Exploring the Neuroplastic and Antidepressant-like Effects of Erythropoietin

by

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ABSTRACT

Depression is a chronic, often debilitating disorder which may be characterized by prolonged periods of mood disruption and negative affect with a high rate of relapse. A growing body of evidence implies that depression may arise in part through a deregulation of neuroplasticity within limbic structures including the hippocampus, and novel rapid-acting antidepressants have shown therapeutic potential by exerting neurogenic and neuroplastic effects. The hematopoietic cytokine erythropoietin (EPO) has antidepressant potential in both humans and animal models, with an ability to regulate neuroplastic as well as cognitive-behavioral outcomes. The present research outlines two experiments undertaken to further the knowledge of the antidepressant potential of EPO, inferred by both cellular and behavioral outcomes. In experiment 1, the effect of 2 week EPO administration (5000IU/kg vs. saline x 4injection) on chronically stressed rodents (a widely accepted animal model of depression) was observed by way of the forced swim test (FST) as well as doublecortin (DCX) immunohistochemistry for immature granule cells in the hippocampal dentate gyrus (DG). Additionally, an analysis of the potential for EPO to modulate structural plasticity within these cells was sought, yet ultimately not undertaken. In experiment 2, whether acute EPO administration (saline, 2500IU/kg, 5000IU/kg) has the potential to synergize with the SSRI citalopram (sal. vs. 5mg/kg) was analyzed using the FST. This research showed no effect of EPO on cellular or behavioral outcomes, and was ineffective in synergizing with citalopram, suggesting a necessity for further studies of this paradigm utilizing alternative models or methods to those used here.

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INTRODUCTION

Stress and Depression

It has been found that experiencing stressful events can impart a prominent susceptibility to depressive symptomatology. Both early life stressors as well as prolonged stress throughout adulthood can contribute to the onset of depressive illness, especially in patients with certain genetic predispositions. Moreover, It has been shown through analysis of the brains of humans diagnosed with Major Depressive Disorder that specific morphological and cellular correlates of a depressed human brain are directly mirrored in the brains of animal models subjected to stressful events (de Kloet *et* al, 2005; Anisman *et al*, 2008; Schoenfeld and Gould, 2012). The analysis of chronically stressed animals has thus become a widely accepted and reliable model for the neuroscientific research of depression (Hill *et al*, 2012), and in the present study, rodents subjected to chronic mild stress were employed to model the disorder.

Real and Predicted Stressors

Stressful stimuli can be theoretically divided into two subcategories, outlined in-depth below, describing their etiology as either biologically intrinsic or extrinsic (Herman *et al*, 2003). While a "real" stressor is a biological threat to homeostasis, "predicted" stressors typify environmental factors that have been cognitively appraised as potentially harmful. A prolonged state of stress-based arousal has been found to have vast implications for psychopathology (Gould, 1992), and therefore, the dichotomy of real and predicted stressors is especially pertinent to the study of human disease. While humans may encounter a direct threat to their bodily well-being, a state of endemic psychological

stress due to prolonged appraisal of adverse life factors, such as an inconsistent income, is a characteristically human experience. Importantly, these chronic stressors seem to have profound consequences within the brains of vulnerable individuals and can lead to depression by way of exacerbating both cellular and behavioral symptomatology and directly undermining mechanisms used to effectively cope with stressful events (Holsboer, 2000; Snyder, 2011)

A classic view of "stress", as it applies to a diverse number of organisms, may outline a stressful event as any life experience that poses a threat to homeostasis. A dynamic state of physiological equilibrium (de Kloet *et al*, 2005), homeostasis is the motivating target outcome of ongoing and directed behavior by an organism in order to ultimately maintain its survival. Direct threats to a healthy biological state, or "real" stressors, are detected by physiological mechanisms and thus trigger "reactive" stress responses in order to recruit the necessary endogenous faculties to prevent bodily harm. Pain, fluctuations in cardiovascular tone, or circulating pro-inflammatory cytokines in response to immune challenge for instance may all signal the presence of a potential physiological threat to homeostasis. Therefore, to recognize and ultimately mitigate these factors, appropriate endogenous responses must be activated (Herman *et al*, 2003).

A more contemporary view of stress, pertinent to the psychological state of an organism or individual, refers to the alternative yet complementary "predicted" stressor. While not a direct threat to homeostasis, a predicted stressor stimulates an organism's psychological appraisal of an impending and possibly noxious event or experience, such as the presence of a predator species (Tanapat *et al*, 2001). When an appraisal of the potential impact incurred by a life event is incongruent with the organism's preconceived

cognitive expectations (those formed around past subjective experience), the appraisal results in a marked increase in physiological arousal, known as an "anticipatory" response (Herman *et al*, 2003). This arousal musters endogenous systems that allow for heightened cognitive processing and vigilance, and these factors allow an organism to effectively direct its behavior toward mitigating the impact of the life event. While obvious predicted stressors may be typified by an uncertainty of physical harm to an organism (such as novel environments or the smell of fox urine to a rat), and indeed potentially dangerous experiences will manifest in a typical stress response from a human, predicted stressors may also take the form of social challenges or intricate appraisals of a lack of control over ones environment (Herman *et al*, 2003).

The Human Stress Experience

Predictive stressors and the resulting anticipatory responses are therefore of direct relevance to an understanding of the contemporary human stress experience. Resulting from cognitive capabilities exclusive to humans affording a sophisticated social environment, the ability to plan for the future, and an advanced capability for abstract reasoning, there exists a tremendously intricate climate of circumstances that have the potential to be psychologically stressful to human beings. Thus common human experiences, such as ongoing hostility in the workplace or marital issues, can result in the activation of stress responses directly related to those incurred by interacting with an animal perceived to be dangerous, for example.

Of direct relevance to the current body of work is the typically chronic nature of these stressors, which may in part constitute the substrate for depressive symptomatology

(Pariante and Lightman, 2008). While effective coping, an adaptive experience, is accomplished by a rapid stress response followed by its effective cessation, a chronically activated stress response itself can exert negative pressures upon the body (McEwan, 1999). The hypothalamic-pituitary-adrenal (HPA) axis, discussed below, constitutes the endogenous system within mammals that recruits mechanisms required to cope with stressful events. Both real and predicted stressors may activate the HPA axis, and while acute activity of this system provides adaptive capabilities to the organism, it has been shown that chronic HPA axis activity has the potential to exert deleterious effects within diffuse brain regions, notably to structures of the limbic system (Gould, 19992; de Kloet *et* al, 2005; Anisman *et al*, 2008; Schoenfeld and Gould, 2012).

The HPA Axis

Adverse physical and environmental factors, experienced under both acute and chronic circumstances, characteristically influence an endogenous coping mechanism within the mammalian CNS known as the hypothalamic-pituitary-adrenal (HPA) axis. In the case of predicted stressors, limbic regions of the brain, specifically the hippocampus, prefrontal cortex (PFC), and amygdala, share reciprocal neural connections (Kim *et al*, 2001; Akirav and Richter-Levin, 2002) and are together responsible for the initial appraisal of a stressful event. Broadly, these structures influence the intersection of memory, decision-making and fear-emotions, and in stressful situations, limbic activation signals the initiation of an HPA axis response (Herman *et al*, 2003). The HPA axis constitutes a chemical cascade wherein hypothalamic corticotropin-releasing hormone (CRH) signals the release of adrenocorticotropin hormone (ACTH) from the pituitary,

ultimately signaling the release of glucocorticoids from the adrenal gland, namely cortisol in humans and corticosterone in rodents (de Kloet *et al*, 2005). Glucocorticoid release by way of the HPA axis musters peripheral bodily functions that serve to effectively respond to the stressful stimulus and diverts energy expenditure from processes that are not necessary for coping with the immediately salient stressful event; these mechanisms are classically referred to as “fight-or-flight”.

However, due to the generally high energy costs of these coping processes, the resulting expenditure can exert deleterious effects upon the organism if prolonged HPA axis activity is left unchecked (McEwen, 1999). In acutely stressful situations, negative-feedback inhibition of the HPA axis is signaled by high concentrations of glucocorticoids in the blood (Pariante and Lightman, 2008) effectively signaling to the brain that the stress response has been sufficiently activated. In conditions of chronic stress however, characterized by ongoing activation of the HPA axis, the prolonged presence of blood glucocorticoids may result in the degradation of limbic structures (Gould, 1992) as well as a derailment of adaptive HPA axis functioning in its entirety (Snyder, 2011), effects that will be elaborated upon in the context of depression, below.

It is clear then that an acute stress response is evolutionarily advantageous, and provides the appropriate mechanisms through which an organism may effectively maintain homeostasis, or mitigate the predicted risk of an environmental insult. However, chronic activation of HPA-associated mechanisms results in hypercortisolaemia, and this high blood-cortisol syndrome has the potential to exert negative pressures on diffuse bodily structures (de Kloet *et al*, 2005); of importance to the present body of work, hypercortisolaemia contributes to a break down of structure

and function within limbic regions of the brain (Lupien *et al*, 1998), notably the hippocampus, and it has been shown that such signs of psychopathology are directly paralleled in patients diagnosed with clinical depression (Dranovsky and Hen, 2006; Campbell *et al*, 2004).

The Neurogenesis Hypothesis of Depression

As previously mentioned, a chronically activated HPA axis results in prolonged high levels of blood glucocorticoids. Hypercortisolaemia, as well as structural and functional alterations within limbic structures, have become recognizable characteristics of a depressed brain; specifically, research has shown that circulating glucocorticoids have the potential to exert profound cellular effects upon the hippocampus (Gould, 1992; Ming and Song, 2011). One of only two neurogenic regions in the mammalian brain (Gage, 2000), the hippocampus seems particularly vulnerable to pathological effects induced by HPA axis hyperactivity. Further, while hippocampal modification can result in many maladaptive cellular and behavioral outcomes, alterations within this structure can also exert a reciprocal effect upon the HPA axis, amplifying the reactivity of the stress response system (Snyder, 2011).

The Hippocampus and Neurogenesis

Multipotent neural stem cells, possessing the ability to selectively differentiate into various progenitor cell lineages, have been found to exist within all areas of the mammalian CNS (Palmer *et al*, 1999). While these cells are present in diverse brain structures the process of neurogenesis, which constitutes the maturation of newly born

neurons, is restricted to two CNS structures; namely the subventricular zone of the lateral ventricals, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (Gage, 2000). While the neurogenic properties of each region have been studied in detail, the remainder of the present discussion will be devoted to examining the nature of the hippocampus; both its structure and function have been found to afford considerable implications to the study of stress, as well depressive symptomatology. Indeed, the process of hippocampal neurogenesis, as well as factors that may manipulate its expression, share a close relationship with HPA axis glucocorticoid secretion.

Hippocampal neurogenesis is critical to development of the immature CNS, yet it has been unequivocally established that this process continues into adulthood and indeed throughout the lifespan (Gage, 2000), undergoing a natural senescence as a result of the ageing process (Kuhn *et al*, 1996). In the adult brain, hippocampal progenitor cells are located in the SGZ of the DG and proliferate into immature neurons known as granule cells, ultimately migrating from the SGZ to the granule cell layer (GCL) of the hippocampus, located between the molecular layer and SGZ. Broadly, these granule cells undergo various structural and functional changes throughout the process of maturation, ultimately forming characteristic processes stretching towards the molecular layer and hippocampal CA3 pyramidal neurons (Zhao *et al*, 2006). However, the majority of granule cells are not destined to reach maturity (Zhao, Deng, Gage, 2008); importantly, it has been shown that the fate of an immature granule cell can be affected at the level of its initial proliferation, its subsequent differentiation to a neuronal lineage, or into its mature lifespan (Kempermann and Gage, 2002). Numerous factors, both environmental and

endogenous, have the potential to influence hippocampal neurogenesis at the level of these processes.

Factors influencing Neurogenesis: Focus on Stress and Cognition

As previously stated, the most basic regulator of neurogenesis is the natural course of ageing. Upon administration of Bromodeoxyuridine (BrdU), a marker of DNA synthesis that reliably labels cell proliferation, 21-month old rats were found to exhibit a marked decrease in labeled cells within the hippocampal GCL as compared to 6-month animals (Kuhn *et al*, 1996). This natural neurogenic senescence seems particularly telling; cognitive functions that may become irregular with age such as memory, learning and mood regulation, share a relationship with hippocampal neurogenesis. Importantly, maladaptive regulation of these cognitive faculties is also typical of depression.

It has been postulated that neurogenesis may be the cellular substrate of learning and memory (Snyder, 2001). While research has shown this relationship to be tremendously complex, broadly, it is accepted that hippocampus-dependent learning tasks stimulate granule cell proliferation (Leuner *et al*, 2006). Reciprocally, whether neurogenesis is in fact a necessity for certain types of learning is currently a topic of considerable neuroscientific debate, the investigation of which has demonstrated conflicting results (Ming and Song, 2011). An enriched environment exerts protective effects on hippocampal neurogenesis, promoting the ongoing survival of granule cells (Kempermann *et al*, 1997), while physical exercise enhances cell proliferation within the SGZ (van Praag *et* al,1999). Importantly, Kim *et al* (2010) demonstrated that short-term and spatial memory deficits associated with ageing were mitigated by physical exercise.

This associative evidence does not unequivocally demonstrate neurogenesis as a causative factor; however, since memory and neurogenesis both naturally decline with age and can be promoted by exercise, it is clear that changes in hippocampal neurogenesis share an association with varying aspects of cognition.

As previously stated, mood regulation is another cognitive process associated with changes in neurogenesis (Gould *et al*, 1992). Of particular importance to the current body of work Gould *et al* (1992) demonstrated that the presence glucocorticoids, typical of the HPA axis stress response, results in a suppression of SGZ cell proliferation. Therefore, since 1) both stressed animals and patients diagnosed with clinical depression exhibit hypercortisolaemia, and 2) evidence strongly implicates stress as a contributing factor to depressive illness, it has been postulated that the characteristic mood disturbances of a depressed individual could result in part due to an inhibition of neurogenesis. Accordingly, a large body of research surrounding the nature of neurogenesis has focused on what effects may be afforded by stressful stimuli, and how these factors could together influence mood and depressive-like symptoms.

Neurogenesis Hypothesis and Classic Antidepressants

As previously mentioned, stressful experiences constitute a prominent pre-disposing factor to depression, and chronically stressed animals exhibit physiological and behavioral symptoms that parallel the mood disturbances seen in depression. Importantly, chronic stressors associated with this depressive-like profile such as repeated restraint and social stress in rodents consistently result in a concomitant down-regulation of hippocampal neurogenesis (Gould, 1996; Warner-Schmidt and Duman,

2006; Schoenfeld and Gould, 2012). Chronic treatment with classic antidepressants however stimulates neurogenesis. Malberg *et al* (2000) found that administration of a variety of antidepressant treatments, including ECT, amplified cell proliferation in the DG while non-antidepressants had no such effect. Importantly, upregulation of BrdU expression occurred only after chronic antidepressant treatment, and it is hypothesized that the latency to therapeutic efficacy seen in numerous classic antidepressants, including SSRIs, may reflect the maturation rate of hippocampal granule cells (Malberg, 2000).

Following these lines of evidence, a so-called neurogenesis hypothesis of clinical depression has been proposed that has garnered considerable evidence. The neurogenesis hypothesis effectively bridges the realms of evidence regarding stress, neurogenesis, depression and antidepressant therapy, and has thus proven an invaluable tool for guiding research surrounding the disorder. This hypothesis postulates that stress-based reductions in neurogenesis are responsible for depressive symptomatology, and moreover, that stimulation of hippocampal neurogenesis is necessary for the efficacy of antidepressant therapies (Malberg *et* al, 2000; Warner-Schmidt and Duman, 2006; Wainwright and Galea, 2013). Indeed, when neurogenesis was selectively reduced via irradiation techniques in rodent models of depression, behavioral correlates of effective antidepressant therapy were abolished (Santarelli *et al*, 2003). Additional support for the neurogenesis hypothesis is derived from research showing that reductions in neurogenesis ablate the normal inhibition of glucocorticoid release following a stressor (Snyder *et al*, 2011). This evidence indicates that the HPA axis stress response known to be

maladaptive in depressed patients is itself hippocampally regulated, likely by way of neurogenesis.

Although promising, the neurogenesis hypothesis seems to fall short of encapsulating in its entirety the substrate of depression related symptoms. Indeed, neurogenesis-independent effects of antidepressant drugs have been observed (David *et al*, 2009). Additionally, and directly pertinent to the present research, an increasing body of evidence has accumulated within the past decade implicating overall neuronal plasticity in the etiology of depression (Kempermann and Kronenberg, 2003; Wainwright and Galea, 2013; Hayley and Litteljohn, 2013; Duman, 2014). While it is clear that hippocampal neurogenesis represents a key cellular correlate of both the onset and amelioration of depressive symptoms, trophic factors such as BDNF, which can be anti-apoptic and foster continued survival and function of neurons (Castrén and Rantamäki, 2010), have recently become the target of continued depression research.

The Neuroplasticity Hypothesis: EPO and Novel Antidepressants

While analysis of the neurogenesis effects of antidepressants is imperative to an understanding of the disorder, current research suggests that neurogenesis is one key feature of a more general deregulation of brain plasticity. It has become increasingly clear that the extended survival of neurons as well as their mature structure and function may play a part in depressive symptomatology, susceptible to both the effects of chronic stress as well as antidepressant therapy. As previously mentioned, both chronically stressed animals as well as patients diagnosed with depression have shown evidence of neuronal degradation within limbic structures (Anisman *et al*, 2008). The neural-growth

factor BDNF and its receptor trkB play a prominent role in preserving the structure and function of neurons as well as their synaptic connections (Wanwright and Galea, 2013), and there is strong evidence linking this neurotrophin to depressive symptomatology, as well as its treatment. Thus it seems that neuronal birth, along with structural and synaptic maintenance of these cells, share an intricate relationship with the etiology and treatment of depression.

BDNF has long been implicated in neuroplastic as well as neurogenic mechanisms (Castrén and Rantamäki, 2010), and thus its relationship to depressive illness has garnered significant appraisal. In the mouse DG, cell proliferation as a result of environmental enrichment requires BDNF (Rossi *et al*, 2006), and BDNF blockade decreases neuronal differentiation (Taliaz *et al*, 2010). Much like physical exercise increases neurogenesis, the same has been shown for BDNF expression (Ying *et al*, 2005). Additionally, maternal deprivation associated with hypercortisolaemia decreased both hippocampal neurogenesis and BDNF expression (Kikusui *et al*, 2009). These findings implicate a role for BDNF in depression, and this has indeed been shown. Both central as well as hippocampal infusion of BDNF results in antidepressant-like effects, while chronic treatment with classic antidepressants increases the expression of hippocampal BDNF (Altar, 1999: Shirayama *et al*, 2002). Moreover, it has been shown that reductions in BDNF resulting from a stressful experience can be mitigated by antidepressants (Duman *et al,* 1999), and importantly, reduced platelet and serum BDNF has been observed in humans diagnosed with depression (Pandey *et al*, 2010; Yoshida *et al*, 2012).

A Neuroplastic Hypothesis of Novel Antidepressants

In light of this evidence, and the ability of trophic factors such as BDNF to regulate processes such as cell death, synaptic pruning and neuronal atrophy (Castrén and Rantamäki, 2010), the neuroplasticity hypothesis of depression has been proposed; herein, disturbances of HPA axis reactivity as well as mood dysfunction in depression may be regulated in part by the maintenance of adaptive neuroplasticity within limbic brain structures (Kempermann and Kronenberg, 2003; Wainwright and Galea, 2013; Hayley and Litteljohn, 2013; Duman, 2014). The neuroplasticity hypothesis of depression effectively integrates neurogenic and neurotrophic hypotheses of depressive etiology. Chronically stressed animals, widely used to model depression, demonstrate aberrant dendritic arborization within limbic brain regions, and neuronal atrophy within hippocampal CA3 neurons has been found under these circumstances (Magariños *et al*, 1996). BDNF on the other hand, reduced in the depressed brain, is capable of affording anti-apoptotic and neuroprotective effects to neurons (Castrén and Rantamäki, 2010). Thus, it seems that atypical or apoptotic neuroplastic events can contribute to depressive illness, while mitigating these factors may afford antidepressant effects. This neuroplasticity hypothesis of depression has garnered considerable evidence in recent years, notably upon observation of rapid neuroplastic effects induced by certain novel antidepressants.

For instance, the abused drug ketamine has been found to afford antidepressant effects in humans within as little as two hours, with patients exhibiting a decrease in suicidal ideation (Aan Het Rot, 2012). It has been proposed that these effects of ketamine are mediated in the PFC by way of the mTOR cellular signaling pathway,

which is responsible for the creation of synaptic proteins required to create and maintain synaptic function (Duman, 2014). Indeed, ketamine administration in animals induces rapid synaptogenesis, while rapamycin induced antagonism of the mTOR pathway ablates both the behavioral and synaptogenic effects of ketamine in animals (Li *et al*, 2010). Importantly, the induction of these neuroplastic effects seems to be a BDNF mediated process (Duncan *et al*, 2013). Though ketamine and BDNF both induce antidepressant effects, ketamine is a drug of wide abuse, while BDNF does not appreciably cross the blood-brain barrier (Pardridge, 1998) and may exert unwanted side effects if administered peripherally (Constandil, 2011). Ongoing research has therefore striven to find alternative compounds that enhance neuroplasticity and may prove beneficial in the treatment of depression.

The Antidepressant Potential of Erythropoietin

The hematopoietic cytokine erythropoietin (EPO) regulates peripheral red-blood-cell formation (Jelkman, 1992), and a growing body of evidence has implied that EPO can be beneficial in the treatment of varying states of psychopathology, including depression (Ehrenreich, 2007a, 2007b; Miskowiak, 2014). Produced predominantly in the kidney in adulthood, it has been demonstrated that EPO and its receptor (EpoR) are present in diverse body tissues including neuronal- and non-neuronal cells of the CNS, and it has been shown that central EPO is produced locally within the brain (Digicaylioglu, 1995). Additionally, EPO and its specific receptor (EpoR) are implicated in numerous neuroprotective and neuroplastic processes, and are found within hippocampal neurons (Morishita *et al*, 1997; Yu *et al*, 2002; Genc, 2004;). In light of this

evidence, recent research has afforded much consideration to the possibility of employing EPO as an antidepressant treatment.

Following electroconvulsive therapy (ECT), effective in ameliorating depressive symptoms, EPO levels were elevated in the hippocampal DG. Along with elevating BDNF expression in the region, EPO administration has stimulated hippocampal neurogenesis, effects known to follow depression related ECT therapy as well as classic pharmacological treatment. EPO administration has been found to exert neurotrophic effects in models of ischemic stroke, and numerous studies have shown its antidepressant-like effects in the rodent forced swim test (FST) (Siren *et al*, 2000; Wang *et al*, 2004; Girgenti, *et al*, 2009; Osborn, 2013).

Additionally, both pre-clinical and recent clinical data have shown that EPO can have beneficial effects upon cognitive outcomes associated with depression. Due to its hematopoietic effects, patients undergoing dialysis and those with anemia have been treated with EPO and, importantly, improvements in mood and cognitive function have been seen in these individuals (Jelkmann, 1992; Picket *et al*, 1999). Miskowiak and colleagues have produced much of the human data on the cognitive effects of EPO, and it has been found that EPO has the potential to reduce limbic responses to fear-related stimuli, both in healthy and depressed individuals (Miskowiak, 2012). In a recent clinical trial, Miskowiak (2014) and colleagues found that patients with a Hamilton Depression Rating Scale of 17 or greater at trial initiation exhibited cognitive improvement as well as decreases in composite scores of depression in response to chronic EPO treatment (9-14 weeks).

Our own studies regarding the antidepressant potential of EPO have found that its administration ameliorates stress induced depressive-like symptomatology in rodent behavior models, as well as increases hippocampal neurogenesis in both stressed and non-stressed animals. Additionally, the EPO induced antidepressant-like effect in the FST was ablated by the mTOR antagonist rapamycin, suggesting a role for this pathway in EPO associated antidepressant effects (Osborn, 2013). As this pathway and its relationship to BDNF have been implicated in the rapid antidepressant effects of novel treatments such as ketamine, it is possible that EPO also induces antidepressant-like effects through BDNF and mTOR-pathway related processes. Taken together, the evidence discussed herein points to the possibility that EPO exerts both neurogenic and neuroplastic effects within limbic structures, including the hippocampus, to produce both cellular as well as cognitive-behavioral antidepressant outcomes.

Current Rationale and Hypotheses

To that end, the first experiment of the present study sought to analyze the effect of chronic EPO administration on hippocampal features as well as depressive-related behaviors in chronically stressed animal models of depression. In another study, whether a low dose of EPO will synergize with sub-therapeutic SSRI (citalopram) administration to decrease distress related behaviors in a modified-FST paradigm was analyzed, in the hopes of implicating EPO as a possible adjunct therapy to lower doses of typical antidepressants. While very similar to the classic FST, the modified FST is superior in its detection of SSRIs (Detke and Lucki, 1996). Classic correlates of antidepressive potential were hypothesized, including increases in neurogenesis through DCX analysis,

as well as a decrease in signs of mobility in the FST. Additionally, the first experiment of the current research endeavored to explore possible effects of stress and EPO treatment on granule cell structure in the neurogenic DG.

While the drastic neuronal atrophy of CA3 pyramidal cells seen in stress models has not been observed in granule cells, manipulation of the trkB receptor that binds BDNF has the potential to alter both pre- and post-synaptic granule cell morphology (Danzer, *et al*, 2008). Additionally, mTOR inhibition has been found to suppress granule cell arborization along the mossy-fiber pathway (Buckmaster, 2009), and BDNF, which is increased by EPO administration, has been implicated in the mTOR-related antidepressant-like function of ketamine. Thus, possibly through BDNF and mTOR related neuroplastic mechanisms, it is hypothesized that EPO has the potential to alter structural as well as synapse related morphology of hippocampal granule cells, which may in turn afford its regulatory role in hippocampus related cognitive abnormalities associated with depression. Unfortunately, due to inconsistent primary results that will be discussed in context below, while hippocampal tissue collection and processing was carried out, the planned exploration of granule cell structure in response to EPO treatment was not further pursued.

METHODS

Experimental Design

In experiment 1, to analyze the effects of chronic stress and EPO administration, a 2 (saline, 5000IU/kg EPO) x 2 (stress, no stress) design was employed. Animals were subject to 21 days chronic mild stress while receiving EPO 4 times from day 11 through 20. In experiment 2, a 3 (saline, 2500IU/kg EPO, 5000IU/kg EPO) x 2 (saline, 5ug/kg citalopram) design was employed to analyze possible synergism between EPO and citalopram in the FST, and test swims were carried out both directly following drug administration as well as one week later to analyze any lasting drug effects.

Subjects

Subjects employed in this study were male Long-Evans rats, acquired from the Charles River Company (St. Constant, Quebec, Canada) at approximately 8-10 weeks of age. For the duration of the experimental process, animals were housed individually within polycarbonite cages with wood-chip bedding, in a temperature-controlled vivarium maintained at 21°C with a 12h light/dark cycle, with lights on at 0800 (exception during 36hr light stressor). Both food and water were provided to subjects *ad libitum* throughout the extent of the study. All rats were handled every day beginning approximately 5 days before initiating injections. All experimental procedures were consistent with the standards of the Canadian Council on Animal Care (CCAC) guidelines, and the Carleton University Animal Care Committee approved all protocols.

Stressor Regimen

Half of the animals in experiment 1 were subject to 21 days chronic mild stress, while non-stressed animals remained in their home cage throughout the duration of the

experiment. Two stressors were employed each day, during an AM and PM session. Stressors consisted of 15min wed bedding; 5min tail pinch; 15min restraint in a plastic decap bag; 15min exposure to loud noise (bike horn); 15min exposure to dirty bedding taken from the cages of other animals; 36 hr light exposure. Each week stressors were employed on a varying and unpredictable schedule, with the exception of 36hr light exposure; this stressor took place on the same day each week, and during this period no other stressors were performed. On day 21, only the AM stressor was performed, and animals were sacrificed following this session.

Injections

rhEPO (EMD Chemicals, San Diego) and powdered citalopram (Lundbeck, Norway) were suspended in 0.9% injectable saline solution, and administered by intraperitoneal injection using 26 3/8" gauge needles at a volume of 0.4mL.

Experiment 1

EPO was administered at 5000IU/kg body weight; control rats received saline injections at an equivalent volume. Rats received EPO injections once every 3 days beginning day 11 through 20, and injections took place directly following the AM stressor. The final EPO injection took place 24hrs before sacrifice.

Experiment 2

A 3 (saline, 2500IU/kg EPO, 5000IU/kg EPO) x 2 (saline, 5ug/kg citalopram) design was employed in experiment 2. As per the FST protocol, following day 1 pre-testing both EPO and citalopram injections took place on day 2 before the test session.

EPO and citalopram injections were administered 4hrs and 45mins prior to the test session, respectively.

Forced Swim Test

Rats were placed in an opaque bucket filled ~40cm with water between 23-26°C such that they could not touch the bottom or escape from the bucket. Following each session, rats were towel dried and placed back in their home cages. Each rat was subject to a 15min pre-test session 24hrs before the 5min test session, which was filmed and later scored for displays of immobility, general swimming, and climbing behavior. Immobility was defined as rats remaining still other than to keep their head above water, and climbing was defined as movement of the forepaws up the side of the bucket not associated with swimming. In experiment 1, a separate cohort of animals used exclusively for behavioral testing underwent pre-testing on day 19, and the test session took place 24hrs later following the final EPO injection. In experiment 2, animals were injected with EPO and citalopram 4hrs and 45mins, respectively, before the initial test session. Animals again underwent a test swim session exactly one week following initial testing, to explore any possible lasting drug effects.

Histology

Tissue Preparation

Following anesthetic administration, rats were transcardially perfused with saline. Brains were then collected and hemisected. The right hemisphere of each was immersion fixed with 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) overnight at

4˚C. The following day, this solution was replaced with 30% sucrose in 0.1M PBS, and brains were stored at 4°C until sectioning. Brains were sectioned on a Leica CM1900 cryostat (Weztler, Germany) at 15µm. A 0.1% sodium azide solution in 0.1M phosphate buffer (PB) was utilized to store sections at 4˚C until staining.

DCX

In carrying out immunohistochemistry, brain sections were washed for 3x5 minutes in a 0.01 M phosphate-buffered saline solution (PBS), then blocked in a 0.3% H2O2/PBS-TX solution for 15 mins. Next, incubation in the primary antibody (goat anti-double-cortin, 1:200) occurred overnight at room temperature. The following day, tissue was washed for 3x5 mins in PBS, followed by a 2hr incubation in the secondary flourescent antibody (Goat 488, Invitrogen Alexa). Following development, sections were placed in PBS, and then mounted on glass slides using Flouromount (Sigma).

Composite virtual images of DCX+ cells were taken using StereoInvestigator software (MBF Bioscience) at 20x for counting. Cell counting was carried out using ImageJ software (NIH); individual cell bodies were manually counted in the GCL under the criteria that a stained process could be seen leaving a clearly stained soma. Cells were counted in the inner rime of the GCL, defined as the border between the hilus and granule layers. The area that stained cells of the GCL occupied within the DG was found, and this allowed the density of DCX(+) immature neurons with the DG to be found per section. Slides were coded before counting, and the code was not broken until cell counting was finished. Two sections per brain were counted; these densities were averaged, yielding a mean density per brain.

Golgi-Cox preparation, Staining and Planned Analysis

No less than six days prior to being utilized, a Golgi-fix solution was prepared by mixing solutions A, B, and C. Solution A consisted of 37.5 g of mercuric chloride ($HgCl_2$) (Sigma) in 750 ml of heated dH_2O (95˚C). Solution B consisted of 37.5 g of potassium dichromate ($K_2Cr_2O_7$) (Sigma) in 750 ml warmed dH_2O (60˚C), and solution C consisted of 30 g of potassium chromate ($K_2Cr_2O_4$) (Sigma) in 750 ml dH_2O. Solutions A, B, and C were mixed in a new flask, and 1500 ml of dH_2O was added. This Golgi solution was then transferred into a glass jar, covered in tinfoil and stored in the dark, as the Golgi-fix is light sensitive. The left hemisphere was immersion fixed in Golgi-fix solution for 14 days in the dark. The hemisphere was then washed in dH_2O for 4hrs, followed by a second wash in dH_2O for 3hrs, and a third wash left overnight. The hemisphere was then placed in 10% sucrose for 8 hours, followed by 20% sucrose overnight. The hemisphere was then placed in 30% sucrose and left for a minimum of four days. Brains were sectioned on a Vibratome series 1000 (Ted Pella Inc) at 200 µm; sections were then placed on double-coated gel slides, and stored in a humidified chamber for 24 hours in the dark before staining.

For Golgi staining, slides were washed in dH_2O for 1 min, followed by a 40 min wash in 28% ammonium hydroxide. Slides were then washed in dH_2O for 1 min followed by a 40 min wash in 1:1 Kodak Professional film fix A (Kodak) in dH_2O. Slides were then immersed in a series of 1 min washes as follows: dH_2O, dH_2O, 50% ethanol (ETOH), 70% ETOH, and 95% ETOH. Slides were then washed for 3x5 min in desiccated 100% ETOH (Fluka Analytical). Next, slides were immersed for 10 mins in a 1:1:1 solution consisting of desiccated ETOH, desiccated clearene, and chloroform for 10

mins, followed by 2x15 min washes in desiccated clearene (Surgipath). Finally, slides were coverslipped using Permount (Sigma) and placed in a desiccated box for a minimum of three days.

While ultimately the golgi analysis was not undertaken, the following outlines the planned analysis for this tissue. Golgi stained cells would be selected randomly for tracing from the population of granule cells in the DG meeting the following criteria: (1) dark and consistent impregnation throughout the extent of all of the dendrites, (2) minimal truncation (i.e., no major processes truncated, and not cut due to sectioning), (3) relative isolation from neighboring impregnated cells which could interfere with analysis. The slides containing Golgi-impregnated brain sections were coded, and the code would not have been broken until after the analysis was complete. The cell bodies and entire basal dendritic field of granule cells from the GCL would be traced; ideally, three cells per brain, with five brains per group, would have been used in the analysis. Tracing of cells is carried out using Neurolucida software (MBF Biosciences) under the Olympus BX-51 microscope with 100X oil objective. Dendritic length and spine density would be recorded, with spines only being counted when there is clear impregnation and attachment of the spine to the basal dendritic branch. Neurolucida Explorer (MBF Biosciences) would then be used to obtain data on dendritic lengths and spine density.

Statistical Analyses

All statistical analysis was carried out using IBM SPSS Statistics (SPSS). For all analyzed measures in experiment 1, the 2x2 (stress vs. EPO) design allowed the employment of a two-way ANOVA. One animal in this experiment was lost from DCX

analysis as the tissue was sliced in error. For experiment 2, data for each FST variable was run through a repeated-measures ANOVA. Due to highly variable results, the climbing variable was stricken from subsequent analysis and therefore only the data for immobility is shown below due to its directly complementing mobility measures in this study. Additionally, while two EPO doses were originally employed in the experiment, the high dose was cut from this discussion due to a lack of contribution of any further information.

RESULTS

Experiment 1: DCX

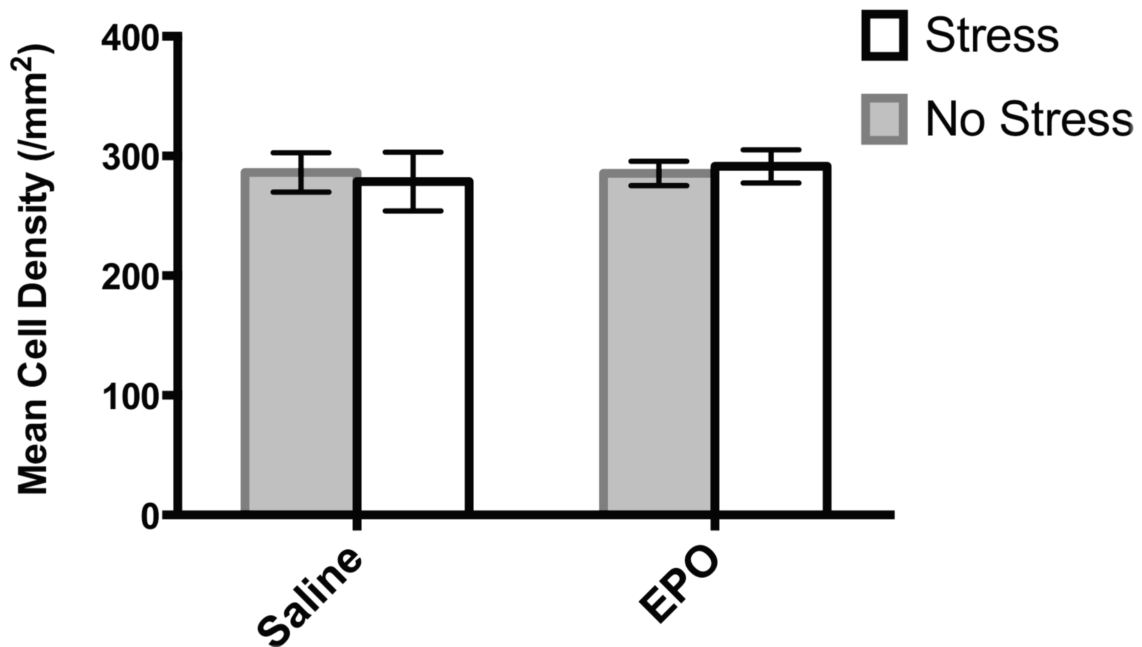

Figure 1: Density of DCX+ cells in the dentate gyrus shows no effect of EPO vs. saline treatment [$p = 0.738$], and no effect of stressor condition [$p = 0.959$]. Data expressed as mean ± SEM; $n = 7$ in EPO-Stress group, all others $n = 8$/group.

As outlined in figure 1 above, there was no evidence of a significant interaction between EPO treatment and stress condition [$F(1, 27) = 0.154$, $p = 0.698$], while neither the stressor regimen [$F(1, 27) = 0.003$, $p = 0.959$] nor EPO treatment [$F(1, 27) = 0.114$, $p = 0.738$] exerted a significant main effect on the density of DCX+ granule cells within the DG.

Experiment 1: Forced Swim Test

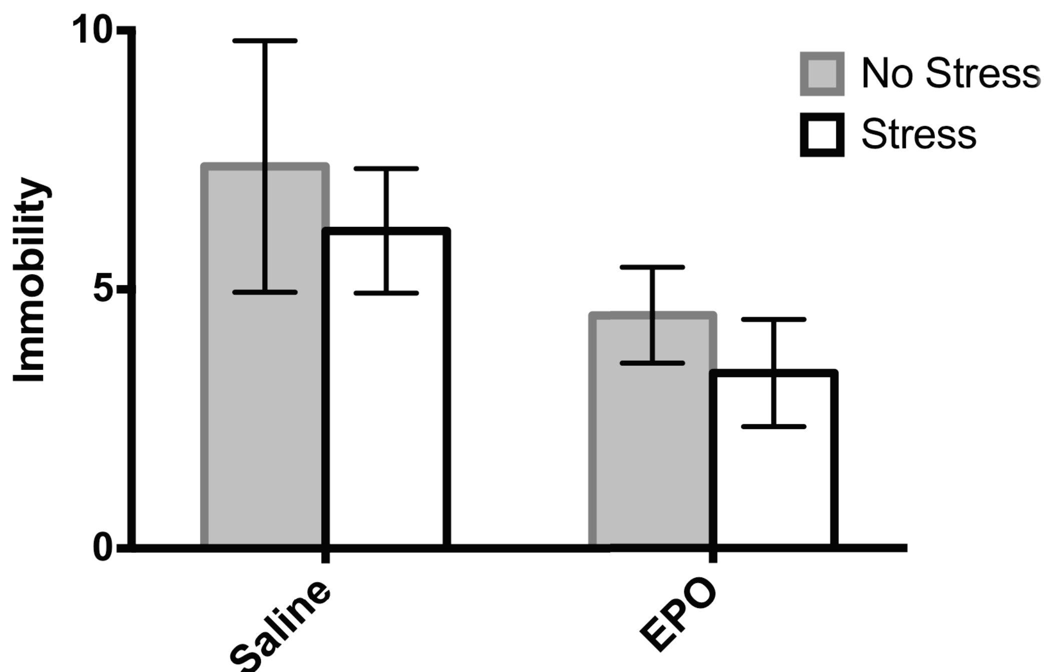

Figure 2: Behavioral displays of immobility in the forced swim test show no effect of EPO vs. saline treatment [$p = 0.075$], and no effect of stressor condition [$p = 0.442$]. Data expressed as mean ± SEM, $n = 8$/group.

Respectively, figure 2 (above), figure 3 (pg. 27) and figure 4 (pg. 28) summarize the behavioral results of the experiment 1 forced swim test for displays of immobility, general mobility, and climbing. For each variable, there was no evidence of a significant interaction between EPO treatment and stressor condition (immobility [$F(1, 28) = 0.002$, $p = 0.968$], mobility [$F(1, 28) = 0.396$, $p = 0.534$], climbing [$F(1, 28) = 0.190$, $p = 0.666$]). Neither EPO treatment nor stress condition exerted a significant main effect on displays of immobility [EPO: $F(1, 28) = 3.416$, $p = 0.075$; Stress: $F(1, 28) = 0.609$, $p = 0.442$], general mobility [EPO: $F(1, 28) = 0.023$, $p = 0.880$; Stress: $F(1, 28) = 0.057$, $p = 0.813$], or climbing [EPO: $F(1, 28) = 0.014$, $p = 0.908$; Stress: $F(1, 28) = 0.190$, $p = 0.666$].

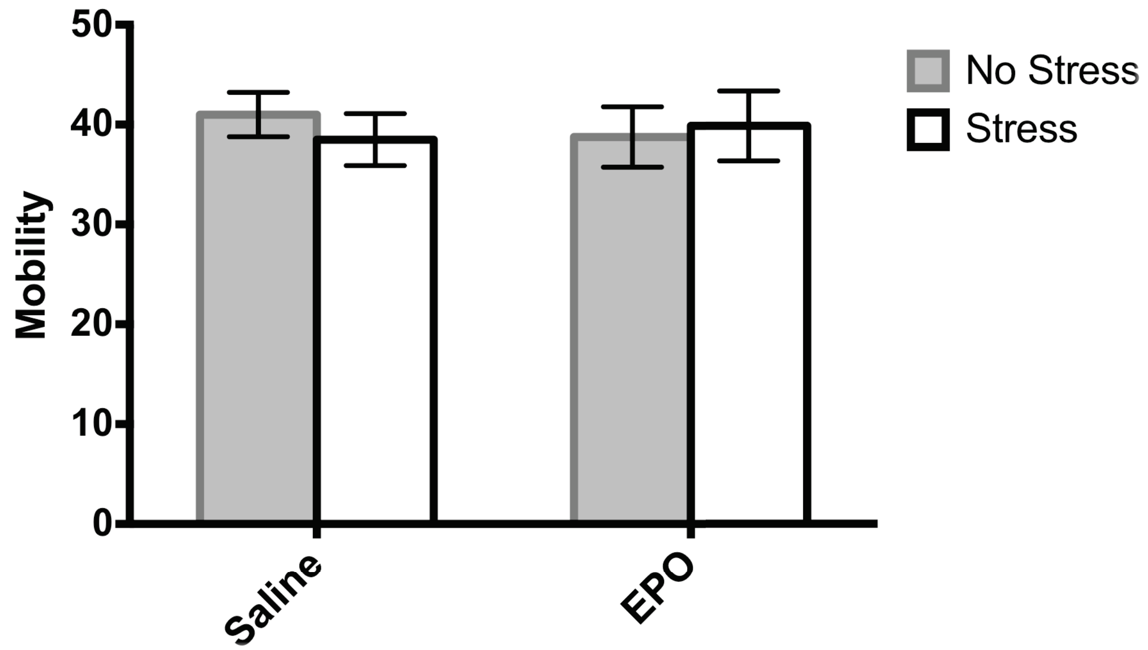

Figure 3: Behavioral displays of general mobility in the forced swim test show no effect of EPO vs. saline treatment [$p = 0.880$], and no effect of stressor condition [$p = 0.813$]. Data expressed as mean ± SEM, $n = 8$/group.

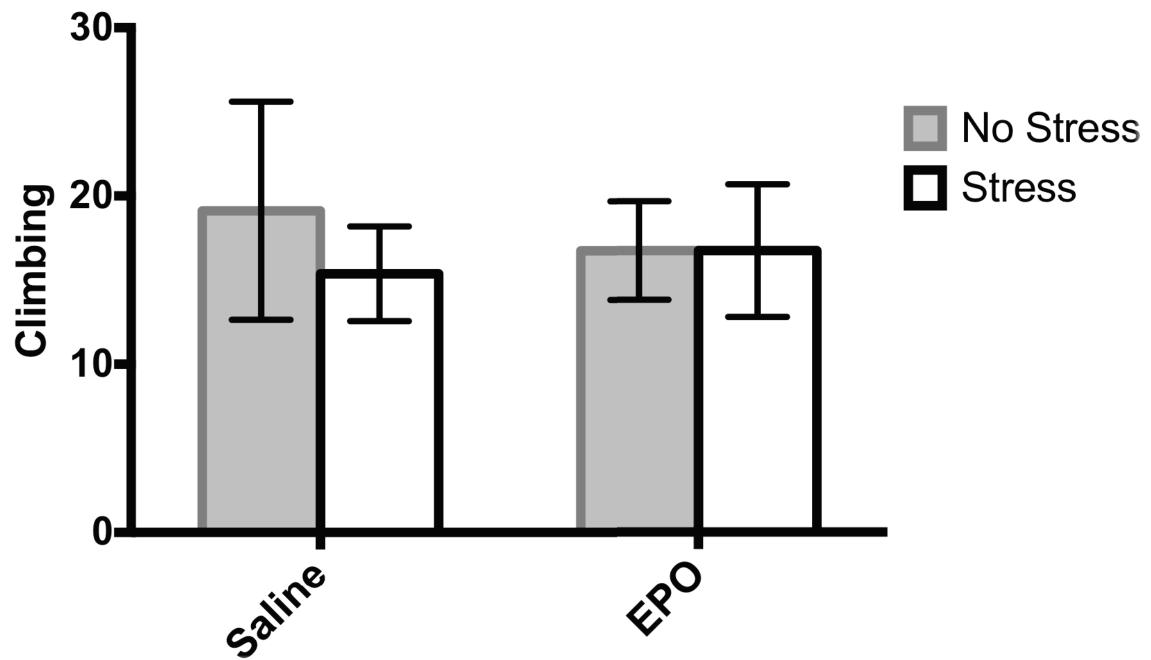

Figure 4: Behavioral displays of climbing in the forced swim test show no effect of EPO vs. saline treatment [$p = 0.908$], and no effect of stressor condition [$p = 0.666$]. Data expressed as mean ± SEM, $n = 8$/group.

Experiment 2: Forced Swim Test

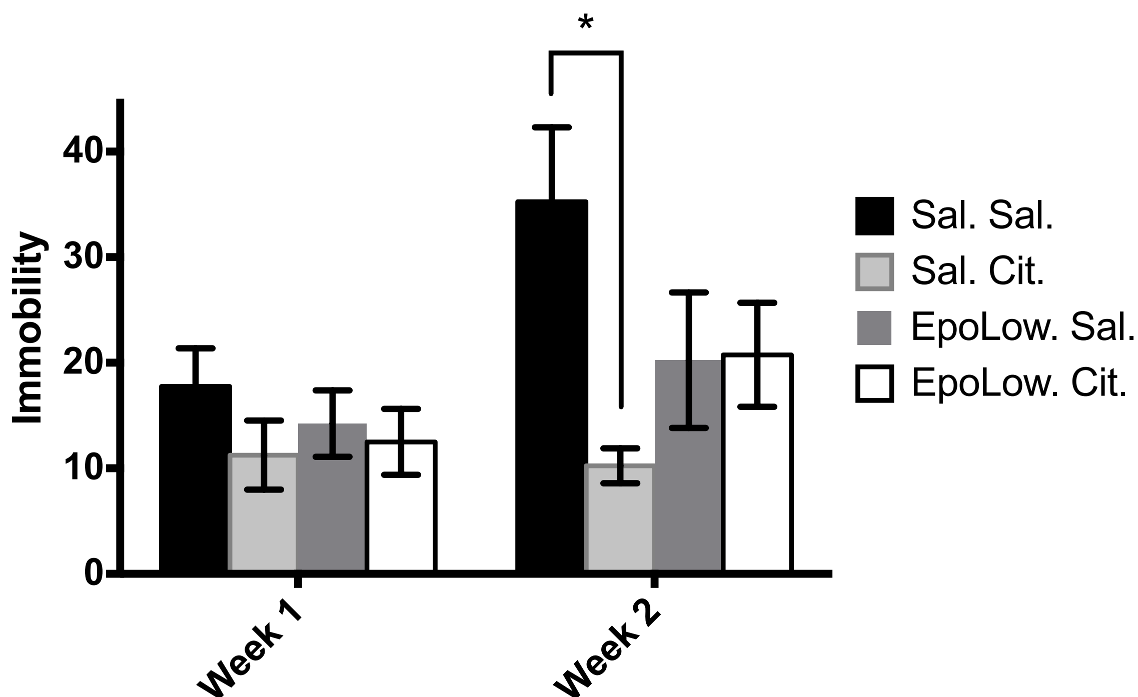

Figure 5: Forced swim test data shows control (Sal. Sal.) animals exhibited significantly greater displays of immobility than animals treated with citalopram alone (Sal. Cit.) during week 2 testing [$p = 0.03$]. Data expressed as mean ± SEM, $n = 4$/group.

Figure 5 (above) depicts the behavioral results of the experiment 2 forced swim test for displays of immobility. In light of the finding of a three-way interaction between EPO treatment, citalopram treatment and test week in the repeated measures ANOVA [$F(1, 12) = 4.875$, $p = 0.047$], a separate two-way ANOVA (EPO x citalopram) for each individual week was performed. While week 1 data showed no significant main effect of either EPO or citalopram treatment, data from week 2 showed a significant interaction between the two drugs [$F(1, 12) = 5.514$, $p = 0.037$]. To analyze this interaction, data based on two drug factors was separated into four individual treatment combinations, and a one-way ANOVA was performed on these groups. Significance of this test [$F(3, 12) = 3.592$, $p = 0.046$] was followed by Tukey post-hoc comparisons, which showed control

(Sal. Sal.) animals displayed greater immobility than animals treated with citalopram alone [$p = 0.03$].

DISCUSSION

Experiment 1

Unfortunately, the analysis of experiment 1 DCX and FST data showed a decisive lack of effect of EPO treatment on both cellular and behavioral outcomes. In light of the lack of compelling evidence offered by these results, it was deemed inappropriate to pursue an analysis of golgi-cox stained granule cells in the hippocampal tissues collected for this experiment. Although an exploration of the structure of these cells in response to EPO administration was among the primary goals of this research, the lack of significant results obtained upon examination of DCX immunohistochemistry as an empirical measure of neurogensis was particularly compelling in reaching the decision to forego the golgi analysis. Additionally, this choice was ultimately bolstered further by a lack of behavioral evidence in the FST.

Although some animal studies have shown evidence of neurogenesis-independent antidepressant-like outcomes (Huang et al, 2008), as previously mentioned it has been widely observed that many effective pharmacological antidepressant therapies such as the SSRIs can increase hippocampal neurogenesis (Malberg, 2000). Additionally, it has been postulated that stimulation of neurogenesis is an *essential* precursor to the efficacy of these antidepressant treatments (Santarelli *et al*, 2003). Within the framework of neurogenic and neuroplastic hypotheses of depression, this consideration of neurogenic vitality would suggest that pharmacological intervention that does not serve to increase

hippocampal neurogenesis will more than likely prove insufficient in yielding antidepressant-related outcomes, as seems to be the case in experiment 1.

As summarized in figure 1, EPO administration had no effect on the density of DCX+ positive cells analyzed within the DG. These results imply that EPO treatment employed within the present paradigm was insufficient in stimulating neurogenesis as compared to saline treatment, in both stressed and non-stressed animals. Intriguingly, these results stand contrary to previous animal studies that show the potential of EPO to modulate neurogenesis (Wang *et al*, 2004; Ransome and Turnley, 2007; Girgenti, *et al*, 2009), including our own (Osborn *et al*, 2013). While the present experiment found no effect of EPO on neurogenesis, previous research has reported that this substance can exert neurogenic effects, in both stressed and non-stressed animals; indeed similar dosing to that used here employed by Osborn *et al* (2013) in mice was sufficient in increasing the number of DCX+ positive cells in the mouse DG, while additionally, EPO was administered over a period of 2 weeks in that study, as was the case here. Unfortunately, this discrepancy in results indicates the possibility of fundamental error in the current research, and this consideration will be examined further below. However, within the theoretical framework of the neurogenesis hypothesis of depression, this result complements the lack of significance found upon analysis of FST data from this experiment.

While the FST is not in itself considered an outright test of antidepressant-like behavioral effects, it has proven a reliable screening tool for compounds that may have the potential to produce such outcomes. Indeed, effective and commonly employed antidepressants have shown an ability to decrease the time an animal spends immobile in

this paradigm (Slattery and Cryan, 2012). In experiment 1, along with non-significant neurogenesis effects, EPO treatment likewise exerted no effect upon the three behavioral outcomes analyzed in the FST. Climbing behaviors have the potential to prove particularly variable in their manifestation (Slattery and Cryan, 2012), and while useful in some cases to separate specific effects of different classes of antidepressants (Detke and Lucki, 1997) this behavior represented a moreso exploratory analysis in its relationship with EPO than did the other FST variables. However, the lack of effect shown by EPO administration on displays of immobility/mobility behavior is, much like the DCX results, puzzling; as is the case with neurogenesis effects, it has been shown in numerous studies including our own that EPO administration indeed has the potential to decrease the time an animal spends immobile in the FST (Girgenti, *et al*, 2009; Osborn, 2013). Unfortunately, the present research failed to reproduce these findings and showed no differences in time spent immobile between groups regardless of drug or stress treatments. Although inconsistent with previous research, since EPO treatment in this study did not increase neurogenesis, it seems appropriate that significant FST results were likewise absent in experiment 1. As it is postulated that stimulation of neurogenesis is a necessary antecedent of antidepressant-like outcomes (Malberg *et al*, 2000; Warner-Schmidt and Duman, 2006), while decreases in immobility in the FST warrants further speculation regarding a drugs antidepressant potential, the non-significant results of both DCX and FST analyses appear supplementary in their mutual lack of effect, albeit elusive in their contrast with previous findings.

Hence, while the lack of significant neurogenesis effects was in itself singularly compelling, the non-significant FST data found herein provided further reason to forego

the analysis of hippocampal granule cell structure. Should the granule cell analysis have been undertaken, and a significant EPO effect been found, these results would be entirely novel. However, in absence of the present research finding any significant effects of EPO upon neurogenesis and FST behaviors that have been shown previously numerous times, such findings would offer entirely inconclusive information in the broader context of the study. Additionally, should the initial hypothesis of this study indeed hold any merit wherein EPO affords its observed antidepressant-like outcomes in part through manipulating granule cell structure, ultimately the previously discussed lack of effect in this experiment indicates that it is unlikely that an analysis of the tissue would have shown this phenomenon, should it exist. To put it bluntly, in light of the lackluster evidence offered by the other measures, significant results of any kind drawn from the golgi analysis would stand alone in this study, and thus not allow for any genuine inference regarding their possible implications.

While EPO administration in this study failed to fulfill expectations, importantly, the stress paradigm employed herein likewise failed to induce significant effects, as is shown by both the DCX and FST results summarized in figures 1-4. Although it is indeed disappointing that EPO administration yielded lacklustre results, the lack of a stress effect displayed in this experiment is troubling, especially in the case of DCX analysis. The theoretical framework of stress as an animal model of depressive-related symptomatology is well established, and while like most models has its drawbacks, is widely used to create a rodent parallel with which to study depression (Hill *et al*, 2012). Studies will often differ in the precise composition of their stress paradigm, yet in general those experiments that achieve a mildly stressful environment over a number of weeks

are expected to decrease neurogenesis (Hill *et al*, 2012). Additionally, in spite of a few anomalous results, chronic stress is expected to reduce immobility in the FST (Willner, 1995).

In experiment 1, while EPO induced no significant effects, likewise the CMS protocol employed was insufficient in modifying the analyzed variables. Thus, it can be reasonably inferred that the CMS paradigm was insufficient in inducing an overall depressive-like profile. There are a number of reasons that could potentially explain this lack of effect, the most obvious being that the stressor paradigm was not intense enough to cause the animals to perceive an endemic threat to their well being, or experience prolonged distress. Although many studies employ a paradigm such as the one used here whereby a multitude of stressors are used in an attempt to maximize the unpredictable nature of the paradigm, other stress studies will utilize only a few or even a single stressor, such as restraint stress, that is reliable in modulating fear related behaviour as well as depressive-related cellular outcomes (Conrad *et al*, 1999; Pham *et al*, 2003). Even further, another alternative is using a social defeat stressor, whereby subjects are exposed to another dominant animal and experience directed aggression, which is accepted as being a highly stressful experience for these animals (Golden *et al,* 2011). Indeed, our own studies show that social defeat can exhibit marked effects upon both neurogenesis as well as FST behaviours, and importantly, EPO was shown to augment these measures (Osborn, *et al*, 2013).

However, while in hindsight a paradigm utilizing a chronic social defeat stressor or more frequent restraint stress may have proven more effective in modulating the results of this study, it is important to note that EPO has been shown to affect both

neurogenesis as well as FST outcomes *regardless* of stress conditions (Osborn, *et al*, 2013). Another possible reason for this discrepancy is that this study employed rat models, while our previous research utilized mouse models. With that in mind, although it is disappointing that the stress paradigm used herein did not have the expected results upon these variables, it stands to reason that should EPO treatment have proven effective in this study it would have done so irrespective of stressor treatment. Indeed, the ultimate goal of experiment 1 was to 1) modulate DCX and FST outcomes through stress, while 2) simultaneously showing an ability of EPO to attenuate such effects; unfortunately, this experiment proved insufficient in achieving either of these outcomes, even while previous research suggests that each result could be reasonably expected regardless of the presence of the other.

Experiment 2

Much like experiment 1, experiment 2 failed to show a beneficial effect of EPO treatment in the synergy FST, either as a main effect or additive contribution to citalopram. Indeed, as discussed further below, superficial examination of the week 2 results would suggest the drugs exerted opposing effects, instead of synergism. Although the study design employed two doses of EPO, both a high dose that has previously shown antidepressant-like potential (the dose employed in experiment 1) as well as half this dose, neither of these served to affect the FST outcomes in the test directly following administration. For this reason the high dose of EPO was stricken from the summarized results shown in figure 5 for the sake of simplicity as it did not provide any further information.

Additionally, while the original intent was to test all animals in a single test swim in response to acute administration of both EPO and citalopram for evidence of synergy, it was ultimately decided to test half the animals again a week following the original swim for any lingering effects of these drugs. Ultimately figure 5 summarizes the data of both weeks only for the 4 animals that were tested at both time points, as the original analysis of week 1 data with all 8 animals/group yielded non-significant results. Additionally, climbing behaviour exhibited highly variable and inconsistent outcomes in the experiment 2 tests, and was therefore stricken from the analysis, leaving only immobility/mobility data. Since the nature of the data remaining for these two variables caused them to be a direct complement of one another, whereby increases in displays of immobility corresponded to proportional decreases in mobility, only the immobility data has been summarized in figure 5.

A shown in figure 5, a three-way EPO, citalopram and test week interaction allowed for the data of each week to be analyzed separately, and there was no evidence of either EPO or citalopram effects on displays of immobility in the original FST, or "week 1" as displayed in the figure. On the other hand, week 2 data showed a significant drug interaction whereby, only in animals not treated with EPO, citalopram treatment served to significantly reduce immobility. Conversely, both animal groups treated with EPO in week 2 showed no difference in displays of immobility as compared to the other groups irrespective of citalopram treatment. Although week 1 data shows no effect of either drug, the data of week 2 seems to suggest that while treatment with citalopram only served to decrease displays of immobility, in the presence EPO co-administration, this effect was nullified while EPO alone showed no effect. Although these results seem to

suggest that EPO may in fact work against citalopram as opposed to offering an additive beneficial effect in the FST as hypothesized, there are worth discussion a number of considerations that warrant caution in interpreting the results in this manner.

While EPO has shown an ability to reduce displays of immobility in the FST upon chronic administration (Girgenti *et al*, 2009; Osborn *et al*, 2013), this evidence has not previously been shown upon acute administration alone. However studies have documented the ability of acute SSRI administration to modulate behavioural responses in the FST specifically reducing time spent immobile by animal models (Slattery and Cryan, 2012). Importantly however, with the end goal of analyzing possible synergism between drugs, a citalopram dose lower than those previously shown to modulate depressive-related symptomatology was utilized here so as not to create a ceiling effect and mask a possible additive contribution of EPO. Taken altogether, previous evidence suggests that it is highly suspect that the citalopram administration alone in this experiment, especially at this dose, should decrease signs of immobility a week after administration while showing no effect in the test carried out immediately following treatment. While it is not entirely inconceivable that acute antidepressant treatment may exhibit lasting behavioural effects over time, it is unlikely that such a lasting effect should occur here due to 1) a lack of significant effects in the first week, and 2) a low dose was used.

This consideration also speaks to the seemingly paradoxical effect of EPO co-administration in the week 2 FST. If the results of week 2 testing were in fact found for the week 1 test, it would become reasonable to infer that EPO may indeed work against the effects of citalopram in the FST rather than synergise with it. Ultimately, the

possibility remains that this delayed “effect” of EPO results from a delay in the drugs efficacy in modulating behavioural outcomes in this test; in light of previous evidence showing an effect of EPO in the FST following chronic administration but no such evidence following acute administration it is possible that EPO may require an extended time period following acute administration before FST effects can be observed.

However, this seemingly “anti-synergism” interpretation yet again becomes problematic in that it is evidenced only in week 2; as previously discussed, the data from week 2 testing remains questionable in its lack of conformity with expectations set by previous research with regard to citalopram. The puzzling citalopram results, namely an effect in week 2 with none in week 1, seem to suggest flaws in the FST employment or analysis in this study. Summarily, the data offered by citalopram are suspect in that the expected effects of this drug are well known, and should be observed to reasonably conclude a sound FST paradigm; while the effects of EPO in week 2 are superficially intriguing, they are dubious at best in light of the paradoxical results offered by citalopram administration, and it would thus be inappropriate to speculate on their implications without further information.

Overall Conclusions and Implications for Future Research

In summary, while EPO has afforded evidence as to its potential as an antidepressant therapy, through both human and animal research, the results of this study do not align with this assertion. However, upon a deeper analysis of the present data in the context of previous research that has enjoyed replication, it becomes increasingly apparent that the lack of conformity displayed here is more likely the result of

inconsistencies in experimental design or execution, rather than a testament to the drugs lack of therapeutic potential. As previously reviewed, EPO has numerous times been observed to increase neurogenesis as well as decrease behavioural immobility in the FST; likewise, it is far more likely that the puzzling results of experiment 2 may be explained by experimental error than a true lack of drug effects, and the inconsistent outcomes of citalopram administration seem to support this claim.

Unfortunately, such a decisive lack of favourable results allows many possible explanations, the most prominent of which will be discussed herein. First, in light of the unexpected neurogenesis effects in experiment 1, it is possible that the effective dose was insufficiently administered. Although the dose chosen was the same that has been employed to significant effect in other studies (Miskowiak, 2012), it is also possible that this specific compound differed in its ability to cross the blood-brain barrier, although this seems unlikely as it is known that EPO reliably crosses the BBB (Sargin *et al*, 2010). Some rat studies exploring EPO have employed rat-recombinant EPO and achieved favourable results (Girgenti *et al*, 2009) while the present study utilized recombinant-human EPO. However, intraperitoneally administered rhEPO has also shown favourable results in other animal studies including stimulating neurogenesis (Ransome and Turnley, 2008) as well dampening ischemic injury (Yu *et al*, 2005). Ultimately, while it is possible that drug/dosage issues may account for the lack of effect seen in this study, should this factor indeed play a prominent role, the nature of this discrepancy remains unclear.

Another possible contributor to the lack of effect of EPO in this study is the strain of rat used. Although our own previous studies examining the antidepressant-like nature

of EPO employed mouse models, and this research successfully demonstrated an effect of EPO in the FST and on neurogenesis, rats have also been employed and seen success in this context (Girgenti *et al*, 2009). However, it seems that no published research that examines EPO in the context of depressive-related outcomes has employed the Long Evans rat, and thus some inter-species variability may have contributed to the difficulty experienced in this study. Should similar research be carried out in our lab in the future, it may be beneficial to consider a mouse model, or perhaps another rat strain that has shown antidepressant-related outcomes in response to EPO treatment in the past, such as the Sprague-Dawley (Girgenti *et al*, 2009), or the Wistar, that has shown EPO effects on fear conditioning (Miu *et al*, 2004).

Finally, the results of this study seem to suggest that the FST paradigm used may not have been sufficient in detecting drug effects. The two main differences between the FST paradigm employed here and the classic method is 1) the time-sampling manner in which the data are collected, and 2) the depth of water which does not allow the rat to support itself using its tail; this design is sometimes referred to as the “modified” forced swim test (Detke and Lucki, 1996; Slattery and Cryan, 2012). While this protocol is accepted as valid in examining the same behaviours that the classic FST does, it is more reliable in detecting SSRI effects than the classic paradigm. As opposed to measuring counts of behaviours in 5s intervals like the modified FST, the classic FST measures total time spent mobile/immobile as continuous variables. Therefore, it is possible that the time sampling analysis or depth of water in this paradigm precluded a reliable analysis of EPO effects. While it may seem beneficial to reproduce this study using the classic FST, in light of the increased difficulty the classic paradigm poses in detecting SSRI effects if

the modified FST does not allow for analysis of EPO then it is possible that EPO and citalopram may be incompatible in this behavioural test, precluding analysis of synergism. A pilot FST test may prove useful in determining which paradigm is more appropriate, and in discovering if the two drugs can indeed be tested together using the FST. Likewise, it would perhaps be beneficial to repeat experiment 1 using the classic paradigm of the FST, however in absence of significant neurogenesis effects it is not likely that this change would make a difference. Alternatively, it is possible that animals in this study did not have sufficient time to manifest drug effects with only 5 mins in the test swim, and therefore increasing the test session time may yield results.

In summary, the present study unfortunately proved ineffective in showing support for the hypotheses put forth at the outset of experimentation. Sadly, the truly novel aspect of this research, the analysis of the golgi-cox stained granule cells in response to EPO treatment, was ultimately scrapped due to unsupportive preliminary data. For this hypothesis to have been sufficiently explored in the present research it would be necessary to ensure beforehand that the EPO treatment being employed is resulting in antidepressant-related outcomes that parallel those found in previous studies. Sadly, here this was not the case. Likewise, the analysis of the experiment 2 FST in hopes of discovering synergism between EPO and citalopram does not seem to allow any reliable conclusions to be drawn. To sufficiently carry out this analysis in the future it is imperative that a reliable FST protocol be formulated that will show the expected effects of established antidepressant “controls”. On the other hand, this analysis may be more beneficially carried out in our lab if the animal model is switched to mice as opposed to rats.

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