, and survival of newborns neurons in the adult hippocampus (Bambico & Belzung, 2012; Banasr & Duman, 2007). However, the strongest support for an association between neurogenesis and depression comes from observations that virtually all antidepressant medications and environmental manipulations that lead to antidepressant-like behavioral effects increase adult neurogenesis (Fournier & Duman, 2012). Indeed, several studies have demonstrated an important role of hippocampal neurogenesis in mediating the behavioral actions of some antidepressant treatments (David et al., 2009; Santarelli et al., 2003). Importantly, these data have fuelled intense research efforts aimed at characterizing the molecular mechanisms governing neurogenesis in the adult brain and their relationship to depression and antidepressant treatment.

Given its connection with a variety of neuropsychiatric disorders (Fatemi et al., 2000), the extracellular matrix protein reelin is of particular interest in helping us understand the etiology of depressive illness. In the adult brain, reelin is synthesized and secreted by a subset of cortical and hippocampal interneurons, where it facilitates neurogenesis, dendrite development, and synaptic plasticity (Herz & Chen, 2006; Teixeira et al., 2012). Interestingly, clinical and preclinical work has shown that reelin is downregulated in the hippocampus of both depressed patients (Fatemi et al., 2000) and in rats exposed to corticosterone (Lussier et al., 2009). In addition, heterozygous reeler mice (HRM) with 50% of normal levels of reelin show increased

vulnerability to the deleterious effects of glucocorticoids (Lussier et al., 2011), suggesting that individual differences in baseline levels of this protein may confer susceptibility to developing depressive illness.

Many of the neurobiological and behavioral effects of chronic stress or glucocorticoids can be blocked or reversed by chronic antidepressant treatment (Bambico & Belzung, 2012). In fact, the degree to which these changes are influenced by antidepressants is often used as an index of their relevance to depressive illness. Recent evidence has shown that antidepressants can increase reelin synthesis in the frontal cortex of rodents, and that reelin overexpression acts as a buffer against the effects of chronic stress (Fatemi et al., 2009; Teixeira et al., 2011). However, it is not known whether antidepressants can influence reelin expression in an animal model of depression, or whether these changes parallel alterations in depressive-like behavior and neurogenesis. Here, we examined this relationship using an animal model of depression, in which daily injections of the stress hormone corticosterone (CORT; 40mg/kg) produce robust increases in depressive-like behavior (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009). We also examined whether changes in reelin expression after CORT and antidepressant treatment are associated with alterations in the number and maturation of immature dentate granule cells. Our findings reveal that CORT increases depressive-like behavior in the forced swim test (FST), decreases the number of reelin+ cells in the subgranular zone (SGZ) and hilus, and alters the number and complexity of immature neurons in the granule cell layer (GCL). Importantly, all of these behavioral and cellular phenotypes were prevented by imipramine, further supporting the role of reelin in depressive illness and as an important target of antidepressants.

2. Materials and Methods

2.1 Subjects

Adult male Long-Evans rats ($N = 73$) were purchased from Charles River Canada (Montreal, Quebec). They weighed approximately 200-250g at the time of their arrival. The rats were housed individually in standard rectangular 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-hr light/12-hr dark cycle. All experimental procedures were conducted in accordance with the

guidelines of the Canadian Council on Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply.

2.2. CORT and Imipramine Treatment

Rats received one of four treatments: repeated CORT injections (CORT rats; *n*= 19), repeated CORT injections plus 10 mg/kg imipramine (CI-10 rats; *n*= 18), repeated CORT injections plus 15mg/kg imipramine (CI-15 rats; *n* = 17), or repeated vehicle injections (vehicle rats; *n*= 19). The injections were delivered once per day for 21 consecutive days; CORT and imipramine injections were delivered 6h apart. CORT (MP Biomedicals) was suspended in physiological saline with 2% Tween-80 (Sigma). Imipramine hydrochloride (Sigma) was dissolved in 0.9% saline. CORT and vehicle injections were delivered subcutaneously at a volume of 1 ml/kg and imipramine was given intraperitoneally at the same volume. All CORT injections were administered at 40 mg/kg. Imipramine doses were chosen based on previous reports indicating their ability to reverse depressive-like behavior and dendritic remodeling in rats (Bessa et al., 2009; Li, Witkin, Need, & Skolnick, 2003; Peng et al., 2008) Separate groups of rats were used for the behavioral and post-mortem analyses.

2.3 Forced Swim Testing

We used a modified FST to assess depression-like behavior (Marks et al., 2009). Rats (vehicle, *n* = 10; CORT, *n* = 10; CI-10, *n* = 10; CI-15, *n* = 9) were placed into a rectangular Plexiglas swim tank (25 cm long x 25 cm wide x 60 cm high) that was filled with 27°C (± 2°C) water to a depth of 30 cm. Each rat was placed individually into the forced swim tank for 10 minutes. Active and inactive components of forced swim behavior were scored; including the time spent climbing, swimming, and immobile. Depression-like behavior was inferred from an increase in time spent immobile and a decrease in time spent swimming or climbing (Porsolt et al., 1978; Gregus et al., 2005).

2.4 Tissue Preparation and Immunohistochemistry

Immunohistochemistry was used to assess the effect of CORT and imipramine on the number of reelin+ cells in the SGZ and hilus, as well as the survival and maturation of immature granule cells (via doublecortin; DCX) in the dentate gyrus (DG). A subset of rats

from each group (vehicle, $n = 9$; CORT, $n = 9$; CI-10, $n = 8$; CI-15, $n = 8$) was deeply anesthetized with sodium pentobarbital and transcardially perfused with saline, followed by 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS: 0.1M, pH 7.4). Brains were post-fixed in 4% paraformaldehyde for 72 hours, and then sectioned on a vibrating microtome at 50μm.

Reelin immunohistochemistry was performed using our previously published methods (Fournier et al., 2010; Lussier et al., 2009). Briefly, free-floating sections were incubated in 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity, blocked in a 0.3% (v/v) Triton X-100 PBS buffer containing 5% (v/v) normal horse serum (NHS) and 1% (w/v) bovine serum albumin (BSA; Sigma) followed by incubation with a mouse anti-reelin monoclonal antibody (1:2000 Chemicon International, 48 hrs, 4^{o}C). Sections were then incubated with a secondary biotinylated antibody (horse anti-mouse, 1:200, 2 h, Vector Laboratories) and then avidin-biotin peroxidase complex (1:200, 1 h, Vectastain ABC Elite, Vector Laboratories), and immunolabeled cells were visualized with a 0.033%3′-diaminobenzidine (DAB, Sigma Aldrich) solution.

DCX immunohistochemistry was also performed according to our previously published methods (Fournier et al., 2010). Briefly, free-floating tissue sections were incubated in 0.5% H_2O_2 for 30 min, 1% H_2O_2 for 1h, and then 0.5% H_2O_2 for an additional 30 min. Sections were then incubated with a goat anti-DCX monoclonal antibody (1:200; Sana Cruz Biotechnology; diluted in 5% NHS and PBSx, 48h, 4°C), followed by incubation with a biotinylated secondary antibody (horse anti-goat; 1:500; Vector Labs; diluted in 5% NHS and PBSx, 2h), then ABC for 1h (1:500, Vector Labs). Immunolabelling was visualized with 0.065% DAB, 4.167% nickel ammonium sulfate, and 0.085% H_2O_2 diluted in 0.175M sodium acetate.

Co-labeling of reelin and DCX in the SGZ and inner GCL was examined via immunofluorescence to determine whether DCX+ cells cluster where reelin+ cells are present and vice versa. Briefly, sections were first subjected to antigen retrieval for 30min (10 mM sodium citrate) at 80°C to reduce protein cross-linking and then blocked in 1% BSA and 5% donkey serum solution for 1hr followed by incubation with the mouse anti-reelin (1:200, Chemicon International, 48h, 4°C) primary antibody. Sections were then incubated with a fluorescent-conjugated donkey anti-mouse (1:500, Alexa 488 Invitrogen) secondary antibody for 3h at room temperature. After a series of PBS washes, sections were blocked a second time (as

above) for 1h and then incubated overnight with the goat anti-DCX primary antibody (1:200, Santa Cruz Biotechnology, 24h, 4°C). Sections were then incubated with a fluorescent-conjugated donkey anti-goat (Alexa 568, 1:500, Invitrogen, 3h) secondary antibody followed by DNA counterstaining (1:1000, Hoeschst 33342, Sigma, 20 min).

2.5 Stereology, Analysis of Dendritic Complexity & Fluorescent Imaging

Reelin+ and DCX+ cells were counted using established stereological procedures. Every sixth section was examined at 400X (reelin) or 1000X (DCX) magnification on a Nikon E800 microscope using a computerized stereology system (MicroBrightfield). The total number of cells was estimated using the unbiased optical fractionator method as described previously (Fournier et al., 2010). Counting parameters included a dissector height of 9 μm for reelin and 15μm for DCX, a sampling area of 70μm X 50 μm (SGZ, reelin), 150 μm X 150 μm (hilus, reelin), and 90 μm X 90 μm (DCX) and a counting frame of 40 μm X 40 μm (SGZ, reelin), 75μm X 75 (hilus, reelin), and 50μm X 540μm (DCX). A guard zone of 2 μm for reelin and 5μm for DCX was used to avoid sectioning artifacts.

Dendritic complexity was assessed at 1000X in a subset of DCX+ cells. Four sections per brain were quantified, and a total of four neurons per section were randomly chosen and reconstructed on a Nikon E800 microscope using Neurolucida software (Microbrightfield). Morphological analysis of the reconstructed neurons was done using NeuroExplorer software (MicroBrightfield), and all measurements were obtained using branched structure analysis. From these measurements, we calculated the average number of dendritic branches per neuron, the average apical and basal dendrite length, and the average apical and basal dendrite branch order.

Fluorescent images were acquired on a Nikon E800 microscope using PictureFrame computer software (version 2.2) with a MicroFire digital camera (Optronics) and a pixel resolution of 1600 X 1200. Monochrome images of reelin, DCX, and Hoescht were taken and imported into Adobe Photoshop (version 8.0) to be colored and merged. To enhance clarity, all digital images were adjusted only for brightness and contrast using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

2.6. Statistical Analysis

Group differences in all measures were assessed using separate one-way ANOVA's with treatment as the between-groups factor. This was followed by Fisher's LSD post-hoc tests when appropriate. All data were analyzed using SPSS (version 13.0, Chicago, IL). The criterion for statistical significance was set at $p < .05$.

3. Results

3.1 Imipramine Prevents the Development of CORT-induced Increases in Depressive-like Behavior

We have previously found that 21 days of CORT administration reliably increases depression-like behavior in rats (Gregus et al., 2005; Johnson et al., 2006; Marks et al., 2009). The results of this experiment confirm these previous findings, as indicated by significant treatment effects on immobility ($F(3,35) = 5.638$, $p = .003$) swimming ($F(3,35) = 3.345$, $p = .03$) and climbing ($F(3,35) = 4.011$, $p = .015$) (see Fig. 2-1). Specifically, CORT-treated rats spent more time immobile ($p < .001$) and less time swimming ($p = .004$) and climbing ($p = .002$) compared to the vehicle rats. Importantly, the effect of CORT on these behaviors was reversed by co-treatment with imipramine. The CI-15 rats spent significantly more time swimming ($p = .035$) and less time immobile ($p = .027$) than the CORT rats. However, the beneficial effect of imipramine was dose-dependent because the CI-10 rats did not differ from the CORT rats on any of the behavioral measures (all p-values > .05).

3.2. Imipramine Prevents CORT-induced Decreases in Hippocampal Reelin

After confirming the presence of depression-like behavior in CORT rats and the therapeutic effect of imipramine, we examined reelin expression in the hippocampus. There was a significant treatment effect on the number of reelin+ cells in both the SGZ [$F(3,30) = 6.582$, $p = .001$] and hilus [$F(3,30) = 6.302$, $p = .002$] (see Fig. 2-2). Specifically, the CORT-treated rats had fewer reelin+ cells in the SGZ ($p = .002$) and hilus ($p = .009$) compared to the vehicle rats. Importantly, this effect was prevented by both doses of imipramine (CI-10: SGZ $p = .001$, hilus $p < .001$; CI-15: SGZ $p = .001$, hilus $p = .002$).

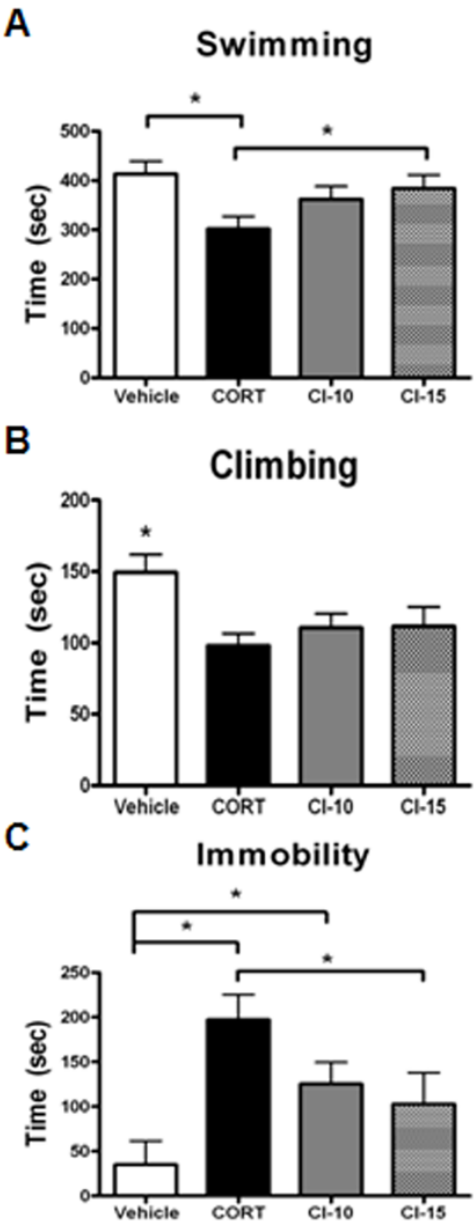

Figure 2-1. The effect of CORT and imipramine administration on depressive-like behavior in the FST. CORT significantly increased depressive-like behaviors in rats, as indicated by a decrease in active behaviors (panels A-B) and an increase in inactive behaviors (Panel C) in the FST. All rats injected with CORT showed a decrease in climbing behavior. Animals treated with CORT alone also showed a decrease in swimming behavior. Imipramine dose-dependently prevented this effect as animals in the CI-15 group spent significantly more time swimming than animals treated with CORT alone. CORT also significantly increased immobility in the FST compared to vehicle and CI-15 treated animals. In addition, animals in the CI-10 group spent significantly more time immobile compared to vehicle but not CI-15 treated animals, again showing that imipramine dose dependently decreases depressive-like behaviors in this task. Asterisks (*) denote a statistical significant difference ($p < 0.05$). Error bars represent the mean ± standard error of the mean.

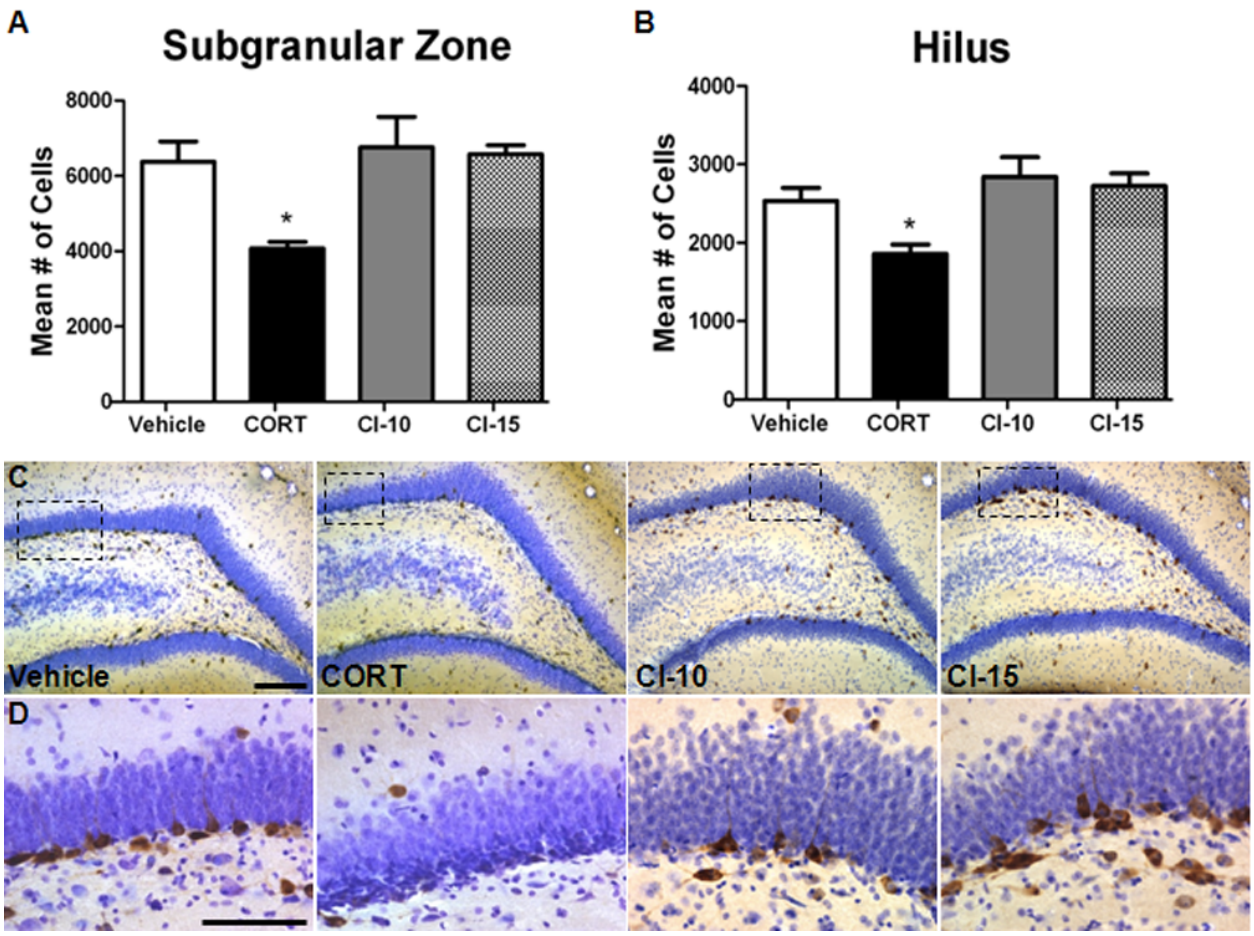

Figure 2-2. The effect of CORT and imipramine on the number of reelin+ cells in the SGZ and hilus. Panels A and B show the number of reelin+ cells between treatment group in the SGZ and hilus respectively. CORT significantly reduced the number of reelin+ cells in both the SGZ and hilus of the hippocampus, while administration of CORT plus 10mg/kg or 15mg/kg of imipramine significantly increased the number of cells in these regions. Error bars represent the mean ± standard error of the mean. Panels C and D show representative photomicrographs of reelin immunoreactivity in each treatment group. Asterisk (*) denotes statistical significance ($p < 0.05$). Scale bar: 200μm.

3.3. Imipramine Prevents CORT-induced Decreases in the Number and Maturation of Immature Neurons

Close examination of the photomicrographs in figure 2-2 show that CORT-treated rats tend to have gaps in the SGZ where no reelin+ cells are present, and that regions of the inner granule cell layer within these gaps tend to contain small darkly stained cells that appear to be immature neurons with a heterochromatic nucleus. These observations suggested to us that the absence of reelin in specific sections of the SGZ might influence the rate of granule cell maturation in associated regions of the granule cell layer. Therefore, we examined the effect of CORT and imipramine on DCX immunoreactivity, as DCX is a marker of immature neurons that are up to 3 weeks old (Brown et al., 2003). As expected, there was a significant treatment effect on the number of DCX+ neurons in the SGZ and GCL ($F(3,29) = 17.207$, $p < .001$) (Fig. 2-3A), with the CORT-treated rats having significantly fewer DCX+ neurons compared to the vehicle rats ($p = .001$). Consistent with our behavioral data, imipramine dose-dependently prevented this effect: The CI-15 rats ($p < .001$), but not the CI-10 rats ($p = .903$), had significantly more DCX+ neurons than did the CORT-treated rats.

To characterize the effect of CORT on cell maturation, we analyzed dendritic morphology in a subset of DCX+ cells (Fig. 2-3B-F). In general, DCX+ cells from the CORT-treated rats had reduced dendritic complexity compared to the vehicle rats. This included fewer dendritic branches per neuron ($p = .006$), a reduced number of higher order branches on apical dendrites ($p = .015$) and decreased apical dendrite length ($p < .001$) (Fig. 2-3 B-D). We also noticed the presence of basal dendrites in DCX+ cells in the CORT, CI-10, and CI-15 rats (Fig 2-3 E-F). This finding is particularly important because the presence of basal dendrites on granule neurons is a sign of immaturity (Lauer, Beckmann, & Senitz, 2003; Ribak, Tran, Spigelman, Okazaki, & Nadler, 2000). This was most pronounced in the CORT-treated rats compared to the vehicle rats, as measured by basal dendrite length ($p = .001$) and basal dendrite branching order ($p < .001$). Importantly, all of these morphological changes were diminished by co-treatment with the higher dose of imipramine. Specifically, the CI-15 rats had significantly more dendritic branches per neuron ($p < .001$), a greater number of higher order apical dendrite branches ($p = .001$), increased apical dendrite length length ($p = .004$), significantly less basal dendrite

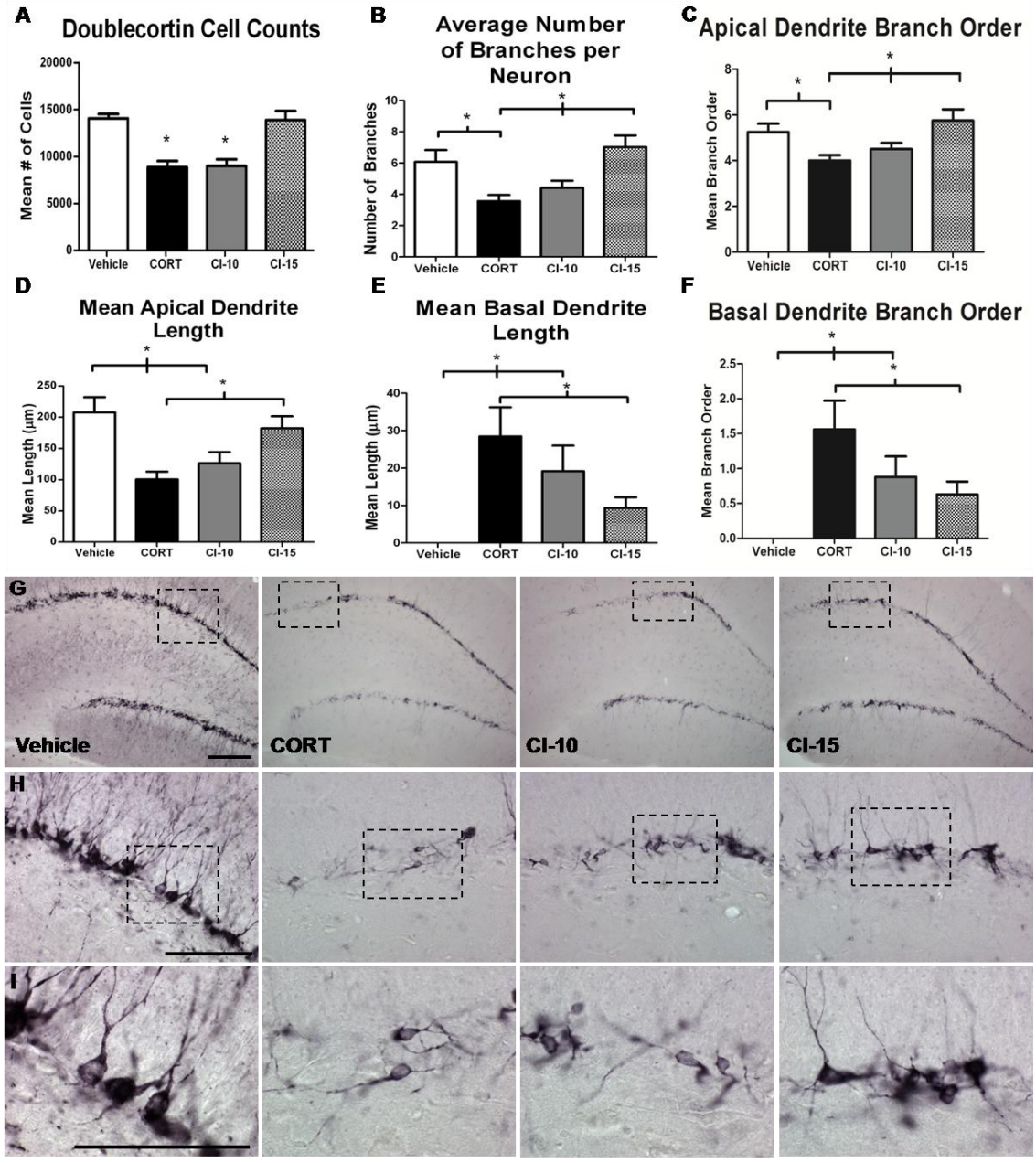

Figure 2-3. The effect of CORT and imipramine on the number and complexity of DCX+ cells in the GCL. Animals in CORT and CI-10 groups had significantly fewer DCX+ cells compared to vehicle and CI-15 treated animals (panel A). CORT also significantly decreased the number of branches and branch points per neuron (panel B-C), and apical dendrite length compared to vehicle and CI-15 animals. CORT and CI-10 animals also had significantly longer (panel E) and more complex (pane F) basal dendrites compared to vehicle treated animals. This effect was prevented in the CI-15 group as these animals had significantly shorter and less complex basal dendrites compared to the CORT-treated animals. Panels G-I show representative photomicrographs of DCX immunoreactivity in each treatment group. Asterisk (*) denotes statistical significance ($p < 0.05$). Scale bar: 200µm.

branching (p = .025) and shorter basal dendrite length (p = .023) compared to CORT treated rats. On these measures, the CI-15 rats were similar to the vehicle rats (all p-values > .05). The CI-10 rats did not differ from the CORT rats on most measures of dendritic complexity (all p-values > .05).

3.4 Surviving DCX Cells Cluster in Regions of the SGZ Where Reelin is Present

To further examine the relationship between the presence of reelin and the maturation of adult-born neurons, we performed double immunofluorescent labeling for reelin and DCX, and examined co-localization in the SGZ and inner GCL. Consistent with our immunohistochemical findings for reelin and DCX alone, CORT decreased both reelin and DCX expression (Fig.2- 4). The lack of dendritic branching in DCX+ cells is particularly apparent under high magnification in the CORT and CI-10 rats, as is the reversal of this effect in the CI-15 rats. Interestingly, in all groups, areas of the SGZ containing reelin+ cells also tended to contain DCX+ cells in the adjacent inner GCL, providing a further indication that reelin+ cells are particularly well located to influence the maturation of newborn granule cells.

4. Discussion

The results of this study make several important points about the relationship between reelin, glucocorticoids, and depression. First, they show that CORT-induced increases in depression-like behavior on the FST parallel changes in reelin expression in the SGZ and hilus. Importantly these behavioral and cellular phenotypes were prevented by co-treatment with imipramine. Although these data are correlational, they support previous reports of resilience to a depressive phenotype in reelin-overexpressing mice (Teixeira et al., 2011) and increased susceptibility in reelin haploinsufficient mice (Lussier et al., 2011). Repeated CORT administration also leads to a significant reduction in the number of immature granule cells in the dentate, along with a marked reduction in the complexity of the ones left surviving. Again, changes in the number and morphology of these cells were prevented with imipramine. Although it is unclear from this experiment whether altered reelin expression is responsible for the morphological changes in dentate granule cells, the fact that we see decreased cell number and complexity in areas devoid of reelin suggests that this possibility is likely.

Recent studies suggest that changes in reelin expression contribute to the pathogenesis of

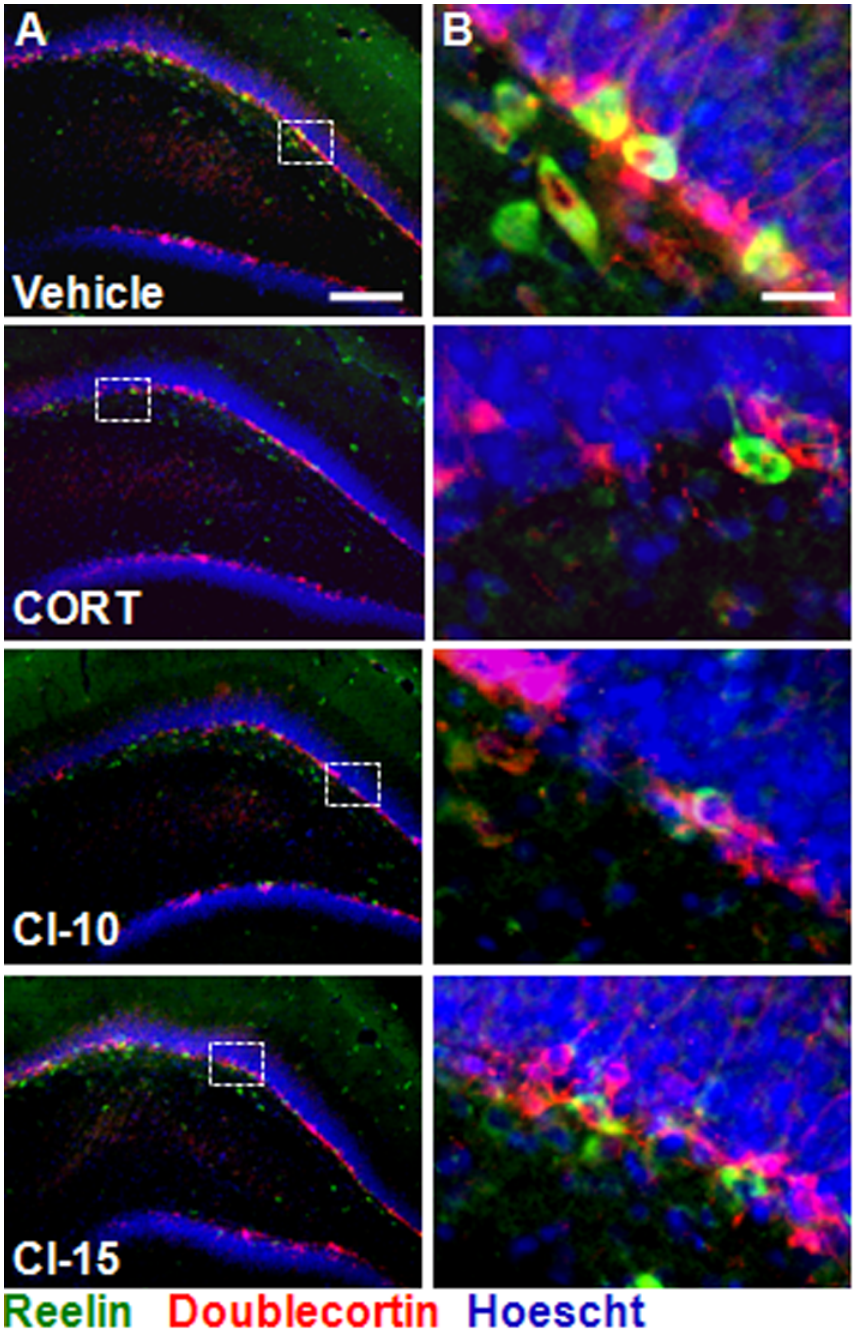

Figure 2-4. The affect of CORT and imipramine on immunofluorescent labeling of reelin and DCX in the DG. Panels A and B show representative immunofluorescent labeling of reelin and DCX between treatment groups. In all groups, areas of the SGZ containing reelin cells also tended to have DCX cells in the adjacent inner GCL. Similar to our cell counts, CORT decreased immunofluorescent expression of both reelin and DCX. The lack of dendritic branching in animals treated with CORT alone and CORT plus 10mg/kg of imipramine is also apparent under high magnification, as is the reversal of this effect in animals treated with CORT plus 15mg/kg of imipramine (Panel B). Scale bar panel A: 200μm. Scale bar panel B: 20μm.

a number of neurpsychiatric illnesses, including depression. Specifically, analyses of post-mortem tissue from depressed patients have revealed general decreases in reelin expression in the hippocampus (Fatemi et al., 2000). In support of these results, we have recently shown that CORT, but not restraint stress, reduces the number of reelin+ cells in SGZ and the stratum-lacunosum moleculare of the CA1 (Lussier et al., 2009). Interestingly, our previous findings indicate that depressive-like behavior is only increased after CORT treatment and not restraint stress (Gregus et al., 2005), suggesting a link between the expression of reelin and the emergence of depressive behaviors. Further support for this relationship comes from work in our lab with HRM. These mice have approximately 50% of normal brain levels of reelin, yet do not have the gross morphological or behavioral abnormalities of reelin deficient mice (Tueting et al., 1999). Importantly, we have found that the effect of CORT on HRM in the FST is more pronounced compared to wild-type animals receiving CORT (Lussier et al., 2011), suggesting that impaired reelin signaling increases vulnerability to the deleterious effects of glucocorticoids. Conversely, mice that overexpress reelin in the forebrain spend less time immobile in the FST compared to their wild-type counterparts treated with CORT (Teixeira et al., 2011), pointing to the antidepressant-like properties of this protein. In the current paper we expand on these data by showing that the antidepressant imipramine prevents CORT-induced decreases in reelin+ cells in the hippocampus and improves performance in the FST. Interestingly, a great deal of overlap exists between the cellular changes induced by antidepressants and the biological activities of reelin (Bambico & Belzung, 2012; Banasr & Duman, 2007; Fatemi, 2005; Fatemi, 2011), suggesting that the behavioral changes observed in this study may be influenced by corresponding changes in reelin expression.

Many of the disorders associated with altered reelin expression are characterized by extensive dendritic remodeling, and abnormalities in neurogenesis (Bambico & Belzung, 2012; Inta et al., 2012). Specifically, reduced hippocampal volume has been repeatedly documented in the brains of depressed patients, with the greatest reductions occurring in those suffering from multiple depressive episodes (MacQueen et al., 2003; Sheline et al., 2003). Similarly, rodent models of the disease have reliably shown decreases in hippocampal cell proliferation and dendritic complexity in surviving cells, and many of these changes are rescued by antidepressant treatment (Banasr & Duman, 2007). Results of the current study support these previous findings, as CORT treated animals had fewer DCX-labeled granule cells, and the ones left surviving

showed reduced dendritic complexity. Importantly, this effect was prevented by co-treatment with imipramine.

Reelin signaling influences various aspects of neurogenesis including cell migration, neural progenitor fate, dendritic development and the integration of dentate granule cells into hippocampal circuitry (Lakoma et al., 2011; Teixeira et al., 2012). For example, deletion of Dab1 in dentate neuroprogenitor cells leads to abnormal orientation and ectopic migration of these cells into the hilus (Teixeira et al., 2012). Moreover, a large number of Dab1 deficient cells in both mice and rats do not develop appropriately complex dendritic processes, as evidenced by marked reduction in dendritic length and reduced number of secondary and tertiary processes (Teixeira et al., 2012). Opposite to this, mice that overexpress reelin in the forebrain show adult generated neurons with enhanced dendritic development (Teixeira et al., 2012). Specifically, adult generated granule cells two weeks of age show significant increases in dendritic tree extension, and in the complexity of their branches, compared to wild-type animals. Two week old granule cells also tend to be located deeper in the GCL. These findings were not evident at 4 or 8 week time points, suggesting that reelin overexpression leads to faster migration and maturation (Teixeira et al., 2012). In the current study, reelin and DCX immunofluorescence revealed that in areas of the SGZ devoid of reelin+ cells, DCX expression tended to be significantly reduced. Moreover, in these reelin-devoid gaps, reduced dendritic complexity in CORT treated animals and reversal with imipramine is particularly apparent at high magnification. Importantly, these observations mirror what we found in our analysis of reelin and DCX cell numbers, as well as our measures of dendritic complexity. As we suspect in our study, CORT-induced reductions in reelin expression leads to slowed maturation of immature dentate granule cells, which can be prevented by antidepressant treatment.

Another important finding in our study was that all CORT treated animals developed prominent basal dendrites on their granule cells. This effect again was dose-dependently diminished by co-treatment with imipramine. Interestingly, increased presence of basal dendrites on granule cells has been associated with a number of neurological disorders and is often associated with a more immature state (Lauer et al., 2003; Ribak et al., 2000). Recent evidence has also shown that disruption of reelin signaling in neural progenitor cells via Dab1 deletion results in the development of basal dendrites in up to 50-78% of these neurons. These cells also develop complex dendritic trees in the hilus, and are being functionally integrated into existing

hippocampal circuitry. Although it is unclear from our study whether the basal dendrites we see develop after CORT treatment are functionally integrated, it is possible that they are, and that this, along with changes in the architecture of existing granule cells, could underlie some of the cognitive deficits we see in depressed patients and animals exposed to stress (Foland-Ross & Gotlib, 2012; Sterner & Kalynchuk, 2010).

Our study provides the first evidence that antidepressant medication protects against stress-induced hippocampal reelin loss. Although we cannot rule out the possibility that changes in other proteins and neurotrophic factors are involved in the behavioral and morphological deficits resulting from CORT treatment, the temporal relationship that exists between altered hippocampal reelin signaling, the development of depression-like behavior and alterations in neurogensis and neuronal maturation in the hippocampus, suggest it likely plays at least some role. More importantly, our data suggest that maintenance of reelin signaling in the hippocampus may be a long-term adaptation required for the efficacy of current treatments for depression.

CHAPTER 3

Immunohistochemical examination of GABAergic interneuron markers in the hippocampus of rats exposed to repeated corticosterone injections

Erin Y. Fenton[a], Brian D. Kulyk[a], Hector J. Caruncho[b], Lisa E. Kalynchuk[c]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[b] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[c] College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Abstract

Reelin is a large protein expressed primarily in inhibitory interneurons in the adult brain. Constitutively secreted into the extracellular matrix, reelin plays a significant role in synaptic function and neuroplasticity. In recent years, converging evidence suggests a role for reelin in the pathophysiology of major depression. In particular, a reduction in the number of reelin+ interneurons in the hippocampus has been documented in preclinical models of depression and in post mortem brain tissue from depressed patients. However, from these data we do not know how the populations of interneurons that express reelin are influenced – do these cells die? Or do they simply no longer synthesize reelin protein? In the current study we used the CORT model of depression to address these questions. Rats received subcutaneous injections of CORT (40mg/kg) or vehicle for 21 consecutive days. The expression of 7 interneuron markers (GAD67, parvalbumin, calretinin, calbindin, somatostain, neuropeptide y and cholecystokinin) was then examined in the dentate gyrus, CA1 and CA3 via immunohistochemistry. Our results show that CORT decreases the number of parvalbumin, calretinin, somatostatin, and neuropeptide-y+ neurons in a number of hippocampal sub-fields, suggesting that the function of these neurons is likely compromised. However, the degradation of these proteins and peptides occurred in the absence of interneuron loss. This finding is particularly important because it suggests that glucocorticoids target an intracellular mechanism to decrease the synthesis and expression of reelin. Moreover, it raises the possibility that drug targets could be developed to reverse reelin-related deficits that occur with chronic neuropsychiatric conditions.

1. Introduction

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter that helps to control the balance of excitation and inhibition in the brain. This neurotransmitter is released by inhibitory interneurons, which unlike their excitatory neuron counterparts, encompass a vast number of different cell types (Freund & Buzsaki, 1996; Mendez & Bacci, 2011). The classification of interneurons is based on the expression of certain calcium binding proteins and/or neuropeptides, their specific electrophysiological characteristics, functional characteristics of synapses that they form and receive, as well as specific anatomical and morphological properties (Freund & Buzsaki, 1996; Mendez & Bacci, 2011). Although inhibitory interneurons make up only 15 to 20% of the total neurons in our brain, they play an integral role in neural function. Overall, these cells provide inhibition to neuronal networks and dictate the temporal pattern of activity of principal cells and other inhibitory neurons (Klausberger & Somogyi, 2008). Ultimately, this control over activity modulates the integration of synaptic inputs important for memory formation and behavior (Buzsaki & Chrobak, 1995; Buzsaki & Draguhn, 2004; Freund & Buzsaki, 1996; Paulsen & Moser, 1998; Somogyi & Klausberger, 2005).

Not surprisingly, a number of neuropsychiatric disorders have been associated with abnormal GABA function (Femenia, Gomez-Galan, Lindskog, & Magara, 2012; Mohler, 2012; Stan & Lewis, 2012). In particular, recent evidence suggests that altered GABA signaling may play a role in the underlying pathophysiology of depression. For example, reduced levels of GABA in the plasma, cerebrospinal fluid, and areas of the prefrontal and occipital cortex have been reported in patients with depression (Gerner & Hare, 1981; Kasa et al., 1982; Petty et al., 1992; Sanacora et al., 2000; Sanacora et al., 1999). Decreases in a number of GABAergic interneuron markers have also been documented in frontal and occipital cortices and in the hippocampus of depressed patients (Fatemi et al., 2000; Khundakar, Morris, & Thomas, 2011; Maciag et al., 2010; Rajkowska et al., 2007). Of particular interest in this regard is the extracellular matrix protein reelin, which is synthesized and released by specific sub-populations of GABAergic interneurons in the hippocampus and cortex of the adult brain (Pesold et al., 1998), and plays an important role in regulating synaptic plasticity, neurogenesis, dendritic development, and glutamatergic neurotransmission (Herz & Chen, 2006; Teixeira et al., 2012). Interestingly, recent evidence suggests that the number of reelin+ interneurons is downregulated in the hippocampus of depressed patients (Fatemi et al., 2000). Putative animal models of the

disorder have reported similar findings (Lussier et al., 2009; Lussier et al., 2011), and show that these changes can be prevented with antidepressant treatment (see chapter 2). However, it remains unknown how the population of interneurons that express reelin are affected in depression. For example, does this subpopulation of interneurons succumb to excitotoxic-mediated cell death associated with repeated exposure to harmful stress hormones? Or, is this subpopulation of GABAergic neurons still present but for some reason no longer synthesizing reelin protein? Although either scenario could essentially produce the same pattern of structural and functional change, the latter situation raises the possibility that drug targets could be developed to reverse reelin-related deficits that occur with chronic neuropsychiatric conditions.

In the current study we addressed these questions by examining how repeated administration of the primary stress hormone corticosterone (CORT), influences interneuron markers that co-localize with reelin in the adult rodent hippocampus. We chose this stress paradigm because 21 days of CORT injections (40mg/kg) is known to produce reliable and robust increases in depression-like behavior in rodents (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009; Sterner & Kalynchuk, 2010). The interneuron markers examined in this study were chosen based on previous co-localization reports in rodents. Specifically, all reelin+ cells in the hippocampus co-localize with GAD67, the enzyme responsible for converting glutamate to GABA (Pesold et al., 1998). A large percentage of reelin+ cells also co-localize with the calcium-binding protein calretinin (CR; 58%) and the peptide somatostatin (SOM; 75%). To a lesser extent, reelin co-localizes with neuropeptide-y (NPY; 30%), the calcium-bindng protein calbindin (CB; 14%), the peptide cholecystokinin (CCK) and the calcium-binding protein parvalbumin (PVB; Alcantara et al., 1998). In light of this evidence, we chose to examine how CORT influences the number of cells expressing each of these markers in the dentage gyrus (DG), CA3, and CA1 regions of the hippocampus. Results of this study will help determine whether the interneuron markers that co-localize with reelin are also susceptible to the deleterious effects of glucocorticoids and shed light on the extent to which the inhibitory network of GABAergic cells in the hippocampus may be disrupted in depression.

2. Materials and Methods

2.1 Subjects

Adult male Long-Evans rats (N = 24) were purchased from Charles River Canada (Montreal, Quebec). They weighed approximately 200-250g at the time of their arrival. The rats were housed individually in standard rectangular 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-hr light/12-hr dark cycle. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply.

2.2 CORT Treatment

Rats received either CORT (n = 12; 40mg/kg) or vehicle (n = 12) injections once per day for 21 consecutive days. CORT (Steraloids) was suspended in physiological saline with 2% Tween-80 (Sigma Aldrich). All injections were delivered subcutaneously at a volume of 1 ml/kg. We chose this paradigm because 21 days of CORT injections at this dose is known to produce reliable and robust increases in depression-like behavior in rodents (Gregus et al., 2005; Johnson et al., 2005; Kalynchuk et al., 2004; Marks et al., 2009).

2.3 Immunohistochemical Analysis of Interneuron Markers

Immunohistochemistry was used to examine the effect of CORT on the number of GABAergic interneuron markers in the hippocampus. Specifically, we examined GAD67 parvalbumin (PVB), calretinin (CR), calbindin (CB), somatostatin (SOM), neuropeptide y (NPY), and cholecystokinin (CCK) expressing cells in the DG, CA1 and CA3 regions of the hippocampus. Rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with saline, followed by 4% paraformaldehyde in phosphate buffered saline (PBS: 0.1M, pH 7.4). The brain was quickly removed and post-fixed in 4% paraformaldehyde for 72 hours. Brains were sectioned on a vibrating microtome at 50µm. Immunohistochemistry was performed using horseradish-peroxidase methodology on free-floating tissue sections.Table 3-1 outlines specific details for each stain. Briefly, heat-induced antigen retrieval (15-30 min at 85-95 degree C in 0.1 sodium citrate buffer, pH 6.0) was performed for CB, SOM, NPY, and CCK to break down formaldehyde cross-linkages thereby unmasking antigenic sites for labeling. Endogenous peroxidase activity was blocked by incubating the tissue in 0.3% H_2O_2 in PBS for 30 min. Tissue was then blocked for 30-90min in normal animal sera and 1% bovine serum

Table 3-1. Immunohistochemistry Details

Antibody	Company	Antigen Retrieval	H_2O_2	Block	Primary Incubation	Secondary Incubation	ABC	DAB
mouse anti-GAD67	Millipore	N/A	0.3% in PBS 30min RT	5% NHS, 1% BSA in PBST, 30min RT	1:2000, 48hrs 4°C	1:500 BHAM in PBST, 2hrs RT	1:500 in PBST, 1hr RT	0.0333% DAB, 2.5% nickel, 0.00786% H_2O_2 in SA
mouse anti-PVB	Sigma Aldrich	N/A	0.3% in PBS 30min RT	1.5% NHS, 1% BSA in PBSx, 30min RT	1:2000, 48hrs 4°C	1:200 BHAM in PBSx, 2hrs RT	1:500 in PBSx, 1hr RT	0.0333% DAB, 0.00786% H_2O_2 in PBS
rabbit anti-calretinin	Millipore	N/A	0.3% in PBS 30min RT	3% NGS, 1% BSA in PBSx, 30min RT	1:4000, 48hrs 4°C	1:200 BGAR, 1% BSA, in PBSx, 2hrs RT	1:500 in PBSx, 1hr RT	0.0333% DAB, 0.00786% H_2O_2 in PBS
rabbit anti-calbindin	Millipore	15min 95°C	0.3% in PBS 30min RT	5% NGS, 1% BSA in PBSx 1.5hrs RT	1:2000, 48hrs 4°C	1:200 BGAR, 5% NGS, in PBSx, 2hrs RT	1:500 in PBSx, 1hr RT	0.0333% DAB, 0.00786% H_2O_2 in PBS
rabbit anti-somatostatin	Peninsula Laboratories	30min 85°C	0.3% in TBS 30min RT	5% NGS, 1% BSA in TBSx 1hr RT	1:1000, 48hrs 4°C	1:500 BGAR, 2% NGS, 1% BSA in TBSx, 2hrs RT	1:500 in TBSx, 1hr RT	0.0333% DAB, 0.00786% H_2O_2 in TBS
rabbit anti-neuropeptide y	Peninsula Laboratories	30min 85°C	0.3% in TBS 30min RT	5% NGS, 1% BSA in TBSx 30min 37°C	1:1000, 48hrs 4°C	1:500 BGAR, 2% NGS, 1% BSA in TBSx, 1.5hrs 37°C	1:500 in TBSx, 1.5hrs 37°C	0.05% DAB, glucose oxidase method
rabbit anti-cholecystokinin	Peninsula Laboratories	30min 85°C	0.3% in TBS 30min RT	5% NGS, 1% BSA in TBSx 30min 37°C	1:1000, 48hrs 4°C	1:500 BGAR, 2% NGS, 1% BSA in TBSx, 1.5hrs 37°C	1:500 in TBSx, 1.5hrs 37°C	0.0333% DAB, 0.00786% H_2O_2 in TBS

NOTE: PBST - 0.2% Tween-20 in PBS; PBSx - 0.3% Triton-X 100 in PBS; TBSx - 0.3% Triton-X 100 in TBS; SA - sodium acetate; BHAM - biotinylated horse anti-mouse; BGAR - biotinylated goat anti-rabbit; NHS - normal horse serum; NGS - normal goat serum

albumin (BSA), followed by incubation for 48h at 4°C in their respective primary antibodies diluted in blocking solution. Following a series of PBS washes, sections were then incubated with a biotinylated secondary antibody specific to the primary host species for 1.5-2h, followed by incubation in an avidin-biotin complex (ABC) for 1-1.5h (1:500, Vectastain ABC Elite, Vector Labs). Immunolabeling was visualized using 0.0333%-0.05% diaminobenzidine (DAB, Sigma Aldrich) and 0.00786% H_2O_2 diluted in either PBS or Tris buffered saline (TBS, pH 7.6). For NPY, ammonium chloride (40 µl per 20ml DAB solution), βd-glucose (160 µl per 20ml DAB solution) and glucose oxidase (60 µl per 20ml DAB solution) were added to the DAB solution instead of H_2O_2 to catalyze the reaction (Long et al., 2011). GAD67 was visualized using 0.0333% DAB, 2.5% nickel ammonium sulfate and and 0.00786% H_2O_2 diluted in 0.175M sodium acetate. Tissue stained for PVB and NPY was counterstained with cresyl violet; all tissue was dehydrated through a series of alcohols, cleared with xylenes, and coverslipped using Entallen resin solution.

The number of GAD67, PVB, CR, CB, SOM, NPY, and CCK+ cells was visualized using a computerized image analysis program (Stereo Investigator, version 8.0, MicroBrightField Inc.) that was attached to a Nikon Eclipse E800 microscope. The following regions were quantified: granule cell layer (GCL), subgranular zone (SGZ), hilus, CA3 strata pyramidale, oriens, lucidum and radiatum, and CA1 strata pyramidale, oriens, and radiatum. An experimenter who was blind to the treatment conditions manually counted cells using a profile counting method, which involved using a meander scan setting to sample non-overlapping fields from each brain region. Five sections per brain were analyzed (from −2.40mm from bregma to approximately −3.94mm from bregma) in both right and left hemispheres. The number of cells counted for each brain region was added across hemispheres and sections, multiplied by 1000000 and divided by their respective areas to obtain the number of cells per mm^2. Data are presented as the average number of cells per mm^2.

2.4 Statistical Analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS, v20.0, Chicago, IL, USA). Differences between CORT and vehicle groups in the number of cells for each marker in each hippocampal region were assessed using separate independent samples t-tests. The criterion for statistical significance was set at $p < .05$.

3. Results

3.1 Interneuron Distribution

The effect of repeated CORT administration on the number of GAD67, PVB, CR, CB, SOM, NPY, and CCK+ cells was examined in the hippocampus in adult male rats. As expected, all markers were present to varying degrees in the DG, CA3 and CA1. Table 3-2 illustrates the distribution of cells throughout the hippocampus for each interneuron marker in both vehicle and CORT treated animals. The results for each area are presented as a percentage, calculated from the total number of cells counted in all hippocampal regions for each treatment group. In general, the distribution of cells for each marker is similar to previous reports in rats (Freund & Buzsaki, 1996).

3.2 CORT Decreases GABAergic Interneurons Markers in a Region-Specific Manner in the Hippocampus

3.2.1 CORT Does Not Influence the Number of GAD67-Positive Cells in the Hippocampus

Figure 3-1 shows the effect of CORT on the number of GAD67+ cells in the hippocampus. We have previously found that 21 days of exogenous CORT administration has no effect on GAD67 protein levels in whole hippocampal homogenates (Lussier et al., 2013). Results of the current study support these data, as CORT had no significant influence on the number of GAD67+ cells in any hippocampal region examined (Fig. 3-1 A-K; all p's > 0.1).

3.2.2 CORT Decreases the Number of Parvalbumin-Positive Cells in the Dentate Gyrus, CA1 and CA3

Figure 3-2 shows the effect of CORT on the number of PVB+ cells in the hippocampus. PVB immunoreactive neurons were present to varying degrees in all sub-fields, with somewhat higher expression in CA1 and CA3 compared to the DG (Table 3-2). Repeated exposure to CORT resulted in a significant decrease in PVB+ cells in a number of regions throughout the hippocampus including the GCL, SGZ, and hilus (Fig. 3-2 B-D), the CA3 strata pyramidale, oriens, and lucidum (Fig. 3-2 E-G), and the CA1 strata pyramidale and oriens (Fig.3-2I-J; all p's < .01).

Table 3-2. Distribution of Interneuron Markers in the DG and CA Regions of the Hippocampus (%)

Marker	GCL	SGZ	Hilus	CA3 Pyramidal	CA3 Oriens	CA3 Lucidum	CA3 Radiatum	CA1 Pyramidal	CA1 Oriens	CA1 Radiatum
GAD67 Vehicle	7.63	11.46	3.71	5.49	4.55	1.23	7.06	24.89	11.92	22.06
GAD67 CORT	7.28	10.63	3.97	5.91	4.78	1.09	7.69	23.80	12.32	22.53
PVB Vehicle	6.58	7.40	4.49	15.16	6.12	1.54	1.93	30.81	22.92	3.06
PVB CORT	7.68	4.52	4.02	12.20	5.15	1.88	2.18	33.88	24.26	4.22
CR Vehicle	5.09	12.53	10.06	15.28	3.68	4.03	6.38	22.14	9.40	11.41
CR CORT	5.10	11.82	9.65	15.93	3.46	4.83	5.59	22.55	9.71	11.35
CB Vehicle	1.87	8.23	1.59	2.70	1.80	5.74	21.44	8.92	10.79	36.93
CB CORT	2.04	7.97	1.36	2.21	1.19	7.38	22.31	6.87	10.09	38.59
SOM Vehicle	0.30	11.98	21.30	3.62	7.89	3.31	5.56	3.63	40.72	1.70
SOM CORT	0.37	12.43	22.42	3.99	8.47	3.40	4.67	3.47	38.87	1.91
NPY Vehicle	4.23	14.34	15.17	7.46	4.73	2.78	4.15	21.17	16.93	9.03
NPY CORT	4.43	13.91	14.80	7.43	5.21	2.85	5.08	20.34	17.36	8.60
CCK Vehicle	2.15	16.67	5.22	1.75	2.61	3.41	13.19	8.86	7.54	38.59
CCK CORT	1.66	14.92	6.66	2.06	3.39	3.64	12.85	9.39	8.13	37.28

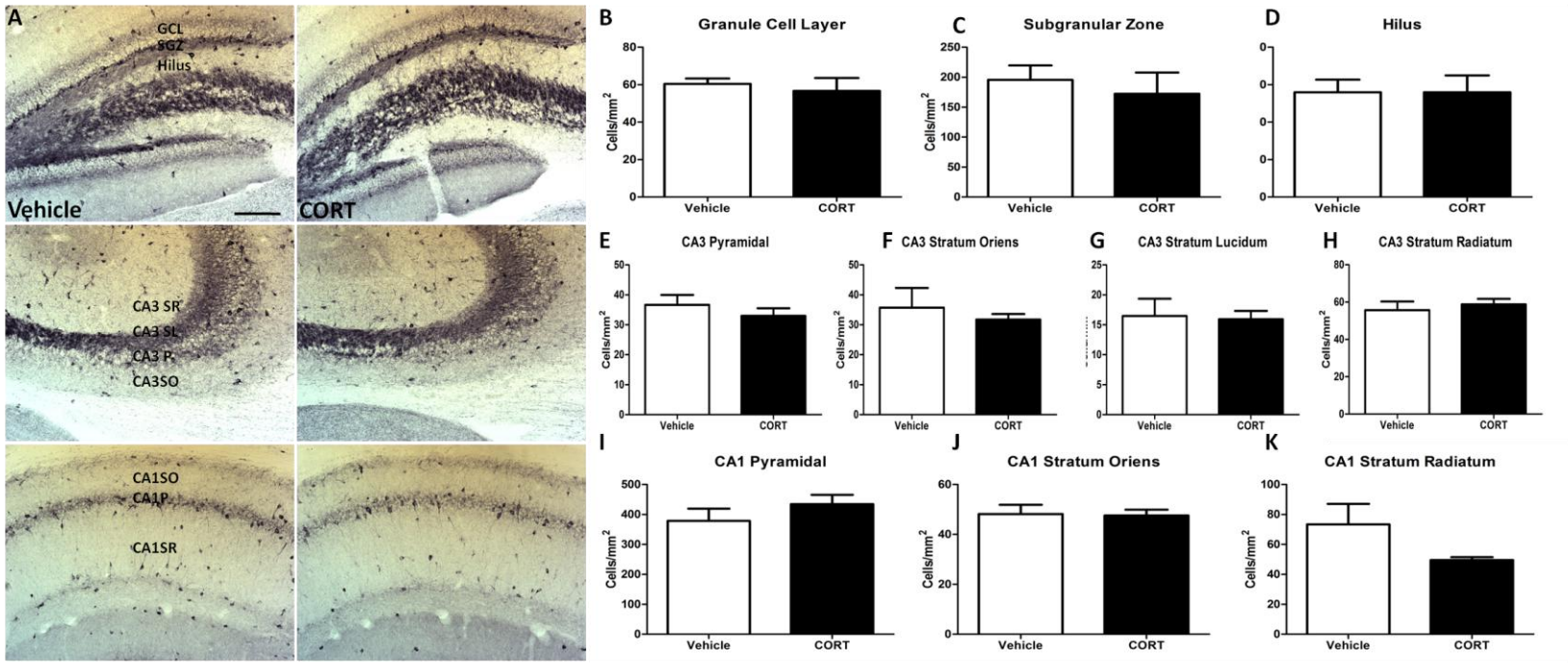

Figure 3-1. Effect of CORT on GAD67-positive cells in the hippocampus. Panel A shows representative photomicrographs of GAD67 immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of GAD67-positive cells mm^2 in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT had no significant influence on the number of GAD67-positive cells in any hippocampal region examined (all p's > 0.1). Error bars represent the mean ± standard error of the mean. Scale bar: 200 µm.

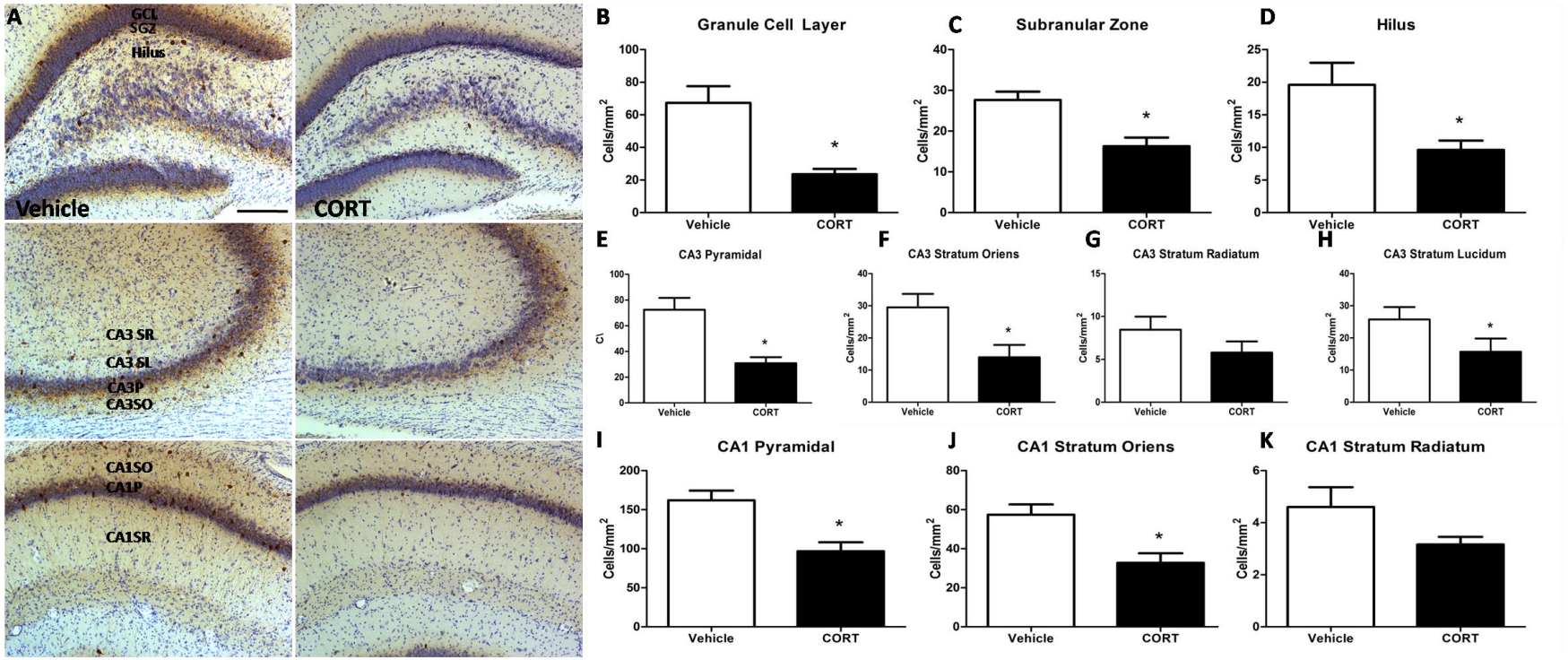

Figure 3-2. Effect of CORT on parvalbumin (PVB)-positive cells in the hippocampus. Panel A shows representative photomicrographs of PVB immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of PVB-positive cells per mm^2 in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT significantly reduced the number of PVB-positive cells in the GCL, SGZ , hilus, CA3 stratum pyramidal, oriens, and lucidum, and CA1 stratum pyramidal and oriens (all p's < .01).. Error bars represent the mean ± standard error of the mean. Scale bar: 200 µm.

3.2.3 CORT Does Not Influence the Number of Calbindin-Positive Cells in the Hippocampus

Figure 3-3 shows the effect of CORT on the number of CB+ cells in the hippocampus. CB immunoreactive neurons were present to varying degrees in all sub-fields examined, with highest expression in CA1 and CA3 compared to the DG (Table 3-2). Repeated CORT exposure had no influence on the number of CB+ cells in the DG, CA1 or CA3 (all *p's* > .05).

3.2.4 CORT Decreases the Number of Calretinin-Positive Cells in the CA3 Stratum Radiatum

Figure 3-4 shows the effect of CORT on the number of CR+ cells in the hippocampus. CR immunoreactive neurons were present in all layers and hippocampal sub-fields examined (Table 3-2). Repeated exposure to CORT resulted in a significant reduction in the number of CR+ cells in the stratum radiatum of CA3 [$t(10) = 2.908$, $p < .05$] (Fig. 3-4 H), but had no effect on any other hippocampal region (all *p*'s > .05).

3.2.5 CORT Decreases the Number of Somatostatin-Positive Cells in the CA1 Pyramidal Layer and the CA3 Stratum Lucidum

Figure 3-5 shows the effect of CORT on the number of SOM+ cells in the hippocampus. SOM immunoreactive neurons were present to varying degrees in all layers and sub-fields examined, with slightly higher expression in the DG and CA1 compared to CA3 (Table 3-2). Repeated exposure to CORT resulted in a significant reduction in the number of SOM+ cells in the CA1 pyramidal layer [$t(10) = 3.208$, $p < .01$] and the CA3 stratum lucidum [$t(10) = 2.298$, $p< .05$] (Fig. 3-5G and I respectively) but had no impact on any other hippocampal region (all *p*'s > .05).

3.2.6 CORT Decreases the Number of Neuropeptide-y-Positive Cells in the Subgranular Zone and Hilus

Figure 3-6 shows the effect of CORT on the number of NPY+ cells in the hippocampus. NPY immunoreactive neurons were present in all layers and sub-fields examined with greatest expression in the SGZ and hilus of the DG and stratum oriens and stratum pyramidale of CA1.

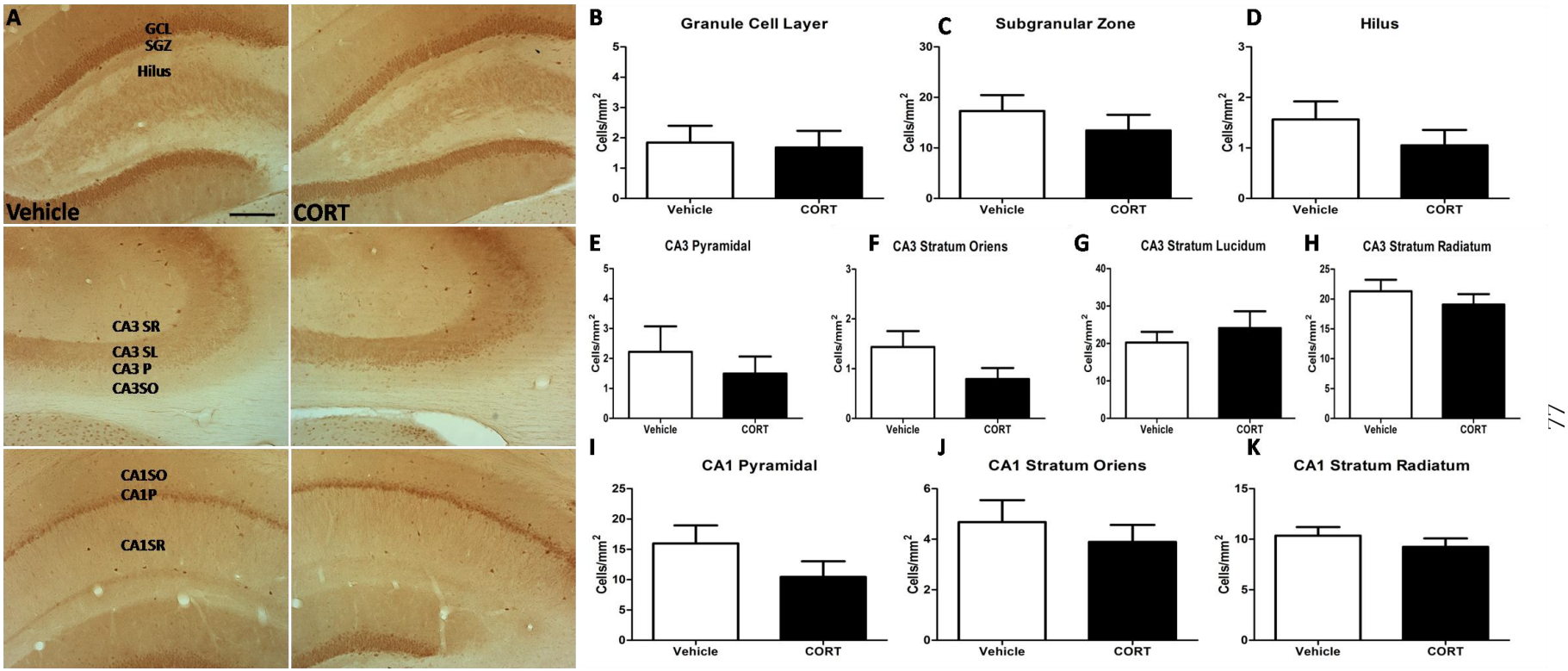

Figure 3-3. Effect of CORT on calbindin (CB)-positive cells in the hippocampus. Panel A shows representative photomicrographs of CB immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of GAD67-positive cells mm^2 in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT had no significant influence on the number of CB-positive cells in any hippocampal region examined (all p's > 0.05). Error bars represent the mean ± standard error of the mean. Scale bar: 200 µm.

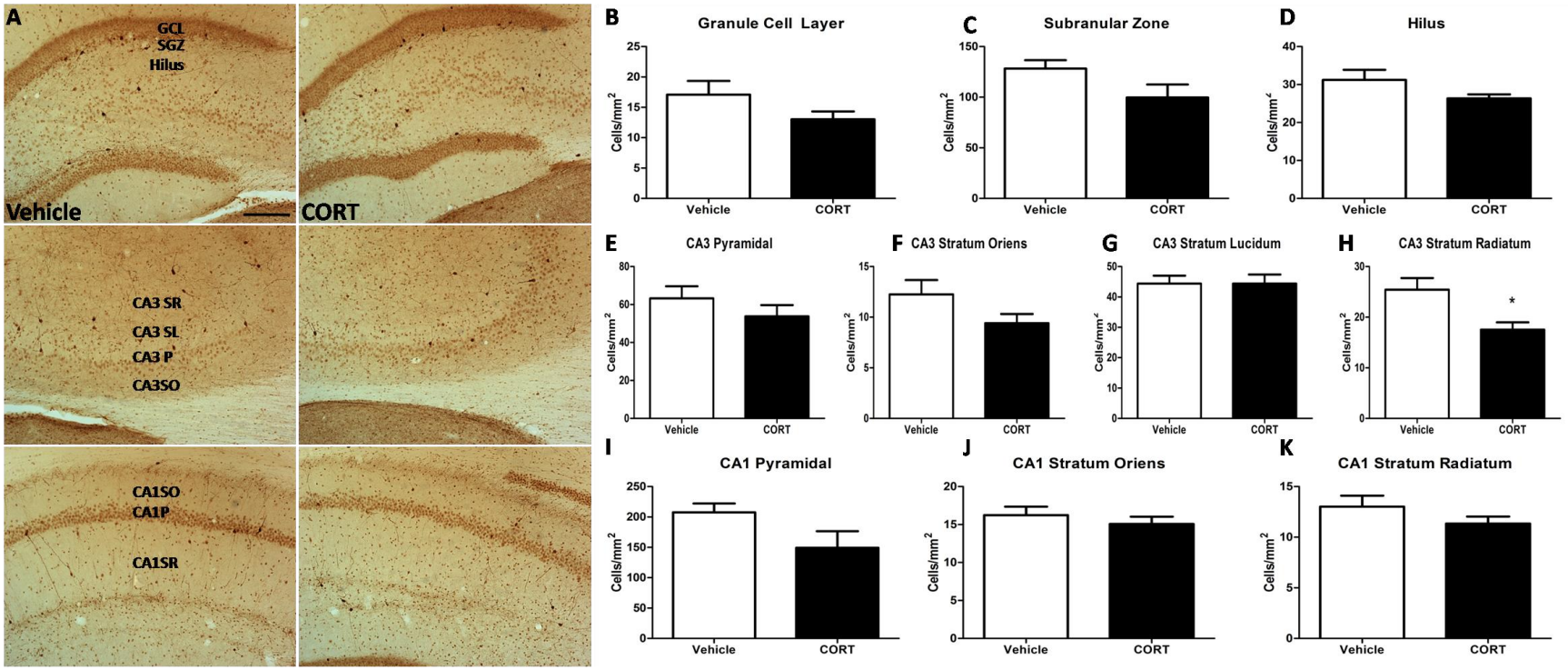

Figure 3-4. Effect of CORT on calretinin (CR)-positive cells in the hippocampus. Panel A shows representative photomicrographs of CR immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of CR-positive cells per mm^2 in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT significantly reduced the number of CR-positive cells in the CA3 stratum radiatum ($p < .05$). CORT had no significant influence on any other region examined in the hippocampus. Error bars represent the mean ± standard error of the mean. Scale bar: 200 µm.

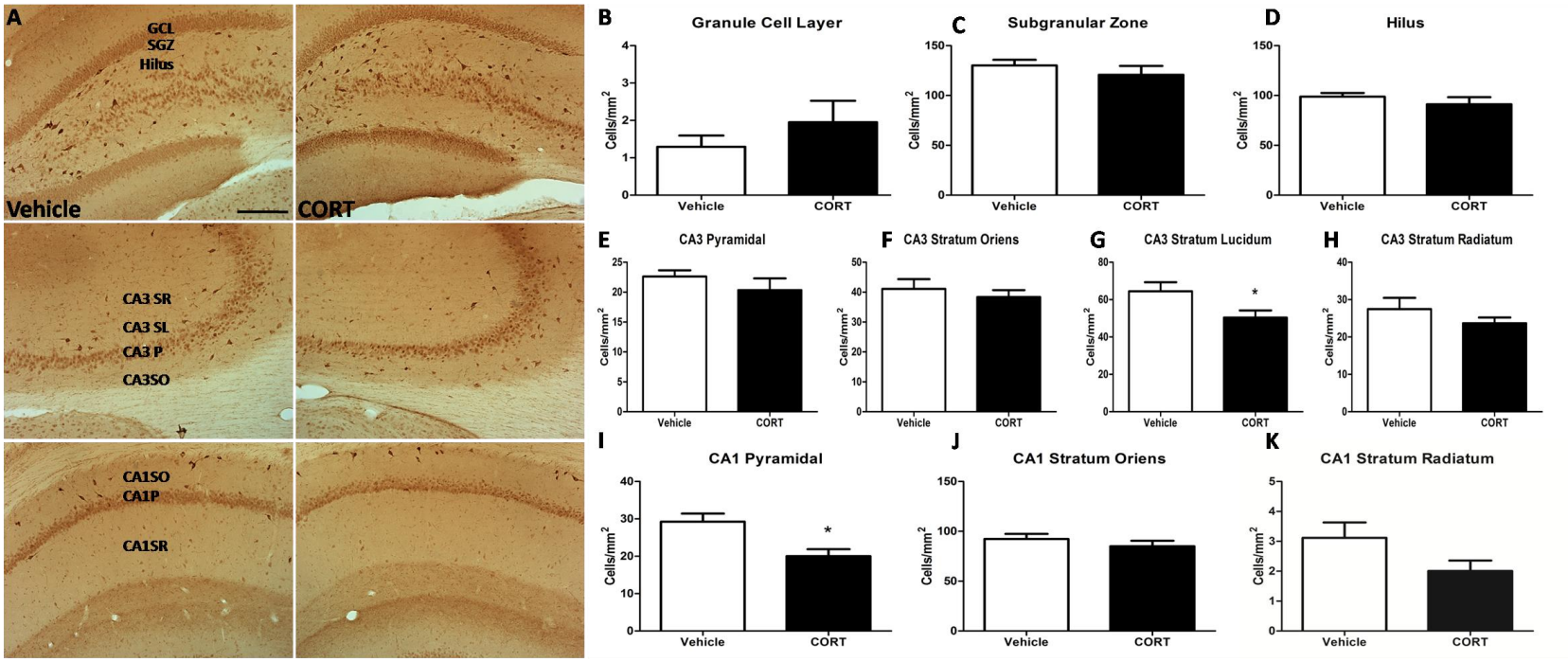

Figure 3-5. Effect of CORT on somatostatin (SOM)-positive cells in the hippocampus. Panel A shows representative photomicrographs of SOM immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of SOM-positive cells per mm² in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT significantly reduced the number of SOM-positive cells in the CA3 stratum lucidum ($p < .05$) and the CA1 pyramidal layer ($p < .01$). CORT had no significant influence on any other region examined in the hippocampus. Error bars represent the mean ± standard error of the mean. Scale bar: 200 μm.

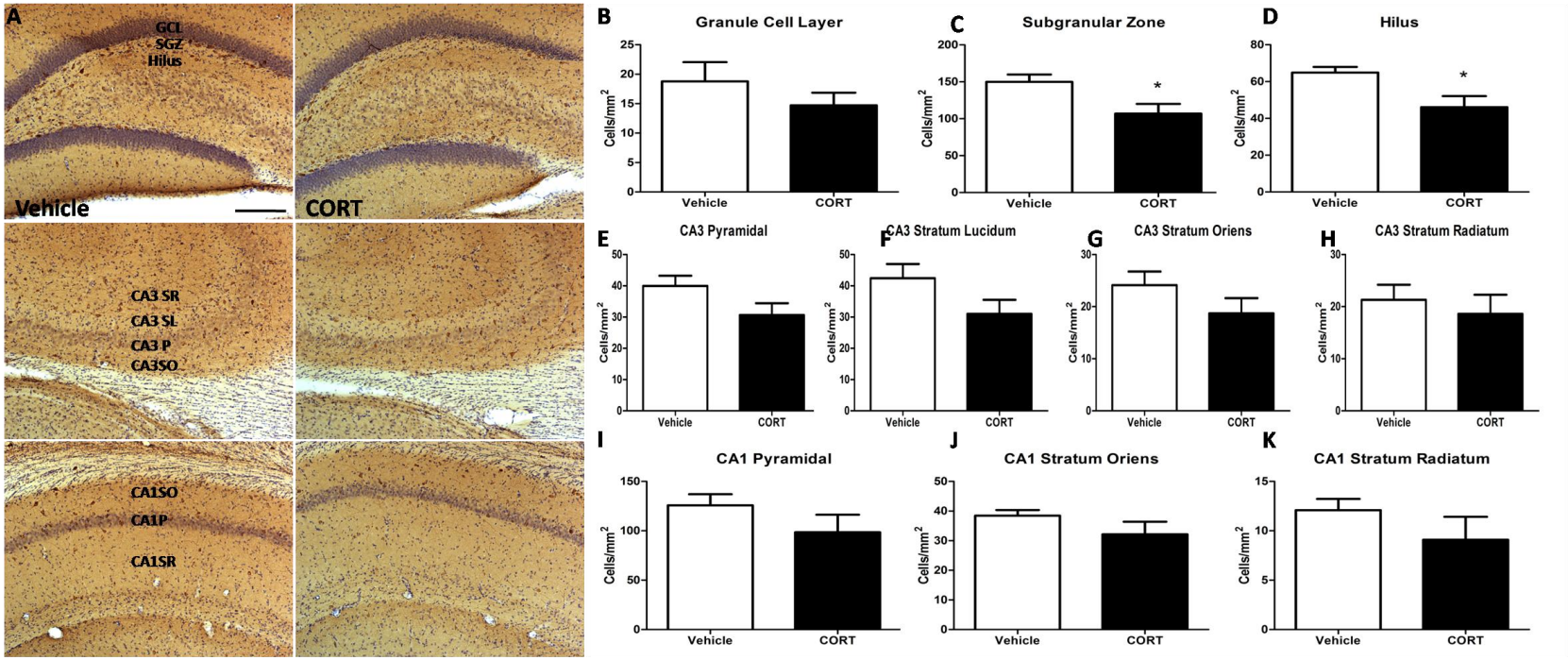

Figure 3-6. Effect of CORT on neuropeptide-y (NPY)-positive cells in the hippocampus. Panel A shows representative photomicrographs of NPY immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of NPY-positive cells per mm^2 in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT significantly reduced the number of NPY-positive cells in the SGZ and hilus (p's < .05), but had no effect on any other region of the hippocampus. Error bars represent the mean ± standard error of the mean. Scale bar: 200 μm.

Repeated CORT exposure resulted in a significant decrease in the number of NPY+ cells in the SGZ [$t(10, 9.363) = 2.602$, $p < .05$] and hilar [$t(10) = 2.801$, $p < .05$] (see Fig. 3-6 C and D respectively) regions of the DG but had no effect on these cells in any other hippocampal region (all p's > .05).

3.2.7. CORT does not Influence the Number of Cholecystokinin-Positive Cells in the Hippocampus

Figure 3-7 shows the effect of CORT on the number of CCK+ cells in the hippocampus. CCK immunoreactive neurons were present to varying degrees in all sub-fields examined in the hippocampus with the greatest expression CA1 compared to the DG and CA3 (Table 3-2). Similar to CB, repeated CORT exposure did not influence the number of CCK+ cells in any hippocampal region examined (all p's > .05; See Figure 3-7).

4. Discussion

The results of this study make several important points about the effect of repeated glucocorticoid exposure on GABAergic interneurons in the hippocampus. First, CORT reduced the number of PVB, CR, SOM, and NPY+ neurons in a number of hippocampal sub-fields, suggesting that the function of these cells has been compromised. However, CORT had no impact on the expression of CB or CCK, indicating that not all interneurons are susceptible to the deleterious effects of CORT. Moreover, CORT had no significant impact on the number of interneurons expressing GAD67, which is an enzyme expressed by all GABAergic interneurons, suggesting that repeated exposure to this stress hormone does not lead to a loss of interneurons in the hippocampus. These results extend past work by providing a comprehensive examination of region-specific alterations in interneuron markers in the DG, CA3 and CA1 after glucocorticoid treatment. More importantly, they suggest that an intracellular mechanism is responsible for the degradation of proteins and peptides expressed in these interneurons, and raise the possibility of reversing these effects.

4.1 CORT Does Not Affect GAD67-Positive Interneurons in the Hippocampus

Glutamate decarboxylase (GAD) is the primary enzyme responsible for converting glutamate to GABA. It exists in two isoforms (i.e., GAD65 and GAD67), each showing distinct

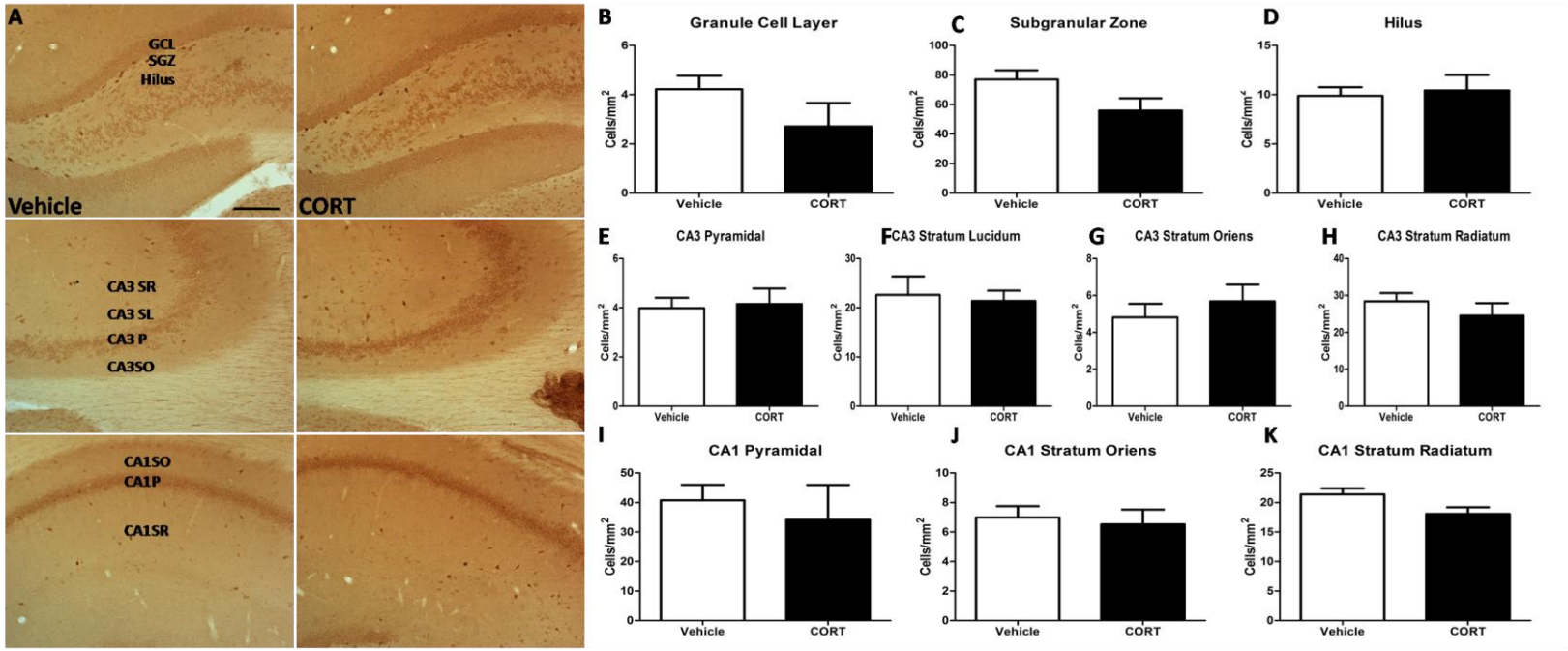

Figure 3-7 Effect of CORT on cholecystokinin (CCK)-positive cells in the hippocampus. Panel A shows representative photomicrographs of CCK immunoreactivity in the dentate gyrus, CA1 and CA3 regions of the hippocampus in CORT and vehicle treated groups. Panels B-K show the mean number of CCK-positive cells mm² in the GCL, SGZ, hilus, CA3 pyramidal, oriens, lucidum and radiatum, and CA1 pyramidal, oriens, and radiatum, respectively. CORT had no significant influence on the number of CCK-positive cells in any hippocampal region examined (all *p's* > 0.05). Error bars represent the mean ± standard error of the mean. Scale bar: 200 µm.

sub-cellular expression and functionality in GABAergic neurons (Kaufman, Houser, & Tobin, 1991). Specifically, GAD67 synthesizes GABA that participates in activities unrelated to neurotransmission (synaptogenesis and protection from neuronal injury), whereas GAD65 participates 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, Martin, Wu, & Espina, 1991; Martin & Rimvall, 1993). In the current study we examined GAD67 because it is primarily expressed in neuronal cell bodies and proximal dendrites and amenable to cell counting, whereas GAD65 is primarily expressed in synaptic terminals (Erlander, Tillakaratne, Feldblum, Patel, & Tobin, 1991; Esclapez, Tillakaratne, Kaufman, Tobin, & Houser, 1994). The results of the current study indicate that CORT does not decrease the number of GAD67+ cells in the DG, CA1 or CA3, supporting previous reports from our laboratory using western blotting techniques (Lussier et al., 2013). However, we have previously found that CORT significantly reduces total protein levels of GAD65 in the hippocampus (Lussier et al., 2013). Together these data suggest that CORT does not result in the loss of GABAergic interneurons, but it may lead to dampened GABAergic neurotransmission in the hippocampus.

4.2 Sub-type Specific Alterations in Calcium-binding Proteins and Peptides: Consequences for Hippocampal Function

4.2.1 Calcium binding proteins: Parvalbumin, calbindin and calretinin

PVB, CB, and CR are all calcium-binding proteins that are present to varying degrees in different sub-types of GABAergic interneurons (Freund & Buzsaki, 1996). All PVB neurons can be classified as either basket or chandelier cells, whose axons innervate the perisomatic domain of pyramidal cells, controlling their output. These cells make up approximately 20-24% of all hippocampal interneurons with slightly higher values for CA1 (22-29%) and CA3 (20-25%) compared to the DG (Aika, Ren, Kosaka, & Kosaka, 1994; Freund & Buzsaki, 1996; Kosaka, Katsumaru, Hama, Wu, & Heizmann, 1987). Unlike PVB, CB protein is found in both principal cells and interneurons in the hippocampus. CB+ interneurons innervate pyramidal cell dendrites, and control the efficacy and plasticity of afferent inputs of principal cells; they make up roughly 10-12% of all hippocampal interneurons (Baimbridge & Miller, 1982; Baimbridge, Miller, & Parkes, 1982; Freund & Buzsaki, 1996; Sloviter, 1989; Toth & Freund, 1992). The precise regional and laminar expression of CR neurons, however, is not as well defined as for PVB. Our

results indicate that CR+ neurons are located in all layers and subfields of the hippocampus and DG (See Table 3-2). These cells are specialized to innervate other interneurons, and may be able to synchronize their firing (Freund & Buzsaki, 1996; Gulyas et al., 1996).

In the current study, a profound decrease in the number of PVB+ cells was found in most regions of the hippocampus, whereas CR+ cells were only decreased in the stratum radiatum of CA3, and the number of CB+ cells was unchanged. These data are similar to a number of previous reports indicating significant reductions of PVB+ neurons in CA1, CA3 and DG regions of the hippocampus after chronic stress (Filipovic, Zlatkovic, Gass, & Inta, 2013; Hu et al., 2010). Contrary to our results, previous reports have indicated a decrease in the number of CB+ cells in the DG after both CMS and CUS; however no significant effects were found in CA1, and no other hippocampal regions were examined (Nowak et al., 2010). Interestingly, neither CMS nor CUS in this study caused alterations in PVB+ neurons, suggesting that differences in stress protocols could account for our findings. Various forms of chronic stress, including CORT treatment, have also been shown to increase CB protein levels in CA1 neurons; it is suggested that this protein acts as a homeostatic regulator of calcium, and could therefore provide protection during times of stress by reducing intracellular calcium levels (Krugers, Koolhaas, Medema, & Korf, 1996; Nowak et al., 2010; Rami, Rabie, & Winckler, 1998). Although it remains to be determined in our study, this mechanism may provide a plausible explanation for our null results. Regarding CR, much less is known about how chronic stress influences the number of interneurons expressing this protein in the hippocampus. Maternal deprivation studies provide most of what we know, and indicate that CR-containing interneurons are resilient to the deleterious effects of stress (Giachino et al., 2007; Xu et al., 2011). To our knowledge, our data are the first to show that repeated CORT administration significantly reduces the number of CR+ cells in the CA3 region of the hippocampus in the adult rodent brain.

What are the potential functional consequences of our findings? Although the current data suggest that glucocorticoids target perisomatic interneurons, no decrease was found in CKK, a peptide also present in interneurons making perisomatic connections with pyramidal cells (Freund & Buzsaki, 1996). Interestingly, these two interneuron subtypes are thought to provide functional dichotomy in the inhibitory network. Specifically, hippocampal PVB interneurons are regarded as a non-plastic precision clockwork for network oscillations, whereas CCK interneurons are considered to function as a plastic fine-tuning device modulating synchronous

activities as a function of sub-cortical inputs (Freund & Katona, 2007; Klausberger et al., 2005). Therefore, dysfunction in PVB+ cells in the hippocampus would likely result in abnormal network oscillations, leading to not only local network dysfunction, but also a reduction in the coordinated activity of large brain networks; this type of dysregulation could contribute to some of the cognitive impairments associated with chronic stress (Sterner & Kalynchuk, 2010). Indeed, recent research has shown that a functional deficit of PVB interneurons and altered γ oscillations are linked to working memory deficits in schizophrenia (Lewis, Hashimoto, & Volk, 2005; Lodge, Behrens, & Grace, 2009).

Alterations in the number of CR+ interneurons in the CA3 stratum radiatum may also have an important influence on hippocampal function. Specifically, the axons of CR+ interneurons make multiple synaptic contacts exclusively on the dendrites of other local GABAergic interneurons in the hippocampus, including other CR+ interneurons, CB+ interneurons, and vasoactive intestinal polypeptide (VIP)-containing basket cells (Gulyas et al., 1996).This unique connectivity of CR cells with other interneurons allows them to play a crucial role in the generation of synchronous, rhythmic hippocampal activity by controlling other interneurons terminating on principal cells (Freund & Buzsaki, 1996; Gulyas et al., 1996). In light of these findings, although the number of CB+ cells was not affected in the current study, it is possible that their function may have been altered by a loss of inhibition from CR+ cells.

We cannot conclude that the observed reduction in PVB and CR+ cells in this study reflects a reduction in interneuron cell number solely on the basis of immunohistochemical examination of these markers. In fact, it is more likely that stress causes a reduction in the perikaryal PVB and CR content in GABAergic cells, without influencing actual cell number. This assumption would support our finding of no change in GAD67+ cells in any region of the hippocampus. Nonetheless, a reduction in such a calcium-buffering system could reflect a functional impairment of PVB+ and CR+ cells in specific sub-regions of the hippocampus.

4.2.2 Neuropeptides: Somatostatin, neuropeptide-y and cholecystokinin

In the current experiment, we examined three neuropeptides, SOM, NPY, and CCK, which are commonly used to identify interneuron populations in the hippocampus and cortex (Freund & Buzsaki, 1996). In the hippocampus, SOM+ cells comprise a heterogeneous population of interneurons, accounting for approximately 14% of all interneurons in the hippocampus. Higher expression of this peptide is found in the DG (16%) compared to CA1

(12.5%) and the CA3 (13%) sub-fields (Bakst, Morrison, & Amaral, 1985; Freund & Buzsaki, 1996). NPY is another peptide expressed in a number of interneuron sub-types in the hippocampus, many of which co-localize with SOM (Kohler, Eriksson, Davies, & Chan-Palay, 1987). In the DG, most of the NPY+ cells (60-70%) are contained within the hilus. In the CA1 subfield, NPY+ neurons are primarily located in the stratum oriens and pyramidal layers, whereas the density of these cells in all CA3 sub-fields is fairly similar (Freund & Buzsaki, 1996). As previously described, CCK is a peptide expressed primarily in basket cells in the hippocampus. GABAergic neurons containing CCK represent approximately 10% of all hippocampal interneurons. The highest number of CCK+ cells are in CA1 subfields (12.5%), with somewhat less in CA3 (10%) and the DG (Freund & Buzsaki, 1996; Kosaka et al., 1985).

In the current study we found a significant decrease in the number of NPY+ cells in the SGZ and hilus, a significant decrease in SOM+ cells in the CA1 pyramidal layer and CA3 stratum lucidum after CORT treatment. However, CORT did not alter the number of CCK+ cells in any hippocampal region. Our data are in support of previous findings for a reduction of NPY mRNA in cells in the dentate gyrus (DG; Lian & An, 2010; Sergeyev et al., 2005; Yu et al., 2010) and no reduction of CCK+ cells in the DG, CA1 or CA3 (Hu et al., 2010) after chronic stress. However, little is known about how chronic stress influences SOM+ interneurons in the hippocampus. One previous report indicates that chronic unpredictable mild stress reduces SOM mRNA in the hippocampus (Zhu, Bai, Zhuo, & Feng, 2009). To our knowledge, no detailed analysis of SOM expression in the hippocampus of CORT-treated rats has been conducted. Therefore, our study is the first to show that repeated CORT treatment significantly reduces the number of SOM+ cells in both the CA1 pyramidal region and CA3 stratum lucidum.

A loss of SOM and NPY+ cells and/or peptide expression may have a significant impact on hippocampal function. For example, the dendrites of SOM+ neurons in CA1 and CA3 make connections with the axon collaterals of local pyramidal cells in these regions, and ultimately help to govern their output (Freund & Buzsaki, 1996). Interestingly, recent work has indicated a correlation between memory performance in hippocampal dependent tasks and the amount of endogenously expressed SOM (Nakagawasai et al., 2003; Nakagawasai, Tadano, Niijima, Tan-no, & Kisara, 2000; Nilsson et al., 1993). NPY has also been implicated in the modulation of important features of neuronal physiology, including neuroprotection and inflammation, calcium homeostasis, neurotransmitter release and cell excitability (Malva et al., 2012). For example, in

the chronic perforant path stimulation model of temporal lobe epilepsy, a marked decrease in SOM and NPY interneurons correlates with reduced inhibition (Sloviter, 1987; Sloviter, 1991), which could ultimately lead to the spread of seizure activity. Therefore, the loss of SOM and NPY seen in our study could contribute to the increased excitatory network proposed in depression (Mathews, Henter, & Zarate, 2012; Serafini et al., 2013). Interestingly, NPY also modulates excitatory and inhibitory synaptic inputs to CCK+ interneurons. Specifically, NPY application leads to decreased excitatory transmission onto CCK+ basket cells, as well as decreased spontaneous and miniature inhibitory post-synaptic currents (Ledri, Sorensen, Erdelyi, Szabo, & Kokaia, 2011). Although we found no change in the number of CCK+ cells in the hippocampus, alterations in NPY expression may change the way these cells process information, which could influence network excitability and hippocampal function.

4.3 Relationship Between Reelin Expression and Interneuron Populations Affected by CORT

Reelin is another protein often used as an interneuron marker because of its exclusive expression in GABAergic interneurons in the adult hippocampus and cortex (Pesold et al., 1998). Importantly, a loss in reelin+ cells has been documented in the hippocampus of depressed patients (Fatemi et al., 2000), and preclinical models indicate that these cells are susceptible to the deleterious effects of glucocorticoids (Lussier et al., 2009). However, there is no current evidence indicating whether the decrease in reelin after stress is due to the loss of interneurons themselves or just a loss in the ability of these cells to synthesize and express reelin protein. Although previous work has demonstrated that reelin+ cells in the hippocampus express glucocorticoid receptors, which could make them susceptible to stress-induced neurodegeneration (Gross et al., 2012), we show that the interneuron markers which predominantly co-localize with reelin (i.e., GAD67, SOM, and CR) are not affected in hippocampal regions where reelin is downregulated by stress (i.e., SGZ and hilus; Lussier et al., 2009). These data suggest that these neurons are in fact not lost. Post-mortem work with tissue from schizophrenic patients also indicates a loss of reelin in the frontal cortex that is not accompanied by a loss in interneuron number (Guidotti et al., 2000a), lending further support for our results. These findings suggest that glucocorticoids harness an intracellular mechanism to

downregulate reelin synthesis and/or protein content within GABAergic interneurons; however, more work is needed to determine exactly how glucocorticoids regulate this process.

Conclusion

Our result show that repeated CORT administration decreases a number of GABAergic interneuron markers in a region-specific manner in the hippocampus, likely leading to the disruption of balance between GABAergic and glutamatergic activity. These results are consistent with observations of decreased interneuron markers and GABA levels in brain and peripheral samples from depressed patients. Importantly, the reduction in these markers occurs independently of interneuron loss. This finding is particularly important because it raises the possibility of restoring the capability of these interneurons to synthesize and express proteins and peptides integral to their function.

CHAPTER 4

Repeated corticosterone administration increases the number of MeCP2-Positive cells in the proliferative subgranular zone but has no influence on global expression of MeCP2 or DMNT1 in the rat hippocampus

Erin Y. Fenton[a], Hector J. Caruncho[b], Lisa E. Kalynchuk[c]

[a] Department of Psychology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[b] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

[b] College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Abstract

Epigenetic processes are important for normal brain function, allowing for long-term regulation of genes involved in establishing and maintaining neuroplasticity. Alterations in epigenetic processes have been associated with the development of a number of diseases and neuropsychiatric conditions, such as major depression. DNA methylation specifically is an important posttranslational epigenetic modification that plays a key role in regulating gene expression during development and in the adult brain. However, the involvement of DNA methylation in the pathophysiology of depression is largely unknown. The current study sought to address this issue by investigating the influence of repeated corticosterone (CORT; 40mg/kg; 21 consecutive days) injections on protein levels of the methyl donor enzyme DNMT1 and the methyl binding protein MeCP2 in the rat hippocampus. As a preliminary examination of regional changes in methylated sites in this region, we also examined how CORT influences the number of MeCP2+ cells in the subgranular zone (SGZ) and hilus. Overall we found no significant changes in the global expression of DNMT1 or MeCP2 protein in the hippocampus. However, the number of MeCP2+ cells was significantly increased in the SGZ of CORT treated rats. These data suggest that glucocorticoids may increase DNA methylation in cells contained within the proliferative zone of the hippocampus. Although we cannot make any definitive conclusions about how gene expression is affected by increased MeCP2 expression in this study, it is possible that increased MeCP2 has an important influence on gene expression within these cells, and may possibly influence neuroplasticity in this hippocampal region.

1. Introduction

Epigenetic regulation is an essential mechanism for normal brain development and function (Kubota et al., 2010). In particular, epigenetic processes are necessary for normal cellular development and differentiation and allow for long-term regulation of genes involved in establishing and maintaining neuroplasticity (Dulac, 2010; Henikoff & Matzke, 1997). Methylation and acetylation are posttranslational modifications to histone proteins and/or DNA that play key roles in regulating gene expression. DNA methylation is one of the most well understood epigenetic mechanisms, and involves stable heritable covalent modifications that alter DNA without changing its sequence. This modification consists of the addition of a methyl group to cytosine residues of the DNA template, is catalyzed by a group of enzymes known as DNA methyltransferases (DNMTs), and is primarily associated with gene silencing (Bredy et al., 2010; Klose & Bird, 2006; Mehler, 2008a,b; Rivera & Bennett, 2010; Suzuki & Bird, 2008). DNA methylation is also associated with histone modifications through methyl-DNA binding proteins [e.g.Methyl-CpG-binding protein 2 (MeCP2)]. These binding proteins help recruit histone modification enzymes such as histone deacetylases (HDACs) that remove acetyl groups from histone proteins (Lachner & Jenuwein, 2002; Tsankova et al., 2007). This removal of acetyl groups leads to the compaction of chromatin which limits the accessibility of transcriptional machinery, and ultimately suppresses gene transcription. In contrast, acetylation of histone proteins reduces the affinity between histones and DNA, and serves to enhance gene transcription by relaxing chromatin structure (Hong et al., 1993). Although epigenetic modifications to DNA and histone proteins are often investigated separately, these modifications work in concert in a number of important ways to regulate gene expression (Miller et al., 2008).

In the past decade there has been an increase in the number of studies demonstrating the dynamic nature of epigenetic processes and their influence on gene regulation and behavior. Importantly, this research has underscored the role of epigenetic events in the control of both normal cellular processes and abnormal events associated with disease (Callinan & Feinberg, 2006; Feng et al., 2007; Henikoff & Matzke, 1997; Mehler, 2008a,b). In particular, recent evidence suggests that transcriptional deregulation may underlie the behavioral manifestation of major depression (Charney & Manji, 2004; Krishnan & Nestler, 2008; Sun et al., 2013). For example, increases in the class II HDAC, HDAC5, in the hippocampus are associated with increased depression-like behavior in rodents (Tsankova et al., 2006). Both HDAC2 and HDAC5

are also increased in peripheral white blood cells of depressed patients in their depressive but not remitted state (Hobara et al., 2010). Conversely, inhibiting histone deacetylation in the rat hippocampus has been shown to reduce depression-like behaviors (Covington, III et al., 2011). Antidepressants also increase acetylation at the promoter region of genes such as BDNF (Tsankova et al., 2006), indicating that epigenetic mechanisms might play an integral role in regulating neuroplasticity.

Although evidence suggests an important role of histone acetylation in the pathophysiology of depression, less is known about the methylation status of the depressed brain. For example, histone methylation in the hippocampus has only been examined in one animal model to date, which found that chronic restraint stress produces a small decrease in histone proteins associated with transcriptional repression (i.e., histone H3 at lysine 9; H3K9) in the DG and CA1 (Hunter, McCarthy, Milne, Pfaff, & McEwen, 2009). Very little is also known about DNA methylation in the brains of depressed patients. One genome-wide DNA methylation study found that, although overall methylation in the frontal cortex was not altered in depressed patients, increased methylation status was observed in or near genes that play important roles in neuronal growth and development (Sabunciyan et al., 2012). Chronic prenatal restraint stress is also known to increase DNMTs in both the hippocampus and frontal cortex in the adult mouse brain, which is associated with increased methylation at gene promoter regions (e.g. reelin) known to be important in regulating neuroplasticity (Matrisciano et al., 2013).There is also evidence suggesting that genes involved in regulating the stress response are impacted directly by epigenetic mechanisms. In particular, it was shown that pups reared by dams providing poor maternal care exhibit DNA-hypermethylation at the promoter regions of the hippocampal glucocorticoid receptor (GR), with a concomitant reduction in GR mRNA and protein expression (Weaver et al., 2004). Importantly, poor maternal care was also associated with an enhanced stress response in offspring, which was related, in part, to differences in GR expression (Weaver et al., 2004) across offspring, suggesting that these animals may be more susceptible to developing stress-induced depression.

Although it has been suggested that DNA methylation is involved in neural plasticity and memory formation in the adult brain (Day & Sweatt, 2010; Day & Sweatt, 2011), the involvement of DNA methylation in the pathophysiology of depression is largely unknown. The current study sought to address this issue by investigating the influence of repeated

corticosterone (CORT) injections on protein levels of the methyl donor enzyme DNMT1 and the methyl binding protein MeCP2 in the hippocampus. As a preliminary examination of regional changes in methylated sites in the hippocampus, we also examined how CORT influences the number of MeCP2+ cells in the dentate gyrus (DG) subgranular zone (SGZ) and hilus. Our results indicate that CORT has no significant effect on global levels of DNMT1 or MeCP2 protein expression in the hippocampus. However, MeCP2+ cells are increased in the proliferative SGZ in CORT treated animals, suggesting that glucocorticoids may increase methylation in a region-specific manner.

2. Materials and Methods

2.1 Subjects

Adult male Long-Evans rats (N = 18) were purchased from Charles River Canada (Montreal, Quebec). They weighed approximately 200-250g at the time of their arrival. The rats were housed individually in standard rectangular 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-hr light/12-hr dark cycle. All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply.

2.2 CORT Treatment

Rats received either CORT (n = 9; 40mg/kg) or vehicle (n = 9) injections once per day for 21 consecutive days. CORT (Steraloids) was suspended in physiological saline with 2% Tween-80 (Sigma Aldrich). All injections were administered subcutaneously at a volume of 1 ml/kg. We chose this paradigm because 21 days of CORT injections at this dose is known to produce reliable and robust increases in depression-like behavior in rodents (Gregus et al., 2005; Johnson et al., 2005; Kalynchuk et al., 2004; Marks et al., 2009).

2.3 Western Blot Analysis

We used western blots to examine the effect of CORT on total levels of DNMT1 and MeCP2 expression in the hippocampus. On day 22, a subset of rats from each group (CORT: n = 4; Vehicle: n = 4) was anesthetised with sodium pentobarbital and then decapitated with a

standard rodent guillotine. The hippocampus was rapidly dissected, flash frozen in liquid nitrogen, and stored at -80ºC until use. Hippocampal tissue was homogenized in 0.3 M sucrose-Tris-EDTA solution on ice containing a protease inhibitor cocktail (Roche Diagnostics) to minimize protein degradation. Tissue samples were then centrifuged at 1000 rcf for 5 min at 4ºC, the supernatant was collected, and protein concentration was measured using the Pierce BCA protein assay kit (Thermo Scientific). A total of 60µg of protein from each sample was diluted in Laemmli Sample Buffer (Bio Rad) and 5% 2-mercaptoethanol at a 1:1 ratio, denatured by heating to 95 ºC,and resolved on 10% sodium dodecyl sulphate-polyacrylamide running gels. Protein was transferred onto nitrocellulose blotting membranes andblocked in tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat milk to prevent non-specific binding. Membranes were then probed with a monoclonal rabbit anti-DNMT1 primary antibody (1:1,000; Cell Signaling Technology) or rabbit anti-MeCP2 (1:1,000; Millipore) overnight at 4ºC, followed by incubation in a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology) diluted in TBST and 5% non-fat milk. The blots were then stripped and re-probed with β-actin primary antibody (mouse anti-β-actin, 1:10,000; Millipore). Immunoreactivebands were visualized with enhanced chemiluminescence (ECL) plus reagents (GE Healthcare) on autoradiography x-ray film (AmershamHyperfilm ECL, GE Healthcare). The films were digitized and optical densities were determined using a computerized image analysis system with a high-powered scanner and Image J software (v1.43u, National Institutes of Health). Single autoradiographic signal bands of appropriate molecular weights (i.e., ~200kDa for DNMT1 and ~75kDa for MeCP2) were quantified and the signal value for each band was normalized to β-actin to verify equivalent loading of protein. Data are represented as a fold change from the control group.

2.4 Immunohistochemical Analysis of MeCP2 Expression

Immunohistochemistry was used to assess the effect of CORT on the number of MeCP2+ cells located in the SGZ and hilus. A subset of rats from each group (vehicle n = 5; CORT n = 5) was deeply anesthetized with sodium pentobarbital and transcardially perfused with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS: 0.1M, pH 7.4). The brain was quickly removed and post-fixed in 4% paraformaldehyde for 72 hours. Brains were sectioned on a vibrating microtome at 50µm. Free-floating tissue

sections were incubated in 0.3% H_2O_2 in PBS for 30 min to block endogenous peroxidase activity, blocked for 30 minutes in 0.3% Triton X-100 in PBS (PBSx), 1.5% normal horse serum (NHS), and 1% bovine serum albumin (BSA), then incubated for 48 h at 4°C with rabbit anti-MeCP2 primary antibody (1:200, Millipore). Sections were then incubated with a biotinylated horse anti-mouse IgG (1:200, Sigma Aldrich) secondary antibody, diluted in PBSx at room temperature (RT) for 2h, followed by incubation in an avidin-biotin complex (ABC) for 1h (1:500, Vectastain ABC Elite, Vector Labs) at RT. Immunolabeling was visualized using 0.033%3′-diaminobenzidine (DAB, Sigma Aldrich) dissolved in 0.00786% H_2O_2 and PBS. Sections were dried overnight, dehydrated in a series of alcohols, cleared in xylenes and coverslipped using Entallen resin solution.

A single examiner who was blind to the group identification of each animal performed the data collection. A total of 5 sections per brain were quantified for each animal. The total number of MeCP2+ cells in the SGZ and hilus of the hippocampas was estimated using the unbiased optical fractionator method (West, Slomianka, & Gundersen, 1991), with assistance from a computerized stereology system (StereoInvestigator, Microbrightfield). All regions were traced at low power (100X) on a Nikon E800 microscope. Images of MeCP2-immunoreactive cells were counted at a high power (400X) magnification and the total number of cells was estimated as: $N_{total} = \Sigma Q^- * 1/ssf * A(x,y\ step)/a(frame) * t/h$; where ΣQ^- is the number of counted cells; ssf is the section sampling fraction (1/12); A(x,y step) is the area associated with each x,y movement (sampling area subgranular zone [SGZ]: 70 μm X 50 μm; hilus: 150 μm X 150 μm); a(frame) is the area of the counting frame (SGZ: 40 μm X 40 μm; hilus: 75 μm X 75); t is the weighted average section thickness; and h is the height of the dissector (9 μm). A guard zone of 2 μm was used during cell counting to avoid sectioning artifacts.

2.5 Statistical Analysis

All data were analyzed using the Statistical Package for the Social Sciences (SPSS, v20, Chicago, IL, USA). Independent samples t-tests (two-tailed) were used to examine the statistical differences between the CORT and vehicle groups for all variables of interest. The criterion for statistical significance was set at $p < 0.05$. Cell count data are represented as means± standard error of the mean and western blot data are represented as the percentage change from control animals.

3. Results

Figure 4-1 shows the effect of repeated CORT administration on protein levels of DNMT1 (A) and MeCP2 (B) in the hippocampus. CORT had no significant effect on global hippocampal levels of DNMT1 [$t(6) = -0.055$, $p = 0.958$] or MeCP2 [$t(6) = 0.36$, $p = .731$] protein. To determine if there may be select region-specific changes in the expression of methylation markers following CORT treatment, we decided to conduct an immunohistochemical examination of MeCP2 in the SGZ and hilus of the hippocampus.

Figure 4-2 illustrates immunohistochemical staining of MeCP2 in the SGZ and hilus of the hippocampus after repeated CORT treatment. All MeCP2+ cells displayed immunoreactivity exclusively in the nucleus, in agreement with this protein binding DNA (Fig. 4-2A). Our results indicate that the number of MeCP2+ cells in the SGZ is significantly increased after 21 days of CORT administration [$t(8) = 2.844$, $p = 0.022$] (Fig. 4-2B). However, no change in MeCP2+ cells was found in the hilar region of the hippocampus [$t(8) = 1.890$, $p = 0.095$] (Fig. 4-2C).

4. Discussion

DNA methylation is catalyzed by a family of DNMTs that include the de novo methltransferases (DNMT 3a, DNMT3b) and a maintenance methyltransferase (DNMT1) (Bestor, 2000; Robertson & Wolffe, 2000). DNA methylation inhibits gene expression by either directly interfering with transcription factors binding to DNA (Watt & Molloy, 1988) or combining with methyl-CpG binding domain proteins (MBPs) which complex with HDACs to transform chromatin to a repressive state (Fan & Hutnick, 2005). Importantly, recent evidence indicates that DNA methylation is reversible, subject to dynamic regulation throughout the postnatal CNS (Feng, Chang, Li, & Fan, 2005), and plays an important role in the pathophysiology of a number of neuropsychiatric disorders, including major depression (Callinan & Feinberg, 2006; Charney & Manji, 2004; Krishnan & Nestler, 2008; Mehler, 2008a,b).

In the current study we examined how repeated administration of the primary stress hormone CORT influences protein levels of DNMT1 and the MeCP2 in the rat hippocampus. The results of this experiment reveal that, although CORT has minimal influence on the global expression of DNMT1 and MeC2 protein in the hippocampus, it does result in region-specific

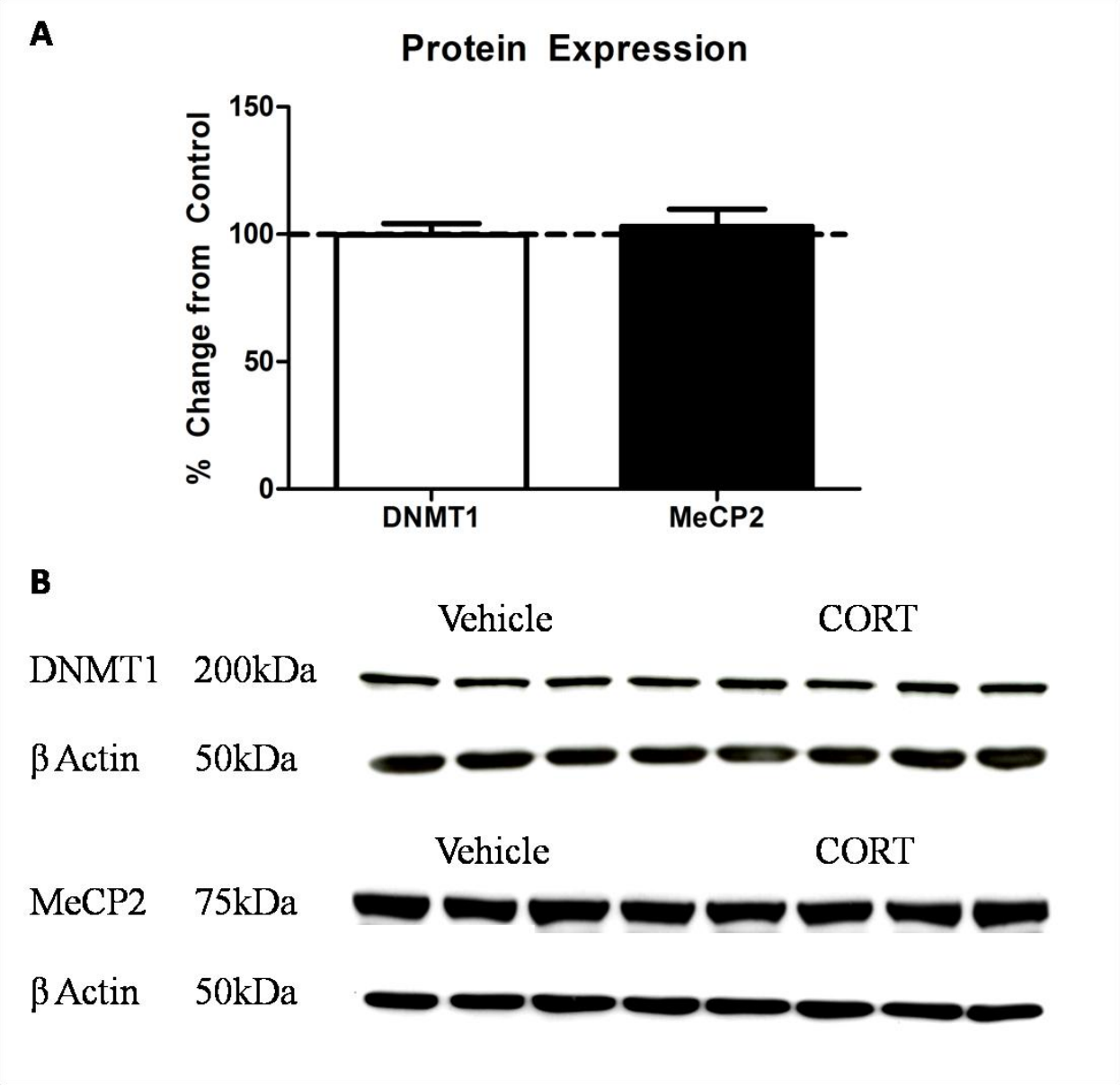

Figure 4-1. Effect of CORT on DNMT1 and MeCP2 protein levels in the hippocampus. The values are shown as a percentage change in optical density scores from the control group (value set to 100 and denoted by the dotted line) ± the standard error of the mean. CORT did not significantly influence total protein levels of DNMT1 or MeCP2 in the hippocamps (all *p*-values > .1). Representative scanned western blots are shown below the histogram.

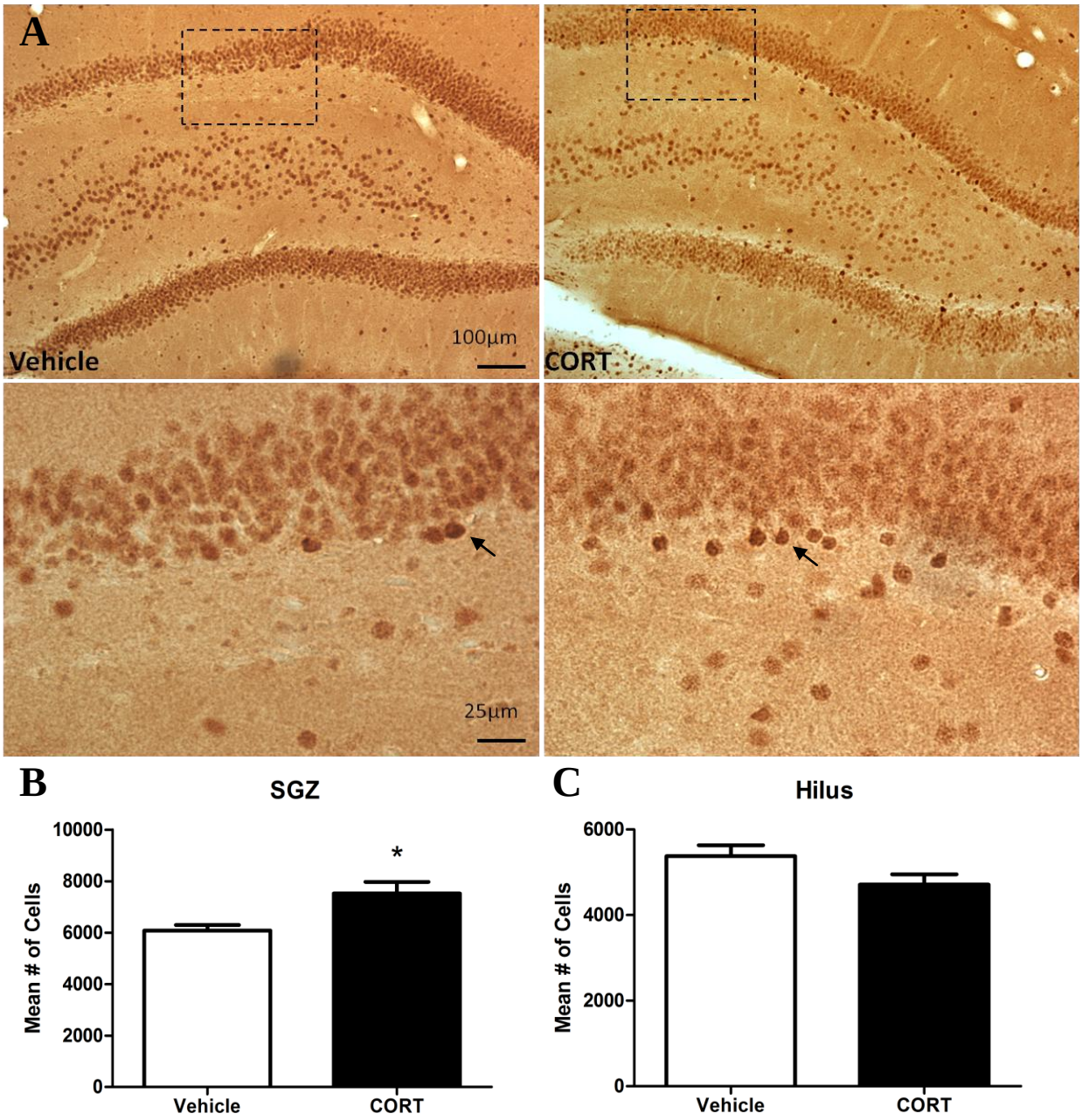

Figure 4-2. The effect of CORT on the number of MeCP2+ cells in the SGZ and hilus of the hippocampus. Panel A shows representative photomicrographs of MeCP2 immunoreactivity in each treatment group. Panels B and C show the number of MeCP2+ cells between treatment group in the SGZ and hilus respectively. CORT significantly increased the number of MeCP2+ cells in SGZ but not in the hilus of the hippocampus. Error bars represent the mean ± standard error of the mean. Asterisk (*) denotes statistical significance ($p < 0.05$).

increases in MeCP2 that may impact gene expression in a meaningful way. In particular, we identified a significant increase in the number of MeCP2+ cells in the SGZ region of the DG. Given that the MeCP2 protein binds to DNA that is methylated (Lewis et al., 1992; Nan, Campoy, & Bird, 1997), our data suggest that glucocorticoids increase the methylation status of cells localized to the proliferative zone of the hippocampus. These results are particularly important because MeCP2 is known to work in concert with DNMTs and HDACs to repress the transcription of proteins and neurotrophic factors important for regulating neurogenesis in the hippocampus (Lachner & Jenuwein, 2002; Matrisciano et al., 2013; Tsankova et al., 2007).

DNMT1 is the most abundant DNA methyltransferase, and is considered to be the key maintenance methyltransferase in mammals (Dhe-Paganon, Syeda, & Park, 2011). Although this enzyme predominantly methylates hemimethylated CpG dinucleotides in the mammalian genome, recent data suggests that DNMT1 can also act as a de novo methyltransferase able to modify unmethylated DNA (Fatemi, Hermann, Gowher, & Jeltsch, 2002). DNMT1 is highly expressed in adult post-mitotic neurons and is essential for keeping proper methylation patterns at certain genomic loci of these cells (Inano et al., 2000). Therefore, disruption of DNMT1 and more generally, DNA methylation, can have profound consequences on neuronal function. For example, DNMT1 knockout mice show decreased DG volume and reduced neuronal size, impaired spatial learning in the morris water maze, and impaired consolidation in contextual fear memory (Feng et al., 2010). Deletion of DNMT1 in neural progenitor cells also causes hypomethylation in post-mitotic neurons leading to multiple defects in neuronal maturation and synaptic transmission (Golshani, Hutnick, Schweizer, & Fan, 2005; Hutnick et al., 2009). Conversely, an increase in DNMT in the striatum and cortex is seen after ischemia, and is associated with neuronal injury. However, blocking DNMT1 activity, either genetically or pharmacologically, is protective to the injured neurons (Endres et al., 2000). Overexpression of DNMT1 in GABAergic interneurons in the frontal cortex has also been identified in schizophrenic patients, and is associated with decreases in proteins important in neural plasticity (e.g. reelin; Veldic et al., 2004). Taken together, this evidence suggests that a precise regulation of DNA methylation levels in the CNS is important for neuronal survival and function.

In the current study we found no global changes in DNMT1 protein expression in the hippocampus after repeated CORT administration. This finding was not entirely surprising given that previous reports have shown no overall changes in DNMT mRNA or protein levels in the

hippocampus of depressed patients (Poulter et al., 2008). Similarly, no global changes in DNA methylation have been found in other brain regions, such as the frontal cortex, in depressed patients (Sabunciyan et al., 2012). It is therefore possible that region-specific alterations in DNMT1 expression occur after chronic stress that escape detection in crude hippocampal homogenate processes. Indeed, mice exposed to prenatal restraint stress show increased levels of DNMT1 in GABAergic interneurons in both the fontal cortex and hippocampus in adulthood (Matrisciano et al., 2013). DNMT1was also found to be overexpressed selectively in GABAergic interneurons in the post-mortem schizophrenic brain (Ruzicka et al., 2007). Taken together, these data suggest that stress-induced changes in DNMT levels are likely cell-type specific.

MeCP2 is another epigenetic regulator of gene expression that is essential for normal brain development and function (Na, Nelson, Kavalali, & Monteggia, 2013). This is underscored by the fact that mutations in the MeCP2 gene have been linked to the neurodevelopmental disorder Rett's Syndrome (RTT). Specifically, RTT is caused by loss of the X-linked MECP2 gene, which ultimately reduces MeCP2 protein expression, and leads to symptoms such as developmental regression, intellectual and learning difficulties, impaired motor skills, loss of language, ataxia, and seizures (Ramocki & Zoghbi, 2008; Zachariah & Rastegar, 2012). In contrast, MeCP2 duplication syndrome is characterized by duplications in the MeCP2 gene, leading to protein overexpression, and symptoms that in some cases resemble those seen in RTT, including mental retardation, impaired development of speech and motor control, and seizures (Ramocki & Zoghbi, 2008). Although much attention has been paid to the role of MeCP2 in brain development, this protein is most highly expressed in mature postmitotic neurons (Balmer, Goldstine, Rao, & LaSalle, 2003) and plays an important role in CNS function in adulthood. For example, recent work has shown that MeCP2 mediates synaptic transmission in the adult CNS (Na et al., 2013). Specifically, MeCP2-deficient neurons have fewer dendritic spines and reduced arborization in the hippocampus and exhibit additional impairments in hippocampal neuronal maturation (Smrt et al., 2007; Zhou et al., 2006). Hippocampal neurons from MeCP2 deficient mice also show a defect in excitatory neurotransmission (Nelson, Kavalali, & Monteggia, 2006). Interestingly, mice that overexpress MeCP2 in CNS neurons show increased anxiety, and impairments in learning and memory that are accompanied by deficits in long-term potentiation (LTP) and short-term synaptic plasticity (Na et al., 2012). These results indicate that MeCP2

plays a vital role in normal synaptic function, again, underscoring the importance of the precise regulation of DNA methylation in the development and maintenance of a healthy CNS.

In the current study, similar to DNMT1, global levels of MeCP2 were not significantly altered in the hippocampus after repeated CORT injections. However, we did find a significant increase in the number of MeCP2+ cells in the SGZ of CORT treated animals. This finding is particularly intriguing given that the SGZ is one of two regions in the mammalian brain capable of generating new neurons (Lennington, Yang, & Conover, 2003). Also important to this is the fact that the production and maturation of new neurons in the hippocampus is severely disrupted in the brains of chronically stressed rodents (Bambico & Belzung, 2012; Banasr & Duman, 2007; Sterner & Kalynchuk, 2010), and hippocampal volume is often reduced in depressed patients (Sheline et al., 2003; Sheline et al., 1996). With these findings in mind, it is possible that MeCP2 is a mechanism by which glucocorticoids facilitate the repression of genes that regulate these adaptive cellular processes in the hippocampus. Indeed, recent evidence suggests that MeCP2 is directly related to the regulation of the reelin and BDNF genes (Martinowich et al., 2003; Matrisciano et al., 2013; Tsankova et al., 2006), both of which are found to be decreased in depressed patients, and are known to regulate neurogenesis, neuronal maturation, and synaptic plasticity in the adult brain (Fatemi et al., 2000; Karege et al., 2002, Lussier et al., 2009; Teixeira et al., 2012). Specifically, it has been shown that MeCP2 can modulate BDNF gene expression and function by interacting with methylated chromatin and suppressing promoter IV transcription (Martinowich et al., 2003). Conversely, activity-dependent induction of BDNF expression in cortical neurons is accompanied by the dissociation of a repression complex including MeCP2 and histone deacetylases (Chen et al., 2003; Martinowich et al., 2003). An increase in MeCP2 binding has also been identified at the promoter region of the reelin gene in the frontal cortex and hippocampus of adult mice exposed to prenatal restraint stress; this increase was associated with hypermethylation of the reelin gene promoter and decreased reelin expression in these regions (Matrisciano et al., 2013). Importantly, in vitro enhancement of reelin signaling can cause the dissociation of MeCP2 from the reelin promoter (Kundakovic et al., 2007), suggesting that MeCP2 may play an important role in the regulation of this protein in the hippocampus. However, we did not test this assumption directly and therefore cannot make any definitive conclusions about which genes may be targeted by increased MeCP2 expression in this

model of depression. Nonetheless, our findings are novel and suggest a role for MeCP2, and more generally DNA methylation, in the neurobiology of depression.

Although mounting evidence suggests that deregulation of the epigenome could lead to various neuropsychiatric disorders, including major depression (Bredy et al., 2010; Sun et al., 2013), many challenging questions remain to be resolved. Specifically, more work is needed to understand what signaling cascades are regulated by epigenetic processes and how that relates to the behavioral manifestation of major depression. The current study was a preliminary examination of how prolonged glucocorticoid exposure influences the expression of the methylation-associated markers in the rat hippocampus. Although we found no global changes in DNMT1 or MeCP2 expression, the increase in MeCP2+ cells in the SGZ of the DG after CORT treatment suggests that stress does exert some influence on DNA methylation. Future work with this model of depression should examine whether CORT influences the binding of MeCP2 and DNMT1 to the promoter region of genes known to be disrupted in depression (e.g. reelin and BDNF). It would also be of interest to determine the methylation status of these gene promoters, and correlate that with changes in actual mRNA and protein levels in a number of hippocampal sub-regions. Although increased MeCP2 is often associated with gene silencing, recent work has indicated that MeCP2 is a multifunctional nuclear protein that also has the ability to activate transcription by influencing chromatin architecture and RNA splicing (Chahrour et al., 2008; Hite, Adams, & Hansen, 2009). Therefore, it is possible that increased MeCP2 in the SGZ seen in this study is related to the upregulation of certain genes, and that this may play an important role in the depressed brain. Recent work in animal models has also recognized the potential use of drugs that directly inhibit DNMT1 and increase histone acetylation in treating neuropsychiatric conditions such as depression and schizophrenia (Guidotti et al., 2011; Sun et al., 2013), suggesting that this area of research could prove fruitful for the development of novel treatment strategies for neuropsychiatric disorders in the future.

CHAPTER 5
General Discussion

1. Summary of Main Findings

The objective of this dissertation was to gain further understanding of how the extracellular matrix protein reelin is influenced by prolonged glucocorticoid and antidepressant treatment, and how these alterations might relate to neurobiological and behavioral measures of depression. In order to study this, I examined the effects of CORT on several different outcomes including behavioral measures of depression, reelin expression, neuronal maturation, and interneuron and methylation marker expression.

In **Chapter 2**, I examined the effect of co-administering CORT and imipramine (10mg/kg or 15mg/kg) on the number of reelin+ cells in the SGZ and hilus of the hippocampal dentate gyrus (DG). Changes in reelin expression were examined in relation to the number and complexity of immature dentate granule cells, as well as measures of depression-like behavior in the FST. Consistent with previous reports (Lussier et al., 2009), CORT significantly reduced the number of reelin+ cells in both the SGZ and hilus. This downregulation was accompanied by reduced complexity of immature neurons in the DG and an increase of depressive-like behaviors in the FST. Importantly, co-treatment with imipramine largely prevented the deleterious effects of CORT on depressive-like behavior, reelin expression, and neuronal maturation, with the 15mg/kg dose having the most positive influence. Collectively, these data provide the first evidence that antidepressant medication protects against stress-induced hippocampal reelin loss. The temporal relationship that exists between alterations in reelin expression, the development of depression-like behavior and alerations in neurogenesis and neuronal maturation in the hippocampus, also suggest this protein likely plays some role in the pathophysiology of the disorder.

This experiment was an important starting point for all subsequent experiments in this dissertation because it was the first preclinical study to show that antidepressants can protect against the downregulation of reelin expression in the hippocampus. These results further substantiated a role for reelin in depression and suggested that this protein may be an important molecular target of antidepressant medications. However, I was unable to determine from this study exactly how CORT and imipramine were influencing reelin signaling in the hippocampus.

Considering 1) the wealth of evidence on neuronal atrophy and cognitive deficits in depressed patients and animals exposed to chronic stress (Boldrini et al., 2013; Conrad, 2010; McIntyre et al., 2013), 2) reelin's integral role in regulating synaptic plasticity and neuronal development in the adult brain (Niu et al., 2004; Niu et al., 2008; Teixeira et al., 2012; Weeber et al., 2002), and 3) the association between reelin deficits and major depression in clinical populations (Fatemi et al., 2000; Knable et al., 2004; Torrey et al., 2005) and in animal models of the disorder (Lussier et al., 2009; Lussier et al., 2011), it was clear that investigating how this protein is influenced by glucocorticoids would be a fruitful avenue for further research.

In **Chapter 3** I examined the influence of prolonged glucocorticoid exposure on reelin-expressing GABAergic interneurons in the hippocampus. The goal of this project was two-fold: 1) to further characterize the extent to which inhibitory interneuron markers in the hippocampus are impacted by repeated CORT treatment, and 2) to determine whether the loss of reelin+ cells in the hippocampus seen in chapter 2 reflects an actual loss of interneurons in this region. Results of this experiment showed that repeated CORT treatment significantly reduces the expression of parvalbumin, calretinin, somatostatin and neuropeptide-y in a number of hippocampal regions. However, not all interneuron markers examined were affected by CORT (i.e., calbindin and cholecystokinin), indicating that some interneuron markers are more resistant than others to the deleterious effects of glucocorticoids. Moreover, GAD67 expression was not altered in any region of the hippocampus examined, indicating that CORT does not cause interneurons in these regions to die. These results are particularly important because they suggest that glucocorticoids act via an intraceullar mechanism to reduce reelin synthesis and expression in the hippocampus, raising the possibility that drug targets could be developed to reverse reelin-related deficits that occur with chronic neuropsychiatric conditions.

Chapter 4 was a preliminary examination of the effects glucocorticoids have on DNA methylation in the hippocampus. Specifically, the goal of this project was to determine if any global or regional changes in DNMT1 and MeCP2, two markers associated with DNA methylation, exist in this region after prolonged CORT exposure. Although CORT had minimal influence on the global expression of DNMT1 and MeCP2, it significantly increased the number of MeCP2+ cells in the SGZ of the DG. Given that MeCP2 protein binds to DNA that is methylated (Lewis et al., 1992; Nan, Campoy, & Bird, 1997), these results suggest that CORT increases the methylation status of cells localized to the proliferative zone of the hippocampus.

This finding is particularly important because the SGZ is one of two sites in the mammalian brain where neurogenesis takes place (Lennington et al., 2003). Given that repeated exposure to glucocorticoids is known to significantly reduce cell proliferation and maturation of hippocampal neurons (Bambico & Belzung, 2012; Banasr & Duman, 2007; Charney & Manji, 2004; Sterner & Kalynchuk, 2010), it is possible that MeCP2 is a mechanism by which these stress hormones facilitate the repression of genes that regulate adaptive cellular processes in the hippocampus. For example, increased binding of MeCP2 to the reelin gene promoter is associated with reduced reelin expression in the hippocampus (Matrisciano et al., 2013). Interestingly, the exact location where reelin is most significantly decreased by CORT in the hippocampus is where MeCP2 expression is increased. Although these findings are correlational, further examination into the relationship between MeCP2 and reelin in this model of depression may shed light on the mechanisms by which glucocorticoids exert their influence over reelin expression in the brain.

The overall results of this dissertation provide strong evidence to substantiate a role for disrupted reelin signaling in depression. Not only do these data show that reelin is downregulated in the hippocampus of rats exposed to glucocorticoids, they show that antidepressants currently used to treat major depression can prevent stress-induced degradation of reelin in the hippocampus. These data also suggest that stress has a direct influence on the synthesis and expression of reelin by acting via an intracellular, possibly epigenetic, mechanism in inhibitory cells of the hippocampus.

2. The Role of Reelin in Depression

A deficit in reelin expression has been associated with a number of neuropsychiatric disorders, including schizophrenia, bipolar disorder, autism, epilepsy, and major depression (Eastwood & Harrison, 2006; Fatemi et al., 2000; Guidotti et al., 2000a; Haas & Frotscher, 2010; Hong et al., 2000; Impagnatiello et al., 1998; Saez-Valero et al., 2003). Results from chapter 2 of this dissertation are in accordance with these findings, as CORT produced a significant decrease in the number of reelin+ cells in the proliferative SGZ and hilus of the hippocampal DG. Interestingly, our laboratory has recently shown that only stress paradigms that produce depressive-like behaviors reduce reelin expression in these regions of the hippocampus (Lussier et al., 2009), suggesting that alterations in this protein may be linked to the emergence of depressive symptomatology. Two weeks of maternal separation also reduces reelin expression in

the hippocampus (Qin et al., 2011), and prenatal restraint stress has been shown to decrease reelin levels in the frontal cortex of mice into adulthood (Matrisciano et al., 2013). Importantly, double labeling of reelin and GR have shown that virtually all reelin+ cells in the stratum oriens, stratum-lacunosum moleculare and GCL are also GR+ (Gross et al., 2012), indicating that reelin+ cells can be directly influenced by circulating levels of glucocorticoids. Taken together, these studies suggest that reelin signaling can be altered by a number of stressor types, and that these changes may be enduring in nature.

Work with transgenic mouse models has begun to tell us a great deal about the link between altered reelin signaling in the brain and affective disorders. For example, reelin haploinsufficient mice (i.e., HRM) have been considered as a model of psychosis vulnerability, as they exhibit decreases in dendritic spines, GABAergic defects, as well as a number of behavioral symptoms (e.g. sensorimotor gating deficit, slow acquisition in the radial arm maze) that are reminiscent of what we see in human schizophrenic patients (Liu et al., 2001; Niu et al., 2008; Tueting et al., 1999). Post-mortem analyses of schizophrenic patients have also indicated widespread deficits in this protein in areas of the prefrontal and temporal cortex, hippocampus, caudate nucleus, and cerebellum, much like what we see with HRM mice (Fatemi et al., 2000; Guidotti et al., 2000a; Impagnatiello et al., 1998; Knable et al., 2004; Tueting et al., 1999). Consequently, the downregulation of this gene likely disrupts GABAergic neurotransmission which is purported to be an important part of the molecular mechanism responsible for at least some of the clinical features of schizophrenia (Guidotti et al., 2005). Interestingly, recent work in our laboratory has shown that HRM mice are more susceptible to the deleterious effects of glucocorticoids (Lussier et al., 2011). In particular, under basal conditions, no differences exist between wild-type and HRM mice on FST behavior. However, the effects of CORT on immobility are more pronounced in HRM compared to other CORT-exposed groups. A more marked reduction in reelin+ cells and dendritic complexity in immature dentate granule cells also exist in CORT-treated HRM mice compared to wild-type animals exposed to CORT (Lussier et al., 2011). Importantly, these data provided the first evidence that the downregulation of reelin could increase susceptibility to developing depressive sympomatology.

The recent development of a gain-of-function transgenic mouse line overexpressing reelin in the postnatal and adult forebrain has helped to further our understanding of the role reelin plays in affective disorders (Pujadas et al., 2010; Teixeira et al., 2011). In contrast to

HRM, mice that overexpress reelin show enhanced resilience to the deleterious effects of stress. Under basal conditions, no differences exist in FST behavior between mice that overexpress reelin and wild-type mice. However, after 3 weeks of CORT in their drinking water, overexpressing mice show no increase in depressive-like behaviors on the FST, whereas wild-type animals exposed to CORT show significant increases on these measures. Reelin overexpression also prevents deficits in prepulse inhibition, potentiates NMDA-dependent glutamatergic neurotransmission, and enhances neuronal maturation and neural cell fate differentiation (Pujadas et al., 2010; Teixeira et al., 2011). Taken together, research with transgenic mouse models has shown us that genetic deficits in reelin confer vulnerability to developing many of the symptoms apparent in affective disorders, whereas overexpression of this protein can be protective. Moreover, these data suggest that targeting reelin may be of particular interest for the development of novel therapeutic strategies in the treatment of disorders characterized by neuronal atrophy.

3. Relationship Between Reelin, Neurogenesis and Depression

As discussed previously, decreased neurogenesis is hypothesized to be involved in the pathophysiology of major depression. Support for this assumption comes from a wealth of preclinical evidence indicating that various forms of chronic stress (e.g. CORT, unpredictable chronic mild stress, repeated restraint stress) decrease the proliferation, differentiation, and survival of neurons in the hippocampus (Bambico & Belzung, 2012; Banasr & Duman, 2007). Decreased hippocampal volumes in depressed patients also lead to questions about the importance of neurogenesis in major depression (Sheline et al., 2003; Sheline et al., 1996). Furthermore, antidepressant drugs increase all stages of neurogenesis, including proliferation, maturation, and survival (Banasr & Duman, 2007), suggesting that proper integration of these newly generated neurons may play an important role in the pathophysiology of depression. However, the link between neurogenesis and depression is not so clear cut. For instance, post-mortem studies have not revealed a change in the number of dividing cells in depressed patients (Reif et al., 2006). Recent preclinical research has also shown that antidepressant-induced changes in the FST and open field test are not neurogenesis-dependent (David et al., 2009), suggesting a disconnect between the generation of new neurons and depressive symptomatology. To further complicate this matter, some research supports decreased hippocampal cell

proliferation after stress (Brummelte & Galea, 2010a; Duman, Malberg, & Nakagawa, 2001; Gould, McEwen, Tanapat, Galea, & Fuchs, 1997) whereas other data suggest that stress predominantly affects neuronal differentiation and maturation phases of neurogenesis (Jayatissa, Bisgaard, West, & Wiborg, 2008; Jayatissa, Henningsen, West, & Wiborg, 2009; Lee et al., 2006). Overall these results suggest that a complex relationship exists between stress, depression, neurogenesis and antidepressant treatment, and that further examination is needed to fully understand the role that neurogenesis plays in affective disorders.

Recent work has demonstrated that alterations in reelin signaling can impact various stages of neurogenesis. For instance, reeler mice show a reduction in both cell proliferation and in the number of immature neurons in the hippocampus (Won et al., 2006; Zhao, Chai, & Frotscher, 2007). The number of cells that express a glial phenotype is also increased in these mice, suggesting that a lack of reelin leads to preferential astrocyte differentiation (Zhao et al., 2007a). Similar results have been found in cells lacking Dab1. In particular, deletion of Dab1 in dentate neuroprogenitor cells leads to abnormal orientation and ectopic migration of these cells into the hilus, a failure to develop appropriately complex dendritic processes and the abnormal development of functional basal dendrites. Moreover, deletion of this adaptor protein affects neuronal fate by favoring the differentiation of neural progenitor cells into glial cells rather than granule cell neurons (Teixeira et al., 2012). Opposite to this, mice that overexpress reelin display an enhanced rate of migration and maturation of adult generated neurons in the hippocampus, along with a significant increase in dendritic tree extension and dendritic complexity (Teixeira et al., 2011). Results from chapter 2 also support a relationship between changes in reelin expression and the maturation of immature granule cell neurons. Specifically, CORT-induced downregulation of reelin in the SGZ and hilus paralleled a reduction in the number and complexity of DCX-labeled neurons, and an increase in basal dendrites. Importantly, these changes were prevented by co-treatment with imipramine and paralleled changes in depressive-like behavior. Although it is unclear from this experiment whether altered reelin expression is responsible for the morphological changes in dentate granule cells, the fact that we see decreased cell number and complexity in areas devoid of reelin suggests it is possible; however, more work is needed with the CORT model to confirm that this is the case. Collectively, these data strongly support a role for reelin in all stages of neurogenesis from proliferation to migration, integration and development, and suggest that further examination into the role of this protein in cell

maturation and survival may be of importance for understanding and treating disorders characterized by decreased neurogenesis and neuronal atrophy.

4. Relationship Between Antidepressant Treatement and Reelin Expression

Many of the behavioral and neurobiological consequences of stress can be reversed or protected by antidepressant treatment (Banasr & Duman, 2007; Duman et al., 2001; Sterner & Kalynchuk, 2010). However, the influence of antidepressant medications on reelin expression in the brain is poorly understood. Results from chapter 2 of this dissertation are the first to show that the atypical antidepressant imipramine, protects against CORT-induced downregulation of reelin in the SGZ and hilus of the hippocampal DG. Jaako and colleagues (2011) provide further evidence that reelin is positively influenced by antidepressant treatment, showing that citalopram protects against kainic acid-induced downregulation of reelin in the hippocampus. In this same study, citalopram on its own was able to increase hippocampal reelin mRNA, indicating that the influence of antidepressants on reelin expression is more than protective. Similar results have been found in other brain regions known to be dysfunctional in depression. In particular, a number of psychotropic medications used to treat affective disorders have been found to enhance reelin and its signaling machinery in the frontal cortex (Fatemi et al., 2009). For example, fluoxetine and olanzapine significantly enhance reelin mRNA and VLDLR expression in the frontal cortex of rats. Fluoxetine also enhances Dab1 protein in this region (Fatemi et al., 2009). Collectively this evidence indicates that antidepressant treatment can significantly enhance reelin signaling in the brain. Moreover, they suggest that reelin may play an important role in the long-term adaptations required for antidepressant efficacy. However further work is needed to determine whether this is the case, and to elucidate the mechanisms by which these changes occur.

The evidence described above strongly supports the potential for targeting the reelin signaling system in the treatment of a number of neuropsychiatric disorders. Specific to depression, a growing body of preclinical evidence points to a deficit in reelin signaling in the disorder (Lussier et al., 2009; Lussier et al., 2011; Teixeira et al., 2011) – a finding which is paralleled in post-mortem brain tissue from human patients (Fatemi et al., 2000; Knable et al., 2004; Torrey et al., 2005). Moreover, it seems that enhancing this signaling system can have a positive influence on neurobiology and depressive symptomatology (Teixeira et al., 2011).

However, this area of research is in its infancy and a great deal more work is needed to fully understand the relationship between reelin and affective disorders – in this regard, a few key experiments will be described as future directions.

5. Mechanisms Governing Reelin Expression in the Brain

As described previously, reelin is secreted exclusively by GABAergic neurons in the cortex and hippocampus in the adult brain (Pesold et al., 1998). These GABAergic neurons make up a heterogeneous population of cells important for controlling inhibition in these regions (Freund & Buzsaki, 1996). Interestingly, a growing body of evidence supports a role for GABA dysfunction in major depression. In particular, decreased levels of GABA have been found in brain and peripheral tissue of depressed patients (Gerner & Hare, 1981; Kasa et al., 1982; Petty et al., 1992; Sanacora et al., 1999; Sanacora et al., 2000), and animals subjected to stress (Cullinan & Wolfe, 2000; Gronli et al., 2007; Orchinik et al., 1995). Decreases in a number of GABAergic interneuron markers, including reelin, have also been described in depressed patients (Fatemi et al., 2000; Knable et al., 2004; Zhang, Sun, & Reynolds, 2002), suggesting that the population of cells responsible for inhibition in the brain is functioning abnormally. Indeed in chapter 3 we found decreases in a number of interneuron markers throughout the hippocampus of CORT treated rats, which have significant implications for inhibitory functioning in this region (see chapter 3 discussion). In chapter 3 we also identified that the loss in reelin+ cells in the SGZ and hilus seen in chapter 2 does not reflect an actual loss of GABAergic interneurons in these hippocampal regions. Previous work with post-mortem tissue from schizophrenic and bipolar disorder patients is also in agreement with this assumption. Specifically, the number of reelin+ cells in the PFC is significantly reduced in these patients, with no apparent change in the number of Nissl or NeuN (neuronal nuclear-specific marker) immunostained markers; since all mature neurons express these markers, the decrease in reelin+ cells cannot be interpreted as a decrease in GABAergic neurons (Guidotti et al., 2000a,b). Ruzicka and colleagues (2007) also recently identified a significant reduction in reelin mRNA in GABAergic neurons in the frontal cortex of schizophrenic patients, providing auxiliary evidence that an intracellular mechanism is responsible for the downregulation of reelin content in these neurons.

A growing body of evidence suggests that epigenetic processes are responsible for the downregulation of reelin expression in neuropsychiatric disorders, and that DNA methylation specifically has a role in the dysfunction of GABAergic neurons (Bredy et al., 2010; Costa et al., 2007; Gavin & Sharma, 2010; Graff & Mansuy, 2009; Roth et al., 2009). In particular, hypermethylation of the reelin gene promoter in the frontal cortex of schizophrenic patients is associated with decreased reelin expression (Grayson et al., 2005; Guidotti et al., 2000b). DNMT1 mRNA and protein levels were also shown to be significantly increased in the cortex of schizophrenic patients (Veldic et al., 2004; Veldic et al., 2005) and these increases paralleled deficits in reelin (Ruzicka et al., 2007; Veldic et al., 2005). DNMT1 was also shown to be overexpressed exclusively in GABAergic interneurons in SZ patients (Veldic et al., 2004; Veldic et al., 2005), and to have a direct role in reelin promoter methylation in vitro (Noh et al., 2005). Similar results have been demonstrated after prenatal restraint stress (PRS) in mice (Matrisciano et al., 2013). Specifically, offspring born from non-stressed mothers show high levels of DNMT1 and 3a mRNA expression in the frontal cortex at birth, but these levels progressively decreased at post-natal days 7, 14, and 60. However, offspring born from stressed mothers (PRS mice) show increased levels of DNMTs at all time-points. It was also determined in this study that the overexpression of DNMT in GABAergic neurons was associated with a decrease in reelin and GAD67 expression in PRS mice in early and adult life. PRS mice also had increased binding of DNMT1 and MeCP2, and an increase in 5-methylcytosine and 5-hydroxymethylcytosine (i.e., methylated cytosine) in specific CpG-rich regions of the reelin and GAD67 promoters (Matrisciano et al., 2013). This experiment was the first direct examination of how stress influences the epigenetic regulation of reelin in the frontal cortex and hippocampus. Interestingly, PRS has been associated with an exaggerated stress response and increased depressive-like behaviors in the FST in offspring (Morley-Fletcher et al., 2003; Morley-Fletcher et al., 2011). Although no current data exists supporting the epigenetic regulation of reelin in depressed patients or adult rats exposed to chronic stress, the above results suggest that chronic stress can have a significant impact on DNA methylation in the brain. Similarly, in chapter 3, I found that repeated CORT administration increased the DNA methylation-associated protein MeCP2, in the SGZ region where the reduction in reelin expression is most pronounced after CORT treatment. Although we cannot conclude that the increase in MeCP is specific to reelin-expressing interneurons, our data suggest that glucocorticoid exposure in adulthood may also

influence DNA methylation and that this may be responsible for the downregulation of reelin in this model of depression. Moreover, they highlight the potential use of drugs that target DNA methylation and histone acetylation for treating disorders characterized by deficits in GABA function.

Another possible mechanism by which reelin may be regulated in the brain is through microRNA (miRNA). MicroRNA's are small non-coding RNA molecules that act primarily as negative regulators of gene expression at a post-transcriptional level by base-pairing with complementary sequences in mRNA molecules (Kato & Slack, 2008). Their repressive regulatory activity is performed via either the degradation of their specific target mRNA, or the inhibition of mRNA translation (Eulalio, Huntzinger, & Izaurralde, 2008; Filipowicz, Bhattacharyya, & Sonenberg, 2008). The first miRNAs were characterized in the early 1990s (Lee, Feinbaum, & Ambros, 1993). Since this time, research has revealed multiple roles for miRNA in transcript degradation and sequestering, translational suppression, and even transcriptional and translational activation (Kato & Slack, 2008). Specific to reelin, the neuron-specific miRNA, miR-128, has been shown to directly influence the expression of this protein in vitro (Evangelisti et al., 2009). In particular, ectopic overexpression of miR-128 in neuroblastoma cells significantly reduces reelin expression, whereas knockdown of this miRNA significantly increases it. This study also demonstrated that the three prime untranslated region (3'UTR; region of mRNA immediately following the translantion termination codon containing regulatory regions that influence post-translational gene expression) of reelin is a direct target of miR-128 (Evangelisti et al., 2009). The family of miR-15 and miR-107 may also be potential target regulators of reelin expression in the brain. Specifically, these miRNA's are known to be increased in the superior temporal gyrus and dorsolateral prefrontal cortex of schizophrenic patients, and the predicted targets of these miRNA's consist of a number of genes important for neural connectivity and synaptic plasticity, including reelin (Beveridge, Gardiner, Carroll, Tooney, & Cairns, 2010). In vitro analyses have also revealed that the 3'UTR region of the reelin gene is consistently silenced by both the miR-15 family and miR-107 (Beveridge et al., 2010). Although a great deal of evidence supports the role of DNA methylation in the regulation of reelin expression, the above data suggest it is also conceivable that post-transcriptional gene silencing contributes to reelin deficits in neuropsychiatric patients. However, this area of research is in its infancy and a great deal of clinical and preclinical work is needed to determine

how changes in miRNA may lead to deregulation of genes important for neural plasticity, and how this relates to affective disorders.

6. Limitations

6.1 Clinical Relevance of Using Exogenous CORT Administration to Model Depression

As discussed previously, a common criticism of many preclinical stress models of depression is the unrealistic nature of the stressors used. Repeated restraint stress, for example, is not a realistic simulation of the human experience and often leads to habituation effects (Galea et al, 1997; Gregus et al., 2005). CMS, on the other hand, is said to be the most realistic model of depression because it consists of exposure to a number of mild stressors throughout the paradigm. However, one of the biggest problems with these stress models is a lack of control over individual differences in HPA axis activation and subsequent CORT levels. Stressful stimuli can differ in their physical qualities (i.e., actual qualities) and their psychological qualities (i.e., perceived qualities). This may result in differing CORT levels between different animals exposed to the same stressor, which in turn could lead to increased experimental variability. Furthermore, many animals habituate to the aversive effects of these stressors when they are repeatedly exposed to them (Galea et al., 1997; Gregus et al., 2005; Grissom et al., 2007). This might explain why these paradigms have produced conflicting behavioral results. The validity of the CORT model of depression has also been questioned. Specifically, the injection of stress hormones into the body is not a realistic simulation of how we experience stress in daily life, and the amount of CORT needed to produce a depressive phenotype is often much higher than what is naturally released during a normal stress response (Johnson et al., 2006). However, the CORT model does produce very reliable behavioral and morphological changes similar to the CMS model (Willner, 1997). Moreover, this model allows us to study the direct influence of stress hormones on depressive behavior and neurobiological changes in the brain without the influence of habituation and psychosocial factors.

6.2 Sex Differences in Depression-Like Behavior and Neurobiology

Depression is the most common neuropsychiatric disorder among women, with a prevalence that is 2 to 3 times higher than in men (Gutierrez-Lobos, Scherer, Anderer, & Katschnig, 2002; Sloan & Kornstein, 2003). Although no female animals were used in any of the

experiments in this dissertation, important differences exist between males and females regarding their response to stress and the development of depressive-like behaviors which should be addressed. First, sex differences are evident in certain aspects of HPA axis activity. Female rats, for example, have higher resting levels of CORT and display greater diurnal changes in both ACTH and CORT compared to males (Handa, Burgess, Kerr, & O'Keefe, 1994; Kitay, 1961; Viau, Bingham, Davis, Lee, & Wong, 2005; Weinstock, Razin, Schorer-Apelbaum, Men, & McCarty, 1998). Female rats also have higher glucocorticoid levels following acute and repeated stress exposure and this seems to be dependent on circulating gonadal hormones (Seale et al., 2004; Seale, Wood, Atkinson, Harbuz, & Lightman, 2004). Although these data suggest that females may be more susceptible to the deleterious effects of chronic stress, the behavioral and neurobiological data supporting this is not so clear. In our laboratory, for example, we have found that 40mg/kg of CORT for 21 days increases depressive-like behavior in the FST in both sexes, but to a lesser degree in females (Kalynchuk et al., 2004). Similarly, after 20 days of CORT (20mg/kg) males show increased depressive-like behavior in the FST whereas females do not (Hill et al., 2003). In the CMS model of depression, males also show increased anhedonia compared to female rats (Dalla et al., 2005; Kamper et al., 2009). In terms of neurobiology, CA3 pyramidal neurons in the hippocampus are more severely atrophied after exposure to 21 days of restraint stress (6h/day) in males compared to females (Galea et al., 1997). Neurogenesis is also disrupted to a greater degree in the dorsal hippocampus of male rats after repeated CORT exposure (Brummelte & Galea, 2010a). Collectively, these data imply that females, despite enhanced HPA axis activity, are actually more resilient to stress. One possible explanation for this is the fluctuation in gonadal hormones across the estrous cycle. For example, during proestrus (i.e., the high estrogen stage) female rats show less anxiety-like behaviors in the open field test and a decrease in depressive-like behaviors in the FST compared to male rats and females during the diestrus phase (Frye & Walf, 2002). The postpartum period, a time when estradiol is greatly reduced, is also considered to be the time of greatest risk for women to develop major depression (Brummelte & Galea, 2010b). Moreover, circulating estrogen levels have been shown to positively correlate with cell proliferation and negatively correlate with cell death in the hippocampus (Pawluski, Brummelte, Barha, Crozier, & Galea, 2009; Tanapat, Hastings, Reeves, & Gould, 1999). These data suggest that the relationship between sex, stress, neurobiology and affective behavior is complex and should be studied further. Unfortunately,

between 55 and 65% of experimental studies in pharmacology and neuroscience use male subjects exclusively, and those that do often do not take sex into account in their analyses (Beery & Zucker, 2011); this points to the need for greater use of females in scientific experimentation, especially in models of disease where female prevalence is high.

In regards to sex differences in reelin expression, little work has been done on this topic, and there have been some conflicting results. For example, two previous studies have found the reelin gene to be associated with a greater risk for developing schizophrenia and bipolar disorder in females (Goes et al., 2010; Shifman et al., 2008). A meta-analysis of 12 post-mortem brain expression studies also found reelin levels to be moderately, but significantly, lower in female patients with bipolar disorder compared to males (Goes et al., 2010). However, others have found no such difference in reelin expression between male and female schizophrenic, bipolar, or depressed patients (Fatemi et al., 2000; Guidotti et al., 2000a). In addition, studies with HRM have found significant cell loss in the cerebellum of males, with no evident effect in females (Hadj-Sahraoui, Frederic, Delhaye-Bouchaud, & Mariani, 1996). Taken together these data also suggest that a complex relationship exists between sex and reelin expression in the brain. Further examination of these differences in the context of stress and affective behavior is therefore needed to enhance our understanding of major depression.

6.3 Other Molecular Markers that May be Involved in the Pathogenesis of Depression

The present dissertation provides evidence that disrupted reelin signaling may be involved in the pathophysiology of depression. However, given the complexity of depression and heterogeneity among patients, there is likely a deregulation in a number of genes that occur over the lifetime of an individual with the disorder. In particular, decreases in a number of neurotrophic factors have been identified in both depressed patients and in animals exposed to chronic stress (Jiang & Salton, 2013). For example, analysis of postmortem human brain tissue has revealed a reduction in BNDF mRNA and protein levels in the hippocampus, prefrontal cortex, and amygdala of depressed patients (Guilloux et al., 2012). Moreover, antidepressant treatments were found to increase BDNF protein levels in a number of hippocampal regions (Chen, Dowlatshahi, MacQueen, Wang, & Young, 2001). Similarly, reductions in BDNF have been identified in the sera and plasma of depressed patients (Lee, Kim, Park, & Kim, 2007; Yoshida et al., 2012), and again this is normalized by antidepressant treatment (Shimizu et al.,

2003). In addition, reduced BDNF content in platelets and decreased BDNF mRNA expression in peripheral blood lymphocytes and mononuclear cells were also reported in depressed patients (Lee & Kim, 2009; Lee & Kim, 2010). Preclinical studies have shown similar results in that multiple stressor types, including CORT, restraint, electrical foot shock, forced swimming, social deprivation and chronic social defeat, down-regulate BDNF mRNA and protein levels in a number of brain regions, including the hippocampus (Jiang & Salton, 2013; Suri & Vaidya, 2013). Interestingly, this reduction in BDNF expression is associated with the development of depressive-like behavior, and peripheral and intra-hippocampal administration of BDNF has shown to be antidepressant (Duman & Monteggia, 2006; Schmidt & Duman, 2010). Similar changes are found with vascular endothelial growth factor (VEGF) in preclinical models of depression (Duman & Monteggia, 2006; Fournier & Duman, 2012). These data indicate that neurotrophins also play an important role in the pathophysiology of depression and may be potential targets for treatment. In addition to altered neurotrophin signaling, chronic stress causes a reduction in cell adhesion proteins (Nacher et al., 2004) and cytoskeletal proteins that play an important role in CNS structural plasticity (Cereseto et al., 2006). Importantly, many of the neurotrophins and proteins downregulated in depressed patients and preclinical models affect many of the same signaling cascades that regulate synaptic plasticity and neurogenesis. For example, both reelin and BDNF enhance LTP via post-synaptic activation of CREB (Chen et al., 2005; Ying et al., 2002). Recent evidence has also pointed to an interaction between BDNF and reelin signaling (Lindhorst, Kurz, Sibbe, Meseke, & Forster, 2012; Ringstedt et al., 1998). Therefore, although the focus of the research in this dissertation has been on reelin, I acknowledge that none of these mechanisms are mutually exclusive and that all of them could contribute to the development of depressive symptomatology.

7. Future Directions

7.1 Do Other Animal Models of Depression Alter Reelin Signaling?

Our laboratory has shown that repeated administration of CORT significantly reduces the number of reelin+ cells in the hippocampal DG, and that this reduction is paralleled by increases in depression-like behavior (Lussier et al., 2009). We have also shown that HRM mice are more susceptible to the deleterious effects of CORT (Lussier et al., 2011). However, a recent report from our laboratory indicates that chronic restraint stress has no significant effect on reelin

expression in the hippocampus (Lussier et al., 2009), suggesting that not all types of stress influence reelin signaling equally. Importantly, this restraint stress paradigm did not increase depression-like behavior in the FST (Gregus et al., 2005), implying that reelin may only be downregulated when the stress is sufficient enough to produce a depressive phenotype. Unfortunately this assumption has not been appropriately tested. There is some evidence to suggest that maternal separation for 3hrs per day for 14 days results in decreased reelin mRNA in the hippocampus (Qin et al., 2011). Prenatal restraint stress has also been shown to downregulate reelin mRNA in the frontal cortex (Matrisciano et al., 2013). Together these data provide evidence that other forms of stress can influence reelin signaling in the brain. However, similar maternal separation protocols have also shown no change in reelin+ cells in the hippocampus immediately and two months following stress (Gross et al., 2010). Unfortunately, in these studies depression-like behavior was not examined, and so it cannot be determined if decreased reelin expression was only apparent after stress that increased depressive-like symptoms. Future research should therefore examine how reelin expression is influenced by other repeated stressors (e.g. CMS) to ensure that the decrease found after CORT treatment is replicable across stress models. Of course, these changes should be examined in the context of depressive-like behavior to determine whether a significant reduction in reelin expression is associated with a depressed state, specifically.

7.2 Is Reelin Signaling Altered in Other Brain Regions Following Repeated Stress?

The hippocampus is one of the most extensively studied brain regions in the context of depression, and compromised integrity of the hippocampal circuit has been one of the most well documented findings in both depressed patients and preclinical models (Sheline et al., 2003; Sterner & Kalynchuk, 2010). However, depression is a complex disorder that affects many brain regions. In particular, the pattern of metabolic changes during major depressive episodes suggests that brain structures thought to play a key role in mediating emotional and stress responses (e.g. the amygdala, hippocampus) are pathologically activated; brain areas thought to modulate or inhibit emotional expression are also activated (e.g. subgenual prefrontal cortex); and, areas implicated in attention and sensory processing are deactivated (Drevets, 2001). Therefore, examining how reelin expression is altered in other brain regions affected by

depression is important for further substantiating a role for this protein in the pathophysiology and treatment of the disorder.

The amygdala plays an important role in the interpretation and expression of affect, especially that related to fear (Davis, 1994; Davis, Rainnie, & Cassell, 1994; LeDoux, 2000). The amygdala is also particularly vulnerable to the effects of stress and contributes to stress-related affective disorders (Roozendaal, McEwen, & Chattarji, 2009). Preclinical studies show that chronic stress results in amygdala hypertrophy and hyperactivity, which is accompanied by enhanced anxiety and fear behavior (Chiba et al., 2012; Mitra, Jadhav, McEwen, Vyas, & Chattarji, 2005; Rosenkranz, Venheim, & Padival, 2010). Human studies have also reported amygdala hypertrophy and hyperactivity in patients with affective disorders (Davidson, 2003; Drevets, 1999; Drevets, Price, & Furey, 2008). These findings suggest that chronic stress may precipitate affective disorders, in part, via modification of amygdala function. To date there has been no examination of how chronic stress or glucocorticoids influence reelin expression in the amygdala. Unpublished work from our laboratory suggests that reelin may be decreased in some amygdalar regions (i.e., the medial and cortical amygdala) after repeated CORT exposure (Lussier and Kalynchuk unpublished observation, 2010). However, a more detailed examination of changes in reelin expression in the different sub-nuclei of the amygdala after chronic stress is needed. Further work is also needed to determine the functional implications of altered reelin signaling in the amygdala. One way to examine this directly is by blocking reelin signaling via CR-50 (D'Arcangelo et al., 1997) infusion into the amygdala, and examining changes in amygdala-dependent behavioral tasks (e.g. fear conditioning). Conversely, it would be interesting to see how infusing reelin into the amygdala would influence behavior and neurobiology in this region. These data would provide important insight into the role reelin plays in fear and anxiety-related behaviors.

The PFC is another brain region that functions abnormally in depression. Similar to the hippocampus, the PFC provides negative-feedback on the HPA axis. This region is also rich in GR and MR, making it particularly susceptible to the deleterious effects of glucocorticoids (Herman et al., 2003; Herman et al., 2005). It is not surprising, then, that PFC volume is reduced in animals exposed to chronic stress and glucocorticoid treatment, reminiscent of what is found in clinical populations (Cerqueira et al., 2005; Cerqueira et al., 2007; Seib & Wellman, 2003; Wellman, 2001). Importantly, this reduction is associated with impairments on working memory

and behavioral flexibility tasks, which are reliant on proper PFC function (Cerqueira et al., 2005). Interestingly, decreases in the number of reelin+ cells and reelin mRNA in layer I of the PFC have been found in both schizophrenic and bipolar disorder patients, but not in unipolar depressed patients (Guidotti et al., 2000a). However, there is no current published data that examines how chronic stress influences reelin expression in the adult PFC or how this relates to depressive-like behavior. Interestingly, it has recently been demonstrated that antidepressants can increase reelin mRNA as well as Dab1 and VLDLR protein expression in the rat frontal cortex (Fatemi et al., 2009). These data suggest that targeting the reelin signaling cascade in this region may be important for antidepressant efficacy. However, we still do not know how altering reelin signaling in the PFC, whether it be increasing or decreasing its expression, influences depression-like behavior. Therefore, a more direct examination of how stress influences reelin expression in the PFC, and how changes in reelin signaling in this region relate to depressive symptomatology is needed.

7.3 How do Antidepressants Influence Reelin Signaling in the Hippocampus?

Although clinical and preclinical evidence suggests that reelin signaling is disrupted in depression (Fatemi et al., 2000; Lussier et al., 2009), little is known about how medications currently used to treat this disorder influence reelin signaling in the adult brain. Most of what we do know about this topic comes from a study conducted by Fatemi and colleagues (2009) examining the influence of psychotropic medications on the expression of reelin and its downstream targets in the frontal cortex. Specifically, this study showed that chronic treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine enhances reelin mRNA, as well as protein levels of Dab1 and the VLDLR receptor in the frontal cortex of rats (Fatemi et al., 2009). These data suggest that antidepressants can influence the expression of reelin and downstream target proteins important for synaptic plasticity in brain areas known to function abnormally in depression. However, only one antidepressant was examined in this study, and so, we do not know if other classes of antidepressants that work on different receptors and neurotransmitter systems, influence reelin signaling in the same way. Interestingly, Fatemi and colleagues (2009) found that different classes of antipsychotic medications (i.e., typical and atypical) target different parts of the reelin signaling cascade, and not all of these medications enhance reelin signaling in the frontal cortex. These data suggest the possibility that different

classes of antidepressant drugs could regulate reelin differently depending on their mechanism of action. In addition, we know little about how antidepressants influence reelin signaling in the hippocampus. Results of chapter 2 showed that imipramine can prevent CORT-induced downregulation of reelin+ cells in the proliferative zone of this brain region. Given that CORT does not result in a loss of reelin-expressing interneurons in the hippocampus (see chapter 3), these data suggest that imipramine prevents the disruption in synthesis and expression of reelin in these neurons. Recent work has also shown that chronic treatment with the SSRI citalopram increases total mRNA levels of reelin in the hippocampus (Jaako et al., 2011), indicating that antidepressants can also enhance reelin signaling in this region. However, from these data we cannot determine whether antidepressants can enhance intracellular levels of reelin in the hippocampus. One way to examine this directly is through laser-assisted microdissection. With this method it is possible to extract reelin-expressing interneurons in the SGZ and hilus after chronic antidepressant treatment and measure changes in reelin mRNA. It would also be of interest to employ in-situ hybridization techniques to examine changes in reelin mRNA in a number of sub-fields and laminae of the hippocampus to identify region-specific alterations in reelin after antidepressant treatment. Ultimately, further research is needed with different classes of antidepressants to elucidate the importance of reelin in the therapeutic actions of these medications.

7.4 Do Chronic Stress and Antidepressants Regulate Reelin via Epigenetic Mechanisms?

Our laboratory has reliably shown that repeated CORT administration significantly reduces reelin expression in the hippocampal DG (Lussier et al., 2009; Lussier et al., 2011), and that antidepressant treatment can block this effect (See Chapter 2). However, we still do not know the mechanism by which this downregulation occurs, or how antidepressants increase reelin expression in the brain. As discussed previously, methylation of the reelin gene promoter is suggested to be a primary mechanism by which reelin protein is regulated in GABAergic cells in the brain (Guidotti et al., 2011). Specifically, the reelin gene promoter was shown to be hypermethylated in post-mortem tissue from schizophrenic patients, and this hypermethylation was associated with decreased reelin expression in these patients (Grayson et al., 2005; Guidotti et al., 2000b); similar results have been found in animal models of schizophrenia (Tremolizzo et al., 2002). In chapter 4 I examined the expression of methylation-associated markers in the

hippocampus of CORT-treated rats and found a significant increase in MeCP2+ neurons in the subgranular region of the DG where reelin deficits are most pronounced after CORT treatment. Unfortunately, from this study I could not conclude that the increase in MeCP2 was occurring in reelin-expressing interneurons, nor could I determine if the increase in MeCP2 was associated with greater binding of this protein to the promoter region of the reelin gene. Although this study provides preliminary evidence that glucocorticoids may influence methylation in the hippocampus, further research is needed to determine how they regulate the methylation status of the reelin gene promoter, and whether antidepressants act via this mechanism to enhance reelin expression in the brain. In particular, methylated DNA immunoprecipitation should be done to determine the ratio of 5'methylated cytosines (i.e., methylated DNA) to unmethylated cytosines of the CpG-enriched promoter region of the reelin gene in animals exposed to CORT and antidepressant treatment. A chromatin immunoprecipitation (ChIP) assay of both DNMT1 and MeCP2 would also identify whether these proteins are actually binding to the reelin promoter in stressed animals, and whether antidepressants can reverse or block this effect. Finally, double immunofluorescent labeling of DNMT1 and MeCP2 with reelin and other interneuron markers that co-localize with reelin should be conducted to see whether these methylation-associated markers are overexpressed in GABAergic interneurons in the hippocampus of CORT-treated animals. Together these data would help identify whether glucocorticoids facilitate DNA methylation, and subsequent hypermethylation of the reelin gene promoter in the hippocampus. This knowledge could then be used to examine whether epigenetic regulation of reelin influences depressive-like behavior, and target potential mechanisms of DNA methylation in treating depressive illness.

Another epigenetic mechanism that might influence reelin expression in the hippocampus is miRNA. As previously discussed, a number of miRNA's (i.e., miR-107, 128, and 15) are currently known to target reelin directly, and ultimately function to repress the synthesis and expression of this protein (Beveridge et al., 2010; Evangelisti et al., 2009). However, there is currently no published data examining how the miRNA's that target reelin are influenced in depressed patients, or how chronic stress influences these miRNA's. In the context of the CORT model, it would be interesting to see whether this stress hormone increases these miRNA's in the whole hippocampus, and/or in sub-regions where reelin is known to be decreased. Moreover, it would be interesting to see whether manipulating miRNA levels in the hippocampus of naïve rats

would have an influence on depressive-like behaviors. Although this area of research is still quite new, it holds great potential for identifying new molecular targets that could be exploited to develop alternative treatments for neuropsychiatric disorders.

8. Conclusions

The goal of this dissertation was to provide an examination of the behavioral and neurobiological effects of prolonged glucocorticoid treatment, and more specifically, to further substantiate a role for reelin in the pathophysiology of major depression. In this dissertation, evidence was provided that glucocorticoids significantly reduce reelin expression in the neurogenic zone of the hippocampus, and that this effect is prevented by antidepressant treatment. Importantly, changes in reelin expression paralleled alterations in dentate granule cell maturation, and depressive-like behavior, suggesting a relationship between depressive symptomatology and disrupted reelin signaling. By examining the influence of CORT on GABAergic interneuron markers that co-localize with reelin in the hippocampus, I was able to determine that repeated CORT administration does not cause these interneurons to die. These data suggest that glucocorticoids exploit an intracellular mechanism to downregulate the synthesis and expression of reelin protein in GABAergic cells. Finally, I showed that CORT increases methyl CpG-binding protein MeCP2 in the same hippocampal region where reelin is most significantly reduced, suggesting the possibility that CORT-induced changes in methylation could disrupt reelin signaling. Although I cannot make any definitive claims of this from this study, my results do provide preliminary evidence that glucocorticoids may influence the methylation status of neurons in the hippocampus. These important observations provide a starting point for understanding how glucocorticoids alter reelin signaling in the hippocampus and how this might relate to depressive symptomatology. Because reelin cannot cross the blood brain barrier, there are major obstacles to using this protein as a treatment in clinical populations. Further examination of the mechanisms that influence reelin signaling directly may therefore help identify novel targets that lead to more effective treatments or interventions that limit the deleterious neurobiological and behavioral consequences of chronic stress and depression.

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