Fear Learning as a Component of a Depressive Phenotype in Rodents

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ABSTRACT

Depression is a complex psychiatric illness that affects a large proportion of the population. Many researchers make use of preclinical animal models to study the behavioural and neurobiological characteristics of this disease. However, although a bias towards maladaptive thinking patterns and emotional responses is a cardinal symptom of depression, these symptoms have been rarely considered in preclinical models. One way to investigate maladaptive thinking is through the use of fear conditioning paradigms. Fear conditioning evaluates emotional memory by assessing a rodent's ability to associate neutral cues with an aversive experience. It requires the activation of brain structures critically involved in emotion-related learning and memory processes, most notably the hippocampus and amygdala, to successfully learn the task. The primary goal of this dissertation was to gain a better understanding of the consequences of repeated corticosterone injections—a validated preclinical model of depression-- on emotionally driven behaviour, the involvement of the hippocampus and amygdala in mediating these behaviours, and whether the antidepressant, fluoxetine, can prevent the effects of corticosterone on these behaviours. To begin, in Chapter 2 I confirmed that the depressogenic effects of corticosterone in the forced swim test, which is a traditional behavioural assay for depression in rodents, are not due to procedural differences or non-specific motor effects. I then investigated the impact of repeated corticosterone injections on the learning and memory of delay and contextual fear conditioning. I examined whether altering the order in which rats recall context versus tone cued fear associations determines the magnitude of corticosterone's effect on conditioned fear. I found that corticosterone dose-dependently increased freezing to contextual cues whereas freezing to tone cues was increased regardless of dose. Furthermore, the order of the presentation of context versus tone cues during recall determined whether corticosterone produced significant enhancements in freezing. In Chapter 4, I investigated whether neuronal activity in the hippocampus and amygdala after recall of contextual or tone cued fear was associated with the effects of corticosterone found in

Chapter 3. Recall of contextual cues was associated with neuronal activity in specific sub regions of the amygdala without any observed changes in the hippocampus. In Chapter 5, I investigated whether repeated corticosterone injections would also enhance the learning and memory of trace fear conditioning, a task that is heavily reliant on the hippocampus. I found that corticosterone increased freezing during recall of trace cues and enhanced the acquisition of trace cues. The results from this chapter, taken together with the results from chapters 3 and 4, suggest that repeated corticosterone exposure readily enhances learning and memory processes that evoke emotional arousal. In Chapter 6, I asked whether repeated treatment with the antidepressant, fluoxetine, could prevent increased fear learning produced by repeated corticosterone injections. I found that fluoxetine decreased freezing behaviour in corticosterone rats during recall of tone cues. Overall, the results of this dissertation further our understanding of the effects of corticosterone on learning and memory tasks that evoke emotional arousal, support the use of fear conditioning as a measure of depression-like behaviour, and demonstrate that repeated corticosterone injections reliably produce a depressive phenotype in rats.

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DEDICATION

I would like to dedicate this dissertation to all of my family and friends who have supported me throughout this journey, and to those I have lost along the way. Each of you has inspired me in unique ways that continue to guide me through life's adventures.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropin hormone
AMPAR	amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptor
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CA	Cornu Ammonis
CORT	corticosterone
CRH	corticotrophin-releasing hormone
FST	forced swim test
GR	glucocorticoid receptor
HPA	hypothalamic pituitary adrenal
LA	lateral amygdala
MR	mineralcorticoid receptor
NMDAR	N-Methyl-D-aspartic acid receptor
PBS	phosphate buffered saline

Chapter 1

General Introduction

1. Outline of Thesis

Depression is one of the most prevalent disorders in Canada with a lifetime prevalence rate of approximately 11% (Patten et al., 2009). This disorder is characterized by a number of heterogeneous symptoms including: depressed mood as indicated by sad or empty feelings, irritability, low self esteem, hopelessness, decreased ability to concentrate, change in appetite and weight loss or gain, sleep disturbances, fatigue or increased agitation, anhedonia, and suicidal ideation (Nestler et al., 2002a). The societal burden associated with this disorder is severe and is ranked by the World Health Organization (WHO) as the 4th leading cause of disability across the globe (Murray & Lopez, 1996). Given the prevalence and societal impact of this disorder, it is imperative that research focuses on understanding the etiology of this disorder.

Altered emotional responses to everyday life events appear to be ubiquitous to the pathology of depression. Strong evidence suggests that stress and more specifically the resultant increase in cortisol contributes directly to the development of pathological emotional processing associated with depression. For instance, cortisol treatment in non-depressed individuals alters the processing of emotionally driven tasks (Stark et al., 2006; Tops et al., 2003). Moreover, approximately half of all individuals with depression show abnormally high circulating levels of cortisol and altered cortisol rhythms (Sachar & Baron, 1979). Furthermore, excessive hypothalamic-pituitary-adrenal (HPA) axis activation observed in this subset of depressed individuals is often corrected by treatment with antidepressants (Arborelius, Owens, Plotsky, & Nemeroff, 1999; Holsboer, 2001). Despite the pervasiveness of these changes, the underlying etiology of the altered emotional processing produced by excessive exposure to stress hormones is poorly understood.

This dissertation comprises a collection of studies that systematically investigates the effects of repeated exposure to the stress hormone corticosterone (CORT) on learned

helplessness behaviours, learning and memory recall of tasks that evoke negative emotional arousal, and whether the antidepressant, fluoxetine, reverses some of the CORT-induced effects observed on fear conditioning performance. The first experiment examined how repeated CORT affects behaviour in two versions of a commonly used measure of depression-like behaviour in rodents. The second experiment examined the effects of repeated CORT administration on behaviour in a delay fear conditioning task, a measure of emotionally driven learning and memory. The third experiment examined Fos immunoreactivity in the hippocampus and amygdala after recall of fear memories following repeated CORT administration. The fourth experiment examined the effects of repeated CORT administration on acquisition and recall of trace fear conditioning. And finally, the fifth experiment examined whether the antidepressant, fluoxetine, was able to prevent the effects of repeated CORT administration on delay fear-conditioned behaviour.

The remaining sections of this chapter provide a brief introduction to the hypothalamic-pituitary-adrenal (HPA) axis and how it regulates the response to stress. I then relate altered HPA axis activity to the pathology associated with depression. Following this, I discuss how animal models provide a necessary means for understanding the underlying etiology and neuropathology of depression. I then provide an overview of fear conditioning and how this task can be used to further our understanding of the changes in emotionally driven processing produced by repeated glucocorticoid exposure. Then I include a short discussion of current knowledge about the effects of stress and glucocorticoids on fear conditioned behaviour. This chapter concludes with a specific set of questions that remain unanswered in regards to the characterization of depression-like behaviours in a repeated CORT model of depression.

2. The HPA axis and Stress

Actual or perceived threats (i.e., stressors) trigger activation of the HPA axis. This response initiates a cascade of secretagogues that result in the eventual release of

adrenocortical steroids that are essential to an organism's survival. This cascade begins in the central nervous system where neural impulses carrying information about the stressor culminate in the parvocellular component of the paraventricular nucleus of the hypothalamus. These cells synthesize corticotrophin-releasing hormone (CRH) and release them into the hypophysial-portal circulation where they are carried to the anterior pituitary gland. CRH in the pituitary stimulates the synthesis and release of adrenocorticotrophin hormone (ACTH), which targets the adrenal cortex and stimulates the synthesis and release of glucocorticoids (i.e., cortisol in humans, corticosterone (CORT) in rodents). Glucocorticoids have both metabolic and behavioural effects that help the organism cope with the stressor and equilibrate to pre-stress levels of functioning (Checkley, 1996; Nestler et al., 2002a; Plotsky, Owens, & Nemeroff, 1998). Although acute HPA axis activation is beneficial, chronic HPA axis activation is associated with a number of disorders including sleep deprivation, immunosuppression, memory impairments, decreased sexual behaviour, and chronic dysphoria (Lau et al., 2012; McEwen, 1998; McEwen, 2006).

The HPA axis is self-regulating and dependent on feedback from two types of receptors that mediate the actions of corticosteroids, glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (McEwen, De Kloet, & Rostene, 1986). MR bind to CORT at a higher affinity indicating that low basal corticosteroid levels occupy MR whereas higher levels (i.e., during stress) occupy GR (De Kloet & Reul, 1987; Reul & De Kloet, 1985). The brain is unevenly distributed with GR with the amygdala and hippocampus being particularly abundant in GR expression (Feldman & Weidenfeld, 1999; Morimoto, Morita, Ozawa, Yokoyama, & Kawata, 1996). Thus, the hippocampus and amygdala are situated to perform an important role in regulating HPA axis activity. Indeed, multiple lines of research suggest that the hippocampus and amygdala are highly involved in HPA axis regulation. For instance, in humans electrical stimulation of the hippocampus or amygdala decreased or increased plasma CORT levels respectively (Mandell, Chapman, Rand, & Walter, 1963). Hippocampal stimulation in rats was

also shown to reduce CORT concentration induced by acute stress (Dupont, Bastarache, Endroczi, & Fortier, 1972). Furthermore, in rats with a dorsal hippocampectomy, basal levels of serum CORT are increased (Magarinos, Somoza, & De Nicola, 1987). Thus, multiple lines of evidence suggest that under normal physiological conditions, the hippocampus inhibits HPA activity, whereas the amygdala has an excitatory influence (Jacobson & Sapolsky, 1991; Nestler et al., 2002a). Figure 1-1 provides a schematic representation of the HPA axis response. Although glucocorticoids are essential for the survival of an organism, excessive glucocorticoid release associated with repeated stress can have deleterious consequences. Repeated exposure to stress or glucocorticoids is associated with pathological plasticity in the hippocampus in a number of ways. Rats subjected to repeated restraint stress or repeated CORT treatment have suppressed hippocampal neurogenesis (Leuner & Gould, 2010; Mayer et al., 2006). Furthermore, the CA3 area of the hippocampus seems to be particularly vulnerable as a reduction in CA3 volume is found following repeated stress with evidence that glial processes are increased (Czeh et al., 2001; Sheline, Gado, & Kraemer, 2003; Tata & Anderson, 2010). Regression of apical dendrites of CA3 pyramidal neurons occurs after three weeks of restraint stress or high dose CORT (Magarinos & McEwen, 1995; Watanabe, Gould, & McEwen, 1992; Woolley, Gould, & McEwen, 1990). Changes in CA1 and dentate are also reported (Karst, Wadman, & Joels, 1994; Lambert et al., 1998; Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000). In addition, aberrant synaptic plasticity also occurs with a loss of mossy fiber synapses following repeated restraint or glucocorticoid exposure (Sousa et al., 2000; Tata, Marciano, & Anderson, 2006). Moreover, increases in calcium (Ca2+) influx are found in CA1 neurons along with enhanced mRNA for Ca2+ channel subunits (Karst et al., 1994; Nair et al., 1998). In contrast, repeated stress leads to enhanced morphology in the amygdala, an area essential for the processing of emotional information (Pape & Pare, 2010). For example, repeated immobilization stress enhances dendritic arborization and spine density in basolateral

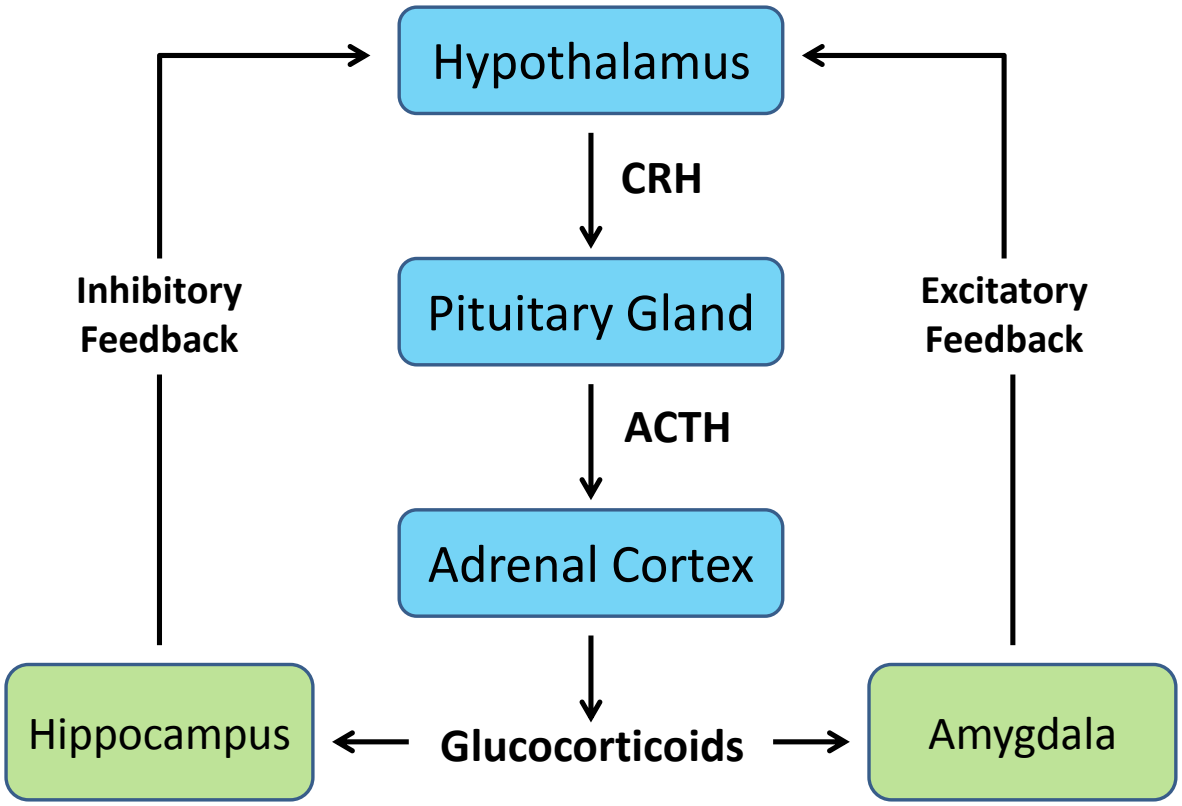

Figure 1-1. Schematic representation of HPA axis regulation. In response to stress the hypothalamus synthesizes and releases corticotrophin-releasing hormone (CRH) into circulation. Cells in the pituitary gland respond to CRH and synthesize and release adrenocorticotrophin hormone (ACTH). ACTH is then detected by cells in the adrenal cortex. In response, the adrenal cortex then synthesizes and releases glucocorticoids into circulation. Glucocorticoids have both metabolic and behavioural affects that help the organism cope with the stressor and return to pre-stress levels of functioning. Glucocorticoid receptor binding in the hippocampus inhibits HPA activity, whereas binding in the amygdala excites HPA activity.

amygdala (BLA) pyramidal neurons in rats (Vyas, Mitra, Shankaranarayana Rao, & Chattarji, 2002; Vyas, Jadhav, & Chattarji, 2006). Pathological morphology and synaptic plasticity in the hippocampus and amygdala resulting from repeated stress and excessive glucocorticoid exposure can lead to maladaptive HPA axis activity. Damage resulting from repeated stress can decrease the inhibitory feedback that the hippocampus has on HPA axis activity, the net effect being a higher than normal increase in circulating glucocorticoids and increased hippocampal and amygdalar damage (Nestler et al., 2002a).

3. The Relationship between Stress and Depression

It is well established that repeated stress and the resultant increase in HPA axis functioning is associated with the development of depression. For instance, major life stressors such as unemployment and divorce are linked to an increased risk of depression (Kessing, Agerbo, & Mortensen, 2003; McEwen, 1998). Also, approximately half of all individuals with depression show hypersecretion of cortisol and altered cortisol rhythms (Sachar & Baron, 1979). Importantly, the excessive HPA activation observed in this subset of depressed individuals is corrected by successful treatment with antidepressants (Arborelius et al., 1999; Holsboer, 2001). Also, more than half of all individuals with Cushing's disease, a syndrome resulting from chronically high circulating levels of glucocorticoids, are also diagnosed with depression (Sonino & Fava, 2002). Research also suggests that healthy individuals at genetic risk for depression already present with mild hypercortisolism and HPA feedback disturbances (Holsboer, Lauer, Schreiber, & Krieg, 1995) suggesting a potential predisposed sensitivity to the effects of stress. Therefore, multiple lines of evidence suggest that chronically high levels of glucocorticoids play a critical role in the development, maintenance, and treatment of depression.

The symptomatology of depression implicates the dysregulation of neural circuits that are involved in emotion-associated processes and stress response. Indeed, grey matter reductions are reported in the anterior cingulated cortex, prefrontal cortex, as well as in the

hippocampus and amygdala (Bora, Fornito, Pantelis, & Yucel, 2012; Du et al., 2012). As well, in patients with depression, the amygdala shows enhanced blood flow in response to sad words (Siegle, Steinhauer, Thase, Stenger, & Carter, 2002) and sad faces (Fu et al., 2004). Structural magnetic resonance imaging revealed that electroconvulsive therapy increased hippocampal and amygdalar volumes in pharmacotherapy resistant depressed individuals with no evidence of a change in total brain volume (Tendolkar et al., 2013).

Similar to animals subjected to repeated stress or glucocorticoid treatment, post-mortem analysis of individuals diagnosed with depression show altered brain morphology in specific sites. Decreases in brain volume following fixation have been noticed in the hippocampus implying decreases in neuropil in depression brains compared to control brains (Stockmeier et al., 2004; Price & Drevets, 2010). Comparatively, reductions in glial density and glia/neuron ratio were also reduced in the amygdala in depressed brains (Bowley, Drevets, Ongur, & Price, 2002; Hamidi, Drevets, & Price, 2004). Furthermore, the expression of genes critical to signal transduction and cell communication was decreased in depressed individuals in the middle temporal cortex (Aston, Jiang, & Sokolov, 2005) and CA1 and dentate gyrus (DG) regions of the hippocampus (Duric et al., 2013). Analysis of post-mortem depression brains has also revealed direct changes in GR and MR expression. Significantly lower MR expression was detected in the hippocampus (Klok et al., 2011), whereas GR protein levels as well as GR-containing astrocytes were significantly increased in the amygdala (Wang et al., 2013).

In summary, a number of lines of research implicate repeated stress as a major precipitating factor in the development and maintenance of depression. More specifically, depression is tightly linked to aberrant plasticity in circuits in the brain that both highly express glucocorticoid receptors and are critical to emotion-related processes as well as stress response. The hippocampus and amygdala are especially sensitive to the effects of repeated stress and are targets of depression related pathology. Thus, the hippocampus and amygdala are particularly relevant to research investigating the underlying relationship between stress and

depression. A systematic investigation into the effects of repeated glucocorticoid exposure on depression-related pathology in the hippocampus and amygdala is warranted.

4. Animal Models of Depression

Although research with clinically depressed populations has vastly increased our understanding of the neural correlates and etiology of this disorder, there are a number of issues that are difficult to study in human patients. For instance, although the literature reviewed above describes a clear link between HPA axis dysregulation and depression, it is also evident that not all individuals with HPA axis disorders develop depression and not all depressed individuals display symptoms of a malfunctioning HPA axis. Therefore, it is difficult to determine, based on analysis of depressed individuals alone, how plasticity associated with chronic stress can lead to the eventual neuropathology seen in post-mortem tissue. Furthermore, clinical research is correlational and thus causal conclusions based on clinical research alone cannot be made. Also, post-mortem tissue of depressed individuals can be affected by factors such as whether the subject had received antidepressant treatment as well as pathologies related to possible co-morbid disorders. In light of the problems associated with human research, animal models have been particularly useful for investigating the pathology associated with depression.

Animal models of depression have the potential to further elucidate the underlying etiology of this disease. However, producing a viable animal model of depression is met with a number of challenges. The first challenge faced stems from the fact that a depressive phenotype is characterized by a range of behavioural symptoms, which likely represent several different types of pathology (Neumann et al., 2011). Thus it is difficult to produce a model that will resemble all the symptoms and pathology associated with depression. Also, some symptoms of depression, such as suicidal ideation, cannot be measured in animals. Despite these inherent downfalls, there are three basic criteria that a satisfactory animal model of depression should fulfill: predictive, face, and construct validity. Predictive validity states that

when a treatment has a known effect on human pathology, either beneficial or negative, the treatment should have a similar effect in the animal model, e.g., antidepressant treatment should produce a similar outcome in the animal model as found in humans. Face validity refers to the similarity between the symptoms presented in the human condition and the behavioural phenotype produced by the animal model. And finally, construct validity stipulates that both the animal model and human disease state are the products of similar underlying neuropathology. With construct validity fulfilled, theories can be developed regarding the etiology and potential progression of a given disorder (Neumann et al., 2011).

Several valid repeated stress models of depression have been developed and include chronic mild stress, social stress, early life stress, repeated restraint stress, and glucocorticoid exposure. In these models, aside from glucocorticoid exposure, the experimenter applies either one or several different forms of environmental stressors over a prolonged period of time to produce a depressive phenotype. In chronic mild stress models, rats are exposed to a variety of mild stressors (e.g., food/water deprivation, 24 hr light cycle, cage tilt) consecutively over a period of several weeks or months (Willner, 1997). Social stress or social defeat refers to exposing an animal to an aggressive and dominant animal either directly or indirectly. An advantage of chronic mild stress and social stress models is that they arguably produce more naturalistic stress than other models (Nestler et al., 2002b). Early life stress involves manipulating an animal's early life environment. These manipulations can include postnatal handling, maternal separation, prenatal stress, or a combination of these manipulations. Repeated restraint stress is one of the most commonly used models of depression (Kim & Han, 2006) and is one of the easiest models to administer. This model involves restraining an animal for a set duration (ranging from minutes to hours/day) repeatedly over several days to weeks. Finally, repeated glucocorticoid exposure models involve administration of glucocorticoids through either pellet implantation, infusions, injections, or through dissolving CORT in drinking water. Repeated CORT injections provide a slight advantage over other methods of

administration as complications can arise from the surgery required for infusion and pellet implantation. In addition, individual differences in motivation to consume water across subjects can create variability in the amount of glucocorticoid exposure within a treatment group.

Investigators invariably have unique reasons for potentially choosing one model over another; however, many experimenter-applied stress models do not produce reliable changes in depression-like behaviour. For example, repeated restraint stress produces variable results on measures of anxiety (Gregus, Wintink, Davis, & Kalynchuk, 2005; Perrot-Sinal, Gregus, Boudreau, & Kalynchuk, 2004) and few increases in depression-like behaviours (Gregus et al., 2005; Perrot-Sinal et al., 2004; Lussier, Caruncho, & Kalynchuk, 2009; Bravo et al., 2009; Dunn & Swiergiel, 2008). It is thought that variability in behaviour produced by experimenter-applied stress models is the result of individual differences in HPA axis responses and potential habituation effects (Gregus et al., 2005; Grissom & Bhatnagar, 2009). Repeated glucocorticoid exposure models simulate exposure to stressful stimuli and confounds associated with habituation effects and individual variability are avoided by ensuring that each animal is exposed to the same amount of glucocorticoid. In light of the drawbacks associated with other models, repeated glucocorticoid exposure offers an ideal way to specifically investigate the effects of glucocorticoids on neuroplasticity and behaviour associated with depression. For these reasons, this dissertation is focused on systematically investigating the effects of repeated glucocorticoid exposure on depression-like behaviour and related brain plasticity.

A number of behavioural tests have been developed that are thought to recapitulate the core symptoms of depression. These measures are used to document the occurrence of depression-like behaviours produced by animal models and thus reinforce the face validity of a given model. The most widely used measure of depression-like behaviour is the forced swim test (FST) (Nestler et al., 2002b). In the FST, rats are forced to swim within an inescapable tank of water. The FST produces a “state of despair” in the rat in which the organism realizes that escape is impossible which renders the rat immobile within the swim tank (Porsolt, Le, & Jalfre,

1977; Porsolt, Anton, Blavet, & Jalfre, 1978). The amount of time spent immobile and latency to immobility are thought to be indices of "learned helplessness" behaviour (Nestler et al., 2002b). Numerous repeated CORT exposure paradigms produce significant increases in depression-like behaviour in the FST. For instance, immobility behaviour is significantly increased following repeated CORT administration via drinking water (David et al., 2009; Gourley, Kiraly, Howell, Olausson, & Taylor, 2008), or subcutaneous pellet implant (Murray, Smith, & Hutson, 2008). A dose dependent increase is also observed following repeated CORT injections for 21 days or more with 40 mg/kg producing consistent increases in immobility behaviour whereas doses of 20 mg/kg and lower produce non-significant increases (Brummelte, Pawluski, & Galea, 2006; Gregus et al., 2005; Kalynchuk, Gregus, Boudreau, & Perrot-Sinal, 2004; Wu et al., 2013). Furthermore, acute injections of CORT do not significantly alter FST behaviour suggesting that chronic exposure is required to produce enhanced depression-like behaviours (Johnson, Fournier, & Kalynchuk, 2006). It is well established that antidepressant treatment decreases immobility behaviour in the FST as this test was originally designed to both resemble depressive illness and selectively respond to antidepressants (Porsolt et al., 1977; Porsolt et al., 1978). Importantly, treatment with the antidepressant, imipramine, also prevents repeated CORT induced increases in immobility behaviour in the FST (Fenton, Fournier, Lussier, Caruncho, & Kalynchuk, 2014), thus providing predictive validity to the repeated CORT exposure model.

It should be noted that exposure to repeated CORT injections decreases body weight gain (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004). This decrease in weight gain is likely due to increased lipolysis and decreased protein synthesis, which, then breaks down muscle into amino acids (Tomas, Munro, & Young, 1979). The net effect of these actions over a period of time is muscle atrophy and weight loss. Therefore it is possible that the effects of CORT observed in the FST are the result of a decrease in muscle strength or non-specific locomotor effects related to ability to maintain active swimming behaviours. Further research investigating the effects of repeated high dose CORT injections on locomotor activity and

muscle strength would help clarify the effects of repeated CORT exposure on behavioural measures, such as the FST, that rely on retained physical aptitude.

Another behavioural measure of learned helplessness involves inescapable shock. In this test, an animal that is repeatedly exposed to a shock and cannot escape will subsequently stop trying to escape when it becomes possible to do so (Nestler et al., 2002a; Willner, 1984). Tail suspension is another test of learned helplessness behaviour where the amount of time an animal spends struggling when suspended by its tail is measured (Nestler et al., 2002a). Although, repeated CORT injections at 20 mg/kg increase immobility behaviour during tail suspension tests (Zhao et al., 2008), future research should determine whether repeated CORT injections in rats increase depression-like behaviours in other learned helplessness paradigms.

Depression-like behaviour can also be demonstrated through natural reward-based tests such as sucrose preference and sexual behaviour. Repeated CORT exposure decreases preference for sucrose consumption (David et al., 2009; Gorzalka, Hanson, Harrington, Killam, & Campbell-Meiklejohn, 2003; Gourley et al., 2008; Wu et al., 2013) as well as food reward responses (Gourley et al., 2008; Gourley et al., 2008). Sexual behaviour is also reduced in males following repeated CORT injections (Gorzalka & Hanson, 1998; Gorzalka, Brotto, & Hong, 1999; Gorzalka, Hanson, & Hong, 2001; Lau et al., 2012). Thus, repeated CORT treatment not only increases helplessness behaviours, but this model also decreases preference for natural rewards, which is indicative of increased anhedonia.

Individuals diagnosed with depression often show cognitive impairments such as impaired recall of autobiographical information (Crane, Barnhofer, Mark, & Williams, 2007) and working memory deficits (Elliott et al., 1996). Similarly, rats repeatedly exposed to high levels of glucocorticoids also show memory deficits specifically on tasks of working memory. This is reflected by deficits in latency to acquire a Morris Water Maze task (Bodnoff et al., 1995) and acquisition of food reward in the radial arm maze (Arbel, Kadar, Silbermann, & Levy, 1994).

In summary, rodents repeatedly exposed to glucocorticoids display a number of behaviours reminiscent of depression including helplessness behaviour, anhedonia, weight fluctuations, and deficits on cognitive tasks involving working memory. Also, antidepressants known to reduce the severity of depression in humans also prevent depression-like behaviours in this animal model. Furthermore, as summarized above, repeated glucocorticoid exposure also produces neuroplasticity that is associated with brain abnormalities observed in depressed individuals in both living and post-mortem tissue. The literature clearly demonstrates that the repeated glucocorticoid exposure model of depression reliably demonstrates all three forms of validity required for a satisfactory model of human pathology. Therefore this model provides a foundation for theory regarding the etiology of this disorder to be developed and examined.

5. Fear Conditioning

Measures of depression-like behaviour in rodent models have focused on the dimensions of a depressive phenotype described above (i.e., helplessness, anhedonia, weight changes, and cognitive impairments). However, one dimension of depression that has been largely overlooked in animal models is the bias towards maladaptive thinking patterns and emotional responses. Numerous lines of research demonstrate that depressed individuals are hyper responsive to negative emotional information. Depressed individuals are more likely to attend to, memorize, and recall information with a negative emotional connotation (Leppanen, 2006). Furthermore, depressed individuals are more likely to label neutral faces with negative emotions (Rubinow & Post, 1992). Depressed individuals are also more likely to ruminate over their negative emotions and experiences. Not only is rumination directly associated with the severity of depressive symptoms, but it also predicts the onset of depressive episodes (Nolen-Hoeksema, 2000). Changes in brain activity are associated with these behavioural abnormalities. Individuals with depression show increased activity in response to sad faces and decreased activity in response to happy faces in the amygdala compared to controls.

Importantly, these emotional processing abnormalities continue into symptom remission and are also shown in individuals at risk for developing the disorder (Grillon et al., 2005; Leppanen, Milders, Bell, Terriere, & Hietanen, 2004; Leppanen, 2006). Research on non-depressed males shows that cortisol administration produces a deficit in memory of neutral and pleasant words (Tops et al., 2003) suggesting that the negative emotional bias seen in depressed individuals is linked to increased circulating glucocorticoids. Therefore it is possible that glucocorticoids place individuals at risk for developing maladaptive thinking patterns and a hyper-responsive memory of information that carries a negative emotional connotation. Unfortunately, the biological bases for these deleterious cognitive processes are poorly understood. Repeated glucocorticoid exposure models may be particularly fruitful in understanding the etiology and underlying mechanism of these particular symptoms of depression.

Pavlovian fear conditioning offers a congruent model for studying changes in memory of negative emotional experiences. Fear conditioning assesses the ability to associate neutral cues, such as a tone or environmental (contextual) cues, with an aversive experience such as a mild foot shock. After a delay, the rodent is presented with the same cues used during training, however the shock is absent, and freezing, which is a rodent's natural defensive behaviour (Blanchard & Blanchard, 1969), is measured. Successful fear learning and memory involves several distinct phases. Acquisition refers to the initial learning of the fear conditioning task. Consolidation refers to the long-term storage of learned fear associations. Recall of fear memory involves testing the conditioned-unconditioned stimulus association when the conditioned stimulus is presented alone (Rodrigues, LeDoux, & Sapolsky, 2009). Expression of fear is also distinct from the other phases of learning in that it refers to the actual production of fear behaviour. Extinction of fear refers to the reduction of a previously acquired fear response resulting from non-reinforced presentations of the conditioned stimulus (Herry et al., 2010). Each phase of memory formation can be affected by experimental manipulations such as lesions, pharmacological interventions, and other procedures that can assess the processes of

emotional memory. For example, if a drug is administered immediately prior to the training phase and impairs short-term and long-term memory, then this drug disrupts the acquisition of fear memory. Alternatively, if training has already occurred and a drug is given immediately afterwards and impairs long-term memory, then this drug disrupts consolidation. If the task has already been acquired and consolidated and a drug is given immediately before testing, the drug can affect expression or recall of fear memory. Extinction of fear memory also includes acquisition, consolidation, expression, and recall phases (Herry et al., 2010; Burghardt & Bauer, 2013). These aspects of fear conditioning provide the opportunity to closely examine exactly which stages in the learning and memory process a particular manipulation, such as stress or glucocorticoid exposure, affects.

Variations of the fear conditioning paradigm have been developed that rely on activation of different brain circuits for successful performance of the task (Phillips & LeDoux, 1992; Raybuck & Lattal, 2011). These modifications allow researchers to assess the functional integrity of different structures, which is another aspect that makes fear conditioning a valuable tool for exploring the processing of emotionally driven memories. Although there are several variations of the fear conditioning task, the three most commonly used are contextual, discrete (cued), and trace conditioning. Contextual fear conditioning, as mentioned above, describes conditioning to the various background stimuli that are present in the conditioning chamber when the unconditioned stimuli occur. Cued conditioning occurs when a discrete cue such as a tone or light is paired or co-terminates with the unconditioned stimulus. Trace conditioning separates the conditioned stimulus, a discrete cue, from the unconditioned stimulus by a temporal gap. Figure 1-2 provides a schematic depiction of these three forms of fear conditioning. Often contextual and cued conditioning are combined in the same task, referred to as delay fear conditioning, allowing researchers to assess how laboratory manipulations influence the memory of tasks dependent on dissociable brain circuits within the same organism.

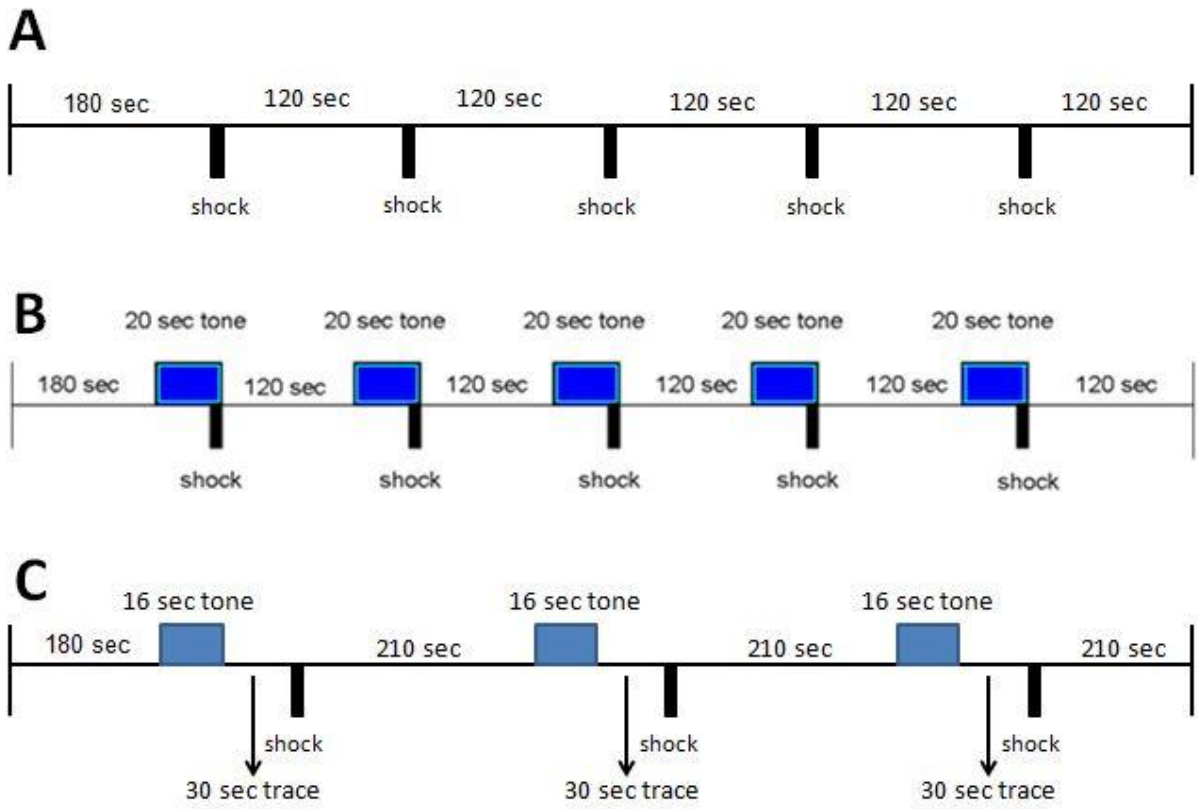

Figure 1-2. Schematic representations of 3 different versions of the fear conditioning task. Panel A shows an example of a contextual fear conditioning training protocol. An animal is placed in a unique environment (context) and learns to associate that context (conditioned stimulus) with noxious foot-shocks (unconditioned stimulus). Panel B shows an example of a delay fear conditioning training protocol. An animal is placed in a unique environment and learns to associate both the context and tone cues with noxious foot-shocks. Tone cues and foot-shocks co-terminate. Panel C shows an example of a trace fear conditioning training protocol. In trace fear conditioning, the tone and shock are separated by a temporal gap.

The key neuronal circuits that underlie contextual and cued fear conditioning (see Figure 1-3) have been elucidated through lesions, electrophysiological studies, pharmacological inactivation and transgenic studies. In particular, the amygdala is well situated to play an integral role in subserving fear conditioning as it has long been implicated in emotional responses (Pape & Pare, 2010). As well, the amygdala receives projections from many brain areas (e.g., hippocampus, thalamus, neocortex) and sends projections to structures that mediate fear responses. Such projected areas include the bed nucleus of the stria terminalis (BNST), which is important for the activation of stress hormones, the periaqueductal gray, which is important for freezing behaviour, and the lateral hypothalamus, which is important for mediating endocrine responses to stress (Kim & Jung, 2006).

Multiple lines of research have implicated subregions of the amygdala in different phases of fear learning and memory. It is widely accepted that the lateral nucleus of the amygdala (LA) is heavily involved in the acquisition of fear associations (Blair, Schafe, Bauer, Rodrigues, & LeDoux, 2001; Maren, 2001; Johansen, Cain, Ostroff, & LeDoux, 2011). Sensory information about the conditioned and unconditioned stimuli, for example auditory information transmitted from the medial geniculate nucleus (Campeau & Davis, 1995; LeDoux, 2000) or somatosensory information transmitted from the thalamic posterior intralaminar nucleus (Shi & Davis, 1999) are projected to neurons in the LA (LeDoux, Sakaguchi, Iwata, & Reis, 1985; LeDoux, Cicchetti, Xagoraris, & Romanski, 1990). As a result, subsequent presentations of the conditioned stimulus without the unconditioned stimulus result in larger responses in LA neurons (Quirk, Repa, & LeDoux, 1995; Repa et al., 2001). Similarly, BLA inactivation with the $GABA_A$ receptor agonist, muscimol, prior to training and retention of contextual and cued fear block acquisition and expression respectively (Helmstetter & Bellgowan, 1994; Muller, Corodimas, Fridel, & LeDoux, 1997). Thus, it is generally believed that the BLA aids in the formation of conditioned fear memory (Kim & Jung, 2006). The central nucleus of the amygdala

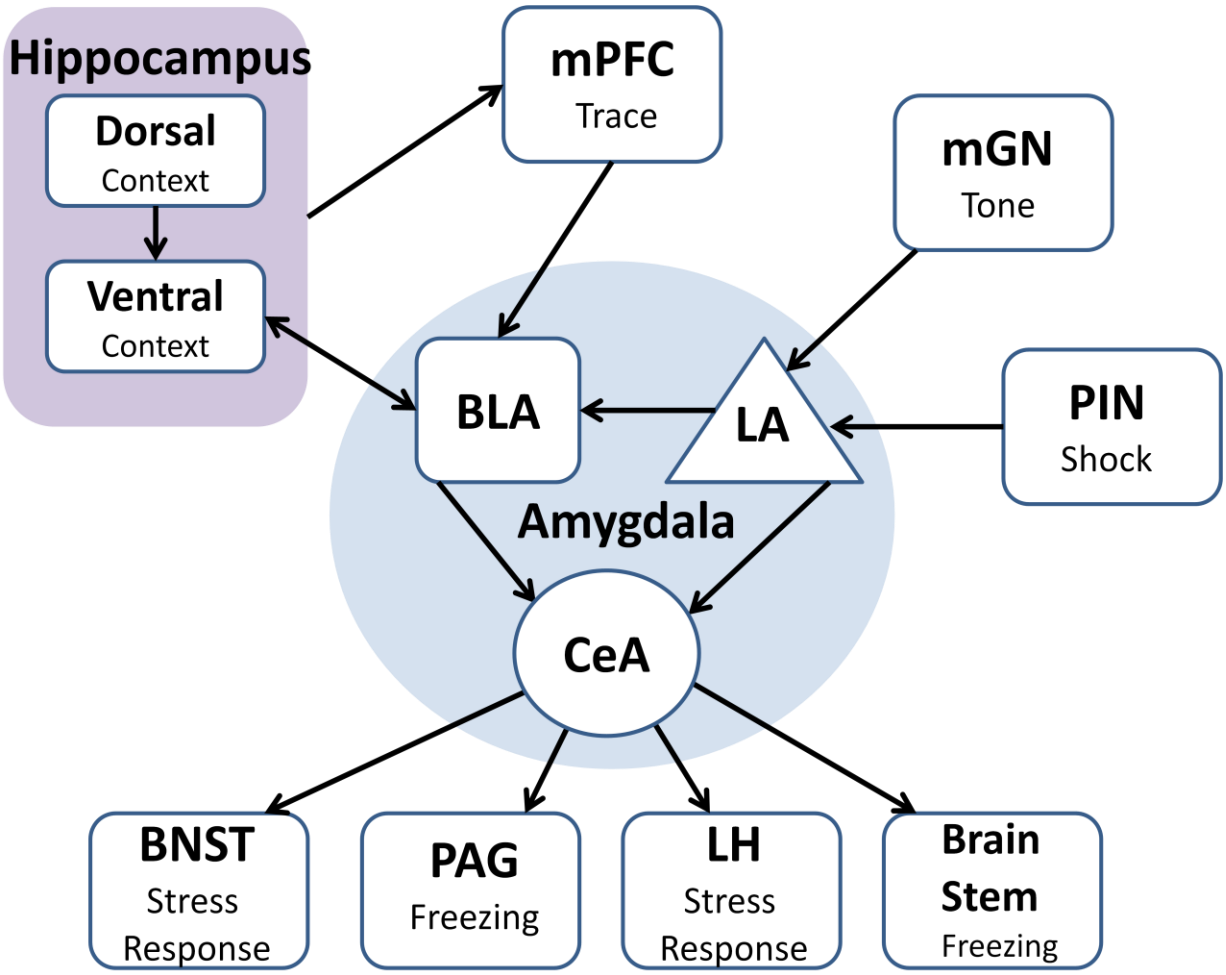

Figure 1-3. Neuroanatomy of fear conditioning circuits. The amygdala can be divided into 3 sub regions. These include the lateral (LA), basolateral (BLA), and central (CeA) nuclei. The LA receives sensory information from a number of sources including the medial geniculate nucleus (mGN) and thalamic posterior intralaminar nucleus (PIN). The BLA also receives and integrates information about the conditioned stimulus and unconditioned stimulus from the ventral hippocampus and medial prefrontal cortex (mPFC). The central nucleus is the primary output of amygdalar activity which results in activation of the lateral hypothalamus (LH), brain stem, periaqueductal gray (PAG), and bed nucleus of the stria terminalis (BNST), areas critical to defensive freezing behaviours and stress response.

(CeA) is the primary output of amygdalar activity. In particular, disinhibition of the medial sub region of the CeA (CeM) results in activation of the hypothalamus and brain stem, areas critical to defensive freezing behaviours and stress response (Francis, Hernandez, & Powell, 1981; Hitchcock, Sananes, & Davis, 1989; Amano, Unal, & Pare, 2010). The lateral sub nucleus of the CeA, (CeL), receives afferents from the BLA, which then are projected to the CeM (Ciocchi et al., 2010). Moreover, fear conditioning results in neuroplasticity of glutamatergic pathways from the LA to CeL neurons which are necessary for fear memory recall (Li et al., 2013). Thus during the conditioned stimulus, both BLA and the CeL neurons disinhibit CeM output (Ciocchi et al., 2010; Li et al., 2013). Functional inactivation methods and inhibition of protein synthesis show that the CeA is not only involved in fear expression and recall, but also acquisition and consolidation (Wilensky, Schafe, Kristensen, & LeDoux, 2006).

Although the amygdala is thought to be necessary for contextual and cued fear associations, other brain regions are critically involved in the fear learning process. The hippocampus plays a key role in associating the contextual cues from the environment of the conditioning chamber with the footshock. As such, lesions of the hippocampus disrupt contextual fear conditioning, but not cued conditioning (Anagnostaras, Maren, & Fanselow, 1999; Maren, Aharonov, & Fanselow, 1997; Phillips & LeDoux, 1992). More specifically, electrolytic lesions of the dorsal hippocampus impair contextual fear conditioning when performed before or after training (Kim, Rison, & Fanselow, 1993; Maren et al., 1997; Phillips & LeDoux, 1992) implying involvement during acquisition of contextual conditioning. However, these results are inconsistent with others showing that the dorsal hippocampus is implicated in the expression of contextual fear as neurotoxic lesions made after training, but not before interrupt freezing behaviour (Maren et al., 1997). The ventral hippocampus is also involved in contextual fear conditioning with pretraining lesions shown to impair contextual conditioning (Richmond et al., 1999). Furthermore, muscimol inactivation of the ventral or dorsal hippocampus shows that this region is required for the formation of a contextual representation

(Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Rudy & Matus-Amat, 2005). Importantly, only the ventral hippocampus projects directly to the amygdala (Pitkanen, Pikkarainen, Nurminen, & Ylinen, 2000). As such, the ventral hippocampus modulates the activity of neurons in the BLA (Pitkanen et al., 2000). Similarly, the BLA is directly connected to the ventral hippocampus and thus can modulate contextual fear conditioning. For instance, muscimol inactivation of the BLA attenuated increased Fos positive cells in the hippocampus following contextual fear training (Huff et al., 2006). The ventral hippocampus can also affect BLA activity through projections to the prelimbic cortex, which then connect with the BLA (McDonald, Mascagni, & Guo, 1996; Vertes, 2006).

Many of the circuits that mediate contextual and cued fear conditioning also mediate trace fear conditioning (see Figure 1-3); however, the temporal discontinuity of trace conditioning requires unique processing. Similar to contextual fear conditioning, hippocampal lesions disrupt trace, but not cued conditioning in rats (McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998a). Furthermore, mutant mice with impaired hippocampal long term potentiation (LTP) display deficits in trace and contextual fear conditioning, but not cued fear conditioning (Bourtchuladze et al., 1994; Huerta, Sun, Wilson, & Tonegawa, 2000). Thus, trace fear conditioning depends on intact hippocampal functioning. More specifically, a number of different research methods (e.g., lesion, pharmacological manipulations, and cell recordings) show that the dorsal hippocampus is particularly important to trace fear (Burman, Starr, & Gewirtz, 2006a; Gilmartin & McEchron, 2005; Huerta et al., 2000; Quinn, Oommen, Morrison, & Fanselow, 2002) although, pre and post-training excitotoxic lesions of the ventral hippocampus are also shown to impair trace conditioning (Yoon & Otto, 2007). The temporal discontinuity of trace fear conditioning requires the mPFC, which is consistent with other tasks that require the processing of temporal gaps between stimuli (Fuster, Bodner, & Kroger, 2000; Runyan, Moore, & Dash, 2004). In general, it is believed that the mPFC and hippocampus preserve information about the tone cue until the shock is delivered (Guimarais, Gregorio, Cruz, Guyon, & Moita,

2011). Unlike cued and contextual fear where multiple lines of evidence show that the amygdala is critical to conditioning, the role of the amygdala in trace fear conditioning is less well understood. In trace fear conditioning, a number of brain areas provide necessary contributions to the formation of learned associations, in particular the hippocampus and mPFC (Raybuck & Lattal, 2013). This produces a more distributed network that may be less reliant on the amygdala. For instance, muscimol inactivation of the amygdala prior to acquisition of trace conditioning provides evidence that trace fear conditioning is amygdala-independent (Raybuck & Lattal, 2011). However, another group of researchers suggest that the amygdala is necessary for the acquisition of trace conditioning (Guimarais et al., 2011). Further work is needed to resolve the discrepancies in the literature focusing on the involvement of the amygdala during trace fear conditioning. In summary, the dissociable circuits of fear conditioning offers the opportunity to examine the effects of experimenter manipulations on specific circuits known to be sensitive to glucocorticoid exposure and aberrant plasticity associated with depression.

6. Fear Conditioning, Stress and Glucocorticoid Exposure

Several lines of research demonstrate that fear conditioning, especially contextual fear, is responsive to alterations in glucocorticoid circulation and receptor binding. Compelling research shows that CORT is required for long-term memory of contextual fear (Pugh, Tremblay, Fleshner, & Rudy, 1997). Adrenalectomized rats showed reduced contextual memory 24 hrs after conditioning, a deficit that was ameliorated by CORT replacement. Alternatively, neither adrenalectomy nor CORT replacement significantly affected tone cued conditioning. Furthermore, plasma CORT levels in rats positively correlate with the degree of freezing during contextual fear testing (Cordero, Merino, & Sandi, 1998). Moreover, infusions of the glucocorticoid antagonist, RU 38486, in the BLA and ventral hippocampus prior to contextual fear training decreased freezing during recall 24 hrs later (Donley, Schulkin, & Rosen, 2005). There is evidence to suggest that glucocorticoids also mediate cued fear conditioning. Deletion

of glucocorticoid receptors in the CeA in knockout mice decreased conditioned fear to both tone and contextual cued fear and attenuated Fos reactivity in the BLA after fear training (Kolber et al., 2008). Thus fear conditioning may be a particularly effective way to systematically investigate the effects of repeated glucocorticoids on performance during learning and memory tasks that are associated with emotional arousal-cognitive processes known to be enhanced in depressed clinical populations (Leppanen, 2006; Nolen-Hoeksema, 2000).

The effects of acute stress or glucocorticoid exposure on fear-conditioned behaviours have been examined in rodents and demonstrate a change in behaviour after even one exposure. CORT injected at a dose of 5mg/kg and at 20 mg/kg 90 minutes before training and testing significantly decreased freezing to contextual cues (Skorzewska et al., 2006; Skorzewska et al., 2007). Alternatively, prior exposure to 4 or 15 footshocks 24 hrs or more before fear conditioning enhanced contextual fear learning (Rau & Fanselow, 2009). Furthermore, rats exposed to a single session of restraint stress showed increased freezing to contextual cues, but not tone cues (Cordero, Venero, Kruyt, & Sandi, 2003). A single glucocorticoid injection immediately after contextual fear training or tone cued fear training increased consolidation of contextual fear and tone fear respectively (Cordero & Sandi, 1998; Hui et al., 2004). Less research has focused on the effects of acute stress and glucocorticoid exposure on trace fear conditioning. The majority of work on the effects of acute stress on trace conditioning has occurred in trace eyeblink conditioning. Similar to fear conditioning, this work has shown that acute stress and glucocorticoid exposure facilitates both the amount and rate of trace eyeblink (Beylin & Shors, 1998; Beylin & Shors, 2003; Weiss, Sametsky, Sasse, Spiess, & Disterhoft, 2005). These findings suggest that a number of factors determine the effects of acute stress or glucocorticoid exposure on fear conditioned behaviours. Interestingly, both the type of stressor and timing of exposure can have differential effects under acute conditions. Glucocorticoid exposure appears to modulate both contextual and tone cued conditioning whereas acute stress more specifically mediates contextual freezing.

The effects of repeated stress and glucocorticoid exposure on fear-conditioned behaviours have also been examined in rodents. Repeated stress and glucocorticoid exposure produce reliable enhancements in fear-related behaviour to contextual cues (Sandi, Merino, Cordero, Touyarot, & Venero, 2001; Skorzewska et al., 2006); although, the effects of repeated exposure to low doses of CORT have shown non-significant effects to contextual fear conditioning (Gourley, Kedves, Olausson, & Taylor, 2009). The effects of repeated stress or CORT administration on other variations of fear conditioning are either unknown or have produced inconsistent results. For instance, few studies have explored the effect of repeated stress and glucocorticoid exposure on context and cue conditioning within the same rats (i.e., delay conditioning). The studies that have explored the effects of repeated stress or glucocorticoid exposure on delay conditioning have produced variable results. Twenty one days of restraint stress enhanced freezing to both tone and contextual cues when testing to both tone and context cues were presented in the same session (Conrad, LeDoux, Magarinos, & McEwen, 1999). However, slight protocol differences (i.e., testing of context cues before tone cues and testing tone cues in a novel environment) resulted in non-significant increases in freezing to both context and tone cues following 21 days of restraint stress (Conrad, Mauldin-Jourdain, & Hobbs, 2001). Moreover, rats exposed to 400 µg/ml of CORT in their drinking water for 21 days show increased fear behaviour to contextual cues but not tone cues when testing to context cues occurred before testing to tone cues (Conrad et al., 2004). Only one study that I am aware of has explored the effect of repeat stress on trace fear conditioning. In this study, 14 days of repeated immobilization stress decreased freezing behaviour; however, this repeated stress model also produced a decrease in freezing in a contextual fear paradigm (Vander Weele, Saenz, Yao, Correia, & Goosens, 2013). A decrease in freezing to contextual fear following repeated stress is not a typical finding indicating that this stress model may not be comparable with other repeated stress or glucocorticoid exposure models. As discussed previously, repeated stress models are known to be subject to habituation effects and variations in HPA

axis response (Gregus et al., 2005; Grissom & Bhatnagar, 2009). Importantly, the effects of 21 days of repeated 40 mg/kg CORT injections on fear conditioned behaviour have not been investigated. This repeated stress model produces robust changes in depression-like behaviour in the FST suggesting that it may also produce significant changes in other measures of behaviour that mirror depressive symptoms in rodents. Research investigating the effects of 21 days of 40 mg/kg CORT injections on fear conditioned behaviour would provide a valuable contribution to the understanding of how repeated glucocorticoid exposure affects learning and memory performance during tasks that evoke fear.

Fear conditioning can also be assessed in humans, which offers the unique ability to make direct comparisons between animal models and performance in humans. Evidence suggests that glucocorticoids in humans modulate fear conditioning similar to effects observed in rodents. Saliva cortisol levels taken after delay fear conditioning significantly correlated with acquisition of conditioning in men (Zorawski, Cook, Kuhn, & LaBar, 2005). Acute stress results in facilitation of fear conditioning in men as well (Jackson, Payne, Nadel, & Jacobs, 2006). However, acute cortisol treatment impaired fear conditioning in men (Stark et al., 2006). Based on the literature, it appears that acute stress in men typically enhances fear-conditioned learning. Importantly, patients diagnosed with depression also show enhanced learning of cued visual fear conditioning as measured through skin conductance (Nissen et al., 2010). Therefore, the effects of repeated stress and glucocorticoid administration in rodents mirrors the behaviours of humans diagnosed with depression.

7. Goals and Objectives

The general goal of this dissertation is to systematically investigate the effects of repeated CORT injections on learned helplessness behaviours in the FST, learning and memory recall of tasks that evoke negative emotional arousal (i.e., variations of fear conditioning), and whether the antidepressant, fluoxetine, prevents some of the CORT-induced

effects observed on fear conditioning performance. Specifically, I compared the ability of repeated glucocorticoid exposure to produce depression-like behaviours in a traditional versus non-traditional version of the FST and whether behavioural effects could be accounted for by changes in nonspecific locomotor activity or muscle strength. Further, I examined the effects of repeated glucocorticoid exposure on several variations of fear conditioning to characterize how this model affects the processing of emotionally driven learning and memory tasks. In addition, I investigated how repeated glucocorticoid exposure mediates the activity of specific brain regions stimulated by the recall of commonly used fear conditioning tasks. Finally, I determined whether fluoxetine treatment could prevent depression-like behaviour produced by repeated glucocorticoid exposure on fear-conditioned behaviours.

Experiment One: Repeated glucocorticoid injections are known to increase helplessness behaviours in one of the most widely used measures of depression-like behaviour in rodents, the FST (Nestler et al., 2002b). However, previous research was conducted using a modified version of the FST in which rats were only tested in the swim tank for one day. It is possible that repeated CORT injections can enhance the acquisition of helplessness behaviours from a one day to the two day traditional version of the FST, thus revealing a larger increase in depression-like behaviour. Furthermore, it has been speculated that increased helplessness behaviour in the FST following repeated glucocorticoid exposure could be the result of nonspecific locomotor effects or decreases in muscle strength. To address these questions, I examined the effects of 21 consecutive days of 40 mg/kg injections of CORT in male rats in both the traditional two day version of the FST and our modified one day version. I also analyzed behaviour in an open-field test and wire suspension test to investigate locomotor behaviour and muscle strength.
Hypotheses: The effects of repeated CORT injections may be more pronounced in a two day version of the FST; however, the main effect of CORT will be seen in both protocols. In addition,

CORT and vehicle injected animals are expected to behave similarly during the open-field and wire suspension test.

Experiment Two: Despite the observation that depression in human patients includes symptoms such as enhancements in fear associated memory (Nissen et al., 2010) and recall of information with a negative emotional connotation (Leppanen, 2006), this is rarely studied in preclinical experiments. Few researchers have explored how repeated glucocorticoid exposure in rats affects memory tasks that evoke emotional arousal. Furthermore, the few studies that have explored the effects of repeated glucocorticoid exposure on a memory task that evokes emotional arousal (i.e., delay fear conditioning) have revealed variable results. I hypothesized that the inconsistencies found in the literature may be the result of protocol differences between studies. Because we have previously shown that the effect of repeated CORT injections on depression-like behaviour in the FST is dose-dependent, (Johnson et al., 2006; Marks, Fournier, & Kalynchuk, 2009), I also wanted to assess whether the effects of CORT on fear conditioned memory would be dose dependent. To investigate this, 21 days of 5 mg/kg and 40 mg/kg of CORT injections were delivered to male rats and freezing behaviour was examined comparing the order of exposure to context and tone cues during testing of delay fear conditioning.
Hypotheses: Freezing behaviour should be increased in CORT injected rats with the greatest effect produced at a dose of 40 mg/kg. Furthermore, I hypothesized that the order in which rats were tested for freezing to context versus tone cues would affect the magnitude of CORT's effects on freezing behaviour.

Experiment Three: Chapter 3 showed that increases in freezing behavior were the most robust in rats exposed to repeated injections of 40 mg/kg of CORT. I wanted to investigate whether altered cellular activity in the hippocampus and amygdala, regions known to be involved in delay fear conditioning, could be mediating the effects of CORT. To examine this question, rats were

injected with 40 mg/kg of CORT or vehicle for 21 consecutive days, followed by delay fear conditioning, and finally sacrificed two hours later to examine Fos immunoreactivity, which is a marker of cellular activation.

Hypothesis: Based on the literature, rats injected with CORT should show enhanced Fos reactivity in the amygdala in response to recall of learned fear associations. Alternatively, the literature is less clear regarding the role the hippocampus has in mediating the effects of repeated CORT during recall of fear associations. Some researchers suggest that hippocampal integrity does not contribute in a direct way to enhanced fear behaviour following repeated stress (Conrad et al., 1999), whereas other researchers have shown altered Fos reactivity in the hippocampus following repeated low dose CORT exposure in response to recall of contextual cues (Skorzewska et al., 2006). Based on these inconsistencies, investigation of Fos in the hippocampus is considered exploratory and specific hypotheses are not made.

Experiment Four: Chapters 3 and 4 revealed that hippocampal dependent contextual fear conditioning is more susceptible to the effects of repeated CORT and that the amygdala may be mediating this effect. Trace fear conditioning provides the opportunity to investigate the effects of repeated CORT on a version of fear conditioning that is less dependent on the integrity of the amygdala (Raybuck & Lattal, 2011; Raybuck & Lattal, 2013). Furthermore, unlike delay fear conditioning where freezing behaviour during acquisition was unaffected by CORT treatment, pilot studies suggested that the acquisition of trace fear conditioning may be altered by CORT. To assess these possibilities, I injected rats with 40 mg/kg of CORT or vehicle for 21 days and subjected them to one of two trace conditioning protocols that allowed for the investigation of acquisition and recall of trace fear conditioning.

Hypotheses: I hypothesized that enhancements would be observed during both the acquisition and recall of trace fear conditioning following repeated CORT treatment.

Experiment Five: To further establish that the behavioural effects of CORT in Experiment 2 are reflective of depression-like behaviour, I assessed whether the antidepressant, fluoxetine, would prevent the effects of CORT on delay fear conditioning. To answer this question, I injected rats with either 40 mg/kg of CORT, 40 mg/kg of CORT + 10 mg/kg fluoxetine, or vehicle for 21 consecutive days and then subjected the rats to a delay fear conditioning task.

Hypothesis: Enhanced recall of fear memories in CORT-treated rats should be prevented by concurrent fluoxetine injections.

CHAPTER 2

Repeated Exposure to Corticosterone Increases Depression-like Behavior in Two Different Versions of the Forced Swim Test Without Altering Nonspecific Locomotor Activity or Muscle Strength

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Abstract

We have recently shown that repeated high dose injections of CORT reliably increase depression-like behavior on a modified one-day version of the forced swim test. The main purpose of this experiment was to compare the effect of these CORT injections on our one-day version of the FST and the more traditional two-day version of the test. A second purpose was to determine whether altered behavior in the FST could be due to nonspecific changes in locomotor activity or muscle strength. Separate groups of rats received a high dose CORT injection (40mg/kg) or a vehicle injection once per day for 21 consecutive days. Then, half the rats from each group were exposed to the traditional two-day FST and the other half were exposed to our one-day FST. After the FST, all the rats were tested in an open field and in a wire suspension grip strength test. The CORT injections significantly increased the time spent immobile and decreased the time spent swimming in both versions of the FST. However, they had no significant effect on activity in the open field or grip strength in the wire suspension test. These results show that repeated CORT injections increase depression-like behavior regardless of the specific parameters of FST, and that these effects are independent of changes in locomotor activity or muscle strength.

1. Introduction

One key mechanism through which the brain reacts to stress is through activation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis initiates a number of neuroendocrine events that culminate in the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex (Nestler et al., 2002a). Glucocorticoids invoke metabolic and behavioral changes that help the organism deal with an acute stressor in an effective manner (Checkley, 1996; Nestler et al., 2002a). However, excessive glucocorticoid release associated with chronic stress can have deleterious consequences. One such consequence appears to be the development of depressive symptomatology. This link between depression and excess glucocorticoids is underlined by observations that patients with depression often exhibit cortisol hypersecretion and disrupted cortisol rhythmicity (Sachar & Baron, 1979). It is also reinforced by the fact that several existing classes of antidepressant drugs act on the neuroendocrine substrates that regulate cortisol secretion (Pariante et al., 2003), and novel antidepressant therapies that inhibit cortisol secretion have shown promise in clinical trials (Belanoff et al., 2002; O'Dwyer, Lightman, Marks, & Checkley, 1995; Young et al., 2004). These observations have spurred a sustained research effort to expose the neurobiological mechanisms by which stress might influence the development of depression.

In order to better understand the relationship between stress, glucocorticoids, and depression, many researchers have turned to animal models. The most useful models in this regard are those in which both behavioral symptomatology and neurobiological mechanisms can be studied in the same animal. However, this is not a simple undertaking, because many experimenter-applied stress models, such as restraint stress, do not produce reliable changes in depression-like behavior, presumably due to individual differences in HPA axis responses and potential habituation effects (Gregus et al., 2005; Grissom & Bhatnagar, 2009). One way to avoid this problem is by using exogenous corticosterone (CORT) administration as a means to "mimic" exposure to stressful stimuli. With this idea in mind, we have shown that rats subjected

to 21 daily CORT injections show reliable and robust increases in depression-like behavior (Gregus et al., 2005). We also discovered that the effects of CORT on depression-like behavior are dose (i.e., high doses produce more depression-like behavior) and time dependent (i.e., repeated, but not acute, injections increase depression-like behavior), and that they occur in both male and female rats (Johnson et al., 2006; Kalynchuk et al., 2004).

In our previous experiments using the CORT-injection paradigm (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004), we employed a number of behavioral tests, but our primary measures of depression-like behavior came from the forced swim test. The forced swim test is a behavioral assay that has been successfully used to screen the efficacy of new antidepressant drugs (Borsini & Meli, 1988; Porsolt et al., 1977; Lucki, 1997). In the traditional version of the forced swim test, rats are placed in an inescapable tank of water for 15 min on day one. Then, they are put back into the tank for 10 min on day two. The general idea is that on day one, exposure to the inescapable tank creates a state of despair, so that when the rat is subsequently placed back into the tank on day two, it adopts behaviors reminiscent of helplessness, such as increased time spent immobile, decreased time spent swimming and a decrease in the latency to become immobile (Porsolt et al., 1977). In our studies using the CORT-injection paradigm, we used a modified version of the forced swim test in which rats were tested in the swim tank for one day only. The rationale for our modification was that by focusing on a single day of testing, we could measure depression-like behavior produced by prior exposure to the CORT injections, rather than prior exposure to the swim tank itself. Indeed, we found that repeated CORT injections do increase immobility behavior and decrease swimming behavior (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004). However, it is possible that the CORT injections would enhance the acquisition of “despair” from day one to day two of traditional forced swim testing, and that using a two-day version of the forced swim test would reveal larger increases in depression-like behavior than a one-day test. Accordingly, the main purpose of this experiment was to compare the effect of repeated CORT injections on

depression-like behavior in a one-day and a two-day forced swim test. Furthermore, because we have previously found that rats receiving repeated CORT injections weigh considerably less than vehicle-injected rats (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004), we also wanted to assess whether increased immobility behavior in the forced swim test could be related to a general reduction in locomotor activity or a decrease in muscle strength. Therefore, in addition to the forced swim test, we also assessed the effect of repeated CORT injections on locomotor activity in an open-field test and muscle strength in a wire suspension test.

2. Materials and Methods

2.1. Subjects

The subjects were 37 male Long-Evans rats (purchased from Charles River Canada, Montreal, Quebec) that weighed approximately 215-240g at the time of arrival. The rats were housed individually in standard polypropylene cages with Purina rat chow and water available ad libitum. The colony room was maintained at a temperature of 21±1°C with a 12h: 12h light/dark cycle (lights on at 8:00 a.m.). All experimental procedures were carried out during the light phase of the light/dark cycle, and they were conducted according to the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care.

2.2 Repeated Corticosterone Injections

The rats were handled for 14 days in the colony room prior to any experimental manipulations. At the end of handling, the rats were weight matched and assigned to either a CORT group (n = 20) or a vehicle group (n = 17). The CORT group received a 40mg/kg injection of CORT once per day for 21 consecutive days and the vehicle group received a vehicle injection along the same time-course. CORT (MP Biomedicals, Solon, OH) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitan

monooleate (Tween-80; Sigma-Aldrich, St. Louis, MO). All CORT and vehicle injections were delivered subcutaneously each day at a volume of 1 ml/kg between 10:00 am and 12:00 pm. The dose of CORT used in this experiment was selected based on previous studies that have employed a repeated CORT injection model to study depression-like behavior using the forced swim test (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004).

2.3 Forced Swim Test

The forced swim test was used to assess depression-like behavior (Porsolt et al., 1977). Two different versions of the forced swim test were used in this experiment—a traditional two-day version of the test (Porsolt et al., 1977; Detke, Rickels, & Lucki, 1995) and a modified one-day version of the test that we have used in past experiments (see (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004)). Half of the rats from the CORT and vehicle groups received the two-day version of the forced swim test and half of the rats received the one-day version of the forced swim test. In the two-day version, rats were subjected to 10 min of forced swim testing one day after their last injection and another 10 min of forced swim testing two days after their last injection. In the one-day version, rats were subjected to 10 min of forced swim testing one day after their last injection and a short period of handling two days after their last injection.

The forced swim test was conducted in a rectangular Plexiglas swim tank (25 cm long x 25 cm wide x 60 cm high), filled with 27°C (± 2°C) water to a depth of 30 cm. Rats were transported from the colony room into an adjacent behavioral testing room. Each rat was placed individually into the forced swim tank for 10 minutes. Behavior in the swim tank was video recorded with a camera mounted on a tripod horizontal to the swim tank. After 10 min, the rat was removed from the tank, dried with a towel and placed back in its home cage to dry under a heat lamp for approximately 15 minutes. The water in the swim tank was changed in between rats.

Both active and inactive components of forced swim behavior were scored from the videotapes by a researcher who was blind to each rat's treatment condition (Detke et al., 1995). The three behavioral measures were: 1) time spent climbing – observed when the rat made vigorous movements in and out of the surface of the water and the rat actively struggled to find a means of escape from the tank; 2) time spent swimming – observed when the rat made active swimming movements that were beyond those necessary to simply maintain its head above water, but less than those observed with climbing (time spent diving was also included here); 3) time spent immobile – observed when the rat made very little movement with its body (only enough to keep from drowning). Rats were engaged in one of these three activities at all times throughout the 10 min session. The latency to immobility was also recorded. Depression-like behaviors were inferred from an increase in total time spent immobile and a decrease in total time spent climbing or swimming (Detke et al., 1995).

2.4 Open-field Test

The open-field test was used to assess general locomotor activity (Kalynchuk, Pinel, Treit, & Kippin, 1997). It was conducted three days after the last injection. The open field was a 70 cm long x 70 cm wide x 60 cm high black wooden box with a transparent Plexiglas bottom and no top. It was located in a small, brightly lit procedure room. The floor of the open field was divided into 36 identical squares by tape attached underneath the floor. Each rat was placed individually into a corner of the open field and allowed to explore for 5 min. A video camera mounted on a tripod above the open field recorded each rats' activity. The number of lines crossed during the 5 min session was calculated by a researcher who was blind to each rat's treatment conditions. A rat received credit for 1 line cross when the center of its back crossed over from one square and into an adjacent square. Locomotor activity was inferred from the number of lines crossed. The open field was thoroughly cleaned between rats using a dilute 0.4% (v/v) acetic acid solution in order to minimize potential olfactory cues.

2.5 Wire Suspension Test

The wire suspension test was used to assess muscle strength (Murphy, Rick, Milgram, & Ivy, 1995). It was conducted two hours after the open field test. This test measures an animal's ability to grasp a horizontal wire with its forepaws and to remain suspended. A 50 cm length metal wire (12 gauge) was suspended between two wooden platforms and was elevated to a height of 80 cm above a cushioned surface. The rat's forepaws were placed on the wire and the latency to fall was measured in sec on two trials (separated by 5 minutes). The maximum score was 30 sec. Muscle strength was inferred from the amount of time the rat held onto the wire.

2.6 Body Weight

All rats were weighed on a daily basis and their weights were recorded for later analysis.

2.7 Statistical Analyses

All data were analyzed using the Statistical Package for the Social Sciences (Chicago, IL, version 13.0). Group differences in forced-swim-test behaviors were analyzed using separate two way ANOVA's for each behavioral measure, with treatment (CORT vs. vehicle) and forced swim test protocol (one day vs. two day) as the between groups factors. Group differences in locomotor activity in the open field were assessed using an independent samples t-test. Finally, group differences in time spent suspended in the wire suspension test and body weight were analyzed using separate repeated measures ANOVA's, with treatment (CORT vs. vehicle) as a between groups factor and time as the repeated measures factor. This was followed by post hoc t-tests when appropriate. The significance level was set at $P \leq 0.05$ for all statistical comparisons.

3. Results

3.1 Forced Swim Test

The CORT injections had a significant effect on behavior in the forced swim test (see Figure 2-1). Our statistical analyses revealed a significant main effect of treatment on the latency to immobility [$F(1, 33) = 6.40$, $p = 0.016$], time spent swimming [$F(1, 33) = 2.78$, $p = 0.001$], and time spent immobile [$F(1, 33) = 8.64$, $p = 0.006$]. Rats that received CORT injections had significantly shorter latencies to immobility, spent significantly less time swimming, and spent significantly more time immobile in the forced swim test than did the rats that received vehicle injections. Our analyses also revealed a significant main effect of forced swim test protocol on latency to immobility only [$F(1, 33) = 7.64$, $p = 0.009$]. Rats that received the one-day version of the forced swim test had significantly longer latencies to immobility compared to rats that received the two-day version of the forced swim test. All treatment x forced swim protocol interactions were non-significant [all p values > 0.476].

3.2 Open Field Test

The CORT injections had little effect on locomotor activity in the open field (see Figure 2-2). There was no significant difference between the groups in total lines crossed during the 5 min open field test [$t(35) = -1.38$, $p = 0.177$].

3.3 Wire Suspension Test

The CORT injections also had little effect on muscle strength in the wire suspension test (see Figure 2-3). There were no significant between [$F(1, 36) = .029$, $p = .865$] or within [$F(1, 36) = .386$, $p = .538$] group differences in the amount of time spent suspended from a steel wire.

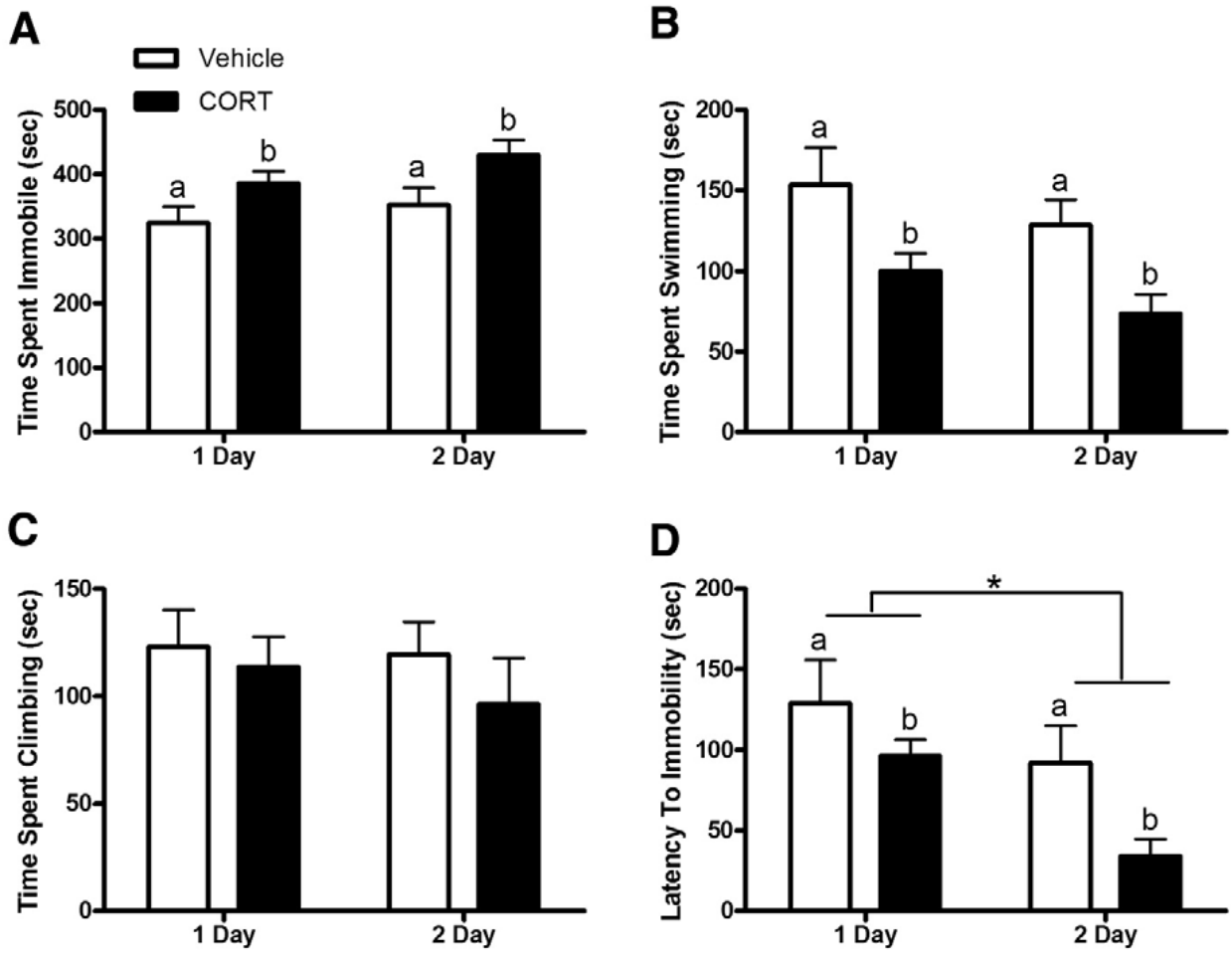

Figure 2-1. The effect of repeated CORT injections on behavior in two versions of the forced swim test. Panel A shows the mean time spent immobile, panel B shows the mean time spent swimming, panel C shows the mean time spent climbing, and panel D shows the mean latency to immobility [vehicle group 1 day, n = 8; vehicle group 2 day, n = 9; CORT group 1 day, n = 10, CORT group 2 day, n = 10]. ^{a} vs ^{b} indicates a significant difference between the CORT and vehicle treated groups ($p < 0.05$). The asterisk indicates a significant difference between Day 1 and Day 2 of the forced swim test ($p < 0.05$). Error bars denote the S.E.M.

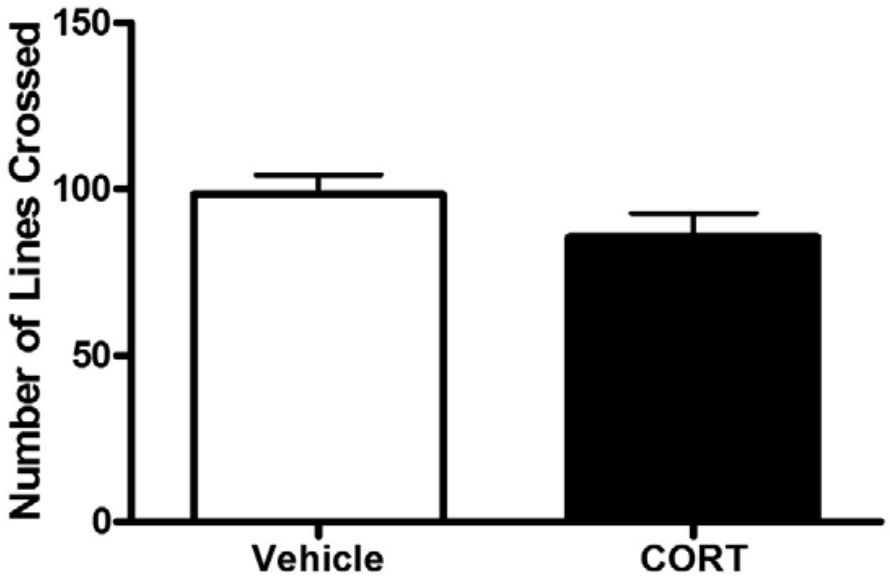

Figure 2-2. The effect of repeated CORT injections on locomotor activity in the open field. There were no significant differences between the groups [vehicle group, n = 17; CORT group, n = 20]. Error bars denote the S.E.M.

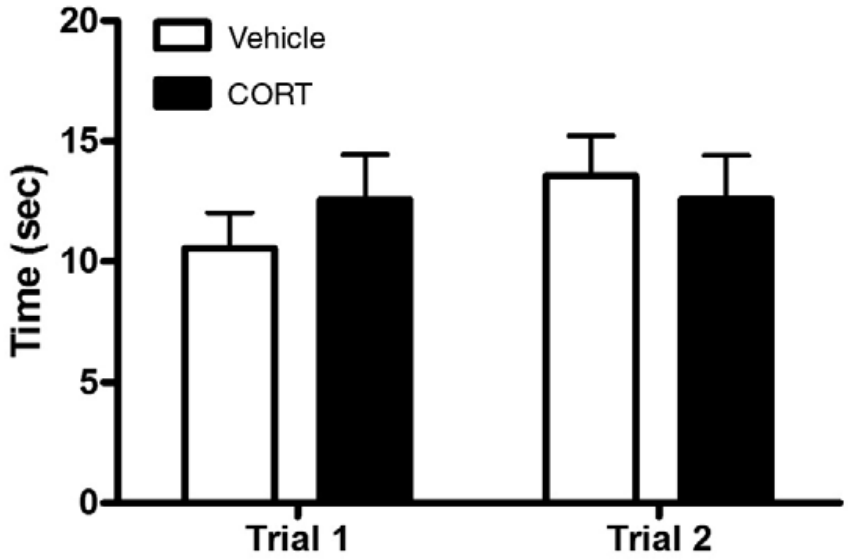

Figure 2-3. The effect of repeated CORT injections on muscle strength in the wire suspension test. There were no significant differences between the groups [vehicle group, n = 17; CORT group, n = 20]. Error bars denote the S.E.M.

3.4 Body Weight

The CORT injections did have a significant effect on body weight (see Figure 2-4). Our repeated measures analysis revealed a significant effect of treatment [$F(1, 35) = 103.016$, $p > .0001$], a significant effect of time [$F(3, 105) = 104.684$, $p < .0001$] and a significant interaction [$F(3, 105) = 220.130$, $p < .0001$]. Post hoc t-tests further revealed that the groups had similar body weights on day 1 of the injections [$t(35) = -0.47$, $p = 0.640$]. However, after 7 days of injections, the CORT-injected rats weighed significantly less than the vehicle-injected rats [$t(35) = -8.43$, $p < 0.001$]. This effect persisted through days 14 [$t(35) = -13.05$, $p < 0.001$], and 21 [$t(35) = -12.32$, $p < 0.001$] of the injection phase.

4. Discussion

Three main findings emerged from the results of this experiment. First, we confirmed that repeated injections of CORT increase depression-like behavior as measured by the forced swim test in rats. Second, we showed that the effects of CORT on depression-like behavior in the forced swim test occur regardless of whether the rats are subjected to a one-day or two-day version of the test. Third, we found that repeated CORT injections have no significant effect on locomotor activity or muscle strength, suggesting that the depressogenic effects of CORT occur independently of changes in nonspecific motor behavior. Each of these findings is discussed in more detail in the following paragraphs.

4.1 Effect of CORT on Depression-like Behavior

The result of this experiment clearly showed that repeated exposure to glucocorticoids can enhance depression-like behavior in rats. The CORT-injected rats showed a decrease in latency to immobility, an increase in time spent immobile, and a decrease in time spent swimming in the forced swim test—all measures typically used to infer a depressive phenotype (Cryan, Valentino, & Lucki, 2005). This finding was not surprising, given that we and others had

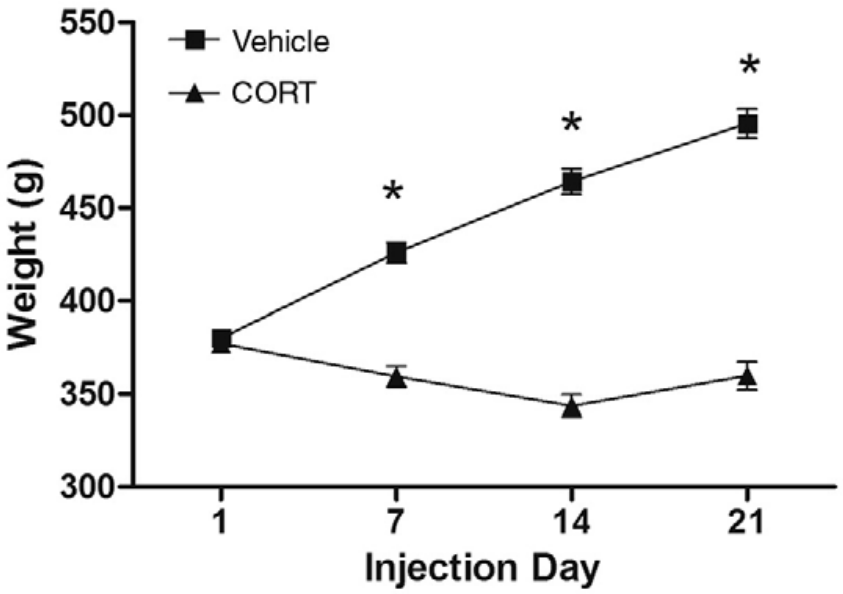

Figure 2-4. The effect of repeated CORT injections on body weight. Asterisks indicate significant differences between groups ($p < 0.01$) [vehicle group, n = 17; CORT group, n = 20]. Error bars denote the S.E.M.

already published similar results (e.g., (Johnson et al., 2006; Hill, Brotto, Lee, & Gorzalka, 2003)). In fact, previous reports have shown that the effect of CORT on depression-like behavior in the forced swim test occurs in both males and females (Kalynchuk et al., 2004), with high but not low doses of CORT (Johnson et al., 2006) and with repeated but not acute administration of CORT (Johnson et al., 2006). Importantly, the depressogenic effects of CORT go beyond behavior on the forced swim test. Rats subjected to CORT-injection paradigms that are similar to the one that we have been using show inhibited sexual behavior (Gorzalka & Hanson, 1998), enhanced conditioned taste aversion (Gorzalka et al., 2003) and impaired cognition (Coburn-Litvak, Pothakos, Tata, McCloskey, & Anderson, 2003; Feldmann, Jr. et al., 2008). Taken together, these findings reinforce the idea that the repeated CORT injection paradigm is a useful animal model for studying the interaction between stress, glucocorticoids, and depression, and suggest that it may be a good tool for investigating the neurobiological mechanisms that underlie specific depressive symptomatology.

A novel aspect of this experiment was comparing the effect of repeated CORT injections on two different versions of the forced swim test. The forced swim test was originally developed by Porsolt (Porsolt et al., 1977) and modified by Lucki and his colleagues (Cryan, Page, & Lucki, 2005; Detke et al., 1995) to act as a reliable behavioral assay for antidepressant drug efficacy. Rats are typically tested on two consecutive days. The first day is an induction phase, in which the rat learns that escape from the cylinder is impossible, and the second day is a test day, in which the degree of “despair” that the rat acquires on day one is reflected in increased immobility behavior and decreased swimming or attempts to escape. This has been extremely successful, as most clinically useful antidepressant drugs reduce immobility and increase swimming time in this test (Borsini & Meli, 1988). More recently, the forced swim test has been increasingly used to detect a depressive phenotype in various animal models of depression (Brummelte et al., 2006; Gregus et al., 2005; Murray et al., 2008; Rygula et al., 2008). Most of these are stress-based models, and rats are subjected to a period of chronic stress prior to

being tested on the forced swim test. The vast majority of these studies use the traditional two-day version of the forced swim test (see (Cryan et al., 2005)). However, a few experimenters, including those within our laboratory, have adopted a one-day version of the test, with the premise that prior exposure to a period of repeated stress should induce “despair’ on its own, making the usual induction day unnecessary (Brotto, Gorzalka, & Barr, 2001; Cryan et al., 2005; Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Kokras et al., 2009; Murray et al., 2008). A one-day version of the test also eliminates any confounding influence of memory from the first day of exposure on behavior during the testing day. Because we had received some criticism for using a one-day forced swim test from previous reviewers, we thought it might be prudent to confirm whether a one-day forced swim test was sufficient for assessing a depressive phenotype in rats previously exposed to stress or whether the two-day test would prove to be more sensitive and therefore necessary. In general, we found comparable results with both a one-day and a two-day version of the forced swim test. Regardless of the test parameters, the CORT-injected rats spent more time immobile and less time swimming than did the vehicle-injected control rats. Therefore, our results show that a one-day forced swim test protocol can be appropriate to assay depression-like behavior in situations that employ prior exposure to a depressogenic manipulation.

We should mention that there were some differences between the one-day and two-day forced swim test with regard to the latency to immobility. The CORT-injected rats subjected to the two-day forced swim test showed a larger decrease in the latency to immobility than the CORT-injected rats subjected to the one-day test. This is likely related to the greater familiarity of the testing situation in rats subjected to the two-day test, rather than a reflection of the intensity of depression-like behavior per se. The fact that the differences in latency to immobility in this experiment did not parallel the result of the other three core measures (i.e., time spent immobile, swimming, and climbing) suggest that latency to immobility may not be a sensitive measure of altered depression-like behavior in rats. Interestingly, in the modified version of the

forced swim test developed by Lucki and colleagues, latency to immobility is not a core measure (Detke et al., 1995). Instead, these authors focus on a sampling procedure of immobility, swimming, and climbing behavior and they show quite convincingly that these measures can be used to assay antidepressant drug efficacy and to distinguish between classes of antidepressant medication with different mechanisms of action (Cryan et al., 2005). The results of this experiment support this idea and suggest that latency to immobility might not be necessary or even useful for assessing depressogenic manipulations.

4.2 Effect of CORT on locomotor activity, muscle strength, and body weight

In addition to altering depression-like behavior, exposure to repeated CORT injections also decreases body weight gain (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004). The CORT-injected rats do not lose weight, but they do stop gaining weight and by the end of the first week of treatment, they always weigh significantly less than the vehicle-injected rats. This observation led us to wonder whether the behavioral changes seen in the forced swim test could be due to muscle atrophy or nonspecific decreases in locomotor activity. It is clear that CORT plays an integral role in metabolic processes (Tempel & Leibowitz, 1994). The effects of CORT on body weight are curvilinear, with optimum weight gain occurring at relatively low levels and weight loss occurring with chronically high levels of either CORT or glucocorticoid receptor agonists (Bridges, Slais, & Sykova, 2008; Devenport, Knehans, Sundstrom, & Thomas, 1989; Santana et al., 1995). The effect of CORT on body weight occurs through its metabolic action on fat and protein stores. CORT has an anabolic action when levels are low to moderate and a catabolic action when levels are chronically high (Tempel & Leibowitz, 1994). Initially, the catabolic actions of high CORT levels are conducive to the survival of the organism because these actions act to provide a substrate for maintaining normal carbohydrate balance after acute stress or times of limited food supplies (Tempel & Leibowitz, 1994). However, prolonged high levels of glucocorticoids promote lipolysis and decreased protein synthesis that in turn amplifies

the breakdown of muscle into constituent amino acids (Tomas et al., 1979). Collectively, these catabolic effects become maladaptive and eventually lead to muscle atrophy and weight loss.

Can the profound effects of CORT on these physiological measures account for the effect of CORT on behavior, more specifically behavior related to physical mobility? The forced swim test requires rats to swim for a period of time and may be overly taxing on rats that are in a chronic catabolic state. However, in this experiment, we detected no behavioral changes to indicate that altered swimming behavior in the forced swim test could be explained by nonspecific effect of CORT. The CORT-injected rats engaged in a similar amount of locomotor exploratory behavior in the open field compared to the vehicle-injected rats. They also performed equally well as the vehicle-injected rats in the wire suspension test. And finally, they showed equivalent amounts of climbing—the most strenuous behavior measured—as the vehicle-injected rats did in the forced swim test. Therefore, although we cannot rule out the possibility that the CORT-treated rats do experience some muscle atrophy, it is unlikely that muscle atrophy caused the decreased swimming seen in the forced swim test in this experiment. Interestingly, other researchers have reported that CORT-treated rats swim the same distance as control rats in a Morris Water maze (Sousa et al., 2000) and that CORT-treated rats actually engage in more lever pressing for electrical stimulation than control rats (Barr, Brotto, & Phillips, 2000), indicating that CORT-treated rats do not always show a decrease in physical behaviors. Taken together, these findings reinforce the idea that altered behavior in the forced swim test in the CORT-injected rats does reflect a depressive phenotype rather than a nonspecific physiological change in locomotion or muscle strength.

4.3 Conclusions

The results of this study show that both the traditional two-day version of the forced swim test and a modified one-day version of the forced swim test can be equally effective in detecting a depressive phenotype in rats subjected to high levels of corticosterone. They also

provide further evidence that the CORT-injection paradigm is a useful animal model for studying the relationship between stress, glucocorticoids, and depression.

CHAPTER 3

The Effect of Repeated Corticosterone on Contextual and Tone Fear Conditioning Depends on Dose and the Testing Protocol

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Author contributions: WNM designed research, collected data, analyzed data, and co-wrote paper; EYF designed research; AJG collected data; LEK designed research and co-wrote paper.

ABSTRACT

Previous research investigating the effects of chronic stress or repeated glucocorticoid administration on fear learning have been equivocal. In this experiment we investigated whether altering the order in which rats are tested for freezing to context vs. tone cues might explain some of the previous inconsistencies in the literature. Male Long-Evans rats received 5 mg/kg of corticosterone, 40 mg/kg of corticosterone, or vehicle injections daily for 21 consecutive days. On day 22, the rats received delay fear conditioning training. On day 23 and 24, rats were tested for recall of fear memories to context and tone cues in a counterbalanced way. Our results revealed dose dependent effects of CORT on freezing: Rats injected with 40mg/kg of corticosterone froze significantly more than the vehicle rats to both context and tone cues regardless of what testing day these cues were presented. However, rats injected with 5mg/kg of CORT froze significantly more than vehicle rats to tone cues only. We also found an order effect in that rats injected with 40mg/kg corticosterone froze significantly more to context cues when these cues were presented on day two and rats injected with 5mg/kg corticosterone froze more to tone cues when they were presented on day two. These results suggest a complex relationship between stress intensity and testing conditions. They also suggest that counterbalancing the presentation of context and tone cues may be the best approach for understanding the effects of chronic stress or glucocorticoids on fear conditioning.

1. Introduction

Activation of the HPA axis and subsequent glucocorticoid release (cortisol in humans, and CORT in rodents) in response to stress is essential to an organism's survival (Checkley, 1996; Nestler et al., 2002a). Rodents subjected to acute stressors show enhanced memory formation and adaptive neuroplastic responses (Shors, 2001; Stamatakis et al., 2008; Zheng et al., 2007). However, prolonged exposure to excessive glucocorticoids can have deleterious consequences. The hippocampus and amygdala are particularly susceptible to the negative consequences of repeated stress/glucocorticoid exposure as both structures contain a large number of glucocorticoid receptors (Feldman & Weidenfeld, 1999; Morimoto et al., 1996). Rats exposed to persistent high levels of CORT display decreased dendritic branching in CA3 pyramidal cells, decreased mossy-fiber synapses, and decreased hippocampal neurogenesis (Caetano et al., 2004; Sousa et al., 2000; Watanabe et al., 1992; Woolley et al., 1990). Prolonged stress is also associated with memory impairments (McEwen & Sapolsky, 1995), and these impairments have been correlated with the dampened hippocampal plasticity produced by excessive glucocorticoids (Luine, Villegas, Martinez, & McEwen, 1994; Sousa et al., 2000). Interestingly, exposure to repeated stress or glucocorticoids seems to facilitate plasticity within the amygdala, which is an important area for the processing of emotional information. Prolonged immobilization stress in rats enhances dendritic arborization and spine density of basolateral amygdala pyramidal neurons (Vyas et al., 2002; Vyas et al., 2006).

Given the divergent effects of glucocorticoids on hippocampal and amygdalar plasticity, it is of interest to examine how glucocorticoids might affect learning and memory events that rely on the integrity of these two brain structures. One way to study this is through the use of fear conditioning, which assesses a rodent's ability to associate neutral cues such as a tone or context (CS) with an aversive experience such as a mild foot shock (US). The CS predicts an aversive outcome and comes to elicit a conditioned response (CR), such as defecation, piloerection, and freezing behavior. The conditioning of fear responses to contextual and tone

cues are processed by different pathways. That is, hippocampal lesions interrupt conditioning of fear responses to contextual cues, whereas amygdala lesions interrupt conditioning of fear responses to both contextual and tone cues (Phillips & LeDoux, 1992). Given that glucocorticoids seem to facilitate synaptic plasticity within the amygdala, one would expect to see enhanced learning and retrieval of fear memories in rats subjected to repeated stress or glucocorticoid exposure. Indeed, previous research has shown that either repeated stress or glucocorticoids can facilitate consolidation of contextual fear memories. However, the effects of glucocorticoids on freezing behaviour in response to the dissociable cues produced by delay fear conditioning are unclear. Few studies have explored the effect of repeated stress on context and cue conditioning within the same rats and the few studies that have explored the effects of repeated glucocorticoid exposure on delay conditioned freezing have reported variable results. For example, 21 days of restraint stress enhanced freezing to both tone and contextual cues when both tone and context cues were presented in the same session (Conrad et al., 1999), but it did not enhance freezing to context or tone cues when testing to context cues were assessed before testing to tone cues or when tone cues were assessed in a novel context (Conrad et al., 2001). Additionally, rats exposed to 400 mg/ml of CORT in their drinking water for 21 days showed enhanced freezing to contextual cues but not tone cues when testing to context cues occurred before testing to tone cues (Conrad et al., 2004). These results suggest that the order of testing (i.e., context first vs tone first) might be an important factor to consider in conducting these types of experiments.

The main purpose of this experiment was to clarify the effect of repeated glucocorticoids on fear learning and memory by examining two important factors: the order of cue presentation during testing and the dose of CORT administered to the rats. As described above, there is reason to believe that some of the inconsistent findings in the literature could be due to methodological differences and order effects in the way that retrieval of fear memories is assessed. Accordingly, we examined the effect of presenting either context cues first or tone

cues first during testing of fear memories. Another possible explanation for previous inconsistent findings could be variability in the severity of stressors or amount of glucocorticoids applied to the rats prior to fear conditioning. Previous work has clearly shown that the effects of repeated CORT on depression-like behavior are dose-dependent (e.g., (Johnson et al., 2006; Marks et al., 2009; Sterner & Kalynchuk, 2010)). Therefore, we also examined the effect of a low dose of CORT (5 mg/kg) and a high dose of CORT (40 mg/kg) on acquisition and retrieval of fear memories.

2. Materials and methods

2.1. Animals

We used 49 adult male Long-Evans rats (purchased from Charles River Canada, Montreal, Quebec) in this experiment. The rats weighed between 215-250 g at the time of arrival from the breeder. They were individually housed in standard polyproplylene cages in a housing room maintained on a 12 hr light/dark cycle (lights on at 07:00 h) at a temperature of 21°C with free access to Purina rat chow and water. All experimental procedures were carried out during the light phase and were conducted according to the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care and Supply.

2.2. Repeated CORT injections

Rats were handled and injected in a procedures room separate from the housing room. Handling occurred daily for 7-14 days prior to the onset of any injections in order to familiarize the rats with the vivarium and researchers. At the end of this handling phase, each rat was assigned to one of three experimental conditions based on body weight (i.e., so that all groups had approximately equivalent average weight). The three experimental conditions were: a 5 mg/kg CORT group (CORT 5 group, n = 14), a 40 mg/kg CORT group (CORT 40 group, n = 14), and a vehicle control group (vehicle group, n = 14). All injections were administered

subcutaneously at a volume of 1ml/kg once per day for 21 consecutive days between 09:00 hr and 11:00 hr. CORT (Steraloids Inc., Newport, RI) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitan monooleate (Tween-80; VWR International, West Chester, PA). The doses of CORT were chosen to mimic physiological levels of endogenous CORT under conditions of low stress and high stress respectively (Johnson et al., 2006; Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994). The high dose of 40 mg/kg reliably increases depression-like behaviour in the forced swim test (Gregus et al., 2005; Kalynchuk et al., 2004; Marks et al., 2009).

2.3. Experimental design

The design of this experiment is shown in Figure 3-1. As mentioned above, one purpose of this experiment was to determine whether some of the discrepant effects of repeated stress or CORT on fear conditioning in past studies could be related to the methods by which rats were exposed to tone vs. contextual cues. Therefore, we used two different testing protocols to examine the effect of order of presentation of contextual versus tone cues in rats treated with a low and high dose of CORT. After 21 days of low CORT, high CORT, or vehicle injections, rats were assigned to one of two protocols. In protocol 1, rats were conditioned to contextual and tone cues on day 22 (training day), and then they were tested with contextual cues on day 23 (contextual testing) and tone cues on day 24 (tone testing). In protocol 2, the testing order was reversed: Rats received the same conditioning to contextual and tone cues on day 22 (training day), but they were tested with tone cues on day 23 and with contextual cues on day 24. This experimental design allowed us to assess two main factors: the effect of CORT dose observed during acquisition and retrieval of fear memories and the effect of order of cue presentation on the magnitude of any CORT-induced changes observed during fear memory recall.

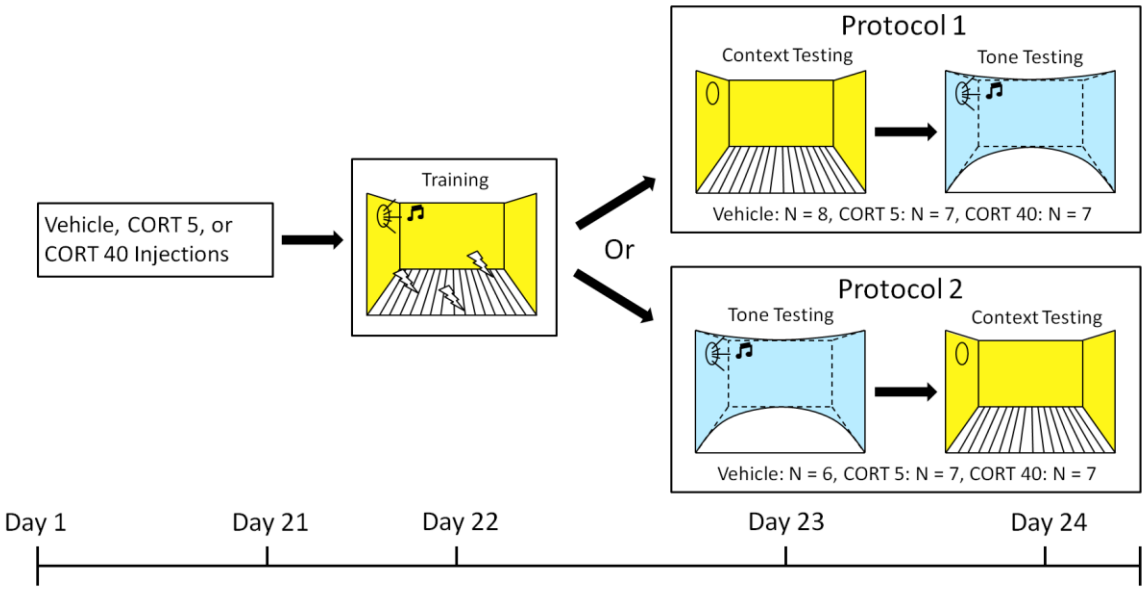

Figure 3-1. Experimental Design. A schematic representation of testing protocol 1 and 2. Rats are injected with vehicle, 5mg/kg CORT or 40 mg/kg CORT for 21 consecutive days prior to delay fear conditioning training. Approximately 24 hr following the final injection all rats are subjected to delay fear conditioning training. The day after training (day 23) rats assigned to protocol 1 are tested for recall of contextual cues whereas rats assigned to protocol 2 are tested for recall of tone cues. The following day (day 24) rats assigned to protocol 1 are tested for recall of tone cues whereas rats assigned to protocol 2 are tested for recall of contextual cues.

2.4. Fear conditioning

2.4.1. Apparatus. The rats were trained and tested in two identical video fear-conditioning chambers (25.5 cm X 32 cm X 25.5 cm) that were attached to a personal computer that controlled all shocks and tone presentations and recorded all rat behavior (Med Associates, St. Albans, VT). The four sidewalls of each chamber were made of aluminum. The front door and ceiling were made of clear Plexiglas, and the back wall was made of white plastic. The floor of each chamber comprised 19 stainless steel rods spaced 1 cm apart; each rod was wired to a shock generator and scrambler that was used to deliver footshocks as unconditioned stimuli (US). The ceiling held a small speaker that allowed for the presentation of individual tones that acted as conditioned stimuli (CS). A stainless steel pan covered by two sheets of paper towel was inserted beneath the grid floor for the collection of urine and fecal matter. The entire chamber was placed inside a white sound-attenuating cubicle (73 cm X 64 cm X 42 cm). Each chamber was lit by a fluorescent lamp.

2.4.2. Training. On day 22 of the experiment (training day), rats in their home cages were transported to the conditioning room on a trolley (2 rats at a time). Rats were placed individually inside one of the chambers and given 180 s to acclimatize to the contextual surroundings. Rats then received 5 tone-shock pairings, with an inter-trial interval (ITI) of 120 s. Each tone was 20 s long, with a volume of 90 dB at a frequency of 2 kHz and a rise time of 50 ms that co-terminated with a 2 s 0.7 mA footshock. Rats remained in the conditioning chamber for 120 s after the 5^{th} pairing before being returned to their home cages and transported back to the housing room. The chamber was cleaned with 0.4% glacial acetic acid in between each training session. All training was conducted between 09:00 hr and 15:00 hr.

2.4.3. Tone Testing in a Novel Context. Testing for retrieval of tone conditioning took place using the same two chambers described above, but the contextual details within the chambers

and testing room were altered in several ways to make the context novel for the rats. First, a pattern containing black horizontal strips was fitted to the outside of the ceiling of the chambers altering both the appearance of the ceiling and the lighting of the chamber. Second, a plastic insert containing two blue vertical stripes and one red vertical stripe was inserted into the chamber covering both the side panels and rear of the chamber giving the chamber a semicircle shape. Third, a white plastic plate was placed over the grid rods to make the floor completely flat and change tactile cues. Fourth, shapes cut from cardboard of various colours were placed on the inner walls of the sound attenuating cubicle to alter visual cues. Fifth, a white noise generator created background noise (65 dB). Sixth, the room where conditioning took place was altered by draping blue curtains from the ceiling changing both the shape and color of the room. A curtain was also draped over the fluorescent ceiling light diming the lighting of the room. Finally, olfactory cues were changed by scenting the room with vanilla coconut pet deodorizer between trials and the chambers were cleaned with 75% ethanol instead of 0.4% glacial acetic acid. Also, transportation from the housing room to the testing room was altered by draping a dark curtain over the home cages and by changing the route of transportation.

Testing for retrieval of tone conditioning was conducted on either day 23 or day 24, depending on whether the rat was in the tone first-context second testing protocol or the context first-tone second testing protocol. Rats were transported to the testing room as described above and placed inside the opposite chamber to the one that they were trained in. Rats were given 180 s to acclimatize to the new contextual surroundings before receiving 5 tones with an ITI of 120 s. Rats remained in the chamber for 120 s after the 5^{th} tone before being returned to their home cages and transported back to the colony room. All testing to tone cues was performed between 14:00 hr and 17:00 hr.

2.4.4. Contextual fear testing. Testing for retrieval of contextual conditioning also took place on either day 23 or day 24, depending on whether the rat was in the tone first-context second

testing protocol or the context first-tone second testing protocol. On contextual testing day, rats were transported in their home cages to the testing room and placed inside the same chamber that they were trained in. Rats remained in the chambers for 14 min, 40 s (equivalent amount of time used for training). At the end of testing, the rats were transported back to the colony room. Conditioning chambers were cleaned with 0.4% glacial acetic acid between trials. All testing to contextual cues was performed between 09:00 hr and 12:00 hr.

2.4.5. Behavioural measures. Behaviour in the conditioning chambers was recorded by a video camera (at a rate of 30 frames/s) mounted to the inside door of the sound attenuating cubicles. Freezing was defined as immobility with the exception of respiratory related movement (Maciejak et al., 2003). All movement within the chambers was recorded by Video Freeze Software (Med Associates, St. Albans, VT) as a change in video pixel composition over time. Freezing was quantified by the software using a motion threshold, measured in activity units (AU), which refers to the limit above which all behavior is registered as movement. We also scored several videos manually and this revealed that determination of motion AU with the software program was strongly correlated ($r > 0.9$) with trained human observation. Video Freeze Software provided the percentage of time spent freezing based on a minimum freeze duration of 1 s.

2.5. Body weight

All rats were weighed daily and their weights were recorded for later analyses.

2.6. Statistical analyses

The data were analyzed using the Statistical Package for the Social Sciences (Chicago, IL, version 18.0). Group differences in freezing behaviour during training were analyzed using separate one-way ANOVAs with treatment (40mg/kg CORT vs. 5mg/kg CORT vs. vehicle) as

the between-groups factor. In addition, group differences in behavior on the two testing days were analyzed using separate two-way ANOVAs with group (i.e., 40mg/kg CORT vs. 5mg/kg CORT vs. vehicle) and protocol (i.e., protocol 1 vs. protocol 2) as the between-groups factors. Unless otherwise stated, significant main effects were followed by Tukey post hoc tests. A priori planned comparisons investigated treatment differences in the percentage of time spent freezing when context and tone cues were presented on day 1 in comparison to day 2. This was accomplished using two separate one-way ANOVAs with treatment (40mg/kg CORT vs. 5mg/kg CORT vs. vehicle) as the between groups factor and a Bonferroni correction (significance level set to $p \leq .025$). The body weight data were analyzed using a two-way mixed design ANOVA with treatment (40mg/kg CORT vs. 5mg/kg CORT vs. vehicle) as the between-subjects variable and injection day (1, 7, 14, and 21) as the within-subjects variable. Simple effects were determined using one-way ANOVAs with a Bonferroni correction (significance level set to $p \leq .016$). Contrasts were conducted using Tukey post hoc tests. Significance for all comparisons was set to $p \leq .05$.

3. Results

3.1 Training Day

The CORT injections did not have a significant effect on behaviour during training (see Figure 3-2). We scored behavior during the 180 s habituation period separately from the period during which tone-shock pairings were presented. There were no significant differences among the groups in the percentage of time spent freezing during the 180s habituation period [$F(2, 39) = 0.375$, $p = .690$, see Figure 3-2A] or during the tone-shock pairings [$F(2, 39) = 2.1$, $p = .136$, see Figure 3-2B].

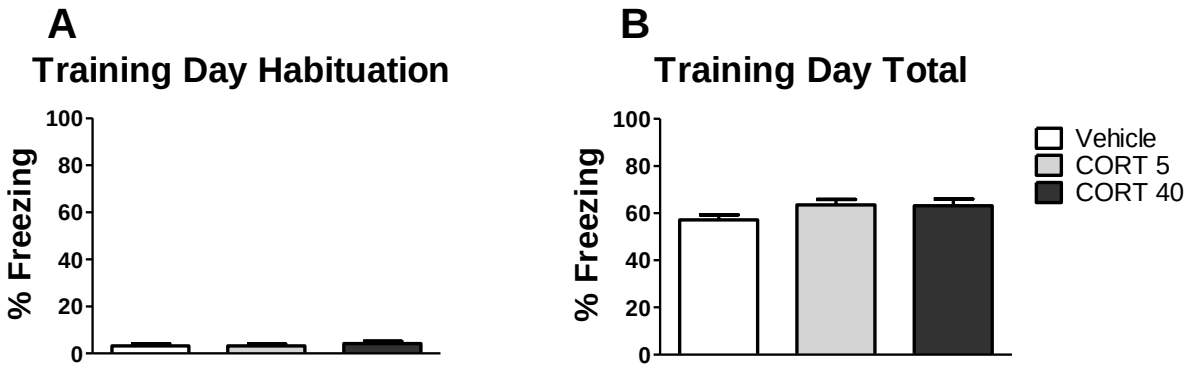

Figure 3-2. The effect of CORT on freezing behaviour during delay fear conditioning training. Panel A shows the mean percentage of time spent freezing during the 3 min habituation period. Panel B shows the mean percentage of time spent freezing over the entire training session [vehicle group, n = 14; CORT 5 group, n = 14; CORT 40 group, n = 14]. Error bars denote the S.E.M.

3.2 Context Testing

In contrast to the training day, the CORT injections did have dose-dependent effects on behaviour during context testing (see Figure 3-3). Our analyses revealed a significant main effect of treatment on the percentage of time spent freezing [$F(2, 36) = 4.738$, $p = .015$; see Figure 3A]. Post hoc analyses further revealed that the CORT 40 rats froze for a significantly greater percentage of time compared to the vehicle rats ($p = .017$). All other between group comparisons were non-significant [all p values ≥ .259].

The order of testing significantly affected the percentage of time rats spent freezing to contextual cues (see Figure 3B). All rats regardless of treatment spent a greater percentage of time freezing to contextual cues when these cues were presented on testing day 1 [$F(1, 36) = 24.253$, $p < .001$; see Figure 3B]. The treatment x fear conditioning protocol interaction was non-significant [$F(2, 36) = 1.798$, $p = .180$]. A priori planned comparisons further probed the difference between protocol 1 and protocol 2. Each protocol was analyzed separately with one-way ANOVAs to isolate the effect of treatment on percent freezing to contextual cues. When contextual cues were presented on testing day 1, the main effect of treatment was not significant [$F(2, 19) = 2.696$, $p = .093$]. However, when contextual cues were presented on testing day 2, the day after testing to tone cues, the main effect of treatment was significant [$F(2, 17) = 5.741$, $p = .012$], with the CORT 40 rats freezing significantly more than the vehicle rats ($p = .036$) and CORT 5 rats ($p = .018$).

3.3 Tone Testing

Freezing behaviour was analyzed for the 3 min period prior to the presentation of the first tone to determine whether the CORT injections had an effect on behaviour in a novel environment. There were no treatment effects on freezing during this time [$F(2, 36) = 1.2$, $p = .313$, see Figure 3-4A]. However, we did find a main effect of fear conditioning protocol on percentage of time spent freezing [$F(1, 36) = 11.997$, $p = .001$, see Figure 3-4A] during the first

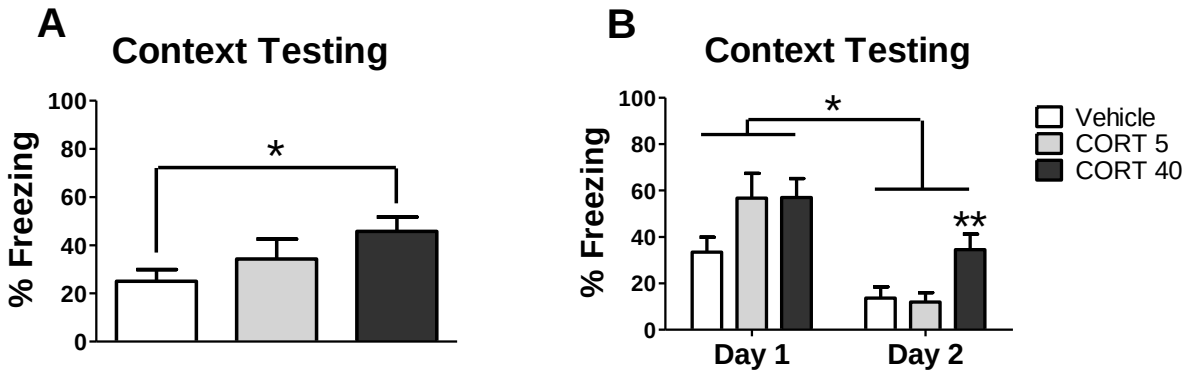

Figure 3-3. The effect of CORT dose and fear conditioning protocol on freezing behaviour during recall of contextual cues. Panel A shows the mean percentage of time spent freezing for each treatment group [vehicle group, n = 14; CORT 5 group, n = 14; CORT 40 group, n = 14]. Asterisk denotes a significant difference between vehicle and CORT 40 rats ($p < .05$). Panel B shows the mean percentage of time spent freezing for each treatment group when recall of context cues was tested one day after training (protocol 1) vs 2 days after training (protocol 2) [vehicle group day 1, n = 8; vehicle group day 2, n = 6; CORT 5 group day 1, n = 7, CORT 5 group day 2, n = 7; CORT 40 group day 1, n = 7; CORT 40 group day 2, n = 7]. Asterisk denotes a significant difference between protocol 1 vs protocol 2 ($p < .05$). Double asterisk denotes a significant difference between the CORT 40 and both the CORT 5 and vehicle treated groups ($p < .05$). Error bars denote the S.E.M.

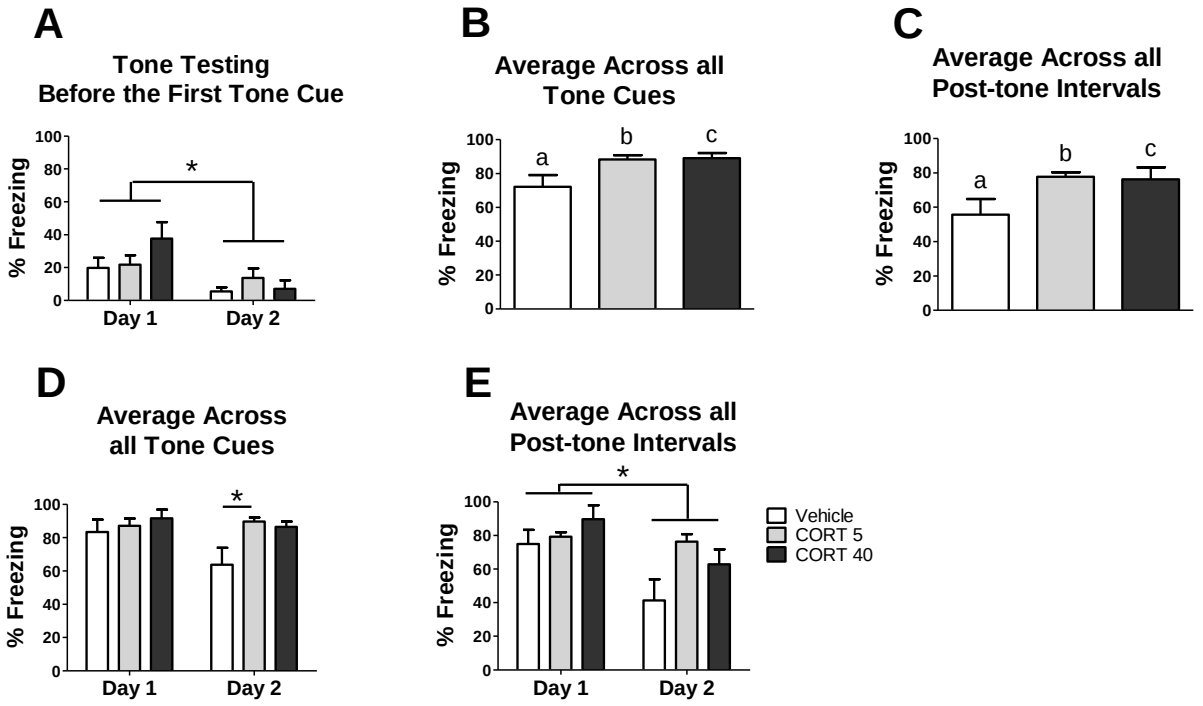

Figure 3-4. The effect of CORT dose and fear conditioning protocol on freezing behaviour during recall of tone cues. Panel A shows the mean percentage of time spent freezing for each treatment group during habituation to the novel tone testing environment when recall of tone cues was tested one day after training (protocol 1) vs 2 days after training (protocol 2) [vehicle group day 1, n = 8; vehicle group day 2, n = 6; CORT 5 group day 1, n = 7, CORT 5 group day 2, n = 7; CORT 40 group day 1, n = 7; CORT 40 group day 2, n = 7]. Asterisk denotes a significant difference between protocol 1 vs protocol 2 ($p < .05$). Panel B shows the mean percentage of time spent freezing for each treatment group during tone cue presentations [vehicle group, n = 14; CORT 5 group, n = 14; CORT 40 group, n = 14]. ^{a}vs^{b} indicates a significant difference between the CORT 5 and vehicle treated groups ($p < .05$); ^{a}vs^{c} indicates a significant difference between the CORT 40 and vehicle treated groups ($p < .05$). Panel C shows the mean percentage of time spent freezing for each treatment group during the intervals between tone cue presentations. ^{a}vs^{b} indicates a significant difference between the CORT 5 and vehicle treated groups ($p < .05$); ^{a}vs^{c} indicates a significant difference between the CORT 40 and vehicle treated groups ($p < .05$). Panel D shows the mean percentage of time spent freezing for each treatment group during tone cue presentations one day after training (protocol 1) vs 2 days after training (protocol 2). Asterisk denotes a significant difference between the vehicle and CORT 5 treated groups ($p < .05$). Panel E shows the mean percentage of time spent freezing for each treatment group during the interval between tone cue presentations one day after training (protocol 1) vs 2 days after training (protocol 2). Asterisk denotes a significant difference between protocol 1 vs protocol 2 ($p < .05$). Error bars denote the S.E.M.

3 minutes of testing. Regardless of treatment, rats showed significantly more freezing behaviour if they were tested in a novel environment the day after training (protocol 2) as opposed to two days after training (protocol 1). The treatment x fear conditioning protocol interaction during the first 3 minutes of testing was non-significant [$F(2, 36) = 1.73$, $p = .191$].

The CORT injections produced a significant increase in freezing behaviour during recall of tone cues in a novel environment (see Figure 3-4). To investigate freezing behaviour, all tones and intervals between tones during testing were summed and averaged. A significant main effect of treatment was found for percentage of time spent freezing during tone cues [$F(2, 36) = 3.728$, $p = .034$; see Figure 3-4B]. Post hoc analyses revealed that this effect was driven by a significant increase in the percent of time spent freezing by the CORT 5 ($p = .04$) and CORT 40 ($p = .031$) rats compared to the vehicle rats. A significant main effect of treatment was found for percentage of time spent freezing during the intervals between tone cues [$F(2, 36) = 3.256$, $p = .05$; see Figure 3-4C]. Post hoc analyses show that both the CORT 5 ($p = .036$) and the CORT 40 ($p = .05$) rats had significantly increased freezing during the intervals between tones.

The percentage of time spent freezing during tone presentation was not significantly affected by testing day [$F(1, 36) = 2.018$, $p = .164$; see Figure 3-4D]. However, testing day did significantly affect percentage of time spent freezing during the intervals between tones [$F(1, 36) = 9.186$, $p = .004$; see Figure 3-4E] where all groups regardless of treatment froze more during the intervals between tones if testing to tone cues occurred on day 1 as opposed to day 2. All treatment x protocol interactions were non-significant [all p values $\geq .184$]. A priori planned comparisons furthered the investigation of whether treatment effects were mediated by testing protocol. Results revealed that when tone cues are tested on day 2, there was a significant treatment effect on the percentage of time spent freezing during tones [$F(2, 19) = 4.493$, $p = .025$] with a trend in the same direction during the intervals between tones that misses significance with the Bonferroni correction [$F(2, 19) = 3.431$, $p = .053$]. Post hoc tests revealed

that rats injected with 5mg/kg of CORT froze significantly more during tone presentations than vehicle injected rats when tone cues were tested on day 2, (p = .035). The trend for rats to freeze significantly more during the intervals between tone cues tested on day 2 resulted from an increase in freezing by rats injected with 5mg/kg of CORT compared to vehicle rats. Interestingly, results revealed that treatment does not significantly affect percentage of time spent freezing during tone presentation or during intervals between tones if tone testing occurs on day 1 [all p values ≥ .315].

3.4 Body Weight

CORT had significant effects on body weight gain (see Figure 3-5). A two-way mixed ANOVA, with Greenhouse-Geisser correction for violation of sphericity, revealed a significant main effect of day [$F(2, 60) = 161.715$, $p < .001$], a significant effect of treatment [$F(2, 39) = 19.718$, $p < .001$] and a significant day x treatment interaction [$F(3, 60) = 77.310$, $p < .001$]. Simple effects analyses confirmed differences in body weight across treatment groups on days 7, 14, and 21 (all p values < .001). Simple contrasts showed that the CORT 40 rats weighed significantly less than the vehicle and CORT 5 rats on days 7, 14, and 21 (all p values < .001).

4. Discussion

The results of this experiment make three main points. First, we confirmed that repeated CORT administration produces enhanced freezing during recall of both contextual and tone fear associations. Second, we found that only the high dose of CORT enhances freezing to contextual cues, whereas both low and high doses of CORT enhance freezing to tone cues. Third, we showed that the order of presentation of testing to tone cues versus context cues determines the magnitude of enhanced freezing following repeated CORT exposure. These findings are discussed in more detail below.

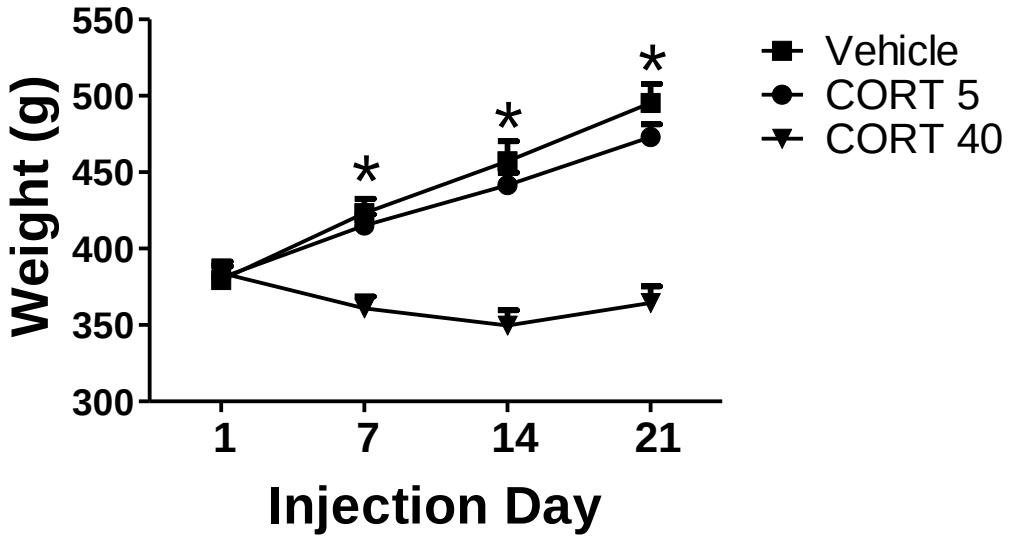

Figure 3-5. The effect of CORT on body weight [vehicle group, n = 14; CORT 5 group, n = 14; CORT 40 group, n = 14]. Asterisks indicate significant differences between CORT 40 and both the vehicle and CORT 5 groups ($p < .001$). Error bars denote the S.E.M.

4.1 Repeated CORT increases freezing to tone and context cues

This experiment clearly demonstrates that repeated exposure to CORT enhances fear related behaviour to both context cues and tone cues associated with noxious foot-shocks. Specifically, the CORT 40 rats froze significantly more to contextual cues than did the vehicle rats. These findings support other research that has found that both repeated stress and CORT exposure enhances freezing or the number of freeze episodes to contextual cues (Conrad et al., 1999; Conrad et al., 2004; Cordero, Kruyt, & Sandi, 2003; Sandi et al., 2001). Interestingly, we only found a significant increase in percentage of time spent freezing in the CORT 40 rats, but not the CORT 5 rats. Other researchers have also found that low doses of CORT dissolved in the drinking water of rats did not increase freezing to contextual cues (Gourley et al., 2009). This dose dependent effect may be predictable given that only high doses of CORT significantly increase depression-like behaviour in the forced swim test (Johnson et al., 2006; Lussier, Romay-Tallon, Kalynchuk, & Caruncho, 2011).

Repeated CORT exposure significantly increased freezing behaviour during tone testing. This effect was significant for both the 5mg/kg and 40mg/kg doses of CORT. Similar results have been reported after 21 days of repeated restraint stress (Conrad et al., 1999) . However, others did not find a significant increase in freezing to tone cues after 21 days of restraint stress (Conrad et al., 2001). Furthermore, repeated CORT dissolved in drinking water did not enhance freezing to tone cues compared to vehicle controls (Conrad et al., 2004). Based on the literature and results from the current study, the effects of repeated stress and CORT exposure on tone fear conditioning appear to be more complex and depend more on both the type of stressor and amount of stress used than contextual fear conditioning. Rats subjected to repeated CORT display more robust increases to tone fear-related behaviour than rats subjected to other repeated stress paradigms such as restraint stress. This effect may not be surprising given that restraint stress also produces variable results in other behavioural tasks, which may be due to potential habituation effects and individual differences in HPA axis response (Gregus et al.,

2005; Grissom & Bhatnagar, 2009). Repeated CORT injection paradigms circumvent these potential problems by consistently ensuring that each animal is consistently exposed to the same amount of glucocorticoid. Interestingly, 5mg/kg of CORT only produced significant enhancements in freezing during recall of tone cues, but not context cues. It is possible that freezing to context related cues may depend more on the dose of CORT (as discussed above) whereas freezing to tone cues may be more dependent on the type of stressor.

It is important to note that the effects of repeated CORT exposure observed in the current study are likely not the result of an increased sensitivity to pain experienced by the CORT-injected rats. Freezing behaviour was analyzed during training to determine whether repeated CORT exposure significantly enhanced reactivity to foot-shock. No significant differences were found between groups implying that both CORT and vehicle treated rats reacted similarly to foot-shock. This finding concurs with other reports showing that repeated CORT treatment neither enhances reactivity to foot-shock (Conrad et al., 2004) nor sensitivity to foot-shock (Dagnino-Subiabre et al., 2012). Although previous work in our lab has shown that the repeated CORT protocol used in this study does not increase anxiety-like behaviour or change locomotor behaviour in the open field test (Gregus et al., 2005; Marks et al., 2009), other researchers have found that acute CORT injections increase locomotion in a novel environment (Sandi, Venero, & Guaza, 1996). Therefore, we thought it would be prudent to investigate possible group differences in freezing behaviour when rats were initially exposed to a novel environment. Our CORT-treated rats did not show enhanced freezing behaviour in a novel environment prior to tone presentation or when first exposed to the fear conditioning chambers on training day. Thus, we are confident that changes in freezing behaviour on the testing days is attributable to the fear association itself and not changes in locomotion or enhanced fear resulting from anxiety in a novel environment.

4.2 Order of tone versus context testing matters

One purpose of this experiment was to determine whether order of testing affects the degree to which stress-induced changes during the recall of fear memories are detectable. The few previous studies investigating the effects of repeated stress or CORT exposure on freezing behaviour to both contextual cues and tone cues in the same animals have reported inconsistent findings. This makes it difficult to draw conclusions about the ways that repeated stress or glucocorticoid exposure might affect these forms of learning and memory. We hypothesized that the variety of testing protocols used to assess the effects of stress or repeated CORT exposure on freezing behaviour could account for variability in past studies. Previous researchers have acknowledged the confounding effects that order of cue presentation can have on testing day behaviour and have attempted to avoid these effects in a number of ways. In light of this, some researchers believe that testing to context cues should be done before testing to tone cues to reduce any lingering associations the context cues may have with the tone cue, even when tone cues are tested for in a novel context (Marchand et al., 2007). Testing fear behaviour to contextual cues before tone cues has been a methodology adopted by many researchers (e.g., (Bast, Zhang, & Feldon, 2003; Sui, Wang, Liu, Wang, & Li, 2006; Toledo-Rodriguez & Sandi, 2007; Kosten, Lee, & Kim, 2006; Pugh, Fleshner, & Rudy, 1997); however, testing to tone cues before contextual cues is common among other researchers (Quinn et al., 2002). Surprisingly, researchers have not counterbalanced testing to context cues and tone cues when studying freezing behaviour to both types of cues in the same animal.

The results of this experiment indicate that the order of presentation of context cues versus tone cues on testing days is a key factor for assessing the effects of repeated CORT treatment during retrieval of fear memories. Specifically, we showed that the CORT 40 rats froze more to context cues compared to the CORT 5 and vehicle rats when the context cues were presented on testing day 2, the day after testing to tone cues. In addition, we also showed that the CORT 5 rats froze more to tone cues when these cues were presented on testing day 2,

the day after testing to context cues. From these data, two important conclusions can be made. First, in this study, habituation to cues associated with foot-shock appear to help tease apart the subtle effects produced by repeated glucocorticoid exposure. Training protocols similar to the one used in the present experiment have been used to elucidate the effects of stress and corticosterone exposure on fear associated behaviour (i.e., consisting of 5 tone and foot-shock pairings at 0.5mA or higher) (Guijarro et al., 2007; Marchand et al., 2007; Morrissey, Mathews, & McCormick, 2011). Despite the frequent use of similar protocols, the training protocol used in this study may produce freezing levels that are too high to reveal the subtle enhancing effects of repeated CORT exposure without habituation to shock associated cues. It is possible that with habituation to shock associated cues prior to training the effects of repeated CORT may be seen to context or tone cues when tested the day after training (day 1). Another potential strategy that might reveal significant effects of repeated CORT on freezing the day after training is to under-train all animals to ensure stressed or CORT treated animals have room to show enhanced freezing. However, restraint stress rats both pre-exposed to the fear conditioning context prior to training and under-trained did not demonstrate a significant difference between stressed and non-stressed rats when testing for freezing to context cues the day after training (Conrad et al., 2001). Admittedly, there is variation in freezing levels between studies occurring in the same lab, using the same protocol, and under the same treatment conditions indicating that fear-associated behaviour is in its nature subject to fluctuation (Conrad et al., 2004; Cordero et al., 1998; Pugh et al., 1997). Based on the literature and the current study it seems that a complex interaction between numerous factors such as habituation, training protocol, treatment conditions, and likely natural variability between studies determines the influence repeated stress or CORT exposure has. It is noteworthy then that the largest effect of treatment found in the current study was comparing the effects of repeated CORT across both protocol types. With the degree of variation found in the literature between protocols and inherent variability seen in fear conditioning itself, the current study shows that counterbalancing

protocols is the way to ensure that valid conclusions are made regarding the effects of repeated stress and glucocorticoid exposure on fear conditioning. Thus the second conclusion that can be made from this study is that without counterbalancing protocols, misinterpretation about the effects of repeated glucocorticoid exposure on the circuits mediating fear-associated behaviours could be made.

4.3 Conclusions

Repeated CORT injections produced changes in freezing behaviour to both contextual and tone associated cues. The repeated CORT injection paradigm used in this study is known to produce robust changes in depression-like behaviour typically leading to a depressive phenotype in rats (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004; Marks et al., 2009). Behavioural measures of depression-like behaviour in rats have focused on tasks such as the forced swim test and sucrose preference test to demonstrate changes in helplessness behaviour and anhedonia. However, depression is a multifaceted disorder that produces symptoms beyond those related to mood such as enhancements in fear associated memory (Nissen et al., 2010) and recall of information with a negative emotional connotation (Leppanen, 2006). Fear conditioning has typically been used to study post-traumatic stress disorder, anxiety related behaviour, and the mechanisms of emotional memory. However, we are proposing that fear conditioning may also be a useful tool in the measure of depression-like behaviour provided that researchers are careful to control for variation in results occurring from order effects.

CHAPTER 4

Repeated Corticosterone Injections Increase Freezing Behaviour and Amygdalar Fos Expression in Response to Recall of Contextual Fear Memories

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Author contributions: WNM designed research, collected data, analyzed data, and co-wrote paper, BDK collected data, LEK designed research and co-wrote paper.

ABSTRACT

Repeated stress and glucocorticoid exposure produce enhancements in fear-related behaviour during contextual fear conditioning, a hippocampal-dependent task. These enhancements are found despite hippocampal atrophy produced during repeated stress. One theory is that the amygdala is mediating the effects of repeated stress and glucocorticoid exposure during fear conditioning. This study examined whether repeated CORT injections increased expression of the neuronal activity marker Fos, in specific areas of the hippocampus and amygdala in response to recall of contextual or tone cues associated with delay fear conditioning. Male Long-Evans rats received 40 mg/kg of CORT or vehicle injections for 21 consecutive days. Rats were then trained and tested in a delay fear conditioning task. On the final day of testing, half the rats were tested for recall of context cues while the other half were tested for recall of tone cues in a novel environment. We found that the CORT injections significantly increased fear-related behaviour to contextual cues, but not tone cues on the final day of testing. We also found significant increases in Fos+ cells in both the central and basolateral nuclei of the amygdala, but not in any areas of the hippocampus. CORT had no significant effect on fear-related behaviour to tone cues or Fos+ cells in any of the examined areas. These results suggest that enhanced amygdala activity could be involved in the effects of CORT on recall of aversive memories.

1. Introduction

Depression is a debilitating psychiatric illness affecting approximately 11% of Canadians at some point during their lifetime (Patten et al., 2009). The social burden of this disorder is severe resulting in significant financial drain associated with diminished worker productivity (Nemeroff, 1998). To reduce the potential impact of this disorder, researchers have developed numerous animal models of depression in an attempt to understand the neural mechanisms underlying this disorder and potentially develop novel therapeutics. Repeated stress models of depression have been successful at recapitulating many of the core symptoms of this disorder. In particular, repeated injections of glucocorticoids, the natural hormone released in response to a stressor (cortisol in humans, and corticosterone (CORT) in rodents), result in robust increases in learned helplessness behaviours, cognitive deficiencies, and anhedonia in rodents (Nestler et al., 2002a; Johnson et al., 2006; Marks et al., 2009; Olausson, Kiraly, Gourley, & Taylor, 2013; Walesiuk & Braszko, 2010). Therefore, evidence suggests that repeated exposure to high levels of glucocorticoids mimics symptoms characteristic of depression in rodents. Given the severity of depression, it is important to understand specifically how elevated glucocorticoids affect plasticity (neurobiology), where these neurobiological changes take place, and how behaviour is influenced by these neurobiological changes.

Measures of depression-like behaviour in rats have focused on understanding changes in mood, appetite and cognitive deficits; however, depression is a complex disorder that is also linked with enhancements in negative emotion associated memories. For instance, in humans diagnosed with depression, enhancements are found in fear associated memory (Nissen et al., 2010) and recall of information with a negative emotional connotation (Leppanen, 2006). Fear conditioning is a test that assesses the ability to associate neutral cues such as a tone or environmental (contextual) cues with an aversive experience such as a mild foot shock. This task affords researchers the ability to explore the processing of negative emotional memoires in animals. In one variation of fear conditioning, delay fear conditioning, a tone and shock are

paired and co-terminate. Delay fear conditioning is particularly useful in investigating emotion regulating circuits as this task produces two associations with the unconditioned stimulus, one to the tone and the other to the context of the conditioning apparatus. The conditioning of fear associations to contextual and tone cues are processed by different neural pathways with tone conditioning being dependent on the amygdala and contextual conditioning being dependent on both the hippocampus and amygdala (Phillips & LeDoux, 1992). The hippocampus and amygdala are abundant in glucocorticoid receptors (Feldman & Weidenfeld, 1999; Morimoto et al., 1996) making these structures particularly susceptible to the effects of repeated glucocorticoid exposure. Rats repeatedly exposed to high levels of glucocorticoids have decreased dendritic branching in CA3 pyramidal cells, decreased neurogenesis, and decreased mossy-fiber synapses (Sousa et al., 2000; Watanabe et al., 1992; Woolley et al., 1990). The amygdala also shows altered morphology after repeated stress in rats; however, contrary to the hippocampus, enhanced dendritic branching and spine density are observed (Vyas et al., 2002; Vyas et al., 2006). Delay fear conditioning provides the opportunity to examine the association between plasticity resulting from repeated glucocorticoid exposure and altered functioning of fear-related memory circuits.

Repeated stress and excessive glucocorticoid exposure produce consistent enhancements in freezing behaviour, a rodent's natural defensive behaviour (Blanchard & Blanchard, 1969), to contextual cues (Sandi et al., 2001; Skorzewska et al., 2006). Less consistent enhancements in freezing behaviour to tone cues are found following repeated stress or glucocorticoid exposure as some studies have found enhancements in freezing (Conrad et al., 1999), whereas others have not (Conrad et al., 2001). One intriguing finding is that despite hippocampal atrophy resulting from repeated stress, rats still show enhanced freezing behaviour to both contextual and tone cued conditioning (Conrad et al., 1999). This has lead to the hypothesis that the amygdala may be overriding the influences of the hippocampus on delay fear conditioning (Conrad et al., 1999).

Few researchers have begun to examine the neural circuits responsible for mediating the effects of repeated glucocorticoid exposure on fear-related memory. One study has examined Fos immunoreactivity in the hippocampus and amygdala in rats repeatedly injected with 20mg/kg of CORT following contextual fear conditioning (Skorzewska et al., 2006). It was shown that an increase in Fos+ cells in the central amygdala (CeA) as well as a decrease in Fos+ cells in the dentate gyrus was associated with enhanced freezing following repeated glucocorticoid injections (Skorzewska et al., 2006). This study suggests that both the hippocampus and amygdala have a strong role in mediating the effects of repeated glucocorticoid exposure to an aversive contextual conditioning task. However, the lower dose of CORT used in the Skorzewska et al. study may not produce the degree of hippocampal dendritic atrophy or increased amygdalar dendritic growth as higher doses (e.g., 40 mg/kg CORT). In addition, the 40mg/kg, but not 20mg/kg dose of repeated CORT is required to produce depression-like behaviours in the forced swim test (Johnson et al., 2006). Therefore, it remains unclear whether the amygdala would override the influence of the hippocampus in a repeated glucocorticoid paradigm that produces robust changes in depression-like behaviour. Also, it is unknown whether similar patterns of neural activation after repeated glucocorticoid exposure would occur following tone cue conditioning. Therefore, the main objective of the current study was to determine the changes in Fos activation associated with repeated injections of 40mg/kg of CORT following both contextual and tone cued fear conditioning.

2. Materials and methods

2.1. Animals

For this study, 28 adult male Long-Evans rats (obtained from Charles River Canada, Montreal, Quebec) were used. Rats weighted between 215-250g on arrival. The rats were individually housed in standard polyproplylene cages in a colony room maintained at a temperature of 21°C on a 12 hr light/dark cycle (lights on at 07:00 hr). All rats had free access to

Purina rat chow and water. Experimental procedures were conducted during the light phase and were carried out according to the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care and Supply.

2.2 Repeated CORT injections

Injections and handling of rats occurred in a procedure room separate from the colony room. Rats were handled daily for 7-14 days prior to injections to habituate them to both the vivarium and researchers. On the completion of handling, rats were assigned to one of two experimental conditions, 40mg/kg CORT (n = 14), and vehicle (n = 14), controlled for body weight. Injections were prepared at a volume of 1ml/kg and were administered subcutaneously once per day for 21 consecutive days between 09:00 hr and 11:00 hr. CORT (Steraloids Inc., Newport, RI) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitan monooleate (Tween-80; VWR International, West Chester, PA). The dose of CORT used for this study, 40mg/kg, in known to consistently increase depression-like behaviour in the forced swim test (Gregus et al., 2005; Kalynchuk et al., 2004; Marks et al., 2009).

2.3. Overall experimental design

After 21 days of CORT or vehicle injections, rats were assigned to one of two protocols. Rats assigned to protocol 1 were conditioned to contextual and tone cues on day 22 (training day), and then were tested for recall of contextual cues on day 23 (context testing) and then for recall of tone cues on day 24 (tone testing). For rats assigned to protocol 2, rats received the same conditioning to contextual and tone cues on day 22, but they then were tested for recall of tone cues on day 23 and contextual cues on day 24. The counterbalancing of contextual versus tone cue presentation was designed to assess whether order of cue presentation significantly changed the magnitude of CORT-induced changes in freezing behaviour and is reported in Chapter 3 of this dissertation. To examine the objectives of this study, only freezing behaviour

on testing day 24 (tone testing in protocol 1 rats, and context testing in protocol 2 rats) is presented and analyzed.

2.4. Fear conditioning

2.4.1. Apparatus. Training was performed in two identical video fear-conditioning chambers (25.5 cm X 32 cm X 25.5 cm) that were attached to a computer that controlled all shock and tone presentations and recorded behaviour (Med Associates, St. Albans, VT). The floor of each chamber contained 19 stainless steel rods that were wired to a shock generator and scrambler that was used to deliver foot-shocks as the unconditioned stimuli. The ceiling of each of the chambers contained a small speaker that allowed for the presentation of tones as the conditioned stimuli. Urine and fecal matter was collected by a stainless steel pan covered by two sheets of paper towel that was inserted beneath the grid floor. The entire chamber was inserted inside a sound-attenuating cubicle (73 cm X 64 cm X 42 cm) and was lit by a fluorescent lamp inside the cubicle.

2.4.2. Training. On day 22 of the experiment, 24 hr after the last CORT injection, rats were transported in their home cages placed on trolley to the conditioning room. Each rat was placed individually into one of the fear conditioning chambers and was given 180 s to acclimatize to the contextual surroundings of the chamber. Each rat then received 5 tone-shock pairings. Each tone lasted 20 s (volume of 90 dB, frequency of 2 kHz, and a rise time of 50 ms) and co-terminated with a 2 s 0.7 mA foot-shock. Inter-trial intervals lasted 120 s. The rats remained in the conditioning chamber for 120 s after the last tone-shock pairing before being transported back to the colony room. The chambers were cleaned with 0.4% glacial acetic acid between each training session. Training was conducted between 09:00 hr and 15:00 hr.

2.4.3. Tone Testing in a Novel Context. Recall of learned tone cue associations took place in the same conditioning chambers described above with a number changes made to the contextual details of the chambers and testing room to make the context novel. First, a white plastic platform was placed over the grid rods to change tactile cues and make the floor completely flat. Second, an insert made of plastic containing one red and two blue vertical stripes was inserted into the chamber. Third, black horizontal strips were placed on the outside of the ceiling of the chamber which changed both the appearance of the ceiling and the perceived lighting inside the chamber. Fourth, rectangular and circular shapes cut from cardboard of various colours were placed on the inner walls of the sound attenuating cubicle to create novel visual cues and a white noise generator created background noise within the cubicle (65 dB). Fifth, a blue curtain was draped over the fluorescent ceiling in the conditioning room which created a dim blue light. Also, blue curtains were draped from the ceiling in the conditioning room which aided in changing the colour of the room to blue and changed the shape of the room. Finally, olfactory cues were altered by cleaning the chambers with 75% ethanol instead of 0.4% glacial acetic acid and the conditioning room was scented with vanilla coconut pet deodorizer between trials. Another aspect that made the testing of tone cue associations novel was altering transportation from the colony room to the conditioning room by draping a dark curtain over the home cages of the rats and by changing their route of transportation.

To test for recall of tone cue associations, rats were transported to the conditioning room as described above and then were placed inside the chamber opposite to the one that they were trained in. Once inside the testing chamber, rats were given 180 s to familiarize themselves with the new contextual surroundings. Then rats received 5 tones (same volume, frequency, and rise time as used during training) with an ITI of 120 s. The rats remained in the conditioning chamber for 120 s after presentation of the last tone and were then returned to their

home cages and transported back to the colony room. All testing was performed between 14:00 hr and 17:00 hr.

2.4.4. Contextual fear testing. Rats were transported in their home cages on a trolley to the conditioning room and were placed inside the same chamber that they were trained in. Rats remained in the chambers for a total of 14 min, 40 s stimulus free (the same amount of time the rats remained in the chambers during training). Once testing was complete, the rats were transported back to the colony room. Testing to contextual cue associations were performed between 09:00 hr and 12:00 hr.

2.4.5. Behavioural measures. All behaviours were recorded by video cameras (30 frames/s) mounted to the inside doors of the sound attenuating cubicles. Movement in the chambers was recorded by Video Freeze Software as a change in video pixel composition over time (version 1.16.0.0., Med Associates, St. Albans, VT). Freezing was computed by the Video Freeze Software using a motion threshold; the motion threshold refers to the limit above which all movement is considered non-freezing behaviour. Freezing behaviour was defined as immobility with the exception of respiratory related motion (Maciejak et al., 2003). The motion index of each rat was individually set after the completion of testing as the amount of movement produced during respiration varied from animal to animal. Individual determination of motion index is shown to correlate strongly [$r > 0.9$] with manual scoring techniques. Percentage of time spent freezing was provided by the Video Freeze Software based on a minimum freeze duration of 1 s.

2.5. Body weight

All rats were weighed daily for later analyses.

2.6. Perfusions and immunohistochemistry

On testing day 24, approximately 2 hr after the completion of behavioural testing, rats were anesthetized with an overdose of sodium pentobarbital (240mg/kg; i.p.) and transcardially perfused with room temperature saline followed by chilled 4% (w/v) paraformaldehyde (pH = 7.4. The brains were removed and postfixed in the same fixative used for perfusions for 72 hr at 4°C. Brains were then transferred to 0.01% sodium azide (w/v) dissolved in phosphate buffered saline (PBS; pH = 7.4) and stored at 4°C until sectioning. Brains were sectioned at 50µm on a vibrating microtome (Vibratome 3000, Vibratome Company) and stored at -20°C in a cryoprotectant solution consisting of 30% (v/v) ethylene glycol, 1% (w/v) polyvinylpyrrolidone, and 30% (w/v) sucrose in 0.1 M PBS (pH = 7.4).

The immunohistochemical reaction was performed on coronal sections free-floating under gentle agitation. The specimens were washed six times (6 x 10 min) in 0.1 M PBS (pH = 7.4) then incubated in 0.3% (v/v) H_2O_2 solution to block the activity of endogenous peroxidise. Specimens were then washed again in PBS (3 x 5 min), and incubated in a blocking solution containing 5% (v/v) normal goat serum (NGS), 1% (w/v) BSA, and 0.3% (v/v) Triton X-100 dissolved in PBS for 60 min at room temperature. Subsequently, sections were incubated for 72 hr at 4°C in Fos anti-rabbit monoclonial primary antibody (1:15,000, Calbiochem) in blocking solution. Following (3 x 5 min) PBS rinses, the specimens were incubated with biotinylated goat anti-rabbit IgG secondary antibody (1:500, Vector Laboratories) for 2 hr at room temperature. Specimens were then rinsed in PBS (3 x 5 min) and incubated for 2 hr in avidin-biotin complex (1:500; Vectastain ABC Elite, Vector Laboratories). Finally, after (1 x 5 min) PBS rinse and (3 x 5 min) 0.175 M sodium acetate (pH = 6.8) rinses, specimens were immunoreacted with a solution containing 0.02% (w/v) 3,3'-diaminobenzidine (DAB), 2.5% (w/v) nickel ammonium sulfate and 0.002% (v/v) H_2O_2. After sufficient staining, the reaction was halted by washing in (3 x 5 min) sodium acetate rinses followed by (1 x 5 min) PBS rinse. The specimens were then transferred to room temperature 0.1 M phosphate buffer (PB) and mounted onto glass slides

which were left to air dry overnight. The slides were then dehydrated by serial alcohol rinses, cleared in xylene, and coverslipped in Permount Mounting Medium (Fisher Scientific).

2.7. Quantification

A researcher blind to experimental conditions examined the sections using a computerized stereology system (StereoInvestigator V 9.0, MicroBrightfield Inc.). The computerized system was connected to a digital camera (MicroFire, Optronics) atop a Nikon Eclipse E800 light microscope with a motorized stage. Six rats from each treatment and protocol group were randomly chosen for immunohistochemical analyses. Fos+ cells were counted in every sixth section throughout both the left and right hippocampus (5 sections total) and amygdala (3 sections total). Only cells with dark, uniformly stained and well defined somas were counted as Fos+. Hippocampal sections were counted between -2.4 to -4.56 relative to bregma, and amygdala sections were counted between -1.8 to -3.36 relative to bregma (Paxinos & Watson, 2009). The granule cell layer (GCL), hilus, CA1, CA3, lateral amygdala (LA), basolateral amygdala (BLA), and central amygdala (CeA) were traced using a 4x objective. The number of Fos+ cells within each traced area was manually counted at 40x magnification using a meander scan profile counting method. The density of Fos+ cells were computed by dividing the total number of Fos+ cells by the total cross-sectional area (Knapska & Maren, 2009). The resulting densities were expressed as the number of Fos+ cells per mm^2.

2.8. Statistical analyses

All data were analyzed using the Statistical Package for the Social Sciences (Chicago, IL, version 18.0). Group differences in freezing behaviour were analyzed using separate *t*-tests (40mg/kg CORT vs. vehicle). *T*-tests were also used to determine group differences in Fos immunoreactivity for each area examined. Group differences in body weight gain were assessed using a mixed design ANOVA with treatment (40mg/kg CORT vs. vehicle) as the

between-subjects variable and injections day (1, 7, 14, and 21) as the within-subjects variable. Contrasts were used to break down significant interactions. Significance was set at $p \leq .05$.

3. Results

3.1 Training Day

Analyses showed that the CORT injections did not have a significant effect on freezing behaviour during training, see Figure 4-1. Freezing behaviour during the 180 s habituation period was analyzed separately to determine whether CORT injections had a significant effect on freezing behaviour during the rat's first exposure to the conditioning chambers. The CORT injections did not significantly affect the percentage of time spent freezing during the acclimatization period ($t(26) = -0.730$, $p = .472$; see Figure 4-1A). Behaviour during the entire training session was also analyzed to determine whether repeated CORT exposure significantly affected freezing behaviour during acquisition. Analyses showed that CORT did not have a significant effect on the total percentage of time spent freezing ($t(26) = -1.730$, $p = .096$; see Figure 4-1B).

3.2 Context Testing

The CORT injections had a significant effect on freezing behaviour to contextual cues, see Figure 4-2A. Repeated CORT significantly increased the percentage of time spent freezing [$t(11) = -2.475$, $p = .031$] during testing to contextual cues.

3.3 Tone Testing

Fear-related behaviours during the 3 min habituation period prior to the first tone presentation were analyzed to determine whether CORT injections affected behaviour in a novel environment. No significant differences were found between CORT or vehicle rats on freezing

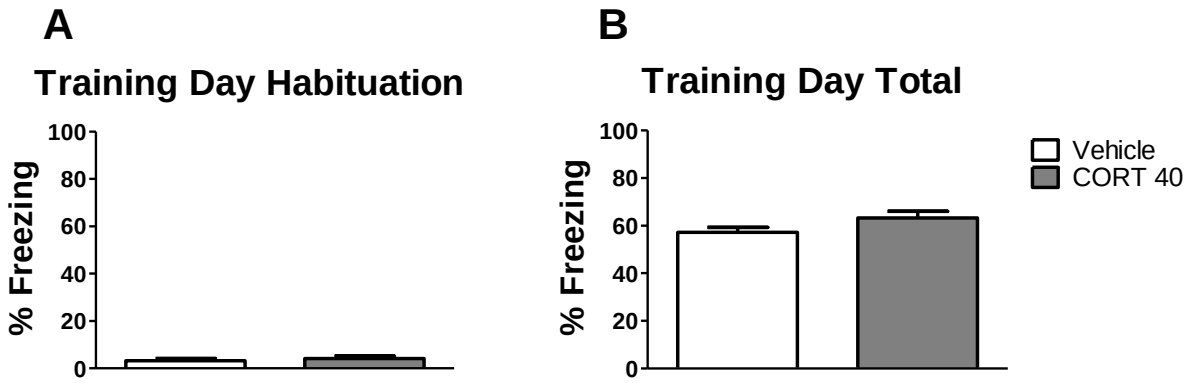

Figure 4-1. The effect of CORT on freezing behaviour during delay fear conditioning training. Panel A shows the mean percentage of time spent freezing during the 3 min habituation period. Panel B shows the mean percentage of time spent freezing over the entire training session [vehicle group, n = 14; CORT group, n = 14]. Error bars denote the S.E.M.

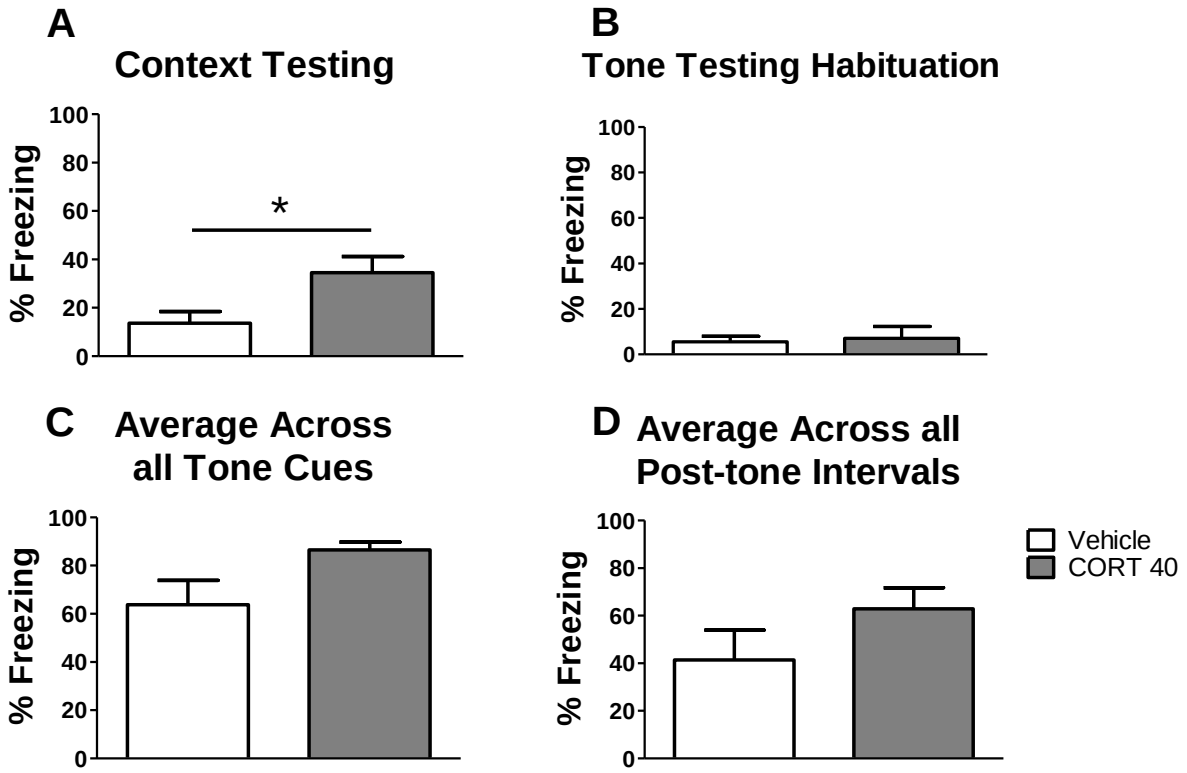

Figure 4-2. The effect of CORT on freezing behaviour during recall of context and tone cues. Panel A shows the mean percentage of time spent freezing during recall of contextual cues for each treatment group [vehicle group, n = 6; CORT group, n = 7]. Asterisk denotes a significant difference ($p < .05$). Panel B shows the mean percentage of time spent freezing during habituation to the novel tone testing environment for each treatment group. Panel C shows the mean percentage of time spent freezing for each treatment group during tone cue presentations. Panel D shows the mean percentage of time spent freezing for each treatment group during the intervals between tone cue presentations [vehicle group, n = 8; CORT group, n = 7]. Error bars denote the S.E.M.

behaviour prior to the first tone presentation on the testing day [$t(13) = -0.278$, $p = .785$; see Figure 4-2B].

Behaviour during all tones and all intervals between tones were summed and averaged for analysis. CORT did not significantly increase fear-related behaviour to tones or the intervals between tones (see Figure 4-2C,D), although there was a trend for CORT to increase the percentage of time spent freezing during tone cues [$p = .065$].

3.4 Fos Immunoreactivity

3.4.1. Contextual Testing. Analyses revealed a significant increase in the number of Fos+ cells in the CeA [$t(9) = -2.319$, $p = .046$; see Figure 4-4A] and the BLA [$t(9) = -2.226$, $p = .05$; see Figure 4-4B] in CORT rats following contextual fear testing, see Figure 4-3 for representative photomicrographs. No significant differences in the number of Fos+ cells were found in the LA, CA1, CA3, hilus, and GCL between CORT and vehicle rats [all p values ≥ .110; see Figures 4-4C-G]. See Figure 4-5 for representative photomicrographs of hippocampus.

3.4.2. Tone Testing. No statistically significant differences were found in the number of Fos positive cells in any of the brain areas examined following recall of tone cue associated fear [all p values ≥ .07; see Figure 4-6].

3.5 Body Weight

CORT significantly slowed the typical increase in body weight over time, see Figure 4-7. A two-way mixed design ANOVA, with Greenhouse-Geisser correction for violation of sphericity, showed a significant main effect of day [$F(2, 40) = 55.424$, $p < .001$] which is qualified by a significant day x treatment interaction [$F(2, 40) = 114.533$, $p < .001$]. These results indicate that rats gained weight over time, but that the change in weight was dependent on whether rats were injected with vehicle or CORT. Contrasts were performed to compare the weight of vehicle and CORT injected rats from injection day 1 to 7, day 7 to 14, and day 14 to 21.

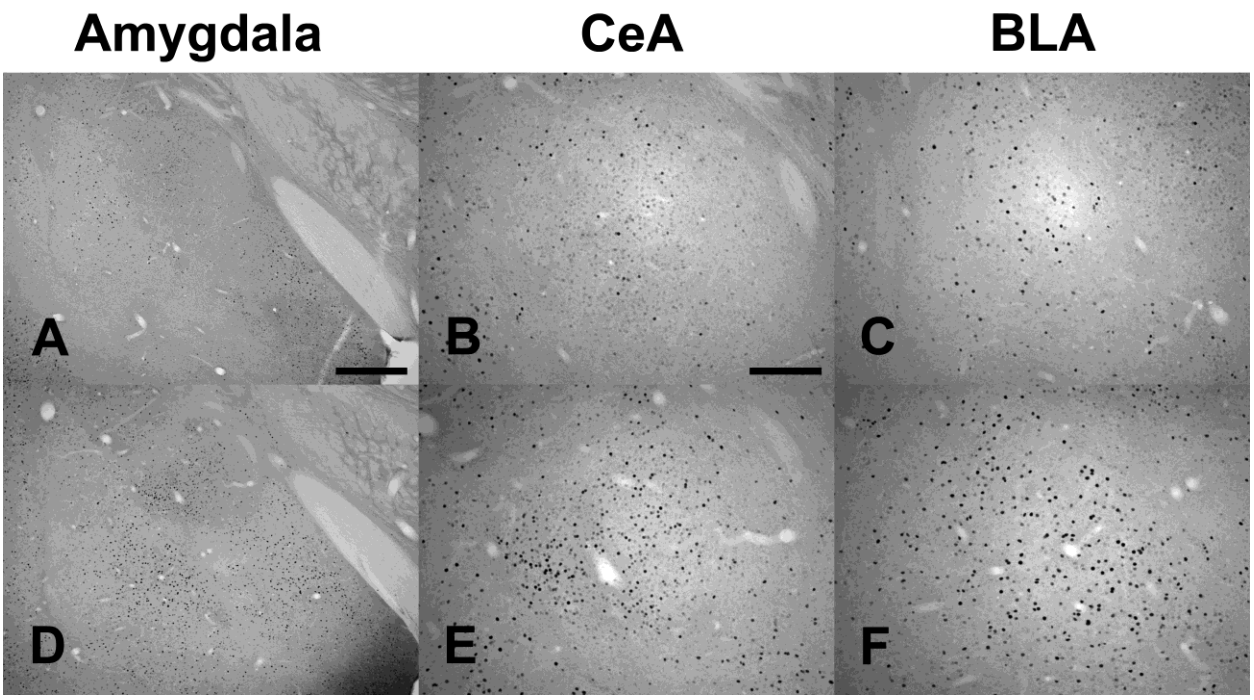

Figure 4-3. Representative photomicrographs of the effect of CORT on Fos expression in the amygdala following recall of contextual cues. (A) Photomicrograph demonstrating the pattern of Fos expression in the amygdala in vehicle injected rats. Scale bar: 500 µm. (B-C) Pattern of Fos expression in the central amygdala (CeA) and basolateral amygdala (BLA) in vehicle rats. Scale bar: 200 µm. (D-F) Photomicrographs demonstrating the pattern of Fos expression in the amygdala (D), CeA (E), and BLA (F) in CORT rats.

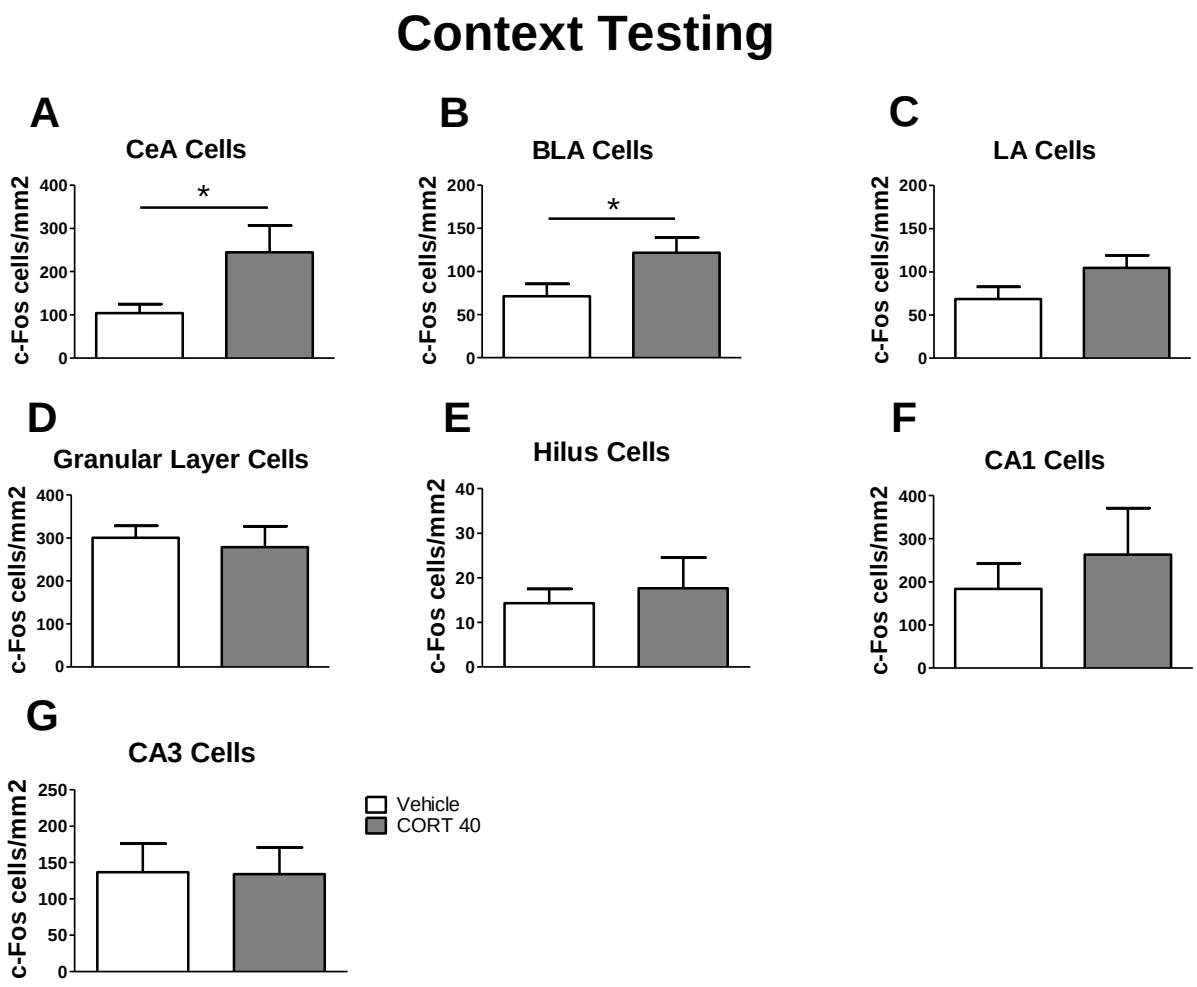

Figure 4-4. The mean number of Fos+ cells in quantified regions of the amygdala (A-C) and hippocampus (D-G) in response to recall of contextual cues for each treatment group [vehicle group, n = 6; CORT group, n = 5]. Asterisks denote a statistically significant difference between vehicle and CORT injected rats ($p \leq .05$). *Abbreviations*: Central amygdala (CeA), basolateral amygdala (BLA), and lateral amygdala (LA). Error bars denote the S.E.M.

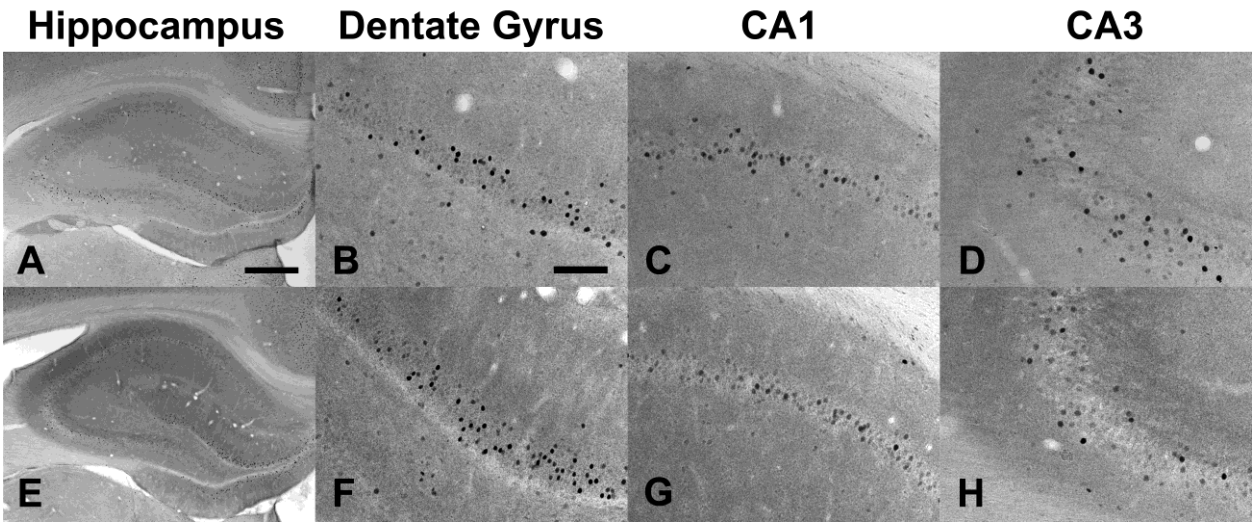

Figure 4-5. Representative photomicrographs of the effect of CORT on Fos expression in the hippocampus following recall of contextual cues. (A) Photomicrograph demonstrating the pattern of Fos expression in the hippocampus in vehicle injected rats. Scale bar: 500 µm. (B-D) Pattern of Fos expression in the granular cell layer, hilus, CA1, and CA3 in vehicle rats. Scale bar: 100 µm. (E-H) Photomicrographs demonstrating the pattern of Fos expression in the hippocampus, granular cell layer, hilus, CA1, and CA3 in CORT rats.

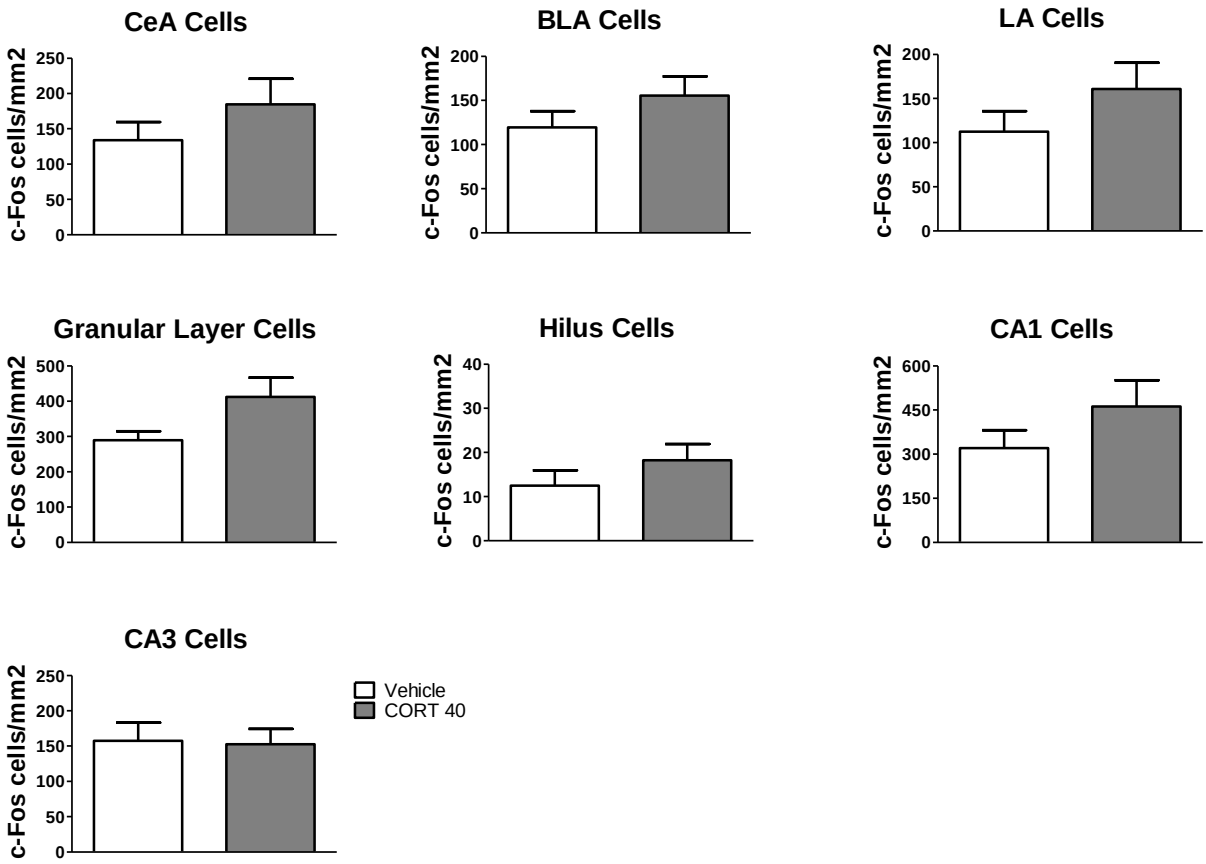

Figure 4-6. The mean number of Fos+ cells in quantified regions of the amygdala and hippocampus in response to recall of tone cues for each treatment group [vehicle group, n = 6; CORT group, n = 6]. *Abbreviations*: Central amygdala (CeA), basolateral amygdala (BLA), and lateral amygdala (LA). Error bars denote the S.E.M.

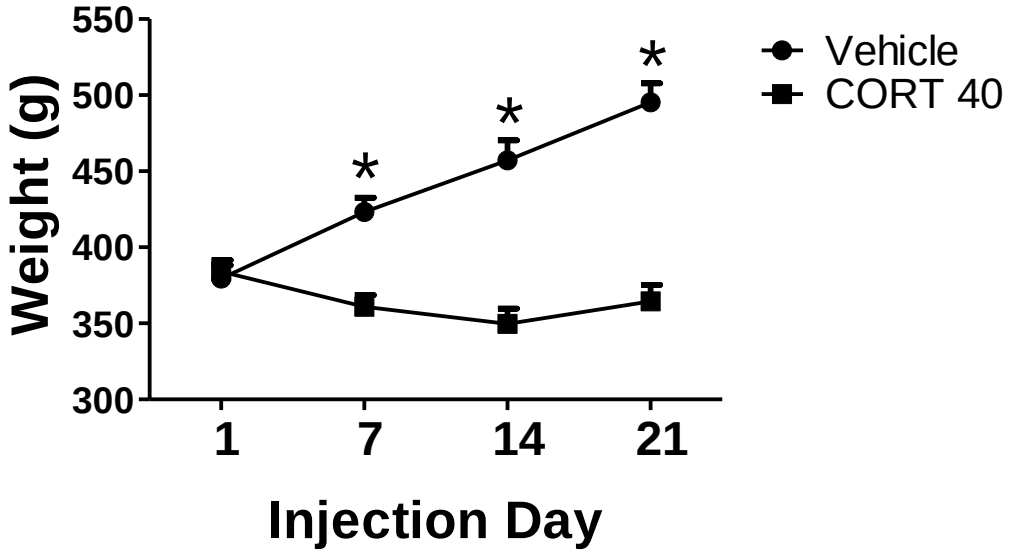

Figure 4-7. The effect of CORT on body weight [vehicle group, n = 14; CORT group, n = 14]. Asterisks indicate significant differences between CORT 40 and the vehicle injected rats ($p < .001$). Error bars denote the S.E.M.

For all contrasts, $p < .001$, indicating that there was a significant difference in the way vehicle injected rats gained weight over time compared to CORT injected rats. Vehicle injected rats had increased weight over time, whereas CORT rats had a stunted change in weight over time.

4. Discussion

This experiment revealed two major findings. First, repeated CORT significantly increased freezing behaviour to contextual cues, but not tone cues on the final day of behavioural testing. Second, CORT also increased Fos expression in both the CeA and the BLA after recall of contextual fear memories, but it did not affect Fos expression in the LA or any areas of the hippocampus investigated. CORT had no effect on Fos expression in any of the brain areas examined following tone cue testing.

4.1. Repeated CORT increases freezing to context tone cues

We found that CORT significantly enhanced freezing behaviours to contextual cues associated with delay fear conditioning. This finding is in agreement with other research that shows enhanced freezing to context cues following repeated stress or glucocorticoid exposure. For example, 21 days of restraint stress (Conrad et al., 1999; Cordero et al., 2003; Sandi et al., 2001), or CORT in the drinking water (Conrad et al., 2004) significantly increased the percentage of time rats spent freezing during recall of contextual fear conditioning. One group of researchers found that repeated exposure to CORT dissolved in the drinking water of rats did not significantly enhance freezing to contextual cues (Gourley et al., 2009); however, a very low dose of CORT was used for this study (1/8 of that which is reported to mimic the endogenous CORT response to stress). Similarly, we found that the percentage of time spent freezing was only significantly increased in rats that were repeatedly injected with 40mg/kg of CORT while a repeated 5mg/kg dose had no significant effect (Marks, Fenton, Guskjolen, & Kalynchuk, 2014a;

in preparation). Therefore, repeated exposure to higher levels of glucocorticoids may be necessary to produce reliable enhancements in contextual fear associated behaviours.

Previous work in our lab has shown that repeated injections of CORT result in HPA axis dysregulation whereby CORT injected rats show blunted increases in CORT following the FST (Johnson et al., 2006). Interestingly, rats subjected to 25 days of 20 mg/kg CORT injections showed decreased serum levels of CORT directly after recall of contextual fear conditioning and enhanced fear-related behaviour during recall (Skorzewska et al., 2006). Similarly, rats that received CORT in their drinking water for 21 days showed enhanced freezing during recall of fear-associated contextual cues and serum CORT levels were negatively correlated with fear behaviours (Conrad et al., 2004). This suggests that the blunted CORT response to a stressful task observed with our repeated CORT injected rats (Johnson et al., 2006) could underlie the changes in freezing behaviour to contextual cues observed in the current study.

4.2. Repeated CORT does not significantly increase freezing to tone cues

We examined the effect of repeated CORT on freezing behaviour to tone cues following delay fear conditioning; unlike contextual cues, freezing behaviour was not significantly enhanced to tone-associated cues. The effects of repeated stress or glucocorticoid exposure on freezing to tone cues following delay fear conditioning are less consistent than the effects on contextual cues. For example, one group of researchers has found enhanced freezing to tone cues in rats exposed to 10 days of CORT in their drinking water (Dagnino-Subiabre et al., 2012). Another group found significantly enhanced freezing to tone cues after 21 days of repeated restraint stress when recall of tone and context cues associations were tested in the same session (i.e. context freezing was considered the interval between tones) (Conrad et al., 1999). However, other researchers have not found enhanced freezing to tone cues following repeated stress and differences in testing protocol is a possible reason for the discrepancy between studies. That is, 21 days of restraint stress did not significantly enhance freezing to tone cues

when these cues were tested after context testing (Conrad et al., 2001; Conrad et al., 2004). Similarly, for the current study, tone cue recall occurred 24 hr after contextual cues recall and we also did not find a significant enhancement in freezing, although there was a trend for CORT injected rats to freeze more than vehicle injected rats during tone cues. These results suggest that the effects of repeated stress and glucocorticoids on learned tone associations are complex and more dependent on factors such as protocol differences than contextual associations.

It should be noted that the effects of repeated CORT injections on freezing behaviour found in this study are likely not the result of an increased sensitivity to pain in CORT-injected rats. Freezing behaviour was analyzed during training to determine whether CORT injections significantly enhanced reactivity to foot-shock. No significant differences were found between CORT and vehicle rats averaged across the training session suggesting similar reactivity to foot-shock overall. This finding agrees with a previous study showing that repeated CORT exposure neither increases reactivity to foot-shock (Conrad et al., 2004) nor sensitivity to foot-shock (Dagnino-Subiabre et al., 2012). Although one group of researchers did find a stress-induced increase in reaction to foot-shock across the entire training session during contextual fear conditioning, these enhancements did not lead to significantly increased freezing during testing (Cordero et al., 2003). Therefore, even when repeated stress does increase reactivity to foot-shock during training, these findings do not explain enhanced freezing behaviour observed during recall. Previous work in our lab has shown that repeated injections of 40mg/kg of CORT do not increase anxiety-like or locomotor behaviours in the open field test (Gregus et al., 2005; Marks et al., 2009); however, it has been shown that acute CORT injections do increase locomotion in a novel environment (Sandi et al., 1996). Therefore, we decided to investigate whether CORT injected rats showed increased freezing behaviour when initially exposed to a novel environment. We found that repeated CORT injections neither increased freezing behaviour when rats were first exposed to the fear conditioning chambers on training day, nor prior to the first tone presentation during recall. Given these findings, we believe the increase in

freezing behaviour found in CORT rats during recall of contextual cues is attributable to the effects of repeated CORT on the fear association itself and not non-specific effects related to increased anxiety or pain reactivity.

4.3. Repeated glucocorticoids enhance Fos expression

In this study, rats repeatedly injected with CORT show significantly increased Fos expression stimulated by the recall of contextual fear-associated cues in a structurally specific way. It is important to note that increases in Fos+ cells only occurred when significant increases in freezing behaviour were also observed. Specifically, aversive context cues increased Fos production in the BLA and CeA, but not the LA or any areas of the hippocampus examined (i.e., GCL, hilus, CA1, and CA3). Similar to our findings, it has been shown that 20mg/kg of CORT injections for 25 consecutive days produced a significant increase in recall of contextual fear-related behaviours and concomitant Fos expression in the CeA, but not CA3, or CA1 of the hippocampus. However, contrary to our findings, enhancements were not found in the BLA and a significant decrease in Fos expression was found in the dentate gyrus (Skorzewska et al., 2006). It is possible that the lower dose of CORT used in this previous research was not sufficient to increase Fos reactivity in the BLA following repeated CORT injections whereas, the CeA may be more sensitive to the enhancing effects of CORT. Interestingly, acute injection of CORT at a dose of both 5mg/kg and 20mg/kg decreased freezing to contextual fear cues and failed to significantly change Fos expression in the CeA or BLA of the amygdala, and CA1, CA3 or dentate gyrus of the hippocampus (Skorzewska et al., 2007). Therefore, significant increases in fear related behaviour following recall of contextual conditioning as well as enhanced Fos immunoreactivity appear to require repeated exposure to glucocorticoids.

The LA, CeA, and BLA are strongly implicated in the processing of fear-associated learning. For instance, neurotoxic lesions to the LA, CeA, and BLA all produced impairments in both contextual and cued fear conditioning (Goosens & Maren, 2001). However, Fos

immunoreactivity following fear conditioning appears to be structurally specific to the BLA and CeA and not the LA. Rats exposed to a shock-paired but not unpaired tone conditioned stimulus showed increased Fos expression in the BLA and CeA, but not the LA or CA1 of the hippocampus (Hall, Thomas, & Everitt, 2001). Also, exposure to an aversive conditioned context increased Fos+ cells specifically in the CeA and BLA (Beck & Fibiger, 1995). The actions of glucocorticoids also appear to mediate fear conditioned behaviour specifically through the CeA and the BLA. In mice, glucocorticoid receptors knocked out in the CeA produced deficits in freezing behaviour during recall of both contextual and cued conditioning with no effect on behaviour during training. In addition, these GR knockout mice showed reduced Fos expression in the CeA and BLA following delay conditioned fear training (Kolber et al., 2008). The GR antagonist, RU 38486, infused into the BLA prior to contextual fear training disrupted retention of fear learning 24hr after training, but did not affect freezing during training (Donley et al., 2005). Similarly, rats exposed to 21 days of CORT in their drinking water and given a BLA infusion of the glucocorticoid antagonist RU 486 prior to delay fear training showed enhanced freezing compared to CORT alone treated rats specifically during recall of contextual cues and not tone cues (Conrad et al., 2004). As discussed above, this suggests that in rats with a history of repeated CORT treatment, a decreased CORT response during training increases freezing to contextual cues during recall. Thus, multiple lines of evidence suggest that the CeA and BLA are particularly involved in mediating the effect of glucocorticoids on fear conditioning with the BLA playing a role specifically in the memory processes of contextual conditioning.

Another possible explanation for the current results is that exposure to repeated CORT injections alone significantly enhance Fos expression in the CeA and BLA. Although we did not investigate the effects of repeated CORT on Fos expression without fear conditioning, it has been shown by other researchers that an acute injection of 20mg/kg of CORT does not significantly modify Fos expression in the amygdala (Skorzewska et al., 2007). Also, in this study, the effects of repeated CORT on Fos expression were limited to specific structures

making a general effect of CORT on Fos expression unlikely. Furthermore, the effects were only significant in association with behaviourally significant results (i.e., contextual conditioning). In addition, control animals received the same experimental procedures as CORT injected rats thereby extinguishing potential non-specific effects on Fos expression. Therefore, the effects of CORT following contextual fear recall are the most likely explanation for the observed increases in Fos expression.

Although the hippocampus is known to play an integral role in both contextual fear memory processes (Kim et al., 1993; Phillips & LeDoux, 1992) and the modulation of stress-induced HPA axis activity, the current study suggests that hippocampal activity may not be directly modulating the effects of repeated stress during contextual fear conditioning. The current results are not consistent with previous research which demonstrated that repeated CORT injections significantly decrease Fos expression in the dentate gyrus following recall of contextual fear conditioning (Skorzewska et al., 2006); however, these authors are unable to account for the meaning of this phenomenon. It has been shown that during a stressful experience, Fos mRNA in the hippocampus was associated with the exploratory potential of the stressful experience rather than the level of HPA axis activity that the stressful experience evoked (Pace et al., 2005). In the present study, we did not find a significant difference in Fos+ cells in CORT rats following recall of either contextual or tone cues in any areas of the hippocampus. Interestingly, a significant increase in Fos+ cells was observed in the CA1 across both vehicle and CORT injected animals following testing of tone cues in a novel environment compared to testing of contextual cues, which occurred in a familiar environment (results not shown). This evidence supports the possibility that the role of the hippocampus in contextual fear conditioning is focused on the processing of environmental cues, and not the modulation of repeated CORT exposure on fear-related behaviours.

4.4. Conclusions

Repeated injections of CORT produced both enhancements in fear-related behaviour to contextual cues and structurally specific increases in Fos expression following recall of contextual conditioning. Specifically, repeated CORT exposure significantly increased Fos expression in the BLA and CeA. Thus, the BLA and CeA show evidence of enhanced neuronal activity stimulated by recall of contextual cues following repeated CORT exposure and are likely mediators of the effects of repeated CORT on aversive memory.

CHAPTER 5

Repeated Corticosterone Enhances Acquisition and Recall of Trace Fear Conditioning

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ABSTRACT

Repeated exposure to high levels of glucocorticoids enhances contextual and discrete fear conditioning in rats. A common belief is that this enhanced freezing behaviour is largely mediated by the amygdala. Both contextual and discrete fear conditioning are dependent on an intact amygdala; however, trace fear conditioning is thought to be largely amygdala independent. In this study we examined whether repeated glucocorticoid exposure would produce memory enhancements in a trace fear conditioning test, despite this task being largely amygdala independent. Male Long-Evans rats received either 40 mg/kg of CORT or vehicle injections for 21 consecutive days. On day 22 half of the rats received 1 trace pairing, the other half received 2 trace-pairings; this was done to determine whether repeated CORT significantly enhanced acquisition of trace fear associations. On day 23 the rats were tested for freezing behaviour to the same tone cues in a novel context. Our results revealed that CORT significantly increased freezing behaviour on the testing day. Furthermore, we found that CORT significantly increased rats' initial fear reaction to tone cues during training as well as acquisition of the trace period. These results suggest that trace fear conditioning is a potential model for investigating how repeated glucocorticoid exposure modifies the physiological substrates that mediate emotionally driven responses without the potential confounds associated with tasks that largely depend on amygdala functioning.

1. Introduction

Activation of the HPA axis and the resultant release of glucocorticoids, cortisol in humans and CORT in rodents, in response to an acute stressor is an adaptive mechanism that allows the organism to cope with the stressor (Nestler et al., 2002a). Despite the initial adaptive effects of acute glucocorticoid release, repeated exposure to glucocorticoids leads to both aberrant brain plasticity and maladaptive behaviours. Some of the most prominent neuroplastic changes associated with repeated glucocorticoid exposure occur in the hippocampus and amygdala as both structures are abundant in glucocorticoid receptors (Feldman & Weidenfeld, 1999; Morimoto et al., 1996). Rats repeatedly exposed to CORT have decreased dendritic branching, decreased mossy-fiber synapses, and show decreased neurogenesis (Sousa et al., 2000; Watanabe et al., 1992; Woolley et al., 1990). Aberrant hippocampal neuroplasticity is associated with deficits in hippocampal dependent memory tasks that are caused by excessive glucocorticoids (Luine et al., 1994; Sousa et al., 2000). Contrarily, the amygdala shows enhanced dendritic arborization and spine density after prolonged immobilization stress in rats (Vyas et al., 2002; Vyas et al., 2006). As well, amygdala-dependent learned fear associations are enhanced after repeated restraint stress (Conrad et al., 1999; Phillips & LeDoux, 1992).

Evidence shows that depressed individuals display similar cognitive deficits and abnormalities as do rodents exposed to repeated stressors and glucocorticoid exposure. For instance, depressed individuals show memory impairments such as impaired recall of autobiographical information (Crane et al., 2007) and also have working memory deficits (Elliott et al., 1996). Also similar to stressed rodents, depressed humans show enhanced learning of tasks involving an emotional component that is specific to stimuli with a negative bias. There is evidence that individuals diagnosed with depression show an attentional bias towards sad faces and enhanced memory for negative emotional information (Leppanen, 2006). Given the strong associations between the effects of repeated stress or glucocorticoid exposure on rodents and

depression in humans, repeatedly exposing rodents to high levels of glucocorticoids offers a valuable model for exploring the underlying neural processes related to depression in humans.

Fear conditioning, a test that assesses a rodent's ability to associate neutral cues with an aversive experience, has been used to explore how glucocorticoids mediate memory tasks that evoke negative emotional arousal. Variations of the fear conditioning paradigm have been developed that require the activation of different brain structures to successfully learn the task (Phillips & LeDoux, 1992; Raybuck & Lattal, 2011). These variations allow researchers to assess the functional integrity of different structures which makes fear conditioning a valuable tool for exploring the processing of negative emotional memories. In a typical fear conditioning protocol, the conditioned stimulus is followed immediately by or co-terminates with the unconditioned stimulus. However, one variation of fear conditioning, trace fear conditioning, separates the conditioned stimulus from the unconditioned stimulus by a temporal gap. Trace fear conditioning is unique in that unlike other forms of classical conditioning such as contextual or delay fear conditioning that are amygdala dependent (Phillips & LeDoux, 1992; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011), evidence suggests that trace fear is amygdala independent (Raybuck & Lattal, 2011). With classical fear conditioning paradigms, such as contextual fear conditioning, repeated stress or glucocorticoid exposure produces a robust increase in fear memory (Sandi et al., 2001; Skorzewska et al., 2006). This memory enhancement is intriguing in that repeated stress and glucocorticoid exposure typically produce memory deficits when the tasks are emotionally neutral (Luine et al., 1994; McEwen & Sapolsky, 1995; Sousa et al., 2000). It is believed that the primary mechanism behind this increase in freezing is an enhancement of amygdala functioning following repeated stress or glucocorticoid exposure (Conrad et al., 1999). Trace fear conditioning provides the opportunity to examine whether repeated glucocorticoid exposure is still able to produce memory enhancements, which are thought to be mediated by the amygdala, despite being a task less dependent on the amygdala (Raybuck & Lattal, 2013).

Surprisingly, very little is known about the effects of repeated stress or glucocorticoid exposure on trace fear conditioning. One study has explored the effects of 14 days of repeated immobilization stress on trace fear conditioning and found a significant decrease in freezing behaviour. However, immobilization stress can produce variable results in tests of depression-like behaviour, possibly due to potential habituation effects and individual differences in HPA axis response (Gregus et al., 2005; Grissom & Bhatnagar, 2009). One way to avoid these potential problems is to repeatedly administer CORT itself thereby ensuring that each animal is exposed to the same amount of glucocorticoid. Therefore, the main objective of this experiment was to determine whether repeated glucocorticoid exposure enhances freezing behaviour to trace fear conditioning cues. Pilot studies performed in our lab suggest that repeated glucocorticoid exposure may increase acquisition of trace fear conditioning, thus a second objective of this experiment was to determine whether repeated glucocorticoid exposure enhances acquisition of trace fear conditioning. A third objective of this experiment was to determine whether any potential increases in acquisition of trace fear memory could account for possible enhancements in freezing behaviour during testing.

2. Materials and methods

2.1. Animals

For this study, 41 adult male Long-Evans rats (purchased from the Animal Resources Center, Saskatoon, Saskatchewan) were used. Rats weighed between 210-250 g at time of arrival to the animal colony. Rats were housed individually in standard polyproplylene cages maintained on a 12hr light/dark cycle (lights on at 07:00 hr) kept at a temperature of 21°C. Rats had free access to water and Purina rat chow. Experimental procedures were conducted during the light phase and were carried out according to the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care and Supply.

2.2 Repeated CORT injections

Rats were handled daily for 7-14 days prior to injections. Handling and injections occurred in a procedure room separate from the housing room. At the completion of handling, rats were assigned to one of two conditions based on body weight (i.e., both groups had approximately equal average weight). The two experimental conditions were: 40 mg/kg CORT group (n = 21), and a vehicle control group (n = 20). Injections were administered once per day subcutaneously at a volume of 1ml/kg for 21 consecutive days between 09:00 hr and 11:00 hr. CORT (Steraloids Inc., Newport, RI) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitan monooleate (Tween-80; VWR International, West Chester, PA). The 40mg/kg dose of CORT was chosen based on previous research showing that this dose reliably increases depression-like behaviour using the forced swim test (Gregus et al., 2005; Kalynchuk et al., 2004; Marks et al., 2009).

2.3. Fear conditioning

2.3.1. Apparatus. Rats were trained and tested in two identical fear-conditioning chambers (25.5 cm X 32 cm X 25.5 cm) that were connected to a computer that controlled both the shock and tone presentations and video recorded all rat behaviour (Med Associates, St. Albans, VT). The front door and ceiling of the chambers were made of clear Plexiglas, the four sidewalls were made of aluminum, and the back wall was made of white plastic. The floor of each chamber comprised 19 stainless steel rods spaced apart by 1cm. Each rod was wired to a shock generator and scrambler that delivered foot-shocks as unconditioned stimuli. The ceiling of the chamber held a speaker that presented individual tones as the conditioned stimuli. A stainless steel pan covered by two sheets of paper towel collected urine and fecal matter under the grid floor. The entire chamber was inserted into a white sound-attenuating cubicle (73 cm X 64 cm X 42 cm) that contained a video camera on the inside door. A fluorescent lamp on the ceiling of the cubicle lit each chamber.

2.3.2. Training. On the last day of injections (day 21) rats from both the CORT and vehicle treatment conditions were randomly assigned to one of two protocols. Rats assigned to protocol 1 (CORT: n = 11, vehicle: n = 10) received 1 tone-trace-shock pairing and rats assigned to protocol 2 (CORT: n = 10, vehicle: n = 10) received 2 tone-trace-shock pairings. This experimental design allowed us to assess three effects: the effect of CORT on the memory of trace fear associations, the effect of CORT on the acquisition of trace fear conditioning, and whether any possible increases in acquisition translated to enhancements in fear-related behaviour. On day 22 of the experiment (training day), rats were transported from their home cages to the conditioning room on a trolley (2 rats at a time). Rats were placed individually into one of the fear conditioning chambers and were then given 180 s to acclimatize to the surroundings in the chamber. After acclimatization rats received either 1 or 2 tone-trace-shock pairings. For the tone-trace-shock pairing, a tone at a volume of 90 dB, frequency of 2kHz, and a rise time of 50ms, played for 16 s followed by a 30 s trace and ended with a 2 s 0.5 mA foot-shock. Rats in the 2 tone-trace-shock pairing protocol had an inter-trial interval of 210 s. Rats remained in the conditioning chamber for 210 s after the final tone-shock-pairing and were then returned to their home cages and transported back to the colony room. The chambers were cleaned with 0.4% glacial acetic acid between each training session. Training was conducted between 09:00 and 13:00hr.

2.3.3. Testing. Retrieval of the tone-trace-shock association took place in the same two chambers described above, however the following contextual details within the chambers and the testing room were altered. First, black horizontal strips were fitted to the outside of the chambers changing both the appearance of the ceiling and the lighting of the chamber. Second, a white plastic plate was placed over the grid rods to change tactile cues and make the floor completely flat. Third, a plastic insert containing two blue vertical stripes and one red vertical stripe was inserted into the chamber giving the chamber a semicircle shape around the side

panels and rear of the chamber. Fourth, cardboard shapes of various colours were placed on the inner walls of the sound attenuating cubicle to alter visual cues and a white noise generator created background noise (65 dB) in the cubicle. Fifth, blue curtains were draped from the ceiling changing both the shape and colour of the room. Lighting in the conditioning room was also changed by draping a blue curtain over the fluorescent ceiling light diming the room. Sixth, olfactory cues were changed by scenting the room with vanilla coconut pet deodorizer. As well, the chambers were cleaned with 75% ethanol instead of 0.4% glacial acetic acid between trials. Finally, transportation from the colony room to the conditioning room was altered by draping a dark curtain over the home cages of the rats and by changing the route of transportation.

During testing, rats were transported to the conditioning room as described above and placed into the opposite chamber they were trained in. Rats were given 180 s to acclimatize to the new contextual surroundings before receiving 5 tones each lasting 16 s (the same volume, frequency, and rise time used for training) with an ITI of 210 s. Rats remained in the chamber for 210 s after the 5th tone before being returned to their home cages and transportation to the colony room. All testing took place between 09:00 hr and 14:00 hr.

2.3.4. Behavioural measures. Behaviour in the conditioning chambers was recorded by a video camera at 30 frames/s (Video Freeze Software, version 1.16.0.0, Med Associates, St. Albans, VT) attached to the inside door of the sound attenuating cubicles. Freezing was quantified by the software using a motion threshold which refers to a minimum change in video pixel composition over time. Freezing was defined as immobility with the exception of movement related to respiration (Blanchard & Blanchard, 1969). The motion threshold of each rat was set individually to account for the variation in movement produced by each animal during respiration. Previous research in our lab shows that individual determination of motion threshold is strongly correlated with manual scoring of behaviour. Video Freeze Software provided the percentage of time spent freezing based on a minimum freeze duration of 1s.

2.4. Body weight

All rats were weighed daily for later analyses.

2.5. Statistical analyses

Data were analyzed using the Statistical Package for the Social Sciences (Chicago, IL, version 18.0). Training day data were analyzed separately for protocol 1 and 2. For protocol 1, group differences in freezing behaviour during the tone, 30 s trace, and post-shock interval were analyzed by separate one-way ANOVAs. For protocol 2, separate mixed design ANOVAs with treatment (vehicle vs. 40mg/kg CORT) as the between-subjects variable and pairing (pairing 1 vs. pairing 2) as the within-subjects variable, were used to analyze freezing behaviour during the tone, 30 s trace, and post-shock interval with profile plots to break down significant interactions. *T*-tests with a Bonferroni correction, significance level set to $p \leq .025$, were used to assess differences between treatment groups during the tone, 30 s trace, and post-shock interval for pairing 1 and pairing 2. Group differences in behaviour on the testing day were analyzed using separate two-way ANOVAs with treatment (vehicle vs. 40mg/kg CORT) and protocol (protocol 1 vs. protocol 2) as the between-group factors. Body weight data was analyzed using a mixed design ANOVA with treatment (vehicle vs. 40mg/kg CORT) as the between-subjects variable and injection day (1, 7, 14, and 21) as the within-subjects variable. Contrasts were performed to break down significant interactions. Significance was set to $p \leq .05$ unless otherwise stated.

3. Results

3.1. Training Day

3.1.1. Habituation. Freezing behaviour prior to the first tone-trace-shock pairing was analyzed separately. Analyses revealed no significant difference between CORT or vehicle injected rats

in percentage of time spent freezing in either protocol 1 (one tone-trace-shock pairing) or protocol 2 (two tone-trace-shock pairings) [all *p* values ≥ .401; see Figure 5-1A,B].

3.1.2. Protocol 1. Freezing behaviour during training was analyzed separately for protocol 1 and 2 to determine whether CORT significantly affected acquisition of trace fear conditioning. No significant differences were found between CORT and vehicle rats in percentage of time spent freezing during the tone, 30 s trace, or post-shock for animals that received only 1 tone-trace-shock pairing [all *p* values ≥ .179; see Figure 5-1A].

3.1.3.Protocol 2. For animals that received 2 tone-trace-shock pairings, a significant main effect of treatment was found for percentage of time spent freezing [$F(1,16) = 11.166$, $p = .004$, see Figure 5-1B] during tone presentations. Collapsed across both tone presentations, the CORT rats froze more than the vehicle injected rats. *T*-tests revealed that although there was no significant difference in percentage of time spent freezing during tone 1, [$t(16) = -1.554$, $p = .140$], there was a significant difference in the percentage of time spent freezing during tone 2, [$t(16) = -3.797$, $p = .002$]. A significant main effect of pairing was found during tones for percentage of time spent freezing [$F(1, 16) = 13.466$, $p = .002$]. A significant pairing x treatment interaction [$F(1,16) = 13.325$, $p = .002$] indicated that CORT and vehicle rats had significantly different patterns of freezing from the first tone presentation to the second tone presentation. Profile plots revealed that rats injected with vehicle maintained the same level of freezing during the tone from the first presentation to the second presentation whereas CORT rats showed increased freezing from the first tone presentation to the second tone presentation.

A significant main effect of treatment was found for percentage of time spent freezing [$F(1,16) = 13.812$, $p = .002$; see Figure 5-1B] during the trace periods for protocol 2. CORT rats froze more across both trace periods compared to vehicle injected rats. *T*-tests revealed that although there was no significant difference in the percentage of time spent freezing during the first trace period, [$t(16) = -1.967$, $p = .067$], there was a significant difference in the percentage

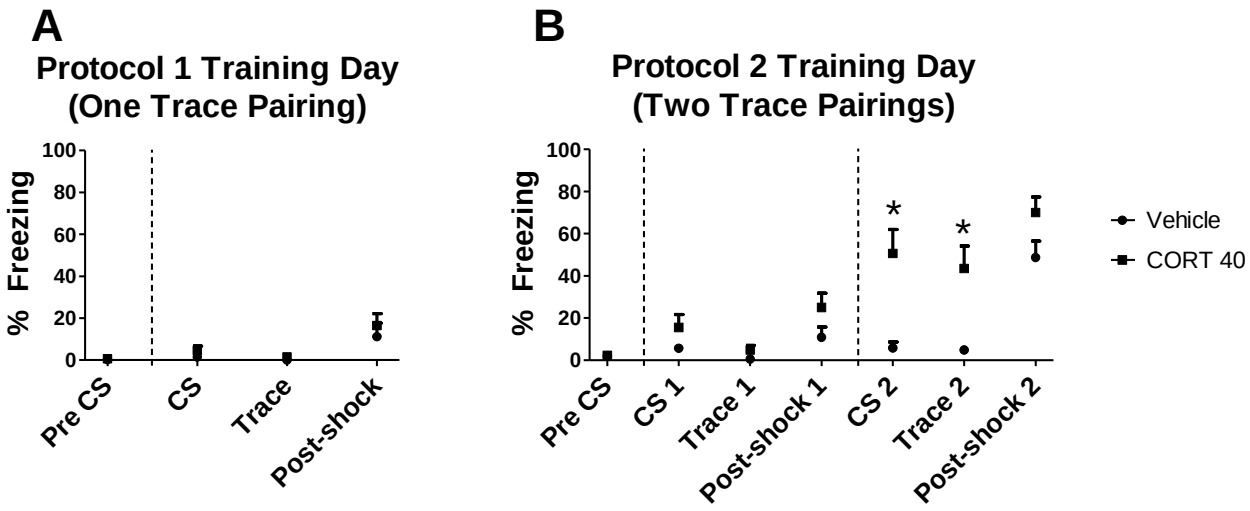

Figure 5-1. The effect of CORT and protocol on freezing behaviour during trace fear conditioning training. Panel A shows the mean percentage of time spent freezing for each treatment group during the habituation period, conditioned stimulus (CS), trace period, and post-shock period for the one trace pairing protocol [vehicle group, n = 9; CORT group, n = 10]. Panel B shows the mean percentage of time spent freezing for each treatment group during the habituation period, CS, trace periods, and post-shock periods for the two trace pairings protocol [vehicle group, n = 9; CORT group, n = 9]. Asterisks denote a significant difference between vehicle and CORT injected rats ($p < .025$). Error bars denote the S.E.M.

of time spent freezing during the second trace period, [$t(16) = -3.559$, $p = .003$]. A significant main effect of pairing was found during trace periods for percentage of time spent freezing [$F(1,16) = 16.569$, $p = .001$]. A significant pairing x treatment interaction for percentage of time spent freezing [$F(1,16) = 10.543$, $p = .005$] revealed that the CORT and vehicle rats had significant differences in their freezing behaviour from the first trace period to the second trace period. Profile plots revealed that the CORT rats significantly increased their percentage of time spent freezing from the first trace period to the second trace period, whereas the vehicle injected rats maintained similar freezing behaviour across both trace periods.

Statistical analyses also revealed a significant main effect of treatment during the post-shock intervals for percentage of time spent freezing [$F(1,16) = 4.860$, $p = .042$; see Figure 5-1B]. CORT rats froze more across both of the post-shock intervals than did the vehicle rats. However, *t*-tests revealed that, analyzed separately, there were no significant differences between CORT or vehicle injected rats in the percentage of time spent freezing during post-shock interval 1, [$t(16) = -1.7$, $p = .109$] or post-shock interval 2 [$t(16) = -1.98$, $p = .065$]. Also, a significant effect of pairing was found during post-shock intervals for the percentage of time spent freezing [$F(1,16) = 61.403$, $p < .001$]. A non-significant pairing x treatment interaction for percentage of time spent freezing [$F(1,16) = 0.459$, $p = .508$] indicated that, although CORT rats showed more freezing behaviour overall, both CORT and vehicle injected animals had increased freezing behaviour from post-shock interval 1 to post-shock interval 2.

3.2. Testing Day

Freezing behaviour was analyzed for the 3 min period on the testing day prior to the presentation of the first tone. There was no significant main effect of treatment on percentage of time spent freezing during initial exposure to the novel testing environment [$F(1, 33) = 3.321$, $p = .077$; see Figure 5-2A]. Similarly, there was no significant effect of protocol and no significant treatment by protocol interaction for the percentage of time spent freezing during the initial 3 min

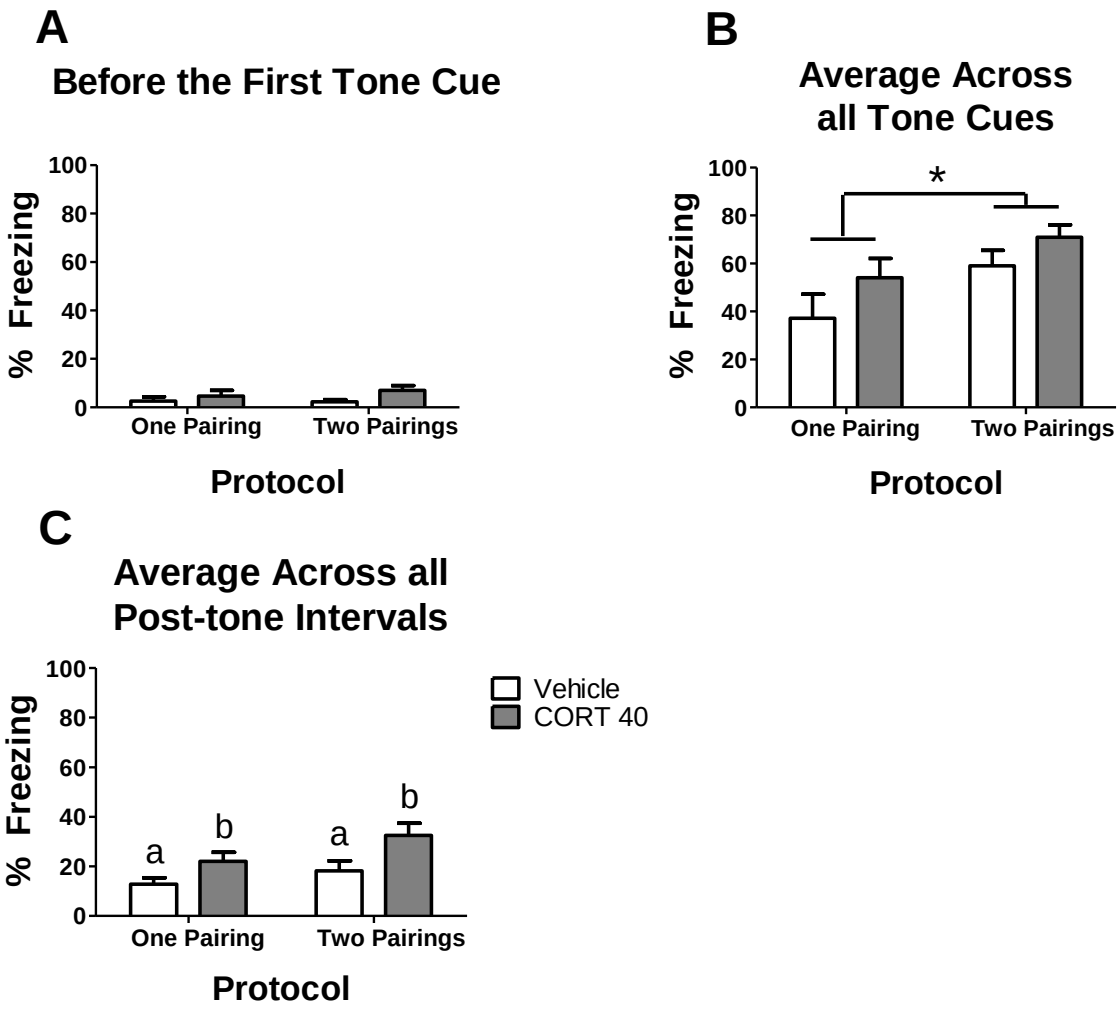

Figure 5-2. The effect of CORT and protocol on freezing behaviour during recall of trace fear cues. Panel A shows the mean percentage of time spent freezing for each treatment group during habituation to the novel testing environment when recall of trace cues was tested for the one trace pairing group (protocol 1) vs two trace pairing group (protocol 2) [vehicle group one pairing, n = 9; vehicle group two pairings, n = 9; CORT group one pairing, n = 10; CORT group one pairing, n = 9] . Panel B shows the mean percentage of time spent freezing for each treatment group during tone cue presentations for the one trace pairing group vs two trace pairing group. Asterisk denotes a significant difference between protocol 1 vs protocol 2 ($p < .05$). Panel C shows the mean percentage of time spent freezing for each treatment group during the interval between tone cue presentations for the one trace pairing group vs two trace pairing group. The letter a vs b indicates a significant difference between the vehicle group and CORT group across protocol 1 and 2 ($p < .05$). Error bars denote the S.E.M.

exposure to a novel environment [both p values ≥ .457].

Analyses revealed that CORT significantly increased freezing behaviour during testing of trace fear associated cues. Freezing behaviour during tones and the intervals between tones were analyzed separately. Results in the proceeding section represent the overall averages of behaviour across all tones or across all intervals between tones. Although a significant main effect of treatment was not found for percentage of time spent freezing during tone cues [$F(1, 33) = 3.470$, $p = .071$; see Figure 5-2B], a significant main effect of treatment was found for percentage of time spent freezing during the intervals between tone cues [$F(1, 33) = 8.985$, $p = .005$; see Figure 5-2C]. CORT rats froze significantly more than vehicle rats during the post-tone intervals.

Freezing behaviour was also significantly influenced by training protocol. A significant main effect of protocol was found for the percentage of time spent freezing during tones [$F(1, 33) = 6.316$, $p = .017$; see Figure 5-2B].The percentage of time spent freezing during the interval between tones neared statistical significance [$F(1, 33) = 4.071$, $p = .052$; see Figure 5-2C]. These results show that animals that received two tone-trace-shock pairings during training showed significantly more freezing behaviours during testing than animals that received only one tone-trace-shock pairing during training. The treatment x protocol interactions were non-significant [both p values ≥ .510].

3.3 Body Weight

CORT significantly delayed the normal increase in body weight over time, see Figure5-3. A two-way mixed design ANOVA, with Greenhouse-Geisser correction for violation of sphericity, revealed a significant main effect of day [$F(3, 76) = 219.186$, $p < .001$] suggesting that rats gained weight over time. However, a significant treatment x day interaction [$F(2, 76) = 170.497$, $p < .001$] qualified the significant main effect of day indicating that CORT and vehicle rats had significantly different changes in weight over time. To break down the treatment x day

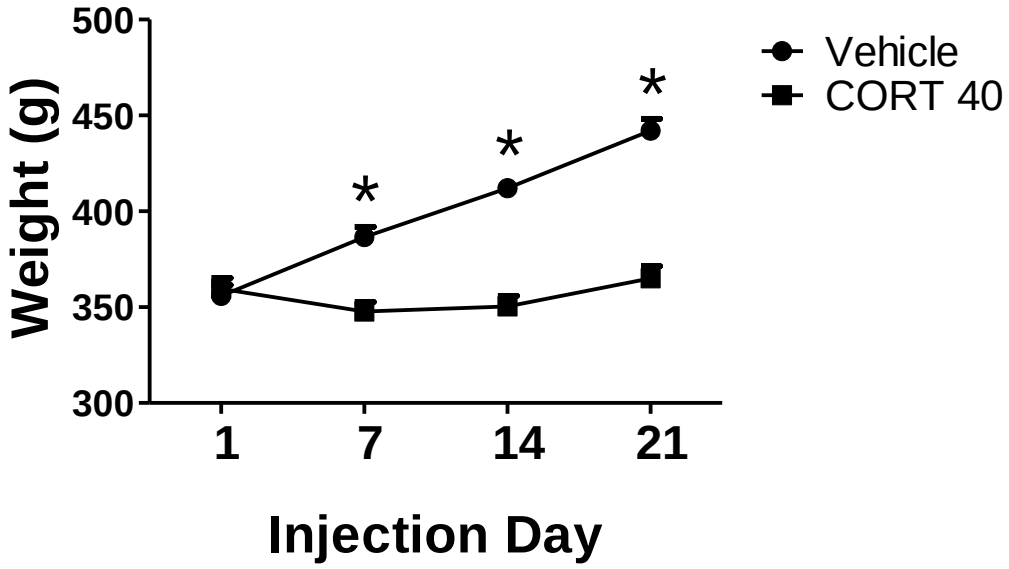

Figure 5-3. The effect of CORT on body weight [vehicle group, n = 18; CORT group, n = 19]. Asterisks indicate significant differences between CORT and the vehicle injected rats ($p < .001$). Error bars denote the S.E.M.

interaction, contrasts were performed that compared the weight of vehicle and CORT rats from injection day 1 to 7, day 7 to 14, and day 14 to 21. For all contrasts, $p < .001$, indicating that vehicle rats gained significantly more weight than CORT rats on days 7, 14, and 21.

4. Discussion

From this study, the following three conclusions can be made. First, repeated CORT exposure significantly enhances freezing behaviour during recall of trace fear associations. Second, repeated CORT exposure significantly increases both fear reaction to trace associated cues as well as freezing behaviour during acquisition of the trace associated cues. Third, CORT-induced enhancements in freezing behaviour observed during recall of trace fear conditioning may be attributed to enhancements in fear behaviour observed in CORT rats during acquisition.

4.1 Repeated CORT increases initial fear reaction and freezing during acquisition of trace associated cues

The results from this experiment show that repeated CORT exposure quickens the initial fear reaction to trace fear associated cues. During training, the significant main effect of treatment to the conditioned stimulus presentations is qualified by a significant interaction between treatment and repeated pairings, which translates to the following: when CORT or vehicle rats received one trace pairing, no differences were seen between the groups in freezing behaviour following the tone-trace-shock pairing. When CORT rats heard the second tone, despite not yet receiving a second shock, they froze significantly more than the vehicle rats. This could be interpreted to indicate that they had already learned to associate fear with the conditioned stimulus after only one pairing, whereas vehicle rats had not. However, after only one pairing, both CORT and vehicle rats showed similar amounts of freezing to tone cues during testing (discussed in greater detail below). Therefore, a more likely explanation is that

CORT rats had a significantly greater initial reaction to the second tone. After vehicle and CORT rats had received the second shock, both groups of rats froze at similarly increased levels suggesting that by the second tone-trace-shock pairing, both groups of rats were reacting similarly to trace associated cues. Similar to these findings, other researchers have shown stress-induced increases in reaction to the unconditioned stimulus during training of contextual fear conditioning (Cordero et al., 2003). In line with our conclusions, these authors also confirm that these increases do not reflect enhanced acquisition as these rats did not show significantly enhanced freezing during recall of context cues (Cordero et al., 2003). However, the results of the current study do not support previous research which showed that chronic immobilization stress did not increase the initial fear reaction to trace fear associated cues (Vander Weele et al., 2013). Although increases in freezing behaviour observed during the conditioned stimuli are likely the result of an increased fear reaction in CORT treated rats, increases in post-tone freezing observed in CORT rats during testing (discussed in greater detail below) could be related to enhanced acquisition of the trace period.

4.2 Repeated CORT increases freezing to trace fear associations

This experiment demonstrated that repeated CORT exposure increases freezing behaviour during recall of trace fear associated cues. Although CORT treated rats did not show increased freezing during the tone conditioned stimulus, they did show enhanced freezing during the intervals between tones. Other paradigms, such as fear-potentiated startle, that have examined the time course of fear expression after trace conditioning indicate that fear is expressed both during the conditioned stimulus and around the time of the scheduled occurrence of the unconditioned stimulus (Burman & Gewirtz, 2004). Therefore it is likely that CORT injected rats are freezing significantly more during the interval between tones as compared to vehicle rats in a greater anticipation of the unconditioned stimulus. These results are consistent with the effects of repeated glucocorticoid exposure during recall of other fear

conditioning paradigms. Previous work by other researchers and in our own lab has shown that repeated CORT injections increase fear-related behaviours during recall of contextual as well as delay fear conditioned cues (Conrad et al., 2004; Skorzewska et al., 2006). Interestingly, the results of the current study are not consistent with research that has shown that repeated immobilization stress causes a decrease in freezing behaviour to trace fear conditioning associated cues (Vander Weele et al., 2013). However, the repeated stress model used in this study also produced a decrease in freezing behaviour during a contextual fear conditioning paradigm. Moreover, these results are not consistent with other reports of the effects of repeated stress on contextual fear conditioning (Conrad et al., 1999; Cordero et al., 2003; Sandi et al., 2001) indicating that this stress model may not be comparable with other repeated stress or glucocorticoid exposure models.

An interesting finding was that CORT injected rats had enhanced freezing behaviour during the intervals between tones and not during the tones themselves. It is likely that learning to associate the temporal gap between the offset of the conditioned stimulus with the onset of the unconditioned stimulus is a more complex task that the vehicle rats had not acquired to the degree that the CORT rats had, whereas both groups of rats similarly acquired the association of the tone with the shock. However, it is also possible that the CORT injections enhanced the consolidation of the temporal gap between the offset of the conditioned stimulus with the occurrence of the shock (i.e., the trace). CORT-induced enhanced expression of freezing behaviour during the post-tone interval is also a possible explanation for these results. Further research is needed to determine whether chronic CORT enhances the acquisition as opposed to the consolidation, or expression of trace associations.

The effects of repeated CORT exposure seen in this study are likely not due to an increased sensitivity to pain or enhanced anxiety in a novel environment. Similar freezing behaviours found between vehicle and CORT rats after the first tone-trace-shock pairing indicate that CORT injected rats did not have an increased initial reaction to the shock itself,

although as mentioned above, CORT injections did increase initial reactions to trace associated cues. This finding supports previous research showing that repeated CORT treatment does not enhance reactivity to foot-shock (Conrad et al., 2004) or sensitivity to foot-shock (Dagnino-Subiabre et al., 2012). Our lab has previously shown that the repeated CORT protocol used for this study does not increase anxiety-like behaviour in the open field test (Gregus et al., 2005; Marks et al., 2009); however, we thought it would be important to determine whether any group differences in freezing behaviour were found when rats were initially exposed to a novel environment which could be indicative of enhanced anxiety. Repeated CORT treated rats did not show increased percentage of time spent freezing in a novel environment on training day or testing day.

4.3 Insights into the neural circuits of trace fear conditioning

Our results have interesting implications regarding the functioning of the neural circuits underlying trace fear conditioning. Trace fear conditioning is dependent on an intact hippocampus during acquisition, consolidation, and expression of the learned association (Burman, Starr, & Gewirtz, 2006b; Quinn et al., 2002; Yoon & Otto, 2007). Yet, despite significant aberrant morphological changes that are observed in the hippocampus following repeated glucocorticoid exposure (Sousa et al., 2000; Watanabe et al., 1992; Woolley et al., 1990), we demonstrate that trace fear associations are enhanced. Previous research has shown that repeated stress enhances learning in delay and contextual fear conditioning, despite also producing hippocampal atrophy (Conrad et al., 1999). This has lead to the belief that the amygdala is driving these learning enhancements (Conrad et al., 1999). Although a number of brain regions are recruited during fear conditioning including the hippocampus, prefrontal cortex, entorhinal cortex, and perirhinal cortex (Esclassan, Coutureau, Di, & Marchand, 2009; Kholodar-Smith, Boguszewski, & Brown, 2008; McEchron, Bouwmeester, Tseng, Weiss, & Disterhoft, 1998b; Runyan et al., 2004), the amygdala is thought to have a particularly prominent role in

mediating the effects of stress and glucocorticoids during fear conditioning. For instance, Fos, a marker of neuronal activation, is increased in the CeA and BLA in CORT-treated rats following contextual fear conditioning (Skorzewska et al., 2006). Also, altering glucocorticoid receptor functioning in the basolateral amygdala of rats exposed to repeated CORT treatment alters contextual fear conditioning (Conrad et al., 2004). Furthermore, inactivation of the BLA with muscimol attenuated increases in *c-fos* mRNA in the hippocampus associated with contextual fear conditioning, but did not affect mRNA levels following a simple context exploration task (Huff et al., 2006). Although trace fear conditioning is fundamentally unique in that it is less dependent on intact amygdala functioning (Raybuck & Lattal, 2011) compared to other variations of fear conditioning, it is possible that the amygdala is mediating repeated glucocorticoid induced enhancements found with trace fear conditioning through either direct or indirect pathways from the amygdala to the hippocampus.

4.3 Conclusions

This study was the first to investigate the effects of repeated glucocorticoid exposure on trace fear conditioning. Previous researchers have focused on investigating the effects of repeated glucocorticoid exposure on fear conditioning tasks that are amygdala dependent with little known about the effects on fear conditioning tasks that are less dependent on the amygdala. We showed that exposure to repeated CORT produced enhancements in freezing behaviour to trace fear associated cues that could be explained by initial increases in freezing behaviour seen during training. Thus, the current study provides a model for investigating how repeated glucocorticoids modify the physiological substrates that mediate emotionally driven responses without the potential ambiguous interpretations associated with tasks that heavily depend on amygdala functioning.

CHAPTER 6

Fluoxetine Prevents the Effects of Repeated Corticosterone on Discrete Tone Cued Fear Conditioning

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Author contributions: WNM designed research, collected data, analyzed data, and co-wrote paper; BDK collected data; LEK designed research and co-wrote paper.

ABSTRACT

Numerous lines of evidence demonstrate that prolonged glucocorticoid exposure can increase freezing behaviour during fear conditioning tasks. Typically fear conditioning tasks are used to model post traumatic stress disorder and anxiety in rodents. However, fear conditioning tasks also demonstrate more general alterations in emotion-associated memories, a symptom of depression in humans. We propose that fear conditioning also be used as a measure of depression-like behaviour in rodents. To validate our proposition, the current study investigated whether the antidepressant fluoxetine is able to prevent freezing behaviour in CORT-treated rats. Male Long-Evans rats received either vehicle, 40 mg/kg of CORT, or 40 mg/kg CORT + 10 mg/kg fluoxetine injections for 21 consecutive days. On day 22, rats received delay fear conditioning training. On day 23, freezing behaviour during recall of contextual cues was measured and on day 24, freezing behaviour during recall of tone cues was measured. Results revealed that fluoxetine significantly decreased freezing behaviour during recall of tone cue associations, but not contextual cues, in CORT-treated rats. However, significant differences in freezing behaviour between CORT and vehicle treated rats were not found. This study demonstrates that fear conditioning is a potential tool for exploring the circuits that mediate pathological alterations in fear-related memory associated with a CORT model of depression in rats.

1. Introduction

The secretion of corticosteroid hormones is one of the most important endocrine responses to stress and is essential for an organism's survival. A key mechanism through which the brain reacts to an acute or repeated stressor is through activation of the HPA axis. Activation of the HPA axis initiates a number of neuroendocrine events that results in the synthesis and release of glucocorticoids (cortisol in humans, CORT in rodents) from the adrenal cortex (Nestler et al., 2002a). Glucocorticoids have both metabolic and behavioural effects that help the organism cope with the stressor and equilibrate the organism to pre-stress levels of functioning via negative feedback control of the HPA axis (Checkley, 1996; Nestler et al., 2002a).

Although glucocorticoids are essential for the survival of an organism, excessive glucocorticoid release associated with repeated stress can have deleterious consequences. Extensive evidence shows a clear link between clinical depression and increased HPA axis functioning. First, significant life stressors such as unemployment and divorce are associated with an increased risk of depression (Kessing et al., 2003; McEwen, 1998). Second, approximately half of all depressed individuals show hypersecretion of cortisol and disrupted cortisol rhythms (Sachar & Baron, 1979). Third, more than half of all individuals with Cushing's disease, a syndrome resulting from repeated glucocorticoid excess, are also diagnosed with depression (Sonino & Fava, 2002). Interestingly, evidence suggests that chronically high levels of glucocorticoids in rodents result in behavioral changes that are associated with human depression. Indeed, repeated stress models of depression in rodents have been particularly successful at capturing a depressive phenotype characterized by learned helplessness behaviours, cognitive deficits, and anhedonia (Johnson et al., 2006; Marks et al., 2009; Nestler et al., 2002b; Olausson et al., 2013; Walesiuk & Braszko, 2010).

Measures of depression-like behaviour in rats have focused on understanding changes in appetite, mood, and cognitive deficiencies; however, less research has focused on attempting

to understand the enhancements in negative emotion associated memories that are linked with the disorder. Not only are depressed individuals more likely to attend to, memorize, and recall information with a negative emotional connotation (Leppanen, 2006; Nissen et al., 2010), they are also more likely to ruminate over their negative emotions and experiences (Nolen-Hoeksema, 2000). Thus, to understand the biochemical link between depression and emotional memory, researchers have begun to focus on determining the role glucocorticoids play in mediating memory tasks that evoke emotional arousal. Fear conditioning is a task that is often used to evaluate emotional memory in rodents. Fear conditioning assesses the ability to associate neutral cues such as environmental (contextual) or tone cues with an aversive experience such as a foot-shock. The rodent is later exposed to the same cues that were present during training with the foot-shock absent and freezing, a rodent's natural defensive behaviour (Blanchard & Blanchard, 1969), is measured. Robust increases in fear-related behaviours to contextual cues are found following repeated stress and excessive glucocorticoid exposure (Sandi et al., 2001; Skorzewska et al., 2006). Enhancements in fear-related behaviours are also seen to tone cue associations although it is noted that these increases are less robust than freezing to contextual cues (Conrad et al., 1999; Conrad et al., 2001).

Fear conditioning tasks are typically used to study anxiety, post-traumatic stress disorder, and the mechanisms of emotional memory. However, enhancements in freezing behaviour following repeated stress and glucocorticoid exposure suggest that fear conditioning may also be a useful tool for measuring depression-like behaviour. One way to reinforce the assertion that conditioned fear associations reflect depression-like behaviour is to show that antidepressants can prevent the effects of repeated glucocorticoid exposure on these behaviours. Therefore, the objective of the current study was to determine whether repeated treatment with the well known and widely used antidepressant, fluoxetine, could prevent the effects of repeated glucocorticoid treatment typically observed during recall of contextual and tone cue associated fear conditioning.

2. Materials and methods

2.1. Animals

For this study, we used 30 adult male Long-Evans rats (purchased from Charles River). Upon arrival rats weighed between 230-250 g. Each rat was individually housed in a standard polyproplylene cage and had free access to Purina rat chow and water. The colony room was maintained at a temperature of 21°C on a 12 hr light/dark cycle with lights on at 07:00hr. All procedures were carried out during the light phase and were conducted according to the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care and Supply.

2.2. Injections

Handling and injections occurred in a procedure room separate from the animal colony. Handling was conducted daily for 7-10 days before the injections began. On the completion of handling, rats were assigned to one of three experimental conditions based on body weight so that all groups had approximately equivalent average weight at first injection: repeated vehicle injections (n = 10), repeated 40mg/kg CORT injections (n = 10), or repeated 40mg/kg CORT + 10mg/kg fluoxetine injections (n = 10). CORT injections were administered subcutaneously for 21 consecutive days at a volume of 1ml/kg between 08:30 hr and 10:30 hr. CORT (Steraloids Inc., Newport RI) was suspended in 0.9% (w/v) physiological saline with 2% (v/v) polyoxyethlene glycol sorbitan monooleate (Tween-80; VWR International, West Chester, PA). The dose of CORT used in this study has shown to reliably increase depression-like behaviour (Gregus et al., 2005; Kalynchuk et al., 2004; Marks et al., 2009). Fluoxetine injections were administered intraperitonially for 21 consecutive days at a volume of 1ml/kg between 14:00 hr and 16:00 hr. Fluoxetine hydrochloride (Spectrum Chemical Manufacturing Corporation, Gardena, CA) was dissolved in 0.9% (w/v) physiological saline. The dose of fluoxetine used in

this study was previously shown to reduce depression-like behaviour in the FST in rats subject to repeated restraint stress (Lapmanee, Charoenphandhu, & Charoenphandhu, 2013). CORT and fluoxetine injection regimes were started and completed on the same day. Rats who received CORT only or vehicle injections received 21 consecutive intraperitoneal injections of 0.9% physiological saline at a volume of 1ml/kg between 14:00 hr and 16:00 hr.

2.3. Fear conditioning

2.3.1. Apparatus. All animals were trained and tested using two identical fear-conditioning chambers (25.5 cm x 32 cm x 25.5 cm). Both chambers were attached to a personal computer that controlled the shock and tone presentations. The computer also recorded the videos of all behaviours in the chambers (Med Associates, St. Albans, VT). The walls of each chamber were made of aluminum with the front door and ceiling made of clear Plexiglas, and the back wall made of white plastic. The floor of each chamber comprised 19 stainless steel rods, each of which was wired to a shock generator and scrambler. The ceiling of each chamber held a small speaker. A stainless steel pan lined with paper towel was inserted beneath the grid floor for the collection of urine and fecal matter. Each chamber was contained within a white sound-attenuating cubicle (73 cm x 64 cm x 42 cm) that contained a fluorescent lamp on the ceiling to light the fear-conditioning chamber.

2.3.2. Training. One day after the final injection (day 22), rats were transported in their home cages to the fear-conditioning room on a trolley (2 rats per trip). Rats were then individually placed inside of one of the chambers and were given 180 s to familiarize themselves with the contextual surroundings of the chamber. Rats then received 5 tones that co-terminated with a footshock, with an inter-trial interval of 120s. Each tone lasted 20 s at a volume of 90 dB, frequency of 2 kHz, and a rise time of 50 ms. Shocks were presented at an intensity of 0.7 mA and lasted for 2 s. The rats then remained in the conditioning chamber for 120 s after the last

tone-shock pairing before being returned to their home cages and transported back to the colony room. Each chamber was cleaned with 0.4% glacial acetic acid between training sessions. All training occurred between 09:00 hr and 14:00 hr.

2.3.3. Contextual fear testing. Testing for recall of contextual conditioning took place on day 23, the day after training. Rats were transported in their home cages (2 rats per trip) on a trolley to the conditioning room and placed inside the same chamber that they were trained in. The rats then remained in the conditioning chamber for a total of 14 min, 40 s stimulus free (the same amount of time used during training). At the completion of testing, rats were placed back in their home cages and transported back to the colony room. The chambers were cleaned with 0.4% glacial acetic acid between trials. Contextual fear testing was performed between 09:00 hr and 13:00 hr.

2.3.4. Tone Testing in a Novel Context. Testing for recall of tone conditioning took place on day 24, the day after testing of contextual conditioning. Testing of tone conditioning took place in the same chambers used during training and contextual conditioning, but environmental cues within the chambers and conditioning room were altered in several ways to create a novel context. First, both the lighting, shape, and colour of the conditioning room was altered by draping blue curtains from the ceiling as well as covering the fluorescent ceiling light with a blue curtain which dimed the lighting to a soft blue colour. Second, the conditioning chambers and cubicles that held the chambers were altered in a number of ways. Shapes cut from cardboard of various colours were taped to the inner walls of the sound attenuating cubicle to alter visual cues. Lighting within of the conditioning chamber was altered by fitting a pattern containing black horizontal stripes to the outside ceiling of the chamber. The shape of the inside of the chamber was altered with a colourful plastic insert that covered both the side panels and rear of the chamber creating a semicircle shape. Also, a white plastic panel was placed over the stainless

steel grid rods to make the floor completely flat. A white noise generator inside the conditioning chambers created novel background noise at 65 dB. Third, olfactory cues associated with both the conditioning room and fear chamber were altered by scenting the room with vanilla coconut pet deodorizer between trials and by cleaning the chambers with 75% ethanol instead of 0.4% glacial acetic acid. Finally, transportation from the colony room to the conditioning room was changed by draping a dark curtain over the home cages and by altering the route taken from the colony room to the conditioning room.

Once rats were transported to the conditioning room (as described above), they were placed inside the opposite chamber to the one they were placed in during training and context testing. Rats then received the same testing protocol used during training; however, the shocks were absent. The rats were given 180 s to acclimatize to the new contextual environment before receiving 5 tones (same frequency, volume, and rise time as used during training) with an inter-trial interval of 120 s. The rats then remained inside the chamber for 120 s after presentation of the 5^{th} tone before being taken out of the chamber and returned to their home cages. Then the animals were transported back to the colony room. Testing to tone cues was performed between 0:900 hr and 13:00 hr.

2.3.5. Behavioural measures. A video camera mounted to the inside door of the sound attenuating cubicles recorded behaviour at a rate of 30 frames/s. Movement, defined as a change in video pixel composition over time, within the chambers was recorded using Video Freeze Software (version 1.16.0.0, Med Associates, St. Albans, VT). Freezing behaviour was defined as immobility with the exception of respiratory related movement (Maciejak et al., 2003). Freezing was measured by the software with a motion threshold, which determined the limit above which all behaviour was registered as movement. The motion threshold of each rat was set individually to account for variations from animal to animal produced during respiration. Freezing behaviour resulting from the individual determination of motion threshold was

determined to correlate strongly (r > 0.9) with trained human observation. Percentage of time spent freezing was measured based on a minimum freeze duration of 1s.

2.4. Body weight

Rats were weighed daily and recorded for later analyses.

2.5. Statistical analyses

All group differences in freezing behaviour were analyzed using one-way ANOVAs with treatment (vehicle vs. 40 mg/kg CORT vs. 40 mg/kg CORT + FLU) as the between-group variable. Significant effects were followed up with Tukey post-hoc tests. Body weight data was analyzed using a mixed design ANOVA with treatment (vehicle vs. 40 mg/kg CORT vs. 40 mg/kg CORT + FLU) as the between-subjects variable and injection day (1, 7, 14, and 21) as the within-subjects variable. Simple effects were conducted using one-way ANOVAs (Bonferroni correction: significance level set to $p \leq .016$) and contrasts were conducted using Tukey post hoc tests. Unless otherwise stated, significance was set to $p \leq .05$.

3. Results

3.1. Training Day

Freezing behaviour during the 180 s habituation period was examined to determine whether treatment significantly affected behaviour in a novel environment (see Figure 6-1). Treatment had no significant effect on the percentage of time spent freezing during habituation [$F(2, 24) = 0.921$, $p = .413$; see Figure 6-1A]. The average percentage of time spent freezing over the entire training session was also analyzed to assess potential group differences in

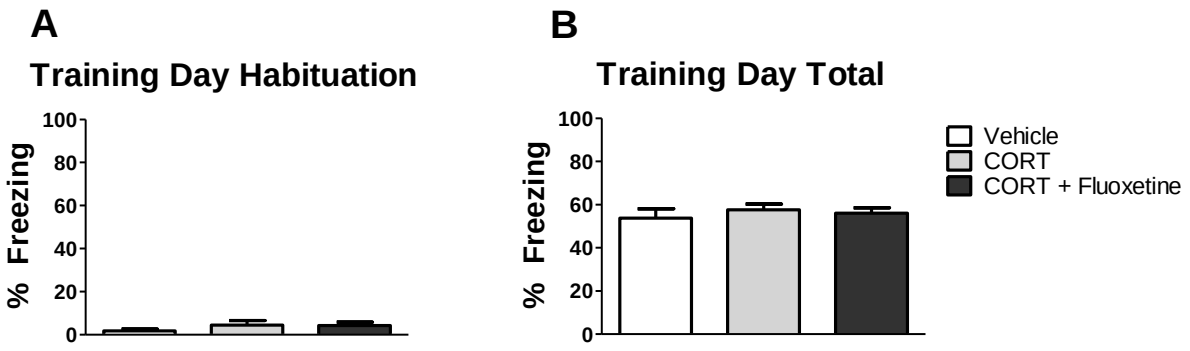

Figure 6-1. The effect of treatment on percentage of time spent freezing during delay fear conditioning training. Panel A shows the mean percentage of time spent freezing during the 3 min habituation period. Panel B shows the mean percentage of time spent freezing over the entire training session [vehicle group, n = 9; CORT group, n = 9; CORT + Fluoxetine group, n = 7]. Error bars denote the S.E.M.

reactivity to foot-shocks. There were no significant group differences in this measure [$F(2, 24) = 0.350$, $p = .709$; see Figure 6-1B].

3.2. Context Testing

Statistical analyses revealed that neither the CORT nor the CORT + fluoxetine injections significantly altered the percentage of time spent freezing [$F(2, 24) = 0.859$, $p = .437$; see Figure 6-2A] during testing of contextual cues.

3.3. Tone Testing

Freezing behaviours were analyzed during the 3 min period prior to the first tone presentation to determine whether either CORT or CORT + fluoxetine injections had a significant effect on behaviour in a novel environment. There were no significant group differences in the percentage of time spent freezing [$F(2, 22) = 0.929$, $p = .411$; see Figure 6-2B] during the pre-tone period.

Freezing behaviour during tone cues was investigated by summing and averaging behaviour during all 5 tones presented during tone testing. There was a significant effect of treatment on the percentage of time spent freezing during tone cues [$F(2, 22) = 6.105$, $p = .009$; see Figure 6-2C]. Post hoc analyses revealed that the CORT + fluoxetine rats had significantly less percentage of time spent freezing during tone cues compared to both vehicle ($p = .011$) and CORT injected rats ($p = .027$). There were no group differences in freezing behaviour in between tone cues [$F(2, 22) = 1.699$, $p = .208$; see Figure 6-2D].

3.4. Body Weight

Repeated injections of 40 mg/kg of CORT and 40 mg/kg of CORT + fluoxetine significantly decreased body weight gain over time, see Figure 6-3. A mixed design ANOVA, with Greenhouse-Geisser correction for violation of sphericity, revealed a significant main effect

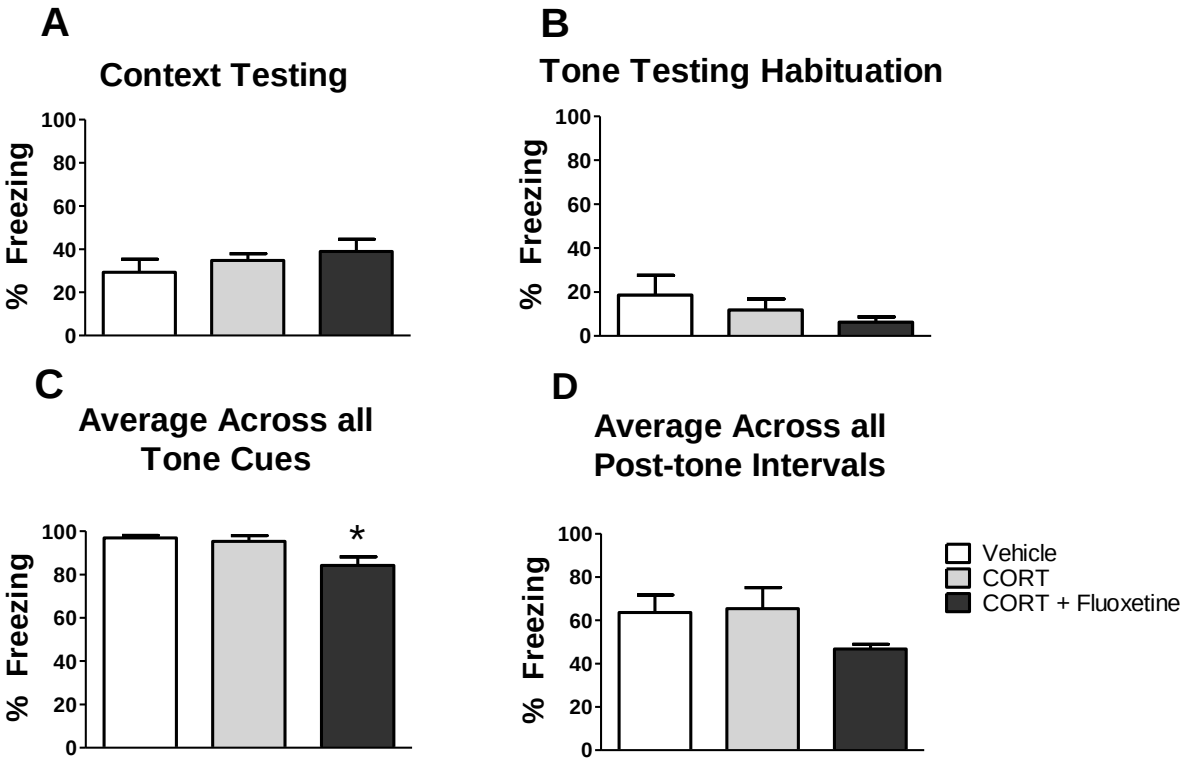

Figure 6-2. The effect of treatment on percentage of time spent freezing during recall of context and tone cues. Panel A shows the mean percentage of time spent freezing during recall of context cues for each treatment. Panel B shows the mean percentage of time spent freezing for each treatment during habituation to the novel testing environment. Panel C shows the mean percentage of time spent freezing for each treatment group during tone cue presentations. Asterisk denotes a significant decrease in freezing in the CORT + fluoxetine rats compared to both the vehicle and CORT treated groups ($p < .05$). Panel D shows the mean percentage of time spent freezing for each treatment group during the intervals between tone cue presentations [vehicle group, n = 9; CORT group, n = 9; CORT + Fluoxetine group, n = 7]. Error bars denote the S.E.M.

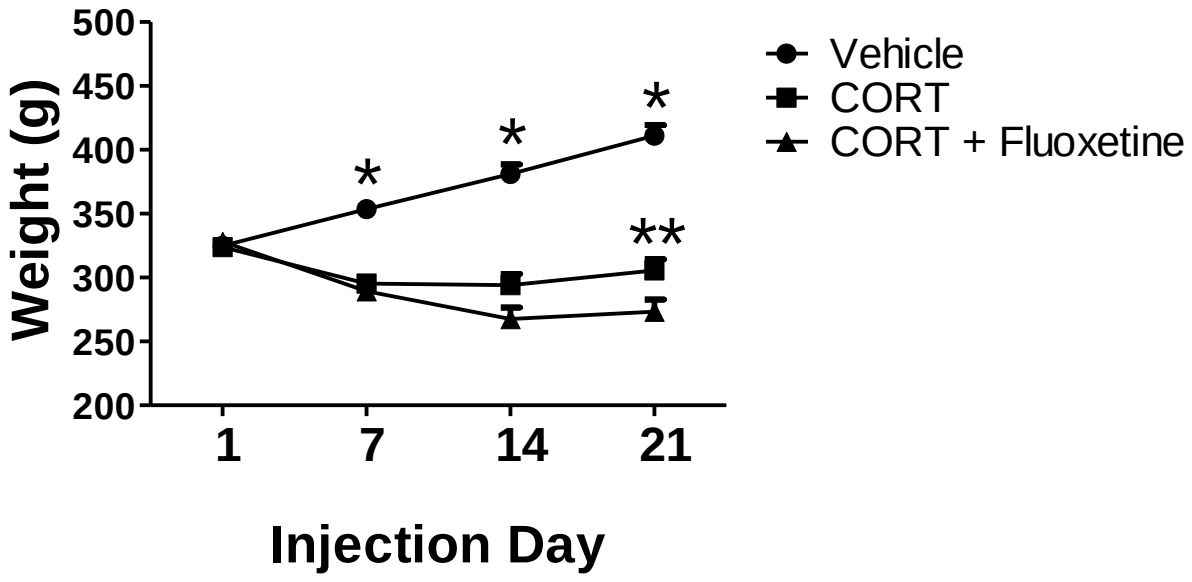

Figure 6-3. The effect of CORT and fluoxetine on body weight [vehicle group, n = 9; CORT group, n = 9; CORT + Fluoxetine group, n = 7]. Asterisks indicate significant differences between both CORT and CORT+ fluoxetine with vehicle-injected rats ($p < .001$). Double asterisk denote a significant difference between CORT and CORT + fluoxetine injected rats ($p < .05$). Error bars denote the S.E.M.

of day [$F(1, 32) = 15.852$, $p < .001$] which is qualified by a significant day x treatment interaction [$F(3, 36) = 77.432$, $p < .001$]. These results suggest that all rats changed weight over time; however, the treatment rats received influenced the magnitude and type of weight change over time. Simple effects analyses confirmed overall differences in body weight between groups on days 7, 14, and 21 (all p values < .001). Simple contrasts indicated that both CORT and CORT + fluoxetine rats weighed less than vehicle rats on days 7, 14, and 21 (all p values < .001). Also, there was no significant difference in body weight between CORT injected and CORT + fluoxetine injected rats on days 7, or 14 (both p values ≥ .099). However, on injection day 21, the CORT rats weighed significantly less than the CORT + fluoxetine rats ($p = .049$).

4. Discussion

The results of this experiment present a mixed bag. We found that fluoxetine significantly decreased freezing behaviour during recall of aversive tone associations in CORT-treated rats. However, fluoxetine did not have this effect for freezing behaviour during recall of aversive contextual cues. We also did not observe significant differences in freezing behaviour between CORT and vehicle rats to either contextual or tone cue associations. These results are discussed in further detail below.

4.1. The effect of CORT and vehicle injections on contextual and tone cued freezing behaviour

The current study did not reveal the typical increases in freezing behaviour seen following repeated glucocorticoid exposure or stress. Previous studies have shown robust increases in fear-related behaviour to contextual cues in response to both repeated CORT treatment (Conrad et al., 2004; Skorzewska et al., 2006) and repeated stress (Conrad et al., 1999; Cordero et al., 2003; Sandi et al., 2001). As well significant enhancements in fear-related behaviour have been observed during testing of tone cue associations following repeated

glucocorticoid exposure (Dagnino-Subiabre et al., 2012) and repeated stress (Conrad et al., 1999), but see (Conrad et al., 2001; Conrad et al., 2004).

One possible explanation for the inconsistencies found between the current study and previous research in our lab relates to the increased stress experienced by both the CORT and vehicle rats with the extra afternoon injection schedule. It has been shown that 3 weeks of daily injections of physiological saline alter spine density in pyramidal neurons in the medial prefrontal cortex (Seib & Wellman, 2003) demonstrating that saline injections are sufficiently stressful to produce neuroplastic changes. Indeed, freezing behaviours of both the CORT and vehicle rats in this experiment are higher than what we have seen previously (Marks et al., 2014a). For instance, the CORT rats had about 10% higher levels of freezing to tone cues than CORT rats from our previous studies, and vehicle rats had about 30% more freezing to tone cues than vehicle rats from our previous studies. Furthermore, the vehicle rats had about a 20% increase in freezing during the interval between tone cues.

Interestingly, the increased freezing observed in this experiment compared to our previous studies did not hold for contextual cues. In fact, the CORT treated rats in this experiment showed about a 20% decrease in freezing during recall of contextual cues compared to our previous results. It is possible that a non-linear relationship exists between the effects of repeated glucocorticoids and recall of contextual freezing. Acute injections of both 5 mg/kg and 20 mg/kg of CORT produce a decrease in freezing to contextual cues (Skorzewska et al., 2006), whereas, our lab has demonstrated that repeated injection of 40 mg/kg of CORT produce increases in freezing to contextual cues (Marks et al., 2014a). Therefore the decrease in freezing to contextual cues observed in CORT treated rats in the current experiment could reflect a non-linear response to contextual fear-associations along a glucocorticoid and stress exposure continuum. Other researchers have shown that both GR agonists and antagonists infused into the basolateral amygdala enhance freezing during recall of contextual cues supporting the possibility that an inverted U-shaped function may exist for the influence of

glucocorticoid receptor stimulation on contextual fear memory performance (Conrad et al., 2004).

It should be noted that both contextual and tone cue associated freezing behaviour following repeated stress and glucocorticoid treatment are sensitive to the order of presentation of cues during testing (see Marks et al., 2014). However, we have previously shown that CORT does enhance freezing to contextual cues with the fear conditioning protocol used in this study (see Chapter 3 of this thesis). It should also be noted that comparable studies using the same conditioning protocol and same treatment conditions have reported fluctuations in freezing of up to 25% (Conrad et al., 2004; Cordero et al., 1998; Pugh et al., 1997). Although it is possible that the inconsistencies in results seen between this study and our previous work could be the result of natural fluctuations in freezing behaviour, the behavioural results in both CORT and vehicle injected rats are more congruent with a stressed phenotype.

4.2. Repeated fluoxetine decreases freezing in repeated CORT treated rats

We are the first to observe a decrease in freezing behaviour during recall of tone cues in rats treated with CORT and fluoxetine concomitantly. These results are consistent with the effects of fluoxetine on other measures of depression following repeated stress or glucocorticoid exposure. Rats subject to repeated restraint stress and concomitant injections of 10 mg/kg of fluoxetine showed reduced depression-like behaviour in the FST as well as prevention of learned fear in an elevated T-maze (Lapmanee et al., 2013). Fluoxetine treatment in non-stressed rats affects fear-related behaviour during recall of contextual cues only at subchronic doses. For instance, subchronic injections of both 10 mg/kg and 20 mg/kg of FLU in rats reduced freezing during recall of contextual fear conditioning (Spennato, Zerbib, Mondadori, & Garcia, 2008). Alternatively, repeated injections of 7 mg/kg of fluoxetine for 3 weeks had no effect on recall of contextual fear cues (Spennato et al., 2008). Therefore it is possible that 3 weeks of repeated fluoxetine treatment only effectively attenuates tone cued associations which

coincides with findings from the current study. Fluoxetine treatment in non-stressed rats also enhances performance in other tasks that measure depression-like behaviours. For instance, 16 days of fluoxetine treatment over a 3 week interval prevented fear reactivation following extinction of tone-cued conditioned fear (Deschaux, Spennato, Moreau, & Garcia, 2011). Also, 3 weeks of fluoxetine treatment at 18 mg/kg increased mobility behaviours in mice in the FST (David et al., 2009). As well, 5 weeks of fluoxetine at a dose of 5 mg/kg reduced the amount of time required to initially acquire the location of a platform in Morris Water Maze testing. However, it should be noted that fluoxetine was not able to prevent this effect in rats that had also received chronic mild stress during antidepressant treatment (First et al., 2011).

The decrease in freezing observed in the CORT + fluoxetine treated rats is likely not related to deficits during acquisition of the conditioning task or enhanced foot-shock sensitivity. Freezing behaviours during acquisition were examined across groups and analyses showed that all treatment groups froze similarly during training. These findings support previous research showing that 16 days of fluoxetine injections at 7 mg/kg spread across 21 days did not significantly alter freezing behaviour during the acquisition of discrete tone conditioning (Deschaux et al., 2011). Also 14 days of fluoxetine injections at a dose of 5 mg/kg do not significantly alter foot-shock sensitivity in mice (Dong et al., 2004). The similar behavioural response to foot-shocks observed during training in the current study and previous research lends support to the conclusion that the findings in the current study cannot be explained by non-specific effects resulting from change in foot-shock sensitivity. Future research should also investigate the effects of 21 days of 10 mg/kg fluoxetine injections on freezing to tone cues to determine whether fluoxetine in the absence of CORT would further decrease the percentage of time spent freezing. It should be noted that the weight of rats concurrently receiving CORT + fluoxetine was significantly reduced compared to CORT rats on injection day 21. Future research should also determine whether the repeated combination of CORT and fluoxetine injections is sufficient to produce liver toxicity in rats.

How does repeated fluoxetine treatment reduce freezing behaviour in rats injected with repeated CORT? A number of lines of research suggest that the morphological changes found in depressed individuals and repeatedly stressed animals are related to alterations in the release and transmission of the excitatory neurotransmitter glutamate. It has been argued that one of the most deleterious effects of excessive glucocorticoid activation is the enhancement of glutamate release and the resulting alterations in neuron morphology (Gorman & Docherty, 2010; McEwen, 2005; Pittenger & Duman, 2008). Two weeks of 10mg/kg of fluoxetine dissolved in rat's drinking water prevented the increase in glutamate release following unpredictable foot-shock stress in the frontal/prefrontal cortex (Musazzi et al., 2010). However, acute stress-induced increases in extracellular glutamate levels in the basolateral and central amygdala were not prevented by a single 10mg/kg FLU injection (Reznikov et al., 2007). Therefore it is possible that chronic fluoxetine treatment may be required to prevent stress-induced increases in glutamate release. We have previously shown that repeated CORT enhances Fos expression, a marker of cellular activation, in the BLA and CeA in response to tone fear conditioning with significant enhancements following contextual fear conditioning (Marks et al., 2014b). Interestingly, neuroimaging studies demonstrate enhanced amygdalar activation in depressed patients (Siegle et al., 2002), which is prevented with short-term selective serotonin reuptake inhibitor (SSRI) treatment such as escitalopram (Godlewska, Norbury, Selvaraj, Cowen, & Harmer, 2012). Future research should determine whether fluoxetine treatment mediates stress-induced alterations in glutamate release in the amygdala to attenuate conditioned fear-related behaviours.

It is also possible that the fluoxetine-induced decrease in freezing in CORT treated rats is directly related to the known pharmacological actions of fluoxetine. Fluoxetine is a SSRI whose pharmacological action leads to an increase in serotonin availability by inhibiting the serotonin transporter (Torres, Gainetdinov, & Caron, 2003). Previous research has demonstrated that either repeated stress or CORT ingestion up-regulates serotonin transporter

protein levels in the hippocampus and amygdala (Zhang et al., 2012). This up-regulation was prevented by adrenalectomy or CORT receptor antagonists. Also, up-regulated serotonin transporter protein levels in the hippocampus and amygdala produced by either repeated stress or CORT administration were reduced by concurrent fluoxetine treatment (Zhang et al., 2012). Thus, the reduction in freezing observed in CORT + fluoxetine treated rats in the current study could be mediated by serotonin transporters.

4.3. Conclusions

The results of this experiment are the first to demonstrate that repeated fluoxetine treatment administered concurrently with CORT decreases freezing behaviour to aversive tone cues compared to rats injected with CORT alone. These results add validity to our proposal that fear conditioning is a valuable tool in the measurement of depression-like behaviour. Future research should further explore how repeated fluoxetine mediates the effects of repeated CORT on fear conditioned behaviours and whether these effects are specific to the acquisition, consolidation, or recall phase of learning.

Chapter 7

General Discussion

1. Summary of the Main Findings

The main objective of this dissertation was to gain a more complete understanding of the depression-like behaviours produced by repeated exposure to glucocorticoids and to gain preliminary insight into the potential brain structures mediating some of these behaviours. To accomplish this, I examined the effects of repeated CORT injections on behaviour in the FST and various versions of the fear conditioning task and investigated the associated changes in regional neuronal activity. In the last study, I examined whether fluoxetine could reduce fear-related behaviour in CORT-treated rats.

In **Chapter 2,** I examined the effects of 21 days of repeated CORT injections on helplessness behaviour in a modified and traditional version of the FST. In addition, to determine whether immobility behaviour in the FST could be related to a general reduction in locomotor activity or muscle strength, activity in an open-field and the ability to remain suspended from a wire were assessed. I found that repeated CORT injections produce helplessness behaviours in the FST regardless of whether a modified or traditional version of the FST is used. I also found that repeated CORT injections had no significant effect on locomotor activity or muscle strength, which suggested that the depressogenic effects of CORT occur independently of potential changes in nonspecific motor behaviour. Importantly, previous research subjecting rats to repeated CORT injection paradigms similar to the one used in this dissertation revealed inhibited sexual behaviour (Gorzalka & Hanson, 1998), impaired cognition (Coburn-Litvak et al., 2003; Feldmann, Jr. et al., 2008), and decreased preference for sucrose consumption (David et al., 2009; Gorzalka et al., 2003; Gourley et al., 2008; Wu et al., 2013). Together, these data reinforce the notion that repeated CORT injections are a useful preclinical paradigm for investigating the interaction between glucocorticoids and depression.

The results from Chapter 2 stimulated a desire to further examine the depressogenic effects of repeated CORT exposure. In **Chapter 3**, I decided to investigate the extent to which repeated glucocorticoids mediate the memory of tasks that evoke emotional arousal (i.e., fear conditioning). Previous research has examined the effects of repeated stress and repeated glucocorticoid exposure on delay fear conditioning (a task that examines both contextual and tone cued fear conditioning within the same animal). However, these studies have revealed inconsistent results thereby complicating interpretation of the effects of repeated stress or repeated glucocorticoid exposure on emotionally driven memory (Conrad et al., 1999; Conrad et al., 2001; Conrad et al., 2004). In this chapter I examined the effects of repeated glucocorticoids on delay fear conditioned behaviours, whether variations in protocol determine the magnitude of these effects, and whether these effects are dose dependent. I found that repeated CORT injections increased freezing behaviour during recall of both context and tone cues associated with delay fear conditioning. Specifically, I showed that freezing during recall of contextual cues is dose dependent with a high dose of CORT significantly enhancing freezing behaviour whereas a low dose of CORT did not. In contrast, both the high and low dose of CORT significantly enhanced freezing behaviour during recall of tone cues. Moreover, the order of the presentation of context versus tone cues during testing determined both the magnitude of freezing behaviour following repeated CORT injections as well as whether the effect was found to be statistically significant. These results show that fear conditioned memory is sensitive to the effects of repeated glucocorticoid exposure and may be useful as a measure of depression-associated changes in emotionally driven memory.

This study initiated a series of experiments that sought to determine how repeated CORT exposure affects different types of fear conditioning and the brain regions involved in mediating these effects. Taking into consideration that depressed individuals are more likely to 1) pay attention to, memorize and recall information with a negative emotional connotation (Leppanen, 2006), 2) label neutral faces with negative emotions (Rubinow & Post, 1992), 3)

ruminate over their negative emotions and experiences (Nolen-Hoeksema, 2000), 4) show increased brain activity in the amygdala in response to sad faces and decreased activity in response to happy faces (Grillon et al., 2005; Leppanen et al., 2004; Leppanen, 2006), and 5) show enhanced learning of fear conditioned cues (Nissen et al., 2010), I reasoned that examining the effects of repeated CORT exposure on the processes of emotionally driven memory would be particularly interesting.

In **Chapter 4**, I investigated whether cellular activity in the hippocampus and amygdala, regions known to be involved the processing of delay fear conditioning and regulation of HPA activity, could be mediating the effects of CORT observed in Chapter 3. I found that repeated CORT injections significantly increased Fos immunoreactivity in response to the recall of contextual fear-associated cues in a structurally specific way. In particular, the number of Fos positive cells was increased in the BLA and CeA, but not the LA or any of the sub regions of the hippocampus examined. However, Fos immunoreactivity was not altered in any of the regions examined in the hippocampus or amygdala following recall of tone-specific cues. These results provide evidence that the CeA and BLA are involved in mediating the effects of repeated glucocorticoids on fear memories particularly during the recall of contextual fear memories.

The results from Chapter 4 implicated the amygdala in mediating the effects of glucocorticoids on fear conditioning, therefore in **Chapter 5** I asked whether trace conditioned freezing, a task heavily reliant on the hippocampus and less on the amygdala (Beylin et al., 2001; McEchron et al., 1998a; Raybuck & Lattal, 2011) is also increased following CORT treatment. Similar to the results from Chapter 4, I found that repeated CORT injections significantly increased fear-related behaviours to trace conditioned cues. Furthermore, I showed that repeated CORT enhances acquisition of fear during the training period. The results from this chapter, taken together with the results from chapters 3 and 4, suggest that repeated CORT exposure reliably enhances fear-related behaviour in at least 3 different fear conditioning tasks

(i.e., contextual, tone, and trace). Therefore, repeated glucocorticoid exposure readily produces enhancements in learning and memory processes that evoke emotional arousal.

For the final experiment, **Chapter 6**, I attempted to provide evidence to support the theory that enhancements in fear-related behaviour following repeated CORT exposure are reflective of a depression-like phenotype in rodents. To provide this evidence, I attempted to prevent freezing behaviour in CORT-injected rats by concomitantly injecting these animals with the antidepressant drug fluoxetine. I found that fluoxetine significantly decreased freezing in CORT-injected rats during recall of tone cued fear associations, but not to context cued fear. Interestingly, I did not find a significant difference in freezing behaviour between CORT and vehicle-injected rats to either tone or contextual cued associations, which limits the interpretability of these findings.

Overall, the results provided in this dissertation provide compelling evidence that repeated glucocorticoid exposure produces behaviours that are reflective of a depressive phenotype. Rats subjected to repeated CORT injections show robust helplessness behaviours and abnormally increased functioning during learning and memory of tasks that invoke a negative emotional experience (i.e., fear). The results obtained also suggest that neural changes within the amygdala may be at least in part responsible for the mediating the effects of repeated glucocorticoids on increased fear behaviours.

2. Repeated Glucocorticoid Exposure Produces Depression-like Behaviour in the FST and Fear Conditioning Tasks

Previous research has shown that the CORT model of depression used in this dissertation (i.e., 21 days of 40 mg/kg of CORT injections) produces helplessness behaviours in the FST (Gregus et al., 2005; Johnson et al., 2006; Kalynchuk et al., 2004); however, a one-day protocol of FST results in less immobility behaviour in rats compared to a two-day protocol (Armario, Gavalda, & Marti, 1988). Therefore, a one-day FST protocol may not be accurately

measuring the effects of repeated CORT injections on helplessness behaviour in this task. The results from my dissertation comparing the effects of CORT on a one day versus two day FST show that repeated high dose glucocorticoid exposure produces robust increases in depressive-like behaviour regardless of FST protocol. Interestingly, I revealed that latency to immobility was dependent on the protocol of FST used where rats subjected to the two-day test showed decreased latency to immobility regardless of treatment used. These results suggest that latency to immobility may not be a sensitive measure of depression-like behaviour in rats, but rather may reflect familiarity to the testing situation that occurs with a two-day protocol.

I replicated previous findings that repeated stress and repeated glucocorticoid exposure produce enhanced freezing behaviours to contextual cues (Conrad et al., 1999; Conrad et al., 2004; Cordero et al., 2003; Sandi et al., 2001; Skorzewska et al., 2006), and tone cues (Conrad et al., 1999; Dagnino-Subiabre et al., 2012), and extended this work to show that repeated CORT can also enhance freezing to trace fear conditioned cues. The question is, are these behaviours reflective of a depressive phenotype or are they reflective of another pathology such as anxiety? There are several reasons to interpret the results from Chapters 3 to 6 as reflecting an increase in depression-like behaviour as opposed to an anxious state. First, several lines of research show that depressed individuals have enhanced performance when attending to, memorizing, or recalling information that has a negative emotional connotation (Leppanen, 2006). Second, because humans can perform fear conditioning tasks, a direct comparison of behaviour can be made between individuals with depression and rodents from preclinical animal models. Indeed, individuals diagnosed with depression show enhanced learning of cued fear conditioning (Nissen et al., 2010), similar to what was reported in this thesis. Third, fear and anxiety are distinct dissociable states. Fear is characterized by arousal and fight or flight responses, such as freezing in response to immediate threats (phasic fear), whereas anxiety is characterized by tension, and worry about the potential that a threat might occur in the distant future (sustained fear) (Davis, Walker, Miles, & Grillon, 2010). Importantly, amygdala lesions

have no effect on the performance of unconditioned tests of anxiety such as the elevated plus maze and black/white two-compartment test (McHugh, Deacon, Rawlins, & Bannerman, 2004; Treit & Menard, 1997). Alternatively, amygdala lesions impair both contextual and tone cued fear conditioning (Phillips & LeDoux, 1992; Sierra-Mercado et al., 2011) suggesting that conditioned fear and unconditioned anxiety are mediated by dissociable brain circuits. Fourth, a study investigating the effects of acute stress on fear conditioned behaviour in humans revealed that self reported anxiety did not predict conditioning levels. In this study, what did predict conditioning was cortisol levels (obtained through saliva) indicating that the stress response may be modulating emotional learning as opposed to anxiety (Jackson et al., 2006). Finally, the antidepressant fluoxetine decreased freezing behaviour in CORT-injected rats during recall of tone cues suggesting that these glucocorticoid-induced behaviours are responsive to widely used antidepressants. Taken together, the overall findings of the research in this dissertation and the literature suggests that repeated exposure to glucocorticoids produces increased fear-related behaviour in various fear conditioning tasks (although there may be some exceptions, see limitations section below)-behaviour that is reflective of a depression-like state. The findings from this dissertation also support the proposal that fear conditioning is a valuable tool in the measurement of depression-like behaviour.

3. The Effects of Repeated Glucocorticoid Exposure on Hippocampal-Dependent Versus Non-Hippocampal Dependent Fear Conditioning

One observation from previous studies and the research presented in this dissertation (Chapters 2 -4) is that repeated stress and repeated glucocorticoid exposure differentially affect hippocampal-dependent fear conditioning (i.e., contextual and trace conditioning) compared to hippocampal-independent fear conditioning (i.e., tone cued conditioning). Previous studies have demonstrated robust increases in fear memory to contextual fear conditioning following repeated stress and repeated glucocorticoid exposure (Conrad et al., 1999; Conrad et al., 2004;

Cordero et al., 2003; Sandi et al., 2001; Skorzewska et al., 2006) whereas less robust effects are found to tone cued conditioning (Conrad et al., 1999; Conrad et al., 2001; Conrad et al., 2004; Dagnino-Subiabre et al., 2012). The results from Chapter 5 suggest that repeated glucocorticoid exposure also produces robust increases in fear behaviours to trace conditioned cues as well. Increased freezing to hippocampal-dependent fear conditioning is intriguing in light of the aberrant neuroplasticity that occurs in the hippocampus following repeated stress and repeated glucocorticoid exposure (Czeh et al., 2001; Magarinos & McEwen, 1995; Sheline et al., 2003; Tata & Anderson, 2010; Watanabe et al., 1992; Woolley et al., 1990) and the results from this dissertation (Chapter 4) and others which suggest that the amygdala may be largely mediating the effects of stress and glucocorticoids on fear conditioning (Conrad et al., 2004; Skorzewska et al., 2006). These results pose two questions: why is hippocampal-dependent contextual fear conditioning more strongly affected by repeated glucocorticoid exposure when the effects of glucocorticoids on fear conditioning are mediated largely by the amygdala? Further, if the amygdala is the region largely responsible for enhanced freezing, then why is tone cued conditioning less robustly affected by repeated stress and repeated glucocorticoid exposure when tone conditioning is also dependent on amygdala functioning?

One explanation for the discrepancy in performance between contextual and tone conditioning could be related to HPA axis dysregulation resulting from repeated glucocorticoid exposure. Research in our lab has shown that repeated injections of CORT result in HPA axis dysregulation whereby CORT-injected rats show blunted endogenous CORT release following an acute stressor (Johnson et al., 2006). Similarly, rats that received 25 days of 20 mg/kg CORT injections showed decreased serum levels of CORT directly after recall of contextual fear memories and enhanced fear-related behaviour during recall (Skorzewska et al., 2006). In addition, rats that received CORT in their drinking water for 21 days showed increased freezing during recall of contextual cues and serum CORT levels were negatively correlated with these fear behaviours (Conrad et al., 2004). Taken together, these results suggest that the blunted

CORT response to a stressful task observed in CORT-treated rats could underlie the changes in fear-related behaviours to contextual cues. The hippocampus is particularly sensitive to the effects of repeated glucocorticoids as it is abundant in glucocorticoid receptors (Feldman & Weidenfeld, 1999). Therefore it may not be surprising that hippocampal-dependent contextual fear conditioning is more robustly affected by repeated glucocorticoid exposure. Furthermore, reciprocal connections between the hippocampus and amygdala are necessary for the processing of contextual fear conditioning but not cued conditioning (Anagnostaras et al., 1999; Huff et al., 2006; Maren et al., 1997; Phillips & LeDoux, 1992). Although the hippocampus did not show preferential activation during recall of contextual cues in CORT-treated rats, hippocampal activity during contextual fear conditioning could still be significantly impacting freezing behaviour through its interactions with the amygdala. It should be noted that other types of neurobiological changes may be occurring in the hippocampus in CORT-treated rats that are not reflected in changed Fos expression. For example, morphological changes in the hippocampus (i.e., spine density changes) or changes in the expression of other genes beyond *c-fos* may be modulating the learning and memory of contextual fear associations. Alternatively, some researchers believe that contextual memories are more vulnerable to the effects of glucocorticoid manipulations because contextual fear memory is considered to be generally weaker (i.e., more difficult to learn and easier to disrupt) than cued conditioned memory (Rodrigues et al., 2009).

It is important to note that Fos is just one measure of neuronal activity and may not capture all the active cells in the hippocampus. Immediate early genes can be divided into two different classes that influence cellular function by either: 1) regulating transcription factors thereby influencing cellular function via the genes they regulate or 2) by directly modulating cellular functions ("effector" immediate early genes). C-fos is a transcription factor immediate early gene, whereas a different type of immediate early gene such as Arc, is an effector gene and has a direct effect on cellular function (Guzowski, Setlow, Wagner, & McGaugh, 2001). It

has been suggested that effector immediate early genes may be involved in the modulation of dendritic scaffolding required for synaptic plasticity after learning. Alternatively, transcription factor immediate early genes may have less of a direct effect on synaptic plasticity (Huff et al., 2006). Future experiments are needed to address whether different types of immediate early genes are more representative of the effects of repeated glucocorticoid exposure on hippocampal cellular activation following recall of fear conditioning tasks.

The results from this dissertation and the literature demonstrate that fear conditioning is a valuable tool for examining alterations in the memory of tasks that evoke emotional arousal produced by preclinical animal models of depression. I have summarized several studies that demonstrate enhanced memory of emotionally arousing tasks in depressed populations. I have also discussed how fear conditioning provides a means to detect differences in performance in emotionally evoked learning and memory in both animals and humans. Furthermore, this dissertation provides evidence that repeated glucocorticoid exposure in rats, which is shown to produce robust depression-like behaviour on other measures, also significantly enhances performance during recall of fear conditioned memories. Moreover, preliminary evidence provided in this dissertation demonstrated that the commonly used antidepressant, fluoxetine, prevents some of the enhancements in conditioned fear produced by repeated glucocorticoid exposure. When taken together, this evidence supports the use of fear conditioning in the measurement of depression-like behaviour in preclinical animal models.

4. Limitations

High Dose CORT Injections are Supraphysiological

One criticism of using repeated high dose CORT injections (i.e., 40 mg/kg) to model depression is that this dose produces supraphysiological circulating levels of glucocorticoids. Indeed, the 5 mg/kg dose of CORT better mimics plasma concentrations produced by an acute stressor (Sandi et al., 1996). Thus a high level of glucocorticoids is needed to reliably produce a

depressive phenotype in commonly used measures of depression-like behaviour such as the FST (Johnson et al., 2006). Contrary to the FST, data presented in Chapter 3 demonstrates that repeated injections of 5 mg/kg of CORT do enhance freezing behaviour to tone cues, which is arguably reflective of depression-like behaviour. However, Chapters 3 – 5 demonstrate that a 40 mg/kg dose of CORT produces more robust freezing during various fear conditioning tasks and is therefore more reliable. Although this criticism is common to repeated high dose CORT models, other repeated stress models of depression also produce unrealistic situations that are unlikely to occur in humans due to the use of physical stressors such as immobilization. Furthermore, habituation effects and individual differences in HPA axis response (Gregus et al., 2005; Grissom & Bhatnagar, 2009) produce variability in these repeated stress models, which are avoided by repeated exogenous administration of CORT. Chronic mild stress is possibly the most realistic representation of the daily stressors experienced by humans in rodents. However, this model also has several criticisms in that it is laborious and can be difficult to establish (Willner, 1997). It would be interesting to examine whether the effects produced by repeated glucocorticoid exposure on fear-conditioned behaviours are also seen in chronic mild stress paradigms.

Is Contextual Fear Also a Measure of Anxiety?

As discussed earlier, fear is characterized by arousal and fight or flight responses to immediate threats (phasic fear), whereas anxiety is characterized by tension about a potential threat that might occur in the distant future (sustained fear) (Davis et al., 2010). Davis et al. (2010) suggest that contextual fear conditioning produces a model of sustained fear. Davis et al. argue that because contextual conditioning uses unpaired shocks, contextual cues become constant reminders of the unconditioned stimulus leaving an organism in a continuous state of fearful apprehension. Conversely, discrete cue conditioning produces phasic fear as the unconditioned stimulus is presented directly after the presentation of the conditioned stimulus

thus producing an immediate threat. This argument suggests the possibility that contextual fear associations may be more representative of a state of anxiety as opposed to as state of enhanced depression-like behaviour. However, it should be noted that the repeated CORT exposure model used throughout this dissertation does not significantly affect anxiety in the open-field or social interaction tests (Gregus et al., 2005). Furthermore, no changes in fear-related behaviour were noted during any of the habituation periods prior to the fear conditioning training sessions (Chapters 3 – 6) suggesting that enhanced freezing in CORT-treated rats during contextual testing is likely not the result of enhanced anxiety. Despite this potential limitation, results presented in Chapter 3 demonstrate that repeated glucocorticoid exposure also produces enhancements to tone cued associations, which is arguably representative of phasic fear and dissociable from anxiety. Indeed, Chapter 6 revealed that fluoxetine specifically attenuated freezing in CORT-treated rats to tone cues and not contextual cues possibly suggesting that discrete cued fear conditioning may be a more direct measure of depression-like behaviour. It should be noted, however, that Davis et al. did not specify whether contextual fear associations acquired during delay conditioning, as occurred in Chapter 3, 4 and 6 of this dissertation (i.e. acquired in conjunction with tone cue associations), produce sustained fear in the same way that strictly contextual fear acquisition does. Therefore, the effects of repeated glucocorticoid exposure on contextual fear observed in Chapters 3 and 4 may not be representative of sustained fear and thus increased anxiety as argued by Davis et al..

Lack of Power in Detecting Significance in Tone Cued Conditioning and Fos Expression?

The research presented in this dissertation demonstrates that fear conditioned behaviour is significantly enhanced regardless of whether the task is dependent on the hippocampus, the amygdala, or both regions (Chapters 3 – 5). Interestingly, we only showed enhanced expression of Fos in the BLA and CeA following contextual fear memory, although Fos expression was not examined following recall of trace conditioning. There was a trend for

increased freezing to tone cues when examined after context testing which was mirrored by an increase in Fos positive cells in the amygdala. It is possible that there may not have been enough statistical power in this group to obtain significance (counterbalanced presentation of cues resulted in significant effects). Thus, increased power in these tests may have revealed that tone cued conditioning is also mediated by the amygdala. Indeed, glucocorticoid receptors in the CeA have been shown to be important to both contextual and tone cued conditioning as CeA specific glucocorticoid receptor knockout mice show deficits in both of these tasks (Kolber et al., 2008).

Can Depression Be Modelled in Rodents?

A number of challenges are faced when attempting to model human psychiatric disorders in rodents. As mentioned in Chapter 1, some of the symptoms of depression such as suicidal ideation cannot be convincingly measured in animal models. Furthermore, behavioural tasks that are used to recapitulate the human depressive phenotype in rodents (e.g., learned helplessness observed in the FST) are only approximate correlates of the human condition (Nestler & Hyman, 2010). Another difficulty arises when determining whether a set of symptoms in rodents sufficiently summates to represent the human psychiatric condition. Depression is characterized by a range of behavioural and cognitive symptoms with a potentially large variation of these symptoms from individual to individual. For instance, one individual may experience decreased interest in pleasurable activities, increased need for sleep, and weight gain. Another individual may experience irritable mood, decreased need for sleep, and weight loss. Therefore, the decision to accept a particular rodent model as representative of the human condition can be quite complex. Despite the difficulties inherent in evaluating rodent models of emotional disorders, three criteria have been commonly accepted to judge the usefulness of preclinical models: predictive, face, and construct validity (discussed in more detail in Chapter 1) (Nestler & Hyman, 2010). Chapters 2 through 5 of this dissertation were largely designed to

further establish face validity for the CORT model. Chapter 6 was designed to provide predictive validity to both the CORT model and fear conditioning as a tool for probing alterations in emotional learning and memory produced by CORT. Future research should continue to evaluate the validity of the CORT model of depression and fear conditioning as a measure of depressive-like behaviour.

5. Future Directions

What Phase of Fear Conditioning is Affected by Repeated Glucocorticoid Exposure?

The data presented in this dissertation should be considered as a starting point for examining the effect of repeated glucocorticoid exposure on fear learning and memory. Chapters 3 through 5 clearly demonstrate that repeated CORT injections significantly enhance freezing behaviour to contextual, tone, and trace cued conditioning. Next it would be interesting to determine what phase of fear learning and memory is significantly affected by repeated glucocorticoid exposure, or whether all phases of learning and memory are affected. As mentioned in the general introduction, learning and memory of fear conditioning can be divided into distinct phases (e.g., acquisition, consolidation, recall) that are mediated by separate brain circuits.

There are, however, some complications in designing experiments that isolate the effects of repeated glucocorticoids on distinct learning and memory phases of fear conditioning. For example, it is difficult to design an experiment that assesses the effects of repeated CORT injections solely on consolidation of learned fear associations. Ideally, with acute stress for example, the subject would acquire the fear association during training then receive an acute stressor directly afterwards to examine whether the consolidation of that memory is affected. A similar protocol would not effectively examine the effects of repeated stress or glucocorticoid exposure on consolidation. If repeated CORT injections were administered following fear training, the consolidation process would be complete well before 21 days of injections were

complete. Future studies will have to examine the effects of repeated glucocorticoid exposure in combination with agents that, for example, alter glucocorticoid receptor functioning, to selectively determine which phases of fear learning and memory are more susceptible to repeated glucocorticoid exposure.

Sex Differences in Response to Repeated CORT Injections During Fear Conditioning

It is well documented that the incidence of depression is different for men and women with women being diagnosed more often than men (Piccinelli & Wilkinson, 2000). Sex differences are also found when examining the effects of acute stress on fear conditioning in men and women. In particular, acute stress exposure facilitates fear conditioning in men; whereas, stress inhibits fear conditioning in women. Salivary CORT levels are associated with freezing levels in men, but not in women (Jackson et al., 2006). Furthermore, acute stress enhances eyeblink conditioning in male rats, but decreases conditioning in females (Wood & Shors, 1998). Sex differences are also noticed following 21 days of restraint stress with females showing significantly decreased freezing during recall of tone cued conditioning whereas males show significant enhancements during recall (Baran, Armstrong, Niren, Hanna, & Conrad, 2009). These studies indicate that males show enhanced performance on emotional memory driven tasks but females show deficits. Interestingly, our lab has examined sex differences in response to repeated high dose CORT exposure in the FST. We found that CORT increased immobility behaviours in both male and female rats, although CORT-injected females showed less immobility than CORT-injected males (Kalynchuk et al., 2004). The culmination of this research suggests a complex relationship between sex, glucocorticoid exposure, and depressive-like behaviour. Future research should investigate the nature of these associations.

The Effects of Repeated Glucocorticoid Exposure on Extinction of Conditioned Fear

As mentioned in the introduction of this dissertation, depressed individuals are more likely to ruminate over their negative emotions and experiences. Furthermore, rumination is directly associated with the severity of depressive symptoms and predicts the onset of depressive episodes (Nolen-Hoeksema, 2000). Despite the extent and impact of rumination and maladaptive thinking patterns on depressed individuals, the biological bases for these deleterious patterns of thinking have received little experimental investigation. Extinction of fear memories is a potential model for studying these maladaptive thinking patterns. Extinction involves new learning of a conditioned stimulus – no unconditioned stimulus association (Bouton & Bolles, 1979; Westbrook, Iordanova, McNally, Richardson, & Harris, 2002) and is measured by the reduction of freezing.

Extinction learning and memory is significantly affected by stress and glucocorticoids. For instance, rats exposed to CORT in their drinking water for 14 days have slowed acquisition of extinction (Gourley et al., 2009). Restraint stress for 3hrs/day for 1 week does not affect acquisition of extinction, but does impair recall of extinction 24 hr later (Miracle, Brace, Huyck, Singler, & Wellman, 2006; Wilber et al., 2011). Also, rats subjected to restraint stress for 6 hrs/day for 21 days (Baran et al., 2009) or chronic mild stress for 21 days (Garcia, Spennato, Nilsson-Todd, Moreau, & Deschaux, 2008) have impaired recall of extinction 24 hr after extinction training without impairments in extinction acquisition. Similar to fear conditioning, studies that have investigated the effects of repeated stress and glucocorticoids on extinction learning and memory, such as 3 weeks of restraint stress, are models shown to be less effective at producing a depressive phenotype than the direct administration of CORT into an organism through subcutaneous injection (Gregus et al., 2005). Therefore, the effects of repeated CORT injections on extinction learning and memory should be investigated at a dose that produces robust changes in depression-like behaviour.

Involvement of Other Brain Regions in Mediating the Effects of Repeated Glucocorticoid Exposure on Fear Conditioning

Although Chapter 4 of this dissertation focused on effects of repeated CORT injections on Fos+ cells in the hippocampus and amygdala, other areas of the brain show evidence of plasticity in response to glucocorticoids and are involved in regulating fear conditioned learning and memory. For instance, the mPFC also contains glucocorticoid receptors (Morimoto et al., 1996) and is vulnerable to the effects of repeated stress. Prolonged stressors are associated with impairments in attentional set-shifting, which is dependent on intact mPFC functioning (Liston et al., 2006). Furthermore, repeated stress produces atrophy of apical dendrites of pyramidal neurons in the mPFC (Radley et al., 2004). Subregions of the mPFC such as the prelimbic cortex have been shown to be involved in fear responses (Burgos-Robles, Vidal-Gonzalez, & Quirk, 2009) and to integrate information from the BLA and ventral hippocampus to regulate fear expression (Sotres-Bayon, Sierra-Mercado, Pardilla-Delgado, & Quirk, 2012). Importantly, depression is also linked to altered mPFC functioning as reductions are found in cerebral blood flow and glucose metabolism in individuals suffering from this disorder (Drevets, 2000). Thus, investigation into the effect of repeated glucocorticoid exposure on mPFC activity in response to fear conditioning is warranted.

Investigation of the Neurobiological Mechanism Underlying the Effects of CORT on Fear Conditioning

The neurobiological mechanisms mediating CORT-induced enhancements in fear memory are not well understood. Chapter 4 of this dissertation presents a potential focus point (i.e., the hippocampus and amygdala) for further investigation into the mechanism underlying these changes. Increasing evidence suggests that the morphological changes found in depressed individuals and stressed animals are related to alterations in the release and transmission of glutamate. During episodes of learning, glutamate accumulates in the synapse

and binds to two types of glutamate receptors—the N-Methyl-D-aspartic acid receptor (NMDAR) and the amino-3-hydroxy-5methyl-4-isoxazolepropionic acid receptor (AMPAR). When glutamate continues to accumulate in the synapse for extended periods of time, however, glutamate becomes excitotoxic (Sapolsky, 2000). Theoretically, this over-activation contributes to many of the neurobiological signatures of depression. The binding of glutamate to NMDARs results in the activation of free cytosolic calcium, which produces changes in synaptic excitability associated with memory (Sapolsky, 2000). However, too much cytosolic calcium results in excessive activity of calcium-dependent enzymes producing cell damage and, in extreme cases, cell death (Sapolsky, 2000; Sapolsky, 2003). Multiple lines of research show that acute stress increases the presynaptic release of glutamate in the mPFC, amygdala, and hippocampus (Hascup et al., 2010; Moghaddam, 1993; Musazzi, Racagni, & Popoli, 2011; Reznikov et al., 2007). Furthermore, repeated stress produces an increase in GluR1 mRNA (an AMPAR subunit) in the hippocampus (Schwendt & Jezova, 2000). Both the acquisition and expression of fear conditioning rely on NMDAR activation (Dalton, Wu, Wang, Floresco, & Phillips, 2012; Maren, Aharonov, Stote, & Fanselow, 1996); therefore, CORT-induced enhancements in fear memory could be partially mediated through synaptic plasticity at glutamatergic synapses. Although recent research has begun to investigate the effects of repeated stress on glutamate transmission and receptor trafficking, these effects are still largely unknown. Fear conditioning offers an ideal tool for exploring the functional consequences of stress on glutamatergic transmission and receptor trafficking.

6. Conclusions

The general goal of this dissertation was to systematically investigate the effects of repeated CORT injections on learned helplessness behaviours in the FST, learning and memory recall of tasks that evoke negative emotional arousal (i.e., variations of fear conditioning), and whether the antidepressant, fluoxetine, reverses some of the CORT-induced

effects observed on fear conditioning performance. Evidence was provided that repeated glucocorticoid exposure results in robust depression-like behaviours in both traditional and non-traditional versions of the FST, one of the most widely used measures of depressive-like behaviours in rodents. Importantly, these changes in behaviour were not accounted for by alterations in locomotor activity or muscle strength. In addition, I demonstrated that repeated CORT injections enhance performance in emotionally driven learning and memory tasks. Specifically, freezing behaviour in contextual, tone cued, and trace fear conditioning paradigms was increased following repeated CORT injections. These findings are consistent with the human literature which shows that tone cued fear conditioning is enhanced in depressed subjects (Nissen et al., 2010). Moreover, cellular activity, stimulated by the recall of contextual fear conditioning, was significantly increased in sub regions of the amygdala implicating these structures in the plasticity associated with enhanced recall of fear conditioned memory. Finally, I showed that the antidepressant fluoxetine can decrease freezing behaviours in CORT-injected rats, thereby providing validity to the idea that fear conditioning is a measure of depression-like behaviour. These experiments provide a starting point for understanding how repeated glucocorticoid exposure alters performance on learning and memory tasks that evoke an emotional response, such as fear. By understanding how learning and memory of emotionally driven tasks are affected by glucocorticoids, selective research on the pathology associated with these behaviours might lead to more effective treatments for depressed individuals afflicted with maladaptive thinking patterns and emotional responses.

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