Antidepressant-like effects of erythropoietin: A focus on
behavioural and hippocampal processes.

by

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Abstract

Depression is a chronic and debilitating condition that is often not treated effectively. Thus, we sought to assess novel “trophic” based treatment strategies. Erythropoietin (EPO), a hematopoietic growth factor, in recent years has emerged as a promising agent to promote neuronal recovery, having neuroprotective consequences in models of stroke and traumatic brain injury. In the current investigation we assessed whether erythropoietin would promote antidepressant-like effects and whether it would diminish the deleterious effects of a social stressor. Indeed, we currently found that EPO did induce antidepressant-like responses (as assessed in a forced swim test, open field, elevated-plus maze, sucrose preference test and a novelty test), and appeared to blunt some of the negative behavioural effects of a social stressor. Furthermore, EPO was found to promote hippocampal neurogenesis; an important feature of effective antidepressants. Further research should be directed towards understanding the mechanisms behind such anti-depressant-like activity.

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Abbreviations

5-HT - serotonin
5-HTT - serotonin transporter
ACTH - adrenocorticotrophic hormone
ANOVA – analysis of variance
BBB - blood brain barrier
BCL-XL - B-cell lymphoma-extra large
BDNF - brain-derived neurotrophic factor
BrdU – bromodeoxyuridine
CA1 - Cornu Ammonis area 1
CA3 - Cornu Ammonis area 3
cEPO - carbamylated EPO
CMS – chronic mild stress
CRH – corticotropin-releasing hormone
DA - dopamine
DG - dentate gyrus
EPM - elevated-plus maze
EPO - erythropoietin
EPOR - erythropoietin receptor
ERK - extracellular signal-regulated kinases
fMRI - functional magnetic resonance imaging
FST - forced swim test
G-CSF - granulocyte colony-stimulating factor
GDNF - glial derived neurotrophic factor
HPA - hypothalamic-pituitary-adrenal
i.p. - intraperitoneal
JAK 2 - janus tyrosine kinase 2
MAP - mitogen-activated protein
mRNA – messenger ribonucleic acid
NE - norepinephrine
NF- κ B - nuclear factor- κ B
NGF - nerve growth factor
NIH - novelty induced hypophagia
NMDA - N-methyl-d-aspartate
NT – neurotrophin
OF - open field
PI3-K - phosphatidylinositol-3-kinase
PTSD - posttraumatic stress disorder
PVN - paraventricular nucleus
RBANS - Repeatable Battery for the Assessment of Neuropsychological Status
SGZ - subgranular zone
SNRI - serotonin–norepinephrine reuptake inhibitors
SSRIs - selective serotonin reuptake inhibitors
STAT-5 - signal transducers and activators of transcription-5
TrkB - tyrosine kinase B

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Antidepressant-like effects of erythropoietin: A focus on behavioural and hippocampal processes

1.1 Depression: behavioral and neurobiological features

Depression is a chronic and debilitating condition that currently lacks adequate treatment options. The two most prominent, and thus diagnostic, symptoms of major depression are depressed mood, or sadness, and anhedonia (an inability to experience pleasure in daily life) (Millan, 2006). In addition to at least one of those two factors, the diagnostic features of major depression also include at least two of the following: significant appetite or weight changes, lethargy, difficulties concentrating, sleep disturbances, psychomotor disturbances, persistent morbid or suicidal thoughts, as well as a feeling of hopelessness, despair, guilt, or worthlessness (Millan, 2006). Depression is also often co-morbid with other serious afflictions, such as Parkinson's disease (Allain et al., 2000), diabetes (Egede, 2004), chronic pain (Wilson et al., 2001), vascular disease (Thomas et al., 2004) and anxiety disorders (Wittchen et al., 2000).

While the mechanisms of depression are not fully understood, substantial attention has been devoted to the neurohormonal aspects of the disorder. In this regard, a dysfunctioning hypothalamic-pituitary-adrenal (HPA) axis is apparent in many cases of depression and possibly other anxious conditions (Millan 2006). The HPA axis involves the intricate system of communication between the hypothalamus, the pituitary gland and the adrenal cortex. This system is responsible for the regulation and release of glucocorticoid hormones which enter the bloodstream and facilitate adaptive responses to acute stressors (Herman and Cullinan, 1997; Sapolsky et al., 2000). However, when this

system is chronically activated the persistent corticoid levels can have damaging effects within the brain, particularly at emotional regulatory regions such as the hippocampus, which have a high density of glucocorticoid receptors (Holsboer, 1999). Significantly, HPA abnormalities have been discovered prior to the onset of clinical symptoms in relatives of major depression patients, suggesting that HPA functioning may be a biomarker of increased risk of developing depression (Holsboer, 2003; Nemeroff, 1996; Nemeroff & Vale, 2005).

It has been known for quite some time that imbalances in central monoamines are involved in depressive behaviors. In particular, norepinephrine (NE), dopamine (DA) and serotonin (5-HT); collectively called the “biogenic amines” have received considerable attention and it is also telling that the currently available standard antidepressant drugs target some combination of these neurotransmitters (Stanford, 1995; Millan, 2006; Anisman et al., 2008). Although all three monoamines have been implicated in depressive disorders, with the rise in popularity of the selective serotonin reuptake inhibitors (SSRIs) in recent decades, most of the recent focus has been directed toward 5-HT (Millan, 2006). Although the literature is somewhat inconsistent, a multitude of studies have linked 5-HT receptor changes to depression (Stockmeier, 2003). For instance, animal studies revealed that 5-HT transporter (5-HTT) knockout mice showed increased immobility in the forced swim test, an indicator for depressive-like behaviour (Wellman et al., 2007). There have also been multiple studies examining 5-HT in the brains of depressed individuals who died by suicide. In some cases, 5-HTT was reported to be diminished in the prefrontal cortex of depressed suicides (Arango et al., 1995), and 5-HTT binding to be reduced in the amygdala, midbrain and brainstem (Malison et al.,

1998). Likewise, the 5-HT_{1A} autoreceptor, in the dorsal raphe nucleus, that influences 5-HT availability in the frontal cortex, has been found to be diminished in the brains of depressed suicides (Boldrini et al., 2007; Drevets et al., 2000; Stockmeier et al., 1998). The gene controlling tryptophan hydroxylase-2 that has been found to translate into reduced 5-HT synthesis, has also been found to occur at high frequencies in depressed patients (Zill et al., 2004).

Perhaps the most convincing link between depression and monoamines stems from the fact that treatment with 5-HT agents or a combination of 5-HT with NE agents can ameliorate depressive symptoms (Millan, 2006; Pineyro and Blier, 1999). While a significant, though complex, relationship between monoamines and depression seems to exist, unfortunately, monoamine-based treatments are still inundated with weaknesses, including the 3-4 week time lag before symptom reduction occurs, as well as the fact that a substantial proportion of patients are treatment refractory or display relapse with continued treatment (Kessler et al., 2003; Montgomery, 2006). Thus, a better understanding of the role of non-monoaminergic pathways in depression is required for the development of novel antidepressant agents.

1.1.1 Neurotrophic factors and depression

Recently, there has been a trend toward examining trophic factors in relation to depression. Trophic factors are generally responsible for cell proliferation, growth, and the prevention of apoptosis (Mufson et al., 1999). For example, brain-derived neurotrophic factor (BDNF), was found to act through its receptor, tyrosine kinase B (TrkB), to enhance neurogenesis and promote neuronal cell survival and neurite growth (Qian et al.,

2006). Likewise, administration of glial derived neurotrophic factor (GDNF) or granulocyte colony-stimulating factor (G-CSF) has been found to enhance neurogenesis in the hippocampus of treated rodents (Kobayashi et al., 2006; Schneider et al., 2005). These findings suggest that trophic factors might hold promise as potential novel clinical anti-depressant utility; particularly as depressive illness has been associated with reduced hippocampal volume (Campbell et al., 2004) and a variety of stressors have been demonstrated to reduce hippocampal neurogenesis (McEwen, 2000; Dranovsky & Hen, 2006). In fact, a correlation was discovered between the degree of hippocampal volume loss and the duration and number of episodes of depression (MacQueen et al., 2003). Additionally, this volume loss was more pronounced in non-remitted compared to remitted patients (Frodl et al., 2004). However, a recent imaging study revealed no evidence that a smaller baseline hippocampal volume was connected to depression, but rather, depression was linked to a faster decline in hippocampal volume (Heijer et al., 2011). Thus, two possibilities exist: 1. That the stress associated with having depression contributes to the hippocampal changes or 2. Some pre-existing hippocampal structural deficit predisposes individuals to developing the illness.

Notably, it has been established that all effective antidepressants increase hippocampal neurogenesis (Malberg et al., 2000). This has best been illustrated in studies that make use of x-ray irradiation to suppress hippocampal neurogenesis. In such studies, the prevention of adult hippocampal neurogenesis inhibited the behavioural actions of antidepressants in rodent behavioural screens for antidepressant activity (Santarelli et al., 2003; Jiang et al., 2005; Airan et al., 2007; Zhu et al., 2010). Importantly, the time course for the maturation of new neurons in the dentate gyrus (DG) region of the hippocampus

correspond with the delayed onset of antidepressant action, providing a further link between antidepressant activity and hippocampal neurogenesis (Ngwenya et al., 2006). It has been suggested that BDNF might be a primary mediator of hippocampal neurogenesis, and that BDNF reductions that occur with stressor exposure give rise to profound neurogenic changes (Duman & Monteggia, 2006; Manji & Duman, 2001). Accordingly, it was also found that the positive effects of several antidepressants were complemented by increased BDNF expression, together with a promotion of hippocampal neurogenesis (Perera et al., 2007; Warner-Schmidt & Duman, 2006) and antidepressant effects were not observed in mice with the targeted deletion of genes for BDNF (Monteggia et al., 2007). Considering this, it appears that trophic factors could play an important role in antidepressant effects and, as will be described in later sections, this is an important focus of the present thesis.

1.2 Stress-depression connection

The one environmental factor that has been unequivocally linked to depression in numerous clinical and experimental based studies has been exposure to stressors (Liu & Alloy, 2010). Yet, it is important to acknowledge that one's genetic constitution, along with the coping strategies employed by an individual, ultimately shape the impact of the stressor and determine whether pathological consequences come about. Thus, there often is not a simple "one-to-one" relationship between stressor exposure and the development of depressive or anxious pathology. That said, there are basic behavioural and biological signatures that are commonly induced following exposure to most stressors.

Stressors may be broadly grouped into categories of either a physical or psychological nature, the former of which is referred to as neurogenic and the latter psychogenic. Neurogenic stressors, which include footshock or tail pinch in the case of rodents, directly challenge and may injure the physical structure of the organism (Lu et al., 1998). In contrast, psychogenic stressors lack a predominant physical component and may include challenges such as exposure to a predatory odour for rodents or public speaking in the human situation (Lu et al., 1998). As well, stressors may be further subdivided into those of which the organism is cognitively aware and those of which it is not. For instance, systemic stressors, coined by Herman and Cullinan (1997), include insults that affect cardiovascular, respiratory and other systemic processes but the organism remains cognitively unaware of these challenges. In this regard, infectious pathogens and associated immunological insults, as well as some environmental toxins, would be considered systemic stressors. Although such systemic stressors likely activate different higher brain regions than those affected by psychogenic type stressors, both classes of stressors have important common final functional outcomes (Ericsson et al., 1994). For instance, the majority of stressful events, regardless of their individual specificity, all provoke similar stereotypical changes in circulating hormonal levels (e.g. glucocorticoids) and behavioural disturbances (e.g. changes in exploration). Animal models of depression that employ social stress might be particularly relevant to the human condition, given that psychosocial stressors would be the primary challenges encountered by depressed individuals on a daily basis (Sachser et al., 1998; Bartolomucci et al., 2005).

One design often used to assess social stress in rodents is the ‘social defeat’ model. This typically involves a naive, experimental rodent being placed with a more aggressive conspecific, and left to interact for a short duration, resulting in a display of submissive behaviours by the naive animal. One variant of this procedure involves intermittent defeat, wherein animals are housed in close proximity, but not tactile contact, such that they still see, hear and smell each other. At various intervals, the barriers separating the animals are removed, and physical interactions are permitted. This brief physical interaction establishes the dominant and submissive pairing, before the barrier is once again replaced. Although the defeat is intermittent, the psychosocial stressor is chronic, as the defeated animal is left in sensory contact, barring tactile contact, with the “bully” (dominant) mouse (Blanchard et al., 2001).

1.2.1 Neuronal and hormonal effects of stressors

Stressors are believed to contribute to depressive-like behavioural pathology, in part, through their impact upon the HPA axis, and central monoamine activity (Keller-Wood & Dallman, 1984; Seyle, 1936, 1946). Indeed, a variety of stressors have been shown to stimulate CRH neurons within the hypothalamus resulting in the liberation of pituitary ACTH and adrenal glucocorticoids (Lightman, 1994; Sapolsky, 2000). Although transient increases in circulating levels of glucocorticoids quickly normalize upon stressor termination by means of negative feedback effects, believed to be mediated, in large part, by corticoid receptors upon hippocampal pyramidal neurons (Leonard, 2005), chronic stressors often de-sensitize neuronal glucocorticoid receptors resulting in corticoid hypersecretion (Pace et al., 2007). Importantly, impaired feedback inhibition

and hyperactive glucocorticoid responses seen in chronically stressed rodents (Herman et al., 1989; Herman et al., 1995; Shiomi et al., 1986) resembles that observed in patients with depressive illness (Young et al., 1991).

Besides these HPA changes, stressors have been reported to induce changes in noradrenergic, dopaminergic and serotonergic activity within the paraventricular nucleus of the hypothalamus (PVN), amygdala, hippocampus, prefrontal cortex and brainstem among other regions (Deutch & Roth, 1990; McIntyre et al., 1999; Pacak et al., 1995; Stanford, 1995). For instance, restraint stress increased NE metabolism within the PVN, the central nucleus of the amygdala and the bed nucleus of the stria terminalis (Pacak et al., 1995). Correspondingly, Morilak et al. (2005) demonstrated that acute immobilization triggered NE release at a number of limbic forebrain regions, (e.g. central and medial amygdala, lateral bed nucleus of the stria terminalis, medial prefrontal cortex, and lateral septum). Stress-induced alterations of NE within these regions is thought to be linked to anxiety-like behaviours, as suggested by variations of arm entry performance in the elevated plus-maze and reductions of social exploratory behaviour in the open field paradigms (File, 1995; Pellow et al., 1985).

Psychogenic stressors were also found to increase DA utilization in the medial prefrontal cortex and the nucleus accumbens (Deutch & Roth, 1990). Given the well established role of DA in mediating the hedonic impact of appetitive events, variations of this transmitter have been suggested to underlie many of the core facets of clinical depression. Indeed, some antidepressants such as pramipexole, a dopamine D2/D3 receptor agonist, elevate mood and alleviate symptoms of depression through their selective actions upon DA circuits, as shown by significantly improved scores on the

Hamilton Depression Rating Scale and the Montgomery-Asberg Depression Rating Scale following antidepressant treatment (Goldberg et al., 2004; Zarate et al., 2004). Renard et al. (2001) even went so far as to argue that the antidepressant effects of various selective SSRIs are mediated by means of D1 and D3 dopamine receptors, suggesting a critical interaction between the serotonergic and dopaminergic systems. In addition, several dopamine reuptake inhibitors were reported to stimulate locomotion and increase swimming activity of mice in the forced swim test, a test in which immobility is considered a reflection of despair (Vaugeois et al., 1996).

Substantial evidence for a deficit in serotonergic pathways in depression is supported by the well-documented ability of SSRIs to effectively treat depressive symptoms (Middlemiss et al., 2002; Tollefson et al., 1995). Moreover, the decreased cortical extracellular concentration of serotonin observed in animals exposed to footshock was completely antagonized by long term administration of the SSRI, fluvoxamine (Dazzi et al., 2005). In addition, SSRIs were shown to normalize 5-HT variations within the medial prefrontal cortex and rectify freezing behaviour of animals subjected to conditioned fear stress, strongly indicating that facilitation of brain serotonergic systems moderates anxiety (Hashimoto et al., 1999). Similarly, decreased serotonergic activity in the prefrontal cortex and increased 5-HT activity in the hippocampus of rats exposed to CMS were both normalized following treatment with the tricyclic antidepressant, imipramine (Bekris et al., 2005). Together these data reinforce the notion that monoaminergic variations are critically involved in stressor induced affective disturbances.

1.3 Stressor provoked alterations of neuroplasticity: Implications for depression

Although it is well established that imbalanced HPA and central monoamine levels contribute to the aetiology of affective states, much less is known of the mechanisms behind these neurochemical changes. In this regard, emerging evidence has suggested that affective disturbances may be characterized by actual structural brain changes or alterations of neuroplasticity, and hence these changes may come to impact upon neurotransmitter activity. In particular, the hippocampus has emerged as an integral region that is extremely susceptible to the impact of stressors and morphological or molecular changes in growth factors or other signalling elements within the hippocampus may be aberrant in depression. Essentially, it is possible that the reduced 5-HT, that is probably the primary neurochemical defining feature of depression, may stem from alterations of hippocampal plasticity (e.g. reduced neurogenesis or dendritic branching). Along these lines, giving monoamine targeting antidepressants would simply manage the symptoms of depression by acting at a very downstream distal level, rather than influence early molecular changes that may be proximal to the origins of the disorder. From this perspective, it is important to consider the possibility that future antidepressant treatments may benefit from targeting neuroplastic processes that may be involved in the origins of depression.

As already mentioned, patients suffering from stress related psychiatric illnesses such as major depression show a decrease in hippocampal volume (Bremner et al., 2000; Sheline et al., 1996; Sheline et al., 2003) and the degree of hippocampal volume reduction was correlated with the total duration of major depression (Sheline et al., 1996). Importantly, findings indicating that hippocampal volume is restored with treatment to

remission (Caetano et al., 2004), provide strong evidence that the depressive state actively contributes to morphological changes. In this respect, it has been posited that the chronic stress associated with dealing with affective illness may in itself provoke changes in hippocampal volume (Sheline et al., 1996; Caetano et al., 2004; Lee et al., 2006; Xu et al., 2006). Yet, it is also possible that pre-existing structural differences, or other deficits, render individuals vulnerable to the disorder in the first place. In fact, Gilbertson and colleagues (2002) provided some indication that volumetric differences in hippocampal volume might predispose individuals to the eventual onset and progression of affective disorders. In their study of monozygotic twins discordant for the psychiatric condition of posttraumatic stress disorder (PTSD), Gilbertson et al. (2002) observed that smaller hippocampal volumes were associated with a greater vulnerability to the development of PTSD following trauma. In addition, Gilbertson et al. found a negative correlation between hippocampal volume and severity of PTSD symptoms, such that twin pairs with severe PTSD had significantly smaller hippocampi than did non-PTSD twin pairs.

1.3.1 Mechanisms of stressor provoked alterations of neuroplasticity

Several mechanisms might account for the loss of hippocampal volume evident in depressed subjects, including a failure of new neuronal birth (neurogenesis) or a loss of existing cells through impairment of trophic support. For instance, postmortem studies have revealed a significant reduction in the expression of BDNF, a growth factor that is critical for the survival and function of neurons, in the prefrontal cortex and hippocampus of depressed individuals that died by suicide (Dwivedi et al., 2003). Postmortem tissue from suicide patients also provided evidence for neurotrophin dysregulation as evidenced

by reduced extracellular signal-regulated protein kinase activity and expression in the hippocampus and cerebral cortex (Dwivedi et al., 2001). Furthermore, mRNA and protein levels of the neurotrophins; nerve growth factor (NGF), NT-3, and NT-4/5 were significantly decreased in hippocampus of suicide victims (Dwivedi et al., 2005). In recent years, attention has shifted towards the proposition that an inhibition of neurogenesis could account for the reduction of hippocampal volume observed in clinically depressed subjects (D'Sa & Duman, 2002; Jacobs et al., 2000; Jacobs, 2002; Kempermann, 2002; Sheline et al., 1996). Indeed, accumulating evidence suggests that stress has potent effects on neuroplastic processes such as neurogenesis and dendritic branching (Kempermann & Kronenberg, 2003). During adult neurogenesis, the generation of new daughter cells from a precursor population occurs via the mitotic cycle in the hippocampus of numerous mammalian species (Christie & Cameron, 2006; Eriksson et al., 1998). Specifically, progenitor cells contained in the subgranular zone (SGZ) of the dentate gyrus divide and migrate to the granule cell layer where they eventually mature and begin to express a neuronal phenotype (Cameron et al., 1993). Stressors of both an acute and chronic nature have been shown to suppress the birth of these dentate gyrus granule neurons (Gould et al., 1998; Kim & Diamond, 2002; Magarinos et al., 1996; McEwen, 1999; McEwen, 2000a; McEwen, 2000b; Stein-Behrens et al., 1994; Tanapat et al., 1998; Watanabe et al., 1992).

It has been found that these social defeat paradigms are particularly effective in provoking hormonal and neurochemical changes. In one experiment, brief repeated social defeat stress over the course of 10 days lead to an inhibition of hippocampal cell proliferation in the submissive but not the dominant animal, although corticosterone

levels were increased in both animals (Yap et al., 2006). This suggests that it is not simply an increase in corticosterone levels that is diminishing the cell proliferation. Likewise, in a social stress experiment in mice, stress was found to decrease cell proliferation preferentially in the hippocampus in the right hemisphere (Mitra et al., 2006). This inhibition of cell proliferation was correlated with individual differences in the frequency of defensive behaviour, not the amount of aggression received or frequency of fleeing (Mitra et al., 2006). Also of interest, is the fact that chronic defeat stress in mice resulted in a significant downregulation in total BDNF mRNA levels, and lasting chromatin remodelling at the BDNF gene in the hippocampus of defeated mice (Tsankova et al., 2006).

Importantly, chronic antidepressant therapy or administration of a single electroconvulsive shock reversed the effects of chronic stressors on neurogenesis (Jacobs, 2002; Malberg et al., 2000). However, chronic antidepressant treatment alone enhanced adult cell proliferation within the dorsal hippocampus (Dremencov et al., 2003; Malberg et al., 2000; Santarelli et al., 2003) suggesting that these drugs have pro-mitotic effects independent of their ability to antagonize the impact of stressors. In this regard, antidepressants, such as fluoxetine, have been suggested to stimulate neurogenesis through their impact upon the 5HT_{1A} receptor (Jacobs & Fornal, 1999). Further support for the importance of the serotonergic system in the birth of new hippocampal cells is provided by studies reporting decreased numbers of progenitor cells in the rat hippocampus after animals underwent a partial serotonergic denervation induced by administration of para-chloroamphetamine (Mamounas et al., 2000; Rosenbrock et al., 2005).

In addition to disturbances of neurogenesis, stressors might influence hippocampal volume by reducing dendritic branching and impairing growth factor expression. In fact, chronic exposure to stressful events provoked morphological changes, including the atrophy and loss of neurons within the CA3 region of the hippocampus (McEwen, 2000a). Specifically, animal studies revealed that chronic exposure to neurogenic and psychogenic stressors, such as footshock and alterations in social housing condition, lead to atrophy of apical dendrites of CA3 pyramidal neurons with concomitant alterations of neurotrophins (Magarinos et al., 1996; McKittrick et al., 2000). Moreover, restraint and footshock induced a down-regulation of the neurotrophin BDNF, which has important pro-survival actions upon neurons and is involved in neuroplastic processes such as long term potentiation (Figurov et al., 1996; Korte et al., 1995; McAllister et al., 1999; Mønborg & Hall, 1995; Thoenen, 2000). Altered expression of hippocampal and cortical BDNF was proposed to be an important factor mediating atrophy and neuronal cell death associated with depression (Duman et al., 2000). Furthermore, antidepressants completely blocked the actions of several stressors upon BDNF expression (Nibuya et al., 1995).

1.4 Novel antidepressant treatments: modulation of neurochemical and neuroplastic processes

While many treatments are available, they are often inadequate or ineffective in the long run. Montgomery (2006) discussed the current need to develop new antidepressants based upon the studies indicating that antidepressants are commonly prescribed at a sub-therapeutic dose and the many negative side effects can lead to non-

compliance. Furthermore, 30% to 40% of those who seek treatment fail to respond above a placebo level to currently employed antidepressants (Kessler et al., 2003; Montgomery, 2006). For those patients who do respond to drug treatment, there is generally a 6 to 8 week delay before the antidepressant drug takes effect; during this time the condition can even worsen.

With this in mind, newer antidepressant approaches have considered the possibility of actually using certain trophic factors themselves to treat depression. In this regard, BDNF was a natural choice to explore as an antidepressant agent, as hippocampal BDNF levels were drastically reduced in experiments using chronically stressed rats (Smith et al., 1995) and mice (Li et al., 2004a,b), and the trophic factor has been employed in animal studies to counter the effects of stress. For instance, a hippocampal BDNF infusion attenuated the stress-induced alterations in glial cells (Ye et al., 2011). Yet, one of the major problems with administering BDNF, or any other neurotrophic protein for that matter, is the fact that they do not appreciably cross the blood brain barrier (BBB) (Yamashita et al., 1997; Pardridge et al., 1998). Moreover, BDNF, as well as GDNF, are known to have substantial side effects, including those affecting pain pathways (Matayoshi et al., 2005; Pezet & McMahon 2006; Constandil et al., 2011). However, one promising candidate trophic factor, erythropoietin (EPO), has recently emerged as having tremendous clinical potential. Indeed, EPO is a hematopoietic growth factor that readily crosses the BBB and is already routinely used in the clinic to treat anemia (Sargin et al., 2010). Recent pre-clinical data also has suggested its possibility as an agent to promote neuronal recovery, having neuroprotective consequences in models

of stroke and traumatic brain injury with over 180 studies reporting a neuroprotective potential for EPO (Sargin et al., 2010).

1.4.1 Erythropoietin as a novel trophic factor with antidepressant properties

The majority of studies that have assessed the impact of EPO upon brain processes have evaluated the potential neuroprotective effects of the cytokine in models of cerebrovascular diseases (Sargin et al., 2010). Indeed, systemic EPO administration was found to reduce infarct volume in response to cerebral artery occlusion; even when delivered 6 hours following the stroke (Matsushita et al., 2003; Villa et al., 2003; Brines et al., 2000). This is significant as it establishes that not only can EPO cross the BBB at a significant concentration to produce neuroprotective effects, but also that EPO can work as a post-treatment, after damage has already begun. However caution should be exercised since other studies, using lower doses with i.p. injections did not always find the same neuroprotective effects (Esneault et al., 2008; van der Kooij et al., 2009).

Many studies have also indicated that EPO provoked cognitive improvements in healthy, as well as neuronal compromised, animals (Hengemihle et al., 1996; Sargin et al., 2010). For instance, it was found that chronic EPO treated mice showed enhanced performance compared to controls in the Morris water maze test for spatial cognitive ability (Hengemihle et al., 1996). While this effect was attributed to increased hematocrit, there have been other studies conducted that find cognitive improvements prior to any changes in haematological status (Miskowiak et al., 2007a,b; Miskowiak et al., 2008). EPO was also found to prevent cognitive impairment in a passive avoidance test after an ischemic challenge (Sakanaka et al., 1998). Furthermore, in a focal stroke model, EPO

enhanced performance in the Morris water maze, in addition to the reduction of cortical infarct volume (Sadamoto et al., 1998).

Cognitive improvements imparted by EPO have also been observed in clinical studies. In a two year, double-blind, placebo controlled, randomized study with schizophrenic patients, EPO had a positive effect on cognition, above that of the placebo control (Ehrenreich et al., 2007a). Patients were tested with the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS), which evaluates delayed memory, semantic fluency, attention and the Wisconsin Card Sorting Test. Patients showed significant improvements in cognitive performance, without effects on psychopathology or social functioning. Furthermore, there was also a significant decline in the glial marker, S100B. Similar results were found with multiple sclerosis patients, with improvements in measures of executive functioning, coding and working memory, and psychomotor speed (Ehrenreich et al., 2007b).

Of importance to the current thesis, besides its cognitive benefits, emerging evidence is beginning to show EPO has potential anti-depressant consequences. One recent study found that EPO administration induced antidepressant-like effects, as measured in the forced swim test (FST) and novelty induced hypophagia (NIH) paradigm (Girgenti et al., 2009). Furthermore, EPO levels were elevated in the DG of the hippocampus after treatment with electroconvulsive shock therapy; a non-traditional antidepressant treatment (Girgenti et al., 2009). While animal models such as these cannot assess mood, it is promising that EPO can produced analogous behavioural outcomes in these tests to those of observed with standard monoamine acting antidepressants (Hengemihle et al., 1996; Girgenti et al., 2009).

Parallel human studies have used functional magnetic resonance imaging (fMRI) to examine the effects of EPO on neural and behavioural aspects of emotional processing that are related to depression (Miskowiak., 2007a). In particular, EPO modulates brain responses to emotional information in a similar manner to that of conventional antidepressants. Specifically, EPO reduced the neural response to fearful faces in the occipito-parietal cortex, consistent with reduced attention to fear (Miskowiak et al., 2007a). Notably, a reduced processing of fear-relevant stimuli has been reported after SSRI and SNRI standard antidepressant regimens (Harmer et al., 2004; Harmer et al., 2006). Miskowiak et al. (2008) also found that the metabolic alterations observed in response to fearful faces in healthy subjects was still apparent 3 days post EPO administration. A series of follow-up experiments, with a clinical major depression population, found bona fide antidepressant-like effects were imparted by EPO, consistent with conventional antidepressants (Miskowiak et al., 2009; Miskowiak et al., 2010). Based on these results, an official large scale clinical trial is currently underway to test the effects of EPO on affective and cognitive symptoms of major depression (Miskowiak et al., 2010).

1.4.2 Signaling pathways of EPO

It is important to better understand the signalling mechanisms through which EPO could impart antidepressant actions. Briefly, the homodimerization of two EPO receptor (EPOR) molecules results in the binding of a single EPO molecule which leads to a conformational change that induces the phosphorylation of the receptor-associated Janus tyrosine kinase 2 (JAK2). Subsequently, several intermediate intracellular factors are

induced, including phosphatidylinositol-3-kinase (PI3-K), akt/protein kinase-B, RAS mitogen-activated protein (MAP) kinases, signal transducers and activators of transcription-5 (STAT-5), and nuclear factor- B (NF-kB) dependent transcription (Ehrenreich et al., 2008). These signalling cascades have been linked to further activation of anti-apoptotic factors, cell differentiation, cellular growth and modulation of plasticity (Sargin et al., 2010; Arcasoy, 2008; Brines & Cerami, 2005). For instance, activation of EPORs was found to prevent N-methyl-d-aspartate (NMDA) induced apoptosis by initiating cross-talk between JAK2 and NF-kB, and the ensuing NF- B-dependent transcription of neuroprotective genes (Digicaylioglu & Lipton, 2001). Similarly, through activation of the ERK-1/-2 and Akt pathways, as well as upregulation of the anti-apoptotic protein, B-cell lymphoma-extra large (BCL-XL), EPO was found to help protect from focal cerebral ischemia (Kilic et al., 2005; Wen et al., 2002). It was also found that in hippocampal neurons, STAT5 and Akt are essential for EPO mediated neurotrophic activity (Byts et al., 2008). Ultimately, EPO appears to exert its effects through many different pathways, having an antiapoptotic, anti-inflammatory, antioxidant, neurotrophic, angiogenetic, and even neuroplastic effects (Ehrenreich et al., 2008).

Given the accumulating data implicating hippocampal disturbances in depression, it is particularly significant that EPO has been found to both protect hippocampal neurons from stress induced apoptosis, and to increase adult hippocampal neurogenesis (Wen et al., 2002; Yis et al., 2008; Zhang et al., 2007). For instance, EPO was able to protect hippocampal CA1 neurons, as well as increase BCL-XL mRNA and protein expression in the CA1 of the hippocampus in an experiment examining chemical hypoxia-induced

damage in gerbils (Wen et al., 2002). Likewise, EPO decreased apoptosis in the CA1, DG and the parietal cortex (Yis et al., 2008). EPO administration following ischemia was found to increase phosphorylation of STAT5 and expression of BCL-XL in the CA1 of the hippocampus as well as coincide with the protection of CA1 neurons (Zhang et al., 2007).

The connection between EPO and BDNF might be particularly promising with regards to potential antidepressant routes of action. As already mentioned, numerous studies have reported that BDNF is adversely affected by stressors and conversely, the trophic factor itself can have antidepressant activity (Jacobs, 2002). In fact, the behavioural effects of several antidepressants were found to be dependent upon a central BDNF elevation (Saarelainen et al., 2003). Furthermore, in clinical populations, depressed patient improvement coincided with BDNF returning to normal levels (Piccinni et al., 2007). It is therefore encouraging that EPO has been shown to increase BDNF levels and synthesis (Wang et al., 2004; Girgenti et al., 2009; Liu et al., 2010).

A major criticism of using EPO to examine antidepressant-like effects in tests such as the forced swim test (FST) is that EPO can have haematopoietic-dependent performance enhancing properties (Hengemihle et al., 1996). However, haematopoietic-independent derivatives of EPO have also been found to have neuroprotective and antidepressant properties (Leconte et al., 2011). As well, EPO induced improvements have been noted prior to any changes in haematological readouts (Miskowiak et al., 2007a,b; Miskowiak et al., 2008). Regardless, it is still important to assess the potential anti-depressant like effects of EPO across a range of behavioural tasks under conditions that are thought to resemble the social stress commonly encountered by humans.

Based on the abundant parallels in neuroprotective, neuroregenerative and behavioural activity between EPO and currently available standard antidepressants, EPO appears to be a promising treatment agent for depression. Also, given that social stress plays an integral role in depressive-like illness, it seems a logical next step to examine any stress modulating effects that EPO may have.

1.5 Aim of the current investigation

In the current thesis, we administered EPO to both stressed (social “bully” paradigm) and non-stressed mice and examined any ensuing antidepressant-like behaviour and biological effects. The focus of the behavioural paradigms was the forced swim test (FST), the open field (OF), the elevated-plus maze (EPM), a novelty task, as well as a sucrose preference test to measure anhedonia. Following this, hippocampal neurogenesis was assessed. We hypothesized that EPO treatment will attenuate the deleterious behavioural effects of the stressor, as well as any reductions in neurogenesis that might be induced by the social stressor. Furthermore, it was expected that even in the non-stressed mice, EPO treatment would promote greater mobility in the FST without a coinciding effect on locomotor activity. Ultimately, we hoped to better understand how EPO might affect neuroplastic aspects of brain functioning that are important for behavioural outcomes thought to be relevant for depressive symptoms.

Methods

2.1 Experimental Animals

64 male CD-1 mice (Charles River) used in the first experiment were from 10-12 weeks of age and weighted approximately 40g. In addition, 16 retired breeder CD-1 mice (Charles River) were used as aggressive conspecifics. As well, a second identical experiment was conducted in order to ascertain a wider range of behavioural effects.

Animals were individually housed in the non-stressed condition in standard (27x21x14 cm³) polypropylene cages. In the stressed condition, animals were housed in identical cages except for a metal mesh divider allotting the cage in half, with an experimental mouse on one side, and an aggressive conspecific “bully mouse” on the other side. All animals were maintained on a 12-h light/dark cycle with lights on at 07:00 h. Mouse chow (Charles River diet, 5071) and water was provided *ad libitum*, and room temperature was maintained at approximately 21 °C. All experimental test paradigms were approved by the Carleton University Committee for Animal Care and were conducted in adherence to guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

A 3 factor ANOVA design was employed: 2 Social stress (stress vs no stress) x 2 Injection (EPO vs vehicle) x 2 Behavior (testing vs naive).

2.2 Injection Treatments

In order to label dividing cells, all mice received an intraperitoneal (i.p) injection of bromodeoxyuridine, (Sigma-Aldrich; lot 060M1224V) (200 mg/kg) on the first day of

the experiment prior to any other treatment and then were randomly assigned to one of 8 conditions (see Figure 1, Appendix B.1)

Either EPO i.p. (R&D systems; recombinant mouse EPO; lot # EUP0409111) or a saline i.p. (Sigma-Aldrich; lot RNBB9031) injection was given 3 times a week for 2 weeks. EPO was delivered at a dose of 5000 U/kg, which was calculated to be 40 µg/kg. Both the saline and the EPO were injected at a volume of 0.4 ml. See the behavioural testing and injection schedule (Figure 2, Appendix B.2).

2.3 Social Defeat Stressor

Thirty two experimental animals were subjected to the social defeat stressor (16 EPO injected and 16 saline injected mice; half of each injection group also underwent behavioural testing). The stressed animals were placed in a cage with an aggressive CD-1 bully mouse, with a mesh divider keeping them separated. Once a day, at a random time after the completion of any required behavioural tests, the divider was lifted and the mice were allowed to physically interact. Once a mouse would show submissive behaviours, or excessive fighting occurred (continuous biting), the divider would be put back into place. Submissive behaviours were defined as standing on back paws while waving front paws in the air, or continuously cowering in a corner and then squeaking and running when the bully comes near. If the mouse was not clearly defeated by the end of 5 minutes the divider would be put back, and they would be placed with a new bully mouse the next day. All interactions were recorded, including latency to fight and submit, submission style, bullies behaviours (fighting style, aggressive tail wagging) and any injuries were

recorded and carefully monitored. No mice needed to be removed from the study due to injuries.

2.4 Behavioural Testing

As already mentioned, 2 identical experiments were conducted the first of which assessed performance in the FST, OF and EPM and the second experiment evaluated sucrose preference and performance on a novelty task. This allowed us to evaluate performance on a series of depression-relevant tasks, without “overloading” mice and inadvertently causing “learning” or stress effects related to the behavioural tests themselves. A second very important point to underline, is the fact that we include the behavioural tests as a separate variable, thereby allowing for us to determine whether any neurogenic changes associated with EPO or stressor exposure were also affected by the testing scenarios.

Thirty two animals received behavioural testing (16 EPO injected and 16 saline injected mice; half of each injection group also underwent social stressing). Behavioural experiments were started by 1 pm and completed before social stressing for the day commenced. Mice were individually moved to the testing area after the previous mouse had already completed its behavioural task. In the first experiment, the FST, OF and EPM were employed; the sequence in which the mice completed the tasks was randomized for each group to prevent order effects. In the second experiment novelty testing and sucrose preference was assessed.

2.4.1 Forced Swim Test

Mice were individually placed in a 2/3rd filled 2000mL beaker with $25^{\circ}\pm 1^{\circ}\text{C}$ water for 10 minutes. The mice could not escape, and their feet could not touch the bottom. After each swim, mice were lightly towel dried and put back in their cage, which was then placed on a heating pad on medium heat for 15 minutes. The water in the beaker was changed between each test. Each FST session was camera recorded, and scored for time spent swimming (pedaling or circular movements around the beaker), time spent climbing (active attempt to escape the beaker) and time immobile (lack of movement beyond those necessary to maintain balance).

2.4.2 Open Field Test

Mice were individually placed in the corner of the OF apparatus which is 30cm³, with an opaque plexiglas arena illuminated by ambient fluorescent ceiling lights. Mice were allowed free exploration of the arena for 10 minutes while a computer system (EthoVision, Noldus, Netherlands) tracked their movements; locomotor velocity, distance travelled, and rearing motions were analyzed. Movement patterns were examined for the whole arena, as well as the large outer square, small outer square, large inner square and small inner square (center). A 10% ethanol solution was used to clean the OF arena between each session.

2.4.3 Elevated-plus Maze

Mice were individually placed in a closed arm of the EPM apparatus for 5 minutes (4 arms total, each 24.8cm long and 7.7cm wide; 2 open arms with no walls and 2 closed arms with 21cm high, opaque, walls). A 10% ethanol solution was used to clean the EPM between each session. Each EPM session was camera recorded, and scored for:

time spent and number of entries in the open arm, closed arm and center, rearing, stretching (keeping feet within closed arm and extending head out to open arm) and head dipping (looking down over the side of the open arm).

2.4.4 Sucrose Preference Test

The sucrose preference test was performed three times in the second week of the experiment. Mice were allowed free choice between two test tubes for 17 hours (1700h-1000h). One test tube contained tap water, the other a 1% sucrose solution. The position of the tubes was altered between trials to prevent place preference in drinking. The mice were exposed to the sucrose preference test 5 times the week prior to the start of the experiment to establish a baseline in sucrose preference. The mice were not food or water deprived before the test. Water and sucrose consumption were measured by weighing the test tubes. Sucrose preference was expressed as a percentage of the total amount of liquid consumed.

2.4.5 Novelty Test

Mice were individually placed in the corner of the OF apparatus, which is 30cm³, with an opaque plexiglas arena illuminated by ambient fluorescent ceiling lights. A novel object was placed inside the OF apparatus prior to the mouse entering the arena. Mice were allowed free exploration of the arena for 5 minutes while a computer system (EthoVision, Noldus, Netherlands) tracked their movements, and measured the time spent and the latency to approach the novel object. A 10% ethanol solution was used to clean the OF arena between each session.

2.5 Immunohistochemistry

On day 14 of the experimental regimen, all mice were anaesthetized with 0.6mL of pentobarbital (Ceva Sante Animale; lot 150A1) and perfused transcardially with saline followed by 4% paraformaldehyde (PFA) in 0.1 M Phosphate Buffer Saline (PBS) (Sigma-aldrich). The brains were removed and stored at 4°C in the PFA mixture for 24 hours. The storage solution was then replaced with a 20% sucrose solution (Sigma-aldrich), and refreshed each day for 2 days, and then once a week for a month. The brains were then sliced to a thickness of 40 microns via the Cryotome FSE (Thermo Scientific). The slices were stained in the hippocampus for doublecortin (DCX). DCX was used as a marker to analyze the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. On the first day of the DCX staining procedure, the slide-mounted brains were washed in a 0.01M PBS solution and then incubated in the primary goat anti-DCX antibody (1:200) at 4°C. Twenty four hours later the slides were washed again and then incubated for 2 hours in the secondary antibody, which was a donkey Alexa 488 anti-goat (1:100). Slides were then washed, air dried and cover-slipped.

2.6 Statistical Analysis

Behavioural data were analysed by a 2 factor ANOVA (EPO injection x Social Stressor treatment), whereas immunohistochemical measures were assessed using a 3 factor ANOVA (EPO injection x Social Stressor x Behavioural Testing) design. Any significant interactions were followed up using Tukey post hoc comparisons. A StatView (version 6.0) statistical software package available from the SAS Institute, Inc was used

for the computations. Any videos scored by hand were scored twice to establish a test re-test reliability.

Results

3.1 Forced Swim Test

There was no difference detected between the time spent swimming and the time spent climbing, so the two behaviours were combined to create a 'time spent mobile' category. EPO significantly increased the time spent mobile in the forced swim test in the context of the social stress exposure, $F(1,28)= 2.07$, $p<.001$ (see Figure 3, Appendix B.3). Overall, mice injected with EPO ($M= 277.81$, $SD=76.72$) spend a greater amount of time swimming, regardless of stress condition compared to the saline controls ($M= 178.49$, $SD=76.72$). However, there was no significant main effect of the stress condition, nor was there a significant interaction between stress condition and injection type on the time spent mobile in the FST.

3.2 Open Field Test

As seen in Figure 4 (Appendix B.4), a significant EPO x stress interaction was apparent with regards to total distance travelled in an open field arena, $F(1,28)= 5.52$, $p<.05$. Specifically, while EPO modestly reduced basal exploration, the trophic cytokine also attenuated the stressor-induced reduction. Table 1 (Appendix A.1) summarizes these results. Likewise, as seen in Figure 5 (Appendix B.5), there was a significant interaction between the stress condition and injection group for velocity in the open field. The social stressor decreased velocity in saline treated mice, whereas EPO injected mice showed no

such stressor induced reduction, $F(1,28)= 5.20$, $p<.05$. See Table 2 (Appendix A.2) summarizes these results.

While there was no main effect of either injection type or stress condition in both mean distance travelled and mean velocity, there was a significant stress effect on the rearing behaviour in the open field. Specifically, stressed animals had a significantly lower rearing frequency ($M=11.60$, $SD=11.86$) compared to the non-stressed animals ($M=22.88$, $SD=15.88$), $F(1,28)= 4.96$, $p<.05$ (see Figure 6, Appendix B.6).

3.3 Elevated-plus Maze

No significant differences were observed between the groups for the measures of rearing, stretching and head dipping in the elevated plus maze. However, the social stressor did significantly reduce the number of entries into the open arm of the elevated plus maze in the saline treated mice, $F(1,14)= 4.51$, $p<.05$ (see Figure 7, Appendix B.7). Importantly, EPO administration reversed the impact of the stressor on entries into the open arm of the maze. Table 3 (Appendix A.3) summarizes these results.

3.4 Sucrose Preference Test

There was a significant interaction between injection type and stressor exposure upon sucrose preference, $F(1,28)= 12.28$, $p<.01$. Indeed, EPO treated mice showed a decrease in sucrose preference in the context of the social stressor; whereas sucrose consumption was increased by the stressor in the absence of EPO exposure ($p < 0.05$) (see Figure 8, Appendix B.8). Table 4 (Appendix A.4) summarizes these results.

3.5 Novelty Test

The social stressor had a significant effect on the time spent around a novel object placed in the open field, $F(1,29) = 4.51$, $p < .05$. Specifically, the stressed animals ($M = 55.75$, $SD = 44.87$) spent significantly less time around the novel object compared to the non-stressed animals ($M = 93.95$, $SD = 55.03$) (see Figure 9, Appendix B.9). However, EPO administration had no effect on novel object exploration.

3.6 Immunohistochemistry Results

Although no significant interaction was evident, there were main effects of social stressor exposure $F(1,24) = 7.75$, $p < .01$, and EPO injection, $F(1,24) = 28.62$, $p < .001$, upon the number of hippocampal neurons expressing doublecortin (DCX; an index of neurogenesis). As shown in Figures 10 (Appendix B.10) and 11 (Appendix B.11), animals in the stress condition ($M = 139.13$, $SD = 25.08$) had a significantly reduced number of DCX positive neurons, relative to animals in the non-stressed condition ($M = 164.75$, $SD = 45.07$). Moreover, mice in the EPO injection group ($M = 176.56$, $SD = 32.86$) displayed a significantly increased DCX cell count compared to their saline injected counterparts ($M = 127.31$, $SD = 25.24$). Importantly, we found that the behavioral testing had no significant influence on hippocampal DCX neuronal count.

Discussion

EPO has previously been associated with neuroprotective consequences and post-trauma neurorecovery, in models of cerebrovascular disease (Sargin et al., 2010; Matsushita et al., 2003; Villa et al., 2003; Brines et al., 2000). Interestingly, EPO has

also been found to provoke cognitive improvements in both animals (Sadamoto et al., 1998; Sakanaka et al., 1998) and humans (Ehrenreich et al., 2007a,b) that had cerebral trauma. As antidepressant activity has been associated with neural changes in areas of the brain that EPO has been found to affect –namely the hippocampus – (Malberg et al., 2000; Wen et al., 2002; Yis et al., 2008; Zhang et al., 2007), and stress plays an important role in the instigation of depressive illness (Anisman et al., 2008; Liu & Alloy, 2010; Keller-Wood & Dallman, 1984; Seyle, 1936, 1946), we sought to assess the connection between EPO, stress and depression. Accordingly, the current investigation endeavored to elucidate possible interactions between antidepressant-like effects of EPO with the broad aim of investigating better treatment options for those afflicted with depressive disorders.

4.1 Antidepressant-like activity, social stress and EPO

Consistent with our hypothesis, EPO was indeed found to have antidepressant-like effects, as measured by the FST. Specifically, EPO injected mice spent more time mobile compared to their saline treated counterparts. Yet, it is important to bear in mind that while the FST is a common screening test for antidepressant activity, it by no means implies clinical significance. It is however, particularly promising that similar results were found using the FST and novelty-induced hypophagia with rats (Girgenti et al., 2009), and fMRI activation comparable to that induced by traditional antidepressant was evident in humans following an EPO injection (Miskowiak et al., 2009, 2010). Importantly, it has been argued that the increased FST mobility might be due to the performance enhancing capabilities of EPO, given its ability to increase red blood cell

count (Hengemihle et al., 1996). However, similar to Girgenti et al. (2009), we found that EPO alone did not increase activity or velocity in the open field, arguing against possible motor enhancing effects.

Contrary to our hypothesis, EPO injected mice did not show an increase but rather a decrease in sucrose preference. Of course, the social stressor also failed to induce the expected decrease in sucrose preference and in fact, appeared to increase sucrose preference. This paradoxical finding appears to be at odds with the substantial literature showing that various types of stressors decrease sucrose consumption in mice and rats (Pothion et al., 2004; D'aquila et al., 1997; Calvo-Torrent et al., 1999; Grønli et al., 2005). Conceivably, one could argue that rather than influencing “neural circuitry” underlying anhedonic behavior, the current stressor preferentially affected processes aligned with carbohydrate craving, as observed in a subset of depressed patients.

To our knowledge, EPO has never been studied in mice in relation to anhedonic-like behaviour and the sucrose preference test. Given that the expected stress effect on anhedonic-like activity that is frequently found in the literature was not observed, while other measures in our study seemed to capture the expected stress effects, it is likely that this inconsistency in our results is due to a difference in our sucrose test design. Perhaps the lack of stress effect is related to the removal of the bully mouse from the cage of the stressed experimental animal for the duration of the sucrose preference testing. It may be that the change in housing expectations altered the animals' sucrose consumption, whereby sucrose was more palatable than it otherwise would have been had the bully still been housed with them. Further experiments are required to better understand how

sucrose preference can be altered by this change in housing, and how injection type may further interact with these variables.

4.2 Anxiolytic-like activity, social stress and EPO

There is a wide range of research that supports a strong co-morbidity between anxiety and depression (American Psychiatric Association, 1994; Estanislau et al., 2011; Hinojosa et al., 2006; Millan, 2006). Indeed, some anxiolytic drug treatments have been found to be moderately effective antidepressants, and treating an individual's anxiety symptoms can often diminish their depressive symptoms (Millan, 2006). As such, we assessed whether EPO might possess anxiolytic-like effects to determine not only its potential as an anxiolytic, but also whether the antidepressant-like activity could have resulted from any anxiety reducing actions.

There are a small number of studies, with varying results, which examine, both directly and indirectly, the potential of EPO to work as an anxiolytic. Leconte et al. (2012) found that repeated mild hypoxia, which initiates an increase in EPO activity, can have mild anxiolytic-like effects, as measured by both the light/dark transition test, and the elevated plus maze (EPM). Likewise, Yet, Leconte et al. (2011) did not find an anxiolytic-like effect of direct EPO administration when they used a light/dark transition test as a measure of anxiety-like behavior. However, Miskowiak et al (2007a) found that EPO reduces neural responses to fear in humans, and that it does so in a way that is also consistent with conventional antidepressants.

Our findings, like the literature, are somewhat mixed. While EPO did not significantly increase the number of entries into the open arms of the EPM, it did appear

to attenuate the stress induced reduction of open arm entries in the EPM. This might indicate that while EPO on its own may not be effective as an anxiolytic, it may act as a buffer against anxiogenic consequences of social stress. Likewise, when we analyzed the movement patterns of the mice in the open field test, we found that EPO was able to attenuate the stress induced reduction in velocity and mean distance travelled in the open field when examining the arena as a whole. However, this pattern did not hold when examining the movement patterns in just the center of the arena. It is presumable that the center of the open field is the most anxiety inducing area of the apparatus, and yet it is in this section that EPO had no discernible effect. EPO also had no effect on the stress reduction in rearing frequency. Similarly, the results of the novelty test also suggest that EPO had weak, at best, anxiolytic-like effect. In this instance, while a clear stress effect was observed, EPO had no stress buffering effect. In summary, our findings seem to most closely match that of Leconte et al. (2011), who did not find an anxiolytic-like effect of EPO. Yet, they did report a mild anxiolytic-like effect of the carboxylated form of EPO, cEPO. This is important in light of the fact that cEPO does not affect red blood cell count, raising the possibility that some beneficial effects of EPO might be compromised in the context of changes in hemocrit. Indeed, Skarstein et al. (2005) found that although the risk of depression increases with decreasing hemoglobin in cancer patients no such connection was evident between hemoglobin and anxiety. In short, more comprehensive research is needed to understand the capabilities and limitations that EPO offers as a buffer to the anxiogenic effects of social stress.

4.3 Neuroplasticity, social stress and EPO

4.3.1 Altered neuroplasticity and depression:

One of the most important but often overlooked aspects of depression is the high degree of relapse that occurs even in individuals that initially responded well to antidepressant drugs (Nutt, 2010; Thase, 1992). This led some investigators and clinicians to speculate that in many individuals depression was a life-long condition (Nutt, 2010; Keller, 1999; Thase, 1992). As such, some enduring neural changes must underlie the presumed pathological brain circuits. In this regard, emerging evidence is beginning to support the contention that protracted deficits in neuroplasticity might occur in depression (Bremner et al., 2000; Campbell et al., 2004; Kempermann et al., 2003; Sheline et al., 2003; Drevets, 2003; Videbech & Ravnkilde, 2004). In particular, major depression has been associated with both structural and functional changes within discrete brain regions, including limbic regions such as the hippocampus and the amygdala (Drevets, 2003; Videbech & Ravnkilde, 2004). Particular attention has focused on the reduced hippocampal volume often observed in patients diagnosed with major depression, and post-mortem analyses indicated that the extent of the hippocampal reduction was related to illness duration (Bremner et al., 2000; Campbell et al., 2004; Kempermann et al., 2003; Sheline et al., 2003; Videbech & Ravnkilde, 2004).

Accompanying the hippocampal deficits, platelet and serum protein levels of the trophic cytokine, brain derived neurotrophic factor (BDNF), were suppressed in depressed subjects, with levels of the growth factor being correlated with symptom severity (Pandey et al., 2010; Cattaneo et al., 2010). Similarly, BDNF mRNA was decreased in leukocytes of depressed patients (Cattaneo et al., 2010) and importantly, the

selective serotonin reuptake inhibitor (SSRI), escitalopram, reversed the BDNF deficits (Cattaneo et al., 2010). In fact, the behavioural effects of several antidepressants were dependent upon central BDNF elevations (Saarelainen et al., 2003). Furthermore, in clinical populations, depressed patient improvement coincided with BDNF returning to normal levels (Piccinni et al., 2007).

Stressor based animal models of depression have extended the animal findings to include impaired hippocampal neurogenesis and aberrant neural morphology (Coyle & Duman, 2003; Sapolsky et al., 1990; Magarinos et al., 1996; Cook & Wellman, 2004). Indeed, impaired hippocampal neurogenesis occurred in rodents exposed to a chronic corticosterone or stressor regimen (Coyle & Duman, 2003) and hippocampal implantation of cortisol pellets induced irregular cell layers, soma shrinkage and dendritic atrophy morphological changes in the hippocampus of monkeys (Sapolsky et al., 1990). Furthermore, psychosocial stress reduced apical dendrite length of pyramidal hippocampal and PFC neurons and dendritic branching points in subordinate male tree shrews (Magarinos et al., 1996; Cook & Wellman, 2004). Animal studies also confirmed that stressor exposure altered central BDNF protein levels and/or mRNA (Duman & Monteggia, 2006; Manji, et al., 2001; MacQueen et al., 2003).

Virtually all effective antidepressant treatments (pharmacological and otherwise) stimulate BDNF and affect neurogenesis. For instance, all SSRIs and tricyclic antidepressants, as well as new alternate treatments such as vagal stimulation and deep brain stimulation positively affect hippocampal neurogenesis (Malberg et al., 2000; Manta et al., 2009; Encinas et al., 2007). Similarly, aerobic exercise in rodents typically increases hippocampal BDNF and has been reported to impart antidepressant-like

behavioural effects, and blunt the neurochemical impact of stressors (Yau et al., 2012). Interestingly, the anti-depressant efficacy of the SSRI, escitalopram, was dependent upon the housing conditions, wherein the presence of a running wheel augmented the effects of the drug (Bjornebekk et al., 2008). Thus, it could be envisioned that the anti-depressant could be enhancing the ability of an organism to properly receive the emotional benefits of a “rich” environment.

Central infusion of BDNF (considered a neurotrophic cytokine itself) was shown to induce an antidepressant-like effect while concomitantly promoting sprouting of central 5-HT neurons (Altar, 1999). Likewise, hippocampal infusion of BDNF produced antidepressant-like effects in the learned helplessness and forced swim tests of depression (Shirayama et al., 2002). As expected, several clinically beneficial treatments, including SSRIs, tricyclics, and electroconvulsive therapy, increased hippocampal BDNF expression (Shirayama et al., 2002; Castrén et al., 2007); antidepressant treatment prevented the reduced BDNF associated with restraint stress (Duman et al., 1999). Conversely, the selective ablation of BDNF resulted in rodents showing heightened vulnerability of stressful challenges (Deltheil et al., 2008), further implicating the trophic factor in depressive-like pathology.

4.3.2 EPO and neuroplasticity:

One substantial complication of using BDNF clinically is the fact that it does not appreciably cross the blood brain barrier (BBB) (Yamashita et al., 1997; Pardridge et al., 1998) and has substantial side effects, including those related to pain pathways (Matayoshi et al., 2005; Pezet & McMahon 2006; Constandil et al., 2011). However, as

indicated earlier, EPO can cross the BBB and is routinely used to treat anemia (Sargin et al., 2010). Although a few studies failed to detect increased EPO CSF levels with systemic administration (Juul et al., 1997, 1999; Marti et al., 1997), others reliably found EPO to be elevated in the CSF of humans and animals after therapeutic systemic doses (Ehrenreich et al., 2002; Brines et al., 2000; Alafaci et al., 2000). Similarly, both the murine and human forms of EPO, as well as the human analog often used clinically, darbepoetin-alpha, all crossed the BBB in untreated naïve mice and accumulated at clinically significant concentrations (Banks et al., 2004). It appeared that EPO crossed the brain through extracellular pathways at about the same rate as albumin (Banks et al., 2004).

As already mentioned, a wide range of studies have implicated stress as having potent deleterious effects on neuroplastic processes (Gould et al., 1998; Kim & Diamond, 2002; Magarinos et al., 1996; McEwen, 1999; McEwen, 2000a; McEwen, 2000b; Stein-Behrens et al., 1994; Tanapat et al., 1998; Watanabe et al., 1992). Our findings were consistent with this, as we found that a social stressor significantly reduced hippocampal neurogenesis.

Fortunately, antidepressants have been found to reverse the effects of chronic stressors on neurogenesis (Jacobs, 2002; Malberg et al., 2000). Indeed, it has been established that all effective antidepressants increase hippocampal neurogenesis (Malberg et al., 2000), independent of the presence of a stressor (Dremencov et al., 2003; Malberg et al., 2000; Santarelli et al., 2003). Accordingly, we found that EPO increased hippocampal neurogenesis. The fact that this increase was independent of whether or not an animal received behavioural testing, eliminates the potential confound that the

observed hippocampal neurogenesis was partially a product of any learning effect associated with repeated testing.

The increase in hippocampal neurogenesis may be a potential mechanism through which EPO could exert its antidepressant-like effects. Indeed it has been found that depressed patients show a decrease in hippocampal volume (Bremner et al., 2000; Sheline et al., 1996; Sheline et al., 2003), and this volume is restored with treatment to remission (Caetano et al., 2004). Furthermore, it has been proposed that this reduction of hippocampal volume could be accounted for by an inhibition of neurogenesis (D'Sa & Duman, 2002; Jacobs et al., 2000; Jacobs, 2002; Kempermann, 2002; Sheline et al., 1996). As stress has been shown to suppress progenitor cells in the hippocampus (Gould et al., 1998; Kim & Diamond, 2002; Magarinos et al., 1996; McEwen, 1999; McEwen, 2000a; McEwen, 2000b; Stein-Behrens et al., 1994; Tanapat et al., 1998; Watanabe et al., 1992), it follows that EPO may be attenuating the negative effects of stress by promoting hippocampal neurogenesis.

Another possible mechanism that might explain EPO's ability to attenuate the effects of the social stressor is through its action downstream on BCL-XL signaling. In this regard, Shishkina et al. (2010) found that rats with a higher endogenous expression of hippocampal BCL-XL spent less time immobile in the FST compared to those rats with lower levels. They suggested individual differences in susceptibility to stress-induced depressive-like behaviours might be partially explained by variations in BCL-XL levels. Considering that EPO has been found to increase BCL-XL mRNA and protein expression in the hippocampus of rodents with ischemic damage (Wen et al., 2002; Zhang et al.,

2007), this represents another viable mechanism through which EPO may be exerting its behavioral consequences.

Other mechanisms of EPO's behavioral actions could include the intermediate intracellular factors that it is known to induce including, phosphatidylinositol-3-kinase (PI3-K), akt/protein kinase-B, MAP kinases, STAT5, and NF-kB (Ehrenreich et al., 2008). Indeed, these signalling cascades have been linked to activation of anti-apoptotic factors, cell differentiation, cellular growth and modulation of plasticity (Sargin et al., 2010; Arcasoy, 2008; Brines & Cerami, 2005). In particular, the connection between EPO and BDNF is particularly promising with regard to a potential antidepressant route of action. In fact, EPO was found to increase BDNF levels and synthesis (Wang et al., 2004; Girgenti et al., 2009; Liu et al., 2010), whereas stressors had the opposite effect, and this action could be attenuated by EPO (Jacobs, 2002). It is possible that the induced endogenous BDNF could synergize with the exogenously applied EPO to further reinforce anti-depressant-like actions. Indeed, BDNF and EPO share some common intracellular signalling mechanisms, in that both act through PI3-K and MAP kinase pathways.

While it is promising that EPO was found to increase hippocampal neurogenesis, future research should be directed towards understanding the downstream factors that EPO may be influencing, and how these may be related to its antidepressant-like effects. Taken together, our findings support the notion that EPO could work as an effective antidepressant treatment, and could have beneficial effects by promoting hippocampal neuroplasticity (Wen et al., 2002; Yis et al., 2008; Zhang et al., 2007).

It should also be emphasized that the current findings are consistent with the notion that EPO could have clinical benefits for not only depression, but a range of conditions in which manipulation of neuroplasticity could have important consequences. Indeed, it is particularly significant that EPO protects hippocampal neurons from stress induced apoptosis (Wen et al., 2002; Yis et al., 2008; Zhang et al., 2007). Through such hippocampal effects EPO might have cognitive enhancing effects, as indicated by improvements in spatial performance in the Morris water maze test (Hengemihle et al., 1996; Sadamoto et al., 1998), as well as improvements in neuropsychological indices of cognitive performance in schizophrenic patients (Ehrenreich et al., 2007a). EPO was also found to prevent cognitive impairment in an avoidance test after an ischemic challenge (Sakanaka et al., 1998). Similar results were found with multiple sclerosis patients who expressed improved executive functioning, coding and working memory, and psychomotor speed (Ehrenreich et al., 2007b). Recent pre-clinical data also suggested its possible use as an agent to promote neuronal recovery, having neuroprotective consequences in models of stroke and traumatic brain injury with over 180 studies reporting a neuroprotective potential for EPO (Sargin et al., 2010).

Ultimately, translating EPO findings using animal models of depression into human clinical trials in a variety of neuropsychiatric patients will be the next important step, albeit a challenging one considering the poor track record with the majority of efforts translating animal studies to human trials being less than successful. That said, at least EPO is already being used successfully to treat anemia; hence, reducing the likelihood of obstacles related to toxicity.

Appendices

Appendix A : Tables

A.1 Table 1. Mean distance travelled in the arena of the open field (n = 8/group)

	Mean Distance Travelled (cm)	SD	SE
No stress, Saline	4839.25	752.00	265.87
No stress, EPO	4159.95	398.28	140.81
Stress, Saline	3766.78	1215.59	429.78
Stress, EPO	4396.17	527.72	265.87

A.2 Table 2. Mean velocity travelled in the arena of the open field (n=8/group)

	Mean Velocity (cm/s)	SD	SE
No stress, Saline	8.07	1.25	.44
No stress, EPO	6.93	.66	.23
Stress, Saline	6.28	2.03	.72
Stress, EPO	7.34	.95	.36

A.3 Table 3. Number of entries into open arm of elevated-plus maze (n=8/group)

	Number Entries into Open Arm	SD	SE
No stress, Saline	21.00	9.47	3.35
No stress, EPO	22.00	12.36	4.37
Stress, Saline	12.71	4.41	1.63
Stress, EPO	19.71	7.37	2.78

A.4 Table 4. Average percent sucrose preference over the 3 days of the sucrose preference test (n= 8/group)

	Average Sucrose Consumption (%)	SD	SE
No stress, Saline	77.01	7.58	2.68
No stress, EPO	82.29	5.49	1.94
Stress, Saline	84.19	6.70	2.37
Stress, EPO	68.92	11.92	4.22

Appendix B : Figures

B.1 Figure 1. The 8, randomly assigned, experimental conditions in the first experiment

B.2 Figure 2. Behavioural testing and injection schedule for the first experiment.

Behavioural tests were started at 1pm. Social stressor exposure was administered once daily (after any behavioural tests for that day were already completed).

B.3 Figure 3. Time spent mobile, in seconds, in the FST. Data are expressed as mean $\pm$ SEM (n = 8/group). *P < 0.001 relative to saline-treated controls according to two-tailed Student's t test.)

B.4 Figure 4. Total distance moved in the arena of the OF. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.05.

B.5 Figure 5. Mean velocity moved in the arena of the OF. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.05.

B.6 Figure 6. Rearing frequency observed in the arena of the OF. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.05.

B.7 **Figure 7.** Number of entries into the open arm of the EPM. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.05.

B.8 **Figure 8.** Average percent sucrose preference observed over 3 days of the SPT. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.01.

B.9 Figure 9. Time spent around the novel object during the novelty test. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.05.

B.10 Figure 10. Total DCX cell count in both hemispheres of the hippocampus. Data are expressed as mean $\pm$ SEM (n = 8/group) *P<0.01, **P<0.001.

B.11 Figure 11. DCX 488 immunofluorescent stain of the hippocampus of CD-1 mice at 20X magnification. a.EPOxNo Stress b.SalinexNo Stress c. EPOxStress d. SalinexStress

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