The Impact of Proinflammatory Cytokines upon Adult Hippocampal Cell Proliferation: Implications for Depression

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Abstract

Accumulating evidence indicates that alterations of hippocampal functioning may influence affective state and cognition. Indeed, disturbances of hippocampal plasticity, including impaired dendritic branching and neurogenesis, are readily provoked by stressful insults and may also occur in clinical depression. It has recently been shown that these neuroplastic disturbances may have behavioural consequences. Although corticoids are likely important in regulating stressor-induced reductions of neurogenesis, several reports suggest that other signalling messengers, including growth factors and cytokines, may also be involved. Proinflammatory cytokines have potent effects upon several processes linked to depression including central monoamine, neuropeptide and glucocorticoid activity. More recently, administration of the immunotherapeutic cytokine, interferon-α, was reported to diminish hippocampal neurogenesis and provoke behavioural signs of depression. Accordingly, we assessed whether 3 proinflammatory cytokines (which may be altered in depressed individuals) influenced hippocampal cellular proliferation (neurogenesis and gliogenesis). As well, social and neurogenic stressors were included for comparison with the cytokines. In this respect, both stressors elicited modest reductions of cellular proliferation that were restricted to the early levels of the hippocampus. Similarly, acute systemic administration of tumor necrosis factor-α (TNF-α) reduced hippocampal cell proliferation, yet, interleukin-1β (IL-1β) and IL-6 had no significant effects. However, chronic but not acute infusion of IL-6 and IL-1β into the hippocampus increased cellular proliferation but TNF-α had no effect in this respect. Thus, the route and chronicity of cytokine administration had a marked influence upon the nature of the alterations of cellular proliferation. These data are discussed in terms of potential mechanisms of cytokine action and functional implications.

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List of Abbreviations

5-HT – serotonin

ACTH – adrenocorticotrophic hormone

AVP – arginine vasopressin

BBB – blood-brain barrier

BDNF – brain derived neurotrophic factor

BrdU – 5-bromo-2-deoxyuridine

CMS – chronic mild stress

CRH – corticotropin releasing hormone

DA – dopamine

DCX – doublecortin

DG – dentate gyrus

GCL – granule cell layer

GFAP – glial fibrillary acidic protein

HPA – hypothalamic-pituitary-adrenal

i.p. – intra-peritoneal

IFN – interferon

IL – interleukin

LPS – lipopolysaccharide

NE – norepinephrine

NK – natural killer

PFA – paraformaldehyde

PTSD – posttraumatic stress disorder

PVN – paraventricular nucleus

SGZ – subgranular zone

SSRI – selective serotonin reuptake inhibitor

SVZ – subventricular zone

TMT – trimethyl thiazoline

TNF – tumor necrosis factor

ZEME – external zone of the hypothalamic median eminence

Introduction

It has become clear that stressful events have repercussions upon a host of physiological and behavioural processes. In fact, repeated exposure to psychological stressors has been closely linked with the provocation of affective and anxiogenic disorders, including clinical depression and generalized anxiety or panic disorders (Anisman, Hayley, Turrin, & Merali, 2002; Post, 1992). With the increasing incidence of affective disorders in society, it has become of incredible importance to outline the hormonal and neurological mechanisms responsible to the initiation, progression and/or exacerbation of these conditions. Indeed, it is not only of heuristic value but also of economic and health concern, given the strain that these pathological states place upon work environments, relationships and social interactions (Rugulies, Bultmann, Aust, & Burr, 2006). The fact that depression and chronic anxiety are highly co-morbid with a number of other medical conditions (e.g. diabetes, cardiovascular and neurodegenerative diseases) highlights the significance of affective states in modulating the morbidity and mortality of such conditions (Haworth et al., 2005; Simon, Von Korff, & Lin, 2005). Finally, it should be emphasized that when examining how behavioural disorders such as depression arise, it is of great importance that the contribution of multiple interacting physiological systems be considered. For instance, for decades the monoamine theory of depressive illness maintained the focus of research upon changes in discrete monoaminergic circuits; however, it has recently become clear that alternate processes involving immune factors, neuropeptides and molecular signals involved in neuroplasticity may all play a role in depression. The present series of studies will assess

the impact of several of these immune factors, as well as stressor exposure upon aspects of neuroplasticity that may be important for depression.

Stressor models of depression

Behavioural consequences: 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, Song, Ravindran, Merali, & Anisman, 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, the relatively new stressor category of systemic stressors, coined by Herman and Cullinan (1997), includes 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, Kovacs, & Sawchenko, 1994). For instance, as will be described in a later section, 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).

In addition to the type of stressor, it is important to consider the effect that chronicity of stressor exposure may have upon behavioural and physiological processes. In this respect, the majority of available evidence suggests that repeated exposure to both neurogenic and psychogenic stressors influences the onset and progression of depressive pathology (Hayley, Merali, & Anisman, 2003). In fact, numerous animal studies have reported that chronic mild stress (CMS) induces depressive-like behaviours. In particular, exposure to a variety of mild unpredictable stressors was shown to reduce the consumption of palatable sweet solutions, an indicator of anhedonia, and a core symptom of depression (D'Aquila, Brain, & Willner, 1994). Exposure to uncontrollable stressors (e.g. inescapable shock) was also reported to provoke a syndrome of learned helplessness, characterized by an animal's inability to utilize appropriate avoidance behaviours when faced with an aversive event (Maier, 1993; Maier, 2001). The observed learned helplessness is believed to be analogous to self-defeating behaviours typically exhibited by depressed individuals following exposure to repeated unavoidable stressors (Keeney & Hogg, 1999). Other investigators using modified versions of the CMS procedure have similarly reported that repeated stressful events had more dramatic consequences than did acute exposure to such challenges (Dwivedi, Mondal, Payappagoudar, & Rizavi, 2005; Zafar, Pare, & Tejani-Butt, 1997). However, it should be mentioned that a certain degree of habituation may occur in situations where the same stressor is repeatedly applied. Along these lines, the predictability of the stressor greatly influences its impact, likely owing to the fact that an individual's perception of their

ability to contend with stressors affects their choice of coping strategies and consequently, whether or not depressive pathology is likely to arise (Lemoine, Armando, Brun, Barontini, & Segura, 1994).

Mechanisms for how chronic stressor exposure may impact upon behavioural pathology may be approached using the recent concept of allostasis (McEwen, 2000b). In this regard, multiple interactions between the neuronal, cardiovascular, and immune systems are envisaged as acting together to maintain a dynamic, ever-changing balance rather than a single homeostatic set-point (McEwen, 2000b). According to this perspective, behavioural pathology follows over-stimulation of allostatic systems in the absence of appropriate coping responses. Changes in neurotransmitter substrates have reliably been noted under such conditions of allostasis and likely underlie behavioural impairment (McEwen, 2000b). In fact, prolonged deficits in monoamine and neuropeptide turnover following chronic stressor exposure may influence the impact of subsequent challenges (Tannenbaum, Tannenbaum, Sudom, & Anisman, 2002). Several studies have also indicated that an initial stressor exposure may sensitize stressor-sensitive central circuits, such that amplified behavioural and neurochemical responses are evident following subsequent stressor exposure. In such a situation, a relatively permanent allostatic set-point change may be sustained which is mediated by sensitized central mechanisms. Such a proposition is consistent with the suggestion that the high rate of relapse observed in depression may reflect the fact that relatively permanent allostatic alterations have occurred (McEwen, 2004).

Effects of stressors upon HPA functioning: One primary mechanism through which stressors can provoke behavioural pathology is by altering the activity of the hypothalamic-pituitary-adrenal (HPA) axis, a system that has long been known to be linked to the stress response (Keller-Wood & Dallman, 1984; Seyle, 1936, 1946). Psychological and physical stressors initiate a cascade of events, beginning with the release of the neuropeptide corticotropin releasing hormone (CRH) from CRH neurons in the paraventricular nucleus (PVN) of the hypothalamus. Corticotropin releasing hormone release then triggers adrenocorticotrophic hormone (ACTH) release from the anterior pituitary gland which subsequently increases levels of glucocorticoids secreted from the adrenal cortex. Activation of the HPA axis in response to an acute stressor produces a transient increase in circulating levels of glucocorticoids that quickly normalizes upon stressor termination by means of self-regulated feedback inhibition (Leonard, 2005). In the presence of chronic stressors however, a desensitization of central glucocorticoid receptors creates a resistance to feedback mechanisms, thereby leading to hypersecretion of adrenal glucocorticoids. The impaired feedback inhibition seen in chronically stressed rodents (Herman, Patel, Akil, & Watson, 1989; Herman, Adams, & Prewitt, 1995; Shiomi, Watson, Kelsey, & Akil, 1986) resembles that observed in patients with depressive illness (Young, Haskett, Murphy-Weinberg, Watson, & Akil, 1991). While HPA overactivity in response to repeated stressors has mainly been attributed to elevated expression of the HPA regulatory hormone CRH, increased levels of arginine vasopressin

(AVP) may also be involved given that chronic immobilization stress activated AVP synthesis in CRH neurons of the parvocellular region of the PVN (de Goeij, Binnekade, & Tilders, 1992). Indeed, both AVP and CRH are co-stored in a subset of

terminals found within the external zone of the hypothalamic median eminence (ZEME), from which they have been shown to synergistically provoke the release of anterior pituitary ACTH into circulation (Torpy et al., 1994). Moreover, elevated central expression of AVP was also reported in post-mortem studies of depressed individuals (Scott & Dinan, 1998). Importantly, these findings are in line with the clinical evidence indicating a hyperactivity of the HPA axis in depressed individuals (Brown, 1993; Frank, Anderson, Reynolds, Ritenour, & Kupfer, 1994; Post, 1992) and may indicate that chronic stressors can influence depressive pathology by provoking protracted changes in hypothalamic CRH and AVP expression.

Effects of stressors upon central neurochemical circuits: In addition to the above-mentioned alterations in neuroendocrine functioning, stressor exposure also leads to numerous changes in central monoamine expression. In particular, stressors have been linked to changes in noradrenergic, dopaminergic and serotonergic activity within the PVN, central nucleus of the amygdala, hippocampus, prefrontal cortex and brainstem among other regions (Deutch & Roth, 1990; McIntyre, Kent, Hayley, Merali, & Anisman, 1999; Pacak, Palkovits, Kopin, & Goldstein, 1995; Stanford, 1995). For instance, restraint stress increased norepinephrine (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, Chopin, File, & Briley, 1985).

Besides the NE changes, psychogenic stressors were found to increase dopamine (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, Burdick, & Endick, 2004; Zarate et al., 2004). Renard, Fiocco, Clenet, Hascoet and Burin (2001) even went so far as to argue that the antidepressant effects of various selective serotonin reuptake inhibitors (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 depressive despair (Vaugeois, Pouhe, Zuccaro, & Costentin, 1996).

Of the many neurotransmitters that have been implicated in the pathophysiology of depression and mood disorders, serotonin (5-HT) has received the most attention. Strong evidence for a deficit in serotonergic pathways in depression is supported by the

well-documented ability of SSRIs to effectively treat depressive symptoms (Middlemiss, Price, & Watson, 2002; Tollefson, 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, Seu, Cherchi, & Biggio, 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, Inoue, & Koyama, 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, Antoniou, Daskas, & Papadopoulou-Daifoti, 2005). Together these data reinforce the notion that monoaminergic variations are critically involved in stressor induced affective disturbances.

Cytokines and depression

Recent studies assessing the mechanisms of depression have gone beyond conventional neurotransmitters and focused upon the possibility that immune factors, particularly cytokines, may be important for the disorder. Cytokines, intercellular chemical messenger proteins, are synthesized and released from several peripheral immune cells, including macrophages, natural killer (NK) cells and T lymphocytes as well as activated glial cells within the brain, following various infections or traumatic events (Vitkovic, Bockaert, & Jacque, 2000). Besides being responsible for communication between immune system cells, these messengers are also believed to act

as immunotransmitters that serve to alert the central nervous system of immunological insults (Dunn, 1992; Hayley & Anisman, 2005; Hopkins & Rothwell, 1995; Weigent, Carr, & Blalock, 1990). The proinflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) induce profound behavioural effects (e.g. anhedonia) that are somewhat reminiscent of those observed in depressed individuals and animals exposed to stressful challenges (Anisman et al., 2002; Bluthé, Dantzer, & Kelley, 1997; Hayley, Brebner, Lacosta, Merali, & Anisman, 1999; Hayley & Anisman, 2005). In fact, these cytokines have been suggested to act as systemic stressors, since, in addition to their potent actions upon circulatory and respiratory processes, they influence many of the same central processes that are affected by more traditional stressors (Anisman, Zalcman, & Zacharko, 1993; Anisman et al., 2002). For instance, social exploration was significantly decreased in rodents having received peripheral or central IL-1β administration (Besedovsky et al., 1991; Bluthé, Beaudu, Kelley, & Dantzer, 1995) and the anhedonic-like effects of the cytokine on short-term food and water intake were proven reversible by intracerebroventricular administration of an IL-1 receptor antagonist (Plata-Salaman & Ffrench-Mullen, 1992).

Effects of cytokines upon HPA functioning: Proinflammatory cytokines such as IL-1β, interleukin-6 (IL-6) and TNF-α stimulate HPA functioning (Besedovsky et al., 1991). As is the case for stressors, such as restraint and footshock, systemic administration of the cytokines IL-1β and TNF-α, has been demonstrated to increase the co-localization of CRH and AVP in the ZEME hypothalamic terminals (Hayley, Merali, & Anisman, 2003; Schmidt, Janszen, Wouterlood, & Tilders, 1995; Schmidt et al., 2001;

Watanobe & Takebe, 1993). The increase in AVP stores within CRH terminals observed following IL-1β treatment was analogous to the increase in the peptide found after prolonged exposure to repeated stressors (de Goeij et al., 1991; de Goeij et al., 1992; de Goeij, Jezova, & Tilders, 1992). Central infusion of IL-1β also increased hypothalamic CRH and AVP expression and increased secretion of these neuropeptides into the pituitary portal system (Watanobe & Takebe, 1993). A long-lasting increase in reactivity of the noradrenergic nerve terminals in the PVN that was elicited by IL-1β was proposed as one mechanism underlying the protracted effects of the cytokine upon HPA functioning (Schmidt et al., 2001).

Similar to IL-1β, systemic administration of TNF-α elevated CRH release from the median eminence and increased circulating ACTH and corticosterone levels (Hayley et al., 1999; Hayley, Lacosta, Merali, van Rooijen, & Anisman, 2001; Hayley, Kelly, & Anisman, 2002; Hayley, Merali, & Anisman, 2003; Webster, Torpy, Elenkov, & Chrousos, 1998). While central infusion of TNF-α was reported to induce a dose-dependent increase in plasma ACTH levels, this cytokine had relatively modest effects on circulating corticosterone when given by this route of administration (Hayley et al., 2001; Hayley et al., 2002). While there is much evidence indicating that TNF-α may stimulate HPA activity by direct actions upon the corticotropic cells within the PVN (Bernardini et al., 1990; van der Meer, Hermus, Pesman, & Sweep, 1996), it was reported that the cytokine may affect HPA processes through actions outside the brain (Turnbull & Rivier, 1996). In this regard, the vasoactive amine, histamine, may be important for some of the protracted central effects of TNF-α upon HPA activity (Hayley et al., 2002). Indeed, antihistamine treatment blocked the sickness and corticoid stimulatory effects of acute

TNF-α administration in mice that had been pre-treated with the cytokine four weeks earlier, suggesting that re-exposure to TNF-α may have induced an inflammatory shock-like state (Hayley et al., 2002).

Effects of cytokines upon central neurochemical circuits: In addition to HPA changes, IL-1β significantly increased NE metabolism and elevated levels of 5-HT metabolite 5-HIAA as well as serotonin precursor tryptophan within hypothalamic and extra-hypothalamic brain regions (Besedovsky, del Rey, Sorkin, & Dinarello, 1986; Dunn, 1988; Merali, Lacosta, & Anisman, 1997). In particular, IL-1β increased 5-HIAA accumulation within the PVN, central amygdala and medial prefrontal cortex (Brebner, Hayley, Zacharko, Merali, & Anisman, 2000). Similarly, systemic TNF-α markedly increased NE and 5-HT activity within the PVN, the central amygdala and hippocampus in addition to increasing 5-HT utilization in the medial prefrontal cortex (Ando & Dunn, 1999; Brebner et al., 2000). In contrast to peripheral administration, central infusion of TNF-α preferentially influenced NE and 5-HT utilization within the hypothalamic PVN and median eminence, while having modest effects upon extra-hypothalamic limbic regions (Hayley, Merali, & Anisman, 2003). Although less is known of the effects of other macrophage-derived cytokines upon central monoamines, at least some evidence indicates that IL-6 may also influence stressor-sensitive neurochemical processes. For instance, systemic and central administrations of IL-6 were reported to dose-dependently increase 5-HT turnover and tryptophan accumulation within the hypothalamus and brainstem (Wang & Dunn, 1999). Taken together, these data indicate that, like stressors,

cytokines have potent effects upon traditional biogenic amines that are known to have a major impact upon mood state.

Stressor provoked alterations of neuroplasticity: Implications for depression

Although it is well established that imbalanced neurochemical 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 (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.

According to recent reports, patients suffering from stress related psychiatric illnesses such as major depression show a decrease in hippocampal volume (Bremner et al., 2000; Sheline, Wang, Gado, Csernansky, & Vannier, 1996; Sheline, Gado, &

Kraemer, 2003). Furthermore the degree of hippocampal volume reduction was correlated with the total duration of major depression (Sheline et al., 1996), raising the possibility that morphological and/or neuroplastic changes occur over the course of depression. Finally, hippocampal volume appeared to be restored with treatment, as patients in remission showed no significant decrease in hippocampal volume when compared to controls (Caetano et al., 2004).

Although findings that hippocampal volume is restored with remission provide strong evidence that depression precedes morphological changes, it is important to examine whether hippocampal variations can also be a cause of the disorder. 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. Yet, it is also possible that pre-existing structural 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.

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 cellular 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 brain-derived neurotrophic factor (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, NT-3, and NT-4/5 were significantly decreased in hippocampus of suicide victims (Dwivedi, Mondal, Rizavi, & Conley, 2005). In recent years, however, 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, Praag, & Gage, 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). However, it remains to be determined what physiological messengers may be responsible for variations of such neuroplastic processes. In this respect, recent evidence from animal models of depression suggests a possible involvement of proinflammatory cytokines and associated inflammatory factors in some of the central effects of stressors (Shintani, Nakaki, Kanba, Kato, & Asai, 1995).

Effects of stressors upon hippocampal neuroplasticity in animals: Disturbed hippocampal neurogenesis may occur in depressed individuals and may be one mechanism through which stressors promote affective and cognitive disturbances. 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 of dentate gyrus divide and migrate to the granule cell layer (GCL) where they eventually mature and begin to express neuronal phenotype (Cameron, Woolley, McEwen, & Gould, 1993). Stressors of both an acute and chronic nature have been shown to suppress the birth of these dentate gyrus granule neurons (Gould, Tanapat, McEwen, Flugge, & Fuchs, 1998; Kim & Diamond, 2002; Magarinos, McEwen, Flugge, & Fuchs, 1996; McEwen, 1999; McEwen, 2000a; McEwen, 2000b; Stein-Behrens, Mattson, Chang, Yeh, & Sapolsky, 1994; Tanapat, Galea, & Gould, 1998; Watanabe, Gould, Cameron, Daniels, & McEwen, 1992). Others have shown that not only do both neurogenic and psychogenic stressors impair neurogenesis in the hippocampus (Gould, McEwen, Tanapat, Galea, & Fuchs, 1997), but also, this effect was reversed by chronic antidepressant therapy or by administration of a single electroconvulsive shock (Jacobs, 2002; Malberg, Eisch, Nestler, & Duman, 2000). In fact, chronic antidepressant treatment alone enhanced adult cell proliferation within the dorsal hippocampus (Lee et al., 2001; 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 recent 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, Bloching, Weiss, & Borsini, 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, Pozzo-Miller, Olafsson, Wang, & Lu, 1996; Korte et al., 1995; McAllister, Katz, & Lo, 1999; Memberg & 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, Malberg, Nakagawa, & D'Sa, 2000). Furthermore, antidepressants completely blocked the actions of several stressors upon BDNF expression (Nibuya, Morinobu, & Duman, 1995).

Alternate mechanisms of stressor induced neuronal damage were suggested by Lee, Ogle, and Sapolsky (2002), wherein neuronal atrophy following stressor exposure was proposed to result from excitotoxicity associated with enhanced glutamate and calcium activity. Indeed, stressful insults are proposed to cause glutamate to accumulate in the synaptic cleft, eventually reaching such excitotoxic concentrations. As a result of the excitotoxic accumulation of glutamate, excess cytosolic calcium is mobilized and promotes overactivity of calcium-dependent enzymes (Lee et al., 2002), resulting in cytoskeletal degradation, protein malfolding and oxygen radical generation, together shown to provoke neuronal damage (Lipton, 1999).

Effects of immunological insults upon hippocampal neuroplasticity: Several sources have suggested that, like stressors, inflammation has inhibitory effects upon neurogenesis (Ekdahl, Claasen, Bonde, Kokaia, & Lindvall, 2003; Monje, Toda, & Palmer, 2003). For instance, body irradiation, such as occurs during cancer treatment, as well as administration of bacterial endotoxin lipopolysaccharide (LPS), upregulated expression of IL-1β and TNF-α while concomitantly suppressing neurogenesis (Monje et al., 2003; Tada, Parent, Lowenstein, & Fike, 2000; Turrin et al., 2001). Furthermore, the effects of LPS and irradiation upon neurogenesis were reversed by either of the tetracycline derivative, minocycline, or the non-steroidal anti-inflammatory compound, indomethacin, which are both potent inhibitors of microglia (Ekdahl et al., 2003; Monje et al., 2003). Importantly, LPS-induced inflammation did not result in the loss of any mature neurons, suggesting that its effects were specific to newborn cells only (Ekdahl et al., 2003). Others have similarly reported decreased overall neurogenesis in the granule

cell layer of the dentate gyrus of young adult transgenic mice overexpressing the proinflammatory cytokine, IL-6 (Vallières, Campbell, Gage, & Sawchenko, 2002) further implicating neuroinflammatory mechanisms in this process.

In addition to their potential effects on neurogenesis, evidence exists supporting the effects of cytokines upon processes linked to neuronal survival. For instance, it is well established that TNF-α influences apoptotic cascades through its intracellular caspase pathways (Varfolomeev & Ashkenazi, 2004) and we and others have previously suggested that such pathways are disturbed following chronic stressor treatment (Hayley, Poulter, Merali, & Anisman, 2005). Alternatively, TNF-α has also been suggested to affect neuroplasticity by provoking the excessive release of glutamate (Allan, 2002; Rothwell, 1999; Silverstein et al., 1997). Similarly, IL-1β was reported to enhance the neurodegenerative effects of an ischemic challenge by activating glutamate-dependent calcium accumulation and the development of excitotoxicity (Rothwell, 1999). It is also possible that proinflammatory cytokines, such as IFN-γ impair neurogenesis and alter dendritic branching by inducing the release of free radicals (Pawate, Shen, Fan, & Bhat, 2004). As well, IL-1β, IL-6 and TNF-α have all been shown to contribute to neurodegenerative processes through their provocation of the release of the superoxide and nitric oxide radicals from microglia and macrophages (Pawate et al., 2004). It is important to underscore that these proinflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ) have been implicated in a host of inflammatory (e.g. heart disease, diabetes, autoimmune disorders) and neurodegenerative (Parkinson's disease, Alzheimer's disease) conditions, which are often co-morbid with depression. Given such co-morbidity, coupled with our assertion that these cytokines have central effects aligned with those of

traditional stressors (and in fact are considered systemic stressors themselves), highlights the importance of evaluating the impact of cytokines upon basic cellular proliferative and neurodegenerative pathways that may be important for depression, as well as a wide range of pathologies that may be co-morbid with the disorder.

The present experiments sought to assess the impact of stressor and cytokine exposure upon hippocampal plasticity by evaluating cell proliferation. In particular, the possibility that cytokines and stressors may impair hippocampal neurogenesis through alterations of growth factors and the activation of inflammatory processes were considered.

Methods

Each of the two experiments involved male CD-1 mice aged 8-10 weeks obtained from Charles River Canada (Laprairie, Quebec). All mice were permitted 2–3 weeks to acclimatize to the laboratory before the start of each experiment. Mice were housed in groups of four in standard (27 x 21 x 14 cm) polypropylene cages and transferred to individual housing 1 week before the initiation of a study. Animals were kept on a 12-h light–dark cycle (lights on: 0800–2000h) in a temperature-controlled vivarium (22-25°C), and were provided free access to mouse chow and water. All experimental procedures complied with the current guidelines stipulated by the Canadian Council on Animal Care and were approved by the Carleton University Animal Care Committee.

Experiment 1 procedures: It was of interest to determine the effect of neurogenic and psychosocial stressor challenges as well as systemic proinflammatory cytokine

administration upon hippocampal neuroplasticity. To this end, a subgroup of mice received a neurogenic stressor mimicking one day of a chronic mild stress protocol. The stressor comprised mice receiving 10 minutes of restraint in a plastic tube followed by a footshock session, wherein the animal was placed in a box containing a metal grid and exposed to a 0.3 mA shock every 30 seconds for a 10 minute period. In all cases, the restraint exposure occurred in the morning between 9:00-11:00 AM and the footshock commenced in the afternoon between 2:00-4:00 PM. The psychosocial stressor treatment involved placing mice in a large cage (rat breeding cage 90 x 60 x 30 cm) divided into four mesh-lined quadrants, in which 3 novel mice were on the other side of the wire mesh. Thus, the experimental mouse could see, smell and hear the 3 novel cagemates but could not make direct contact. This procedure has been reported to provoke marked corticoid and central monoamine variations (Anisman & Matheson, 2005; Czeh et al., 2002). The animal remained in this altered housing condition for 24 hours. In the case of mice receiving the systemic injections, animals were administered either IL-1β [0.1 μg, intra-peritoneal (i.p.) dissolved in 0.2mL vehicle: sterile phosphate buffered saline (PBS)], IL-6 (1.0 μg, i.p. in 0.2mL vehicle), TNF-α (1.0 μg, i.p. in 0.2mL vehicle) or the vehicle alone (0.2mL, i.p.).

In order to later examine and quantify cellular proliferation, animals were injected with 5-bromo-2-deoxyuridine (BrdU), an exogenous cell proliferation agent widely used to label mitotically active cells. Specifically, mice received one i.p. injection of BrdU (200 mg/kg; Sigma-Aldrich, Toronto, ON) dissolved in 0.9% saline immediately prior to the stressor exposure or cytokine injection. This thymidine analogue becomes

incorporated into the DNA in place of endogenous thymidine during the S-phase of the mitotic cell cycle, thereby giving a snapshot of the state of proliferation.

Experiment 2 procedures: To assess the direct impact of cytokines upon hippocampal cellular proliferation, animals were stereotaxically implanted with a 33 gauge guide cannula just above the hippocampus (placement coordinates: A/P = -1.82mm, D/V = -1.25mm, M/L = ± 1.00mm). Following a four day recovery period, mice (n = 6/group) were subjected to either a single infusion or five days of once-daily infusions of either saline, IL-1β (0.01μg), IL-6 (0.05μg) or TNF-α (0.05μg). Cytokine were dissolved in vehicle and in all cases 2 μL of solution was slowly infused over a period of five minutes using a Pico Plus syringe pump (Harvard Apparatus Inc., Holliston, MA). In addition, animal received one i.p. injection of BrdU (200 mg/kg; Sigma-Aldrich, Toronto, ON) immediately prior to the final cytokine/saline injection.

Tissue preparation: At 24 hours after the experimental treatments, animals were overdosed with pentobarbital and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA). Brains were collected, postfixed for 24 hours in PFA and then crytoprotected, for at least 3 days, in a 20% sucrose solution with 0.02% azide. Using a cryostat, 20μm thick coronal hippocampal sections were collected and mounted on gelatin-coated slides. Ten slides were collected (6 sections/ slide) for each animal and stored at -80 °C pending analysis. Hippocampal levels collected were operationally defined as early (bregma -1.22 to -1.82), mid (bregma -1.82 to -2.46) and late (bregma -2.46 to -2.92). Thus, data analyses assessed whether cytokine and stressor treatments

preferentially affected proliferation at specific anatomical levels of the hippocampal dentate gyrus.

Immunohistochemistry:

BrdU: Sections were processed for BrdU labelling by incubation in 1N HCl (2 hours at 50°C) to denature DNA, followed by neutralization with 0.1M borate buffer (pH 8.5) for ten minutes. After rinsing with PBS (0.01 M), the sections were incubated with mouse monoclonal anti-BrdU (1:200 Sigma-Aldrich, Toronto, ON) for 48 hours at 4°C. Thereafter, CY-3 conjugated donkey anti-mouse (1:200; Jackson ImmunoResearch Laboratories Inc., PA) was used as the secondary antibody and visualized by fluorescent microscopy.

DCX: In order to quantify levels of neurogenesis, sections were incubated using anti-doublecortin (DCX) (1:1000; guinea pig polyclonal, Abcam, FL), a marker of immature neurons, for 36 hours at 4°C followed by FITC (1:100; Goat polyclonal to Guinea Pig, Abcam, FL) for two hours at room temperature.

Cd11b: Accurate location of cannula placement in Experiment 2, was verified using an antibody to the microglial marker Cd11b (anti-Cd11b, 1:1000; rat monoclonal, Raleigh, NC). This staining will also give an indication of the degree of inflammation that may have been provoked by the surgery. Briefly, sections were incubated overnight (4°C) in the primary antiserum and the next were followed by two hour incubation at

room temperature with CY-3 conjugated donkey anti-rat secondary antiserum (1:200; Jackson ImmunoResearch Laboratories Inc., PA).

BrdU/DCX double-labelling: Several attempts to double-label brain sections using both BrdU and DCX have proven to be unsuccessful, likely owing to damage of DCX epitopes following the denaturing process necessary for BrdU labelling. Consequently, the double-labelling protocol is presently being refined. To this end, we have recently had some success when sections were first incubated with DCX and FITC according to procedure described above and then subsequently denatured and incubated with BrdU and CY3. However, the FITC staining was still markedly low compared to that of CY3, resulting in a potential underestimation of DCX-positive cells.

Statistics: Quantitative analyses were performed under conditions in which slides were coded, such that the rater was blind to the treatments received. For Experiment 1, the total number of bilaterally labeled BrdU-positive cells were counted within the early, middle and late portions of the dentate gyrus. Indeed, using 40X magnification, each 20μm section was counted and the average of immunopositive cells per section calculated for each hippocampal level. For Experiment 2, BrdU- and DCX-positive cells of the ipsilaterally infused hemisphere were quantified and averaged. Data for experiment 1 were analyzed at each hippocampal level using one-way ANOVAs, with IL-1β, IL-6, TNF-α, acute stress, and control as factor levels for the treatment condition. Data for experiment 2 were analyzed at each hippocampal level using two-way ANOVAs, with chronicity (acute and chronic) and treatment (IL-1β, IL-6, TNF-α, and control) as factors.

Post hoc Tukey tests were performed on these totals with Bonferroni correction for multiple comparisons.

Results

Experiment 1

BrdU staining: The ANOVA for the treatment main effects at the early level of the hippocampus revealed significant differences in BrdU counts between the groups $F(5, 23) = 3.002$, $p = .0314$. Post hoc Tukey tests revealed that mice of the TNF-α and acute stress groups were significantly different from saline-injected animals ($p < .05$). Indeed, as observed in Figures 1 and 3, mice that received i.p. administration of TNF-α had markedly fewer BrdU-positive cells than animals in the remaining groups. In fact, there was an approximately 7 fold reduction of BrdU labelling in the TNF-α treated mice relative to those that received saline (Figures 1 and 3). The neurogenic restraint + footshock stressor treatment also provoked a 50% decrease in BrdU staining ($p < .05$). While the psychosocial and IL-6 treatments seemed to reduce the number of proliferating cells (Figure 3), variability in the data precluded finding statistically significant differences. In terms of morphological aspects of the proliferating cells, BrdU-positive cells were generally small, oval-shaped and had a stippled pattern (as seen in Figure 2). They were mainly in the subgranular zone (SGZ) of the dentate gyrus and the hilus, often in clusters of two or three. These morphological characteristics and pattern of distribution closely resemble that reported in the existing literature (Christie & Cameron, 2006). Although no significant treatment effects were observed in the middle portion of the hippocampus, there was a definite trend towards a cytokine-induced reduction of BrdU labelling (Figure 4). In fact, in this region, IL-1β and TNF-α, but not IL-6, provoked a

modest decrease in BrdU staining (35-40% decrease) relative to saline treated mice. Finally, the treatments had no effect upon BrdU labelling in the more caudal or later part of the hippocampus (Figure 5). Clearly, the impact of these cytokine and stressor treatments varied across hippocampal bregma level.

DCX staining: As depicted in Figure 6, visual inspection revealed a reduction of DCX labelling within the early portion of the hippocampus in the TNF-α and social stressor exposed animals, relative to the vehicle treated controls. Given that DCX is only expressed in immature neurons, we can be confident that these staining variations reflect specific disturbances of neurogenesis, rather than cellular proliferation in general. While this finding remains to be statistically quantified, the observed reduction of DCX staining was evident in multiple animals that received the TNF-α and psychosocial stressor treatments. Presently, ongoing analyses are being conducted to quantify the actual reduction of neurogenesis provoked by the cytokine and stressor treatments.

As seen in Figure 6, The DCX-positive somas were mainly located in the granule hippocampal cell layer and were rarely seen in the subgranular zone. Axons from DCX-positive somas in the dorsal blade of the dentate extended out projecting into CA1 and towards the corpus callosum, while DCX cell bodies in the ventral blade of the dentate extended down towards the ventricles. In addition to obvious reductions of DCX labelling of somas following the stressor and cytokine administrations, it was also apparent that these treatments affected dendritic branching patterns. Specifically, as shown in Figure 6, mice that had received the TNF-α or social stressor treatments had

markedly diminished number of dendritic branches and those that were evident were clearly disordered in pattern. Thus, in addition to reducing the birth of new neurons, the TNF-α and stressor treatments may be disturbing the ability of those neurons that are newly born to integrate and make connections with the appropriate neighbouring mature neurons. Of course this proposition is somewhat speculative at this point. No differences were observed with respect to DCX staining in the middle or caudal levels of the hippocampus (data not shown).

Figure 1: BrdU immunopositive cells in the early hippocampal dentate gyrus (DG; bregma -1.22 to -1.82) of animals subjected to (A) saline i.p. injection, (B) neurogenic stressor exposure [tube restraint (10 minutes) & foot shock (0.3mA shocks administered at 30 second intervals for ten minutes)], (C) psychosocial stressor exposure (placed in large divided cage with 3 conspecifics for 24 hours until sacrifice), (D) TNF-α (1.0 μg, i.p.), (E) IL-1β (0.1 μg, i.p.) or (F) IL-6 (1.0 μg, i.p.). There was a clear reduction of BrdU-labelled cells in mice that received the neurogenic stressor (panel B) or TNF-α (panel D) treatments, relative to saline injected controls (panel A). Fluorescent microscopy; 10X magnification.

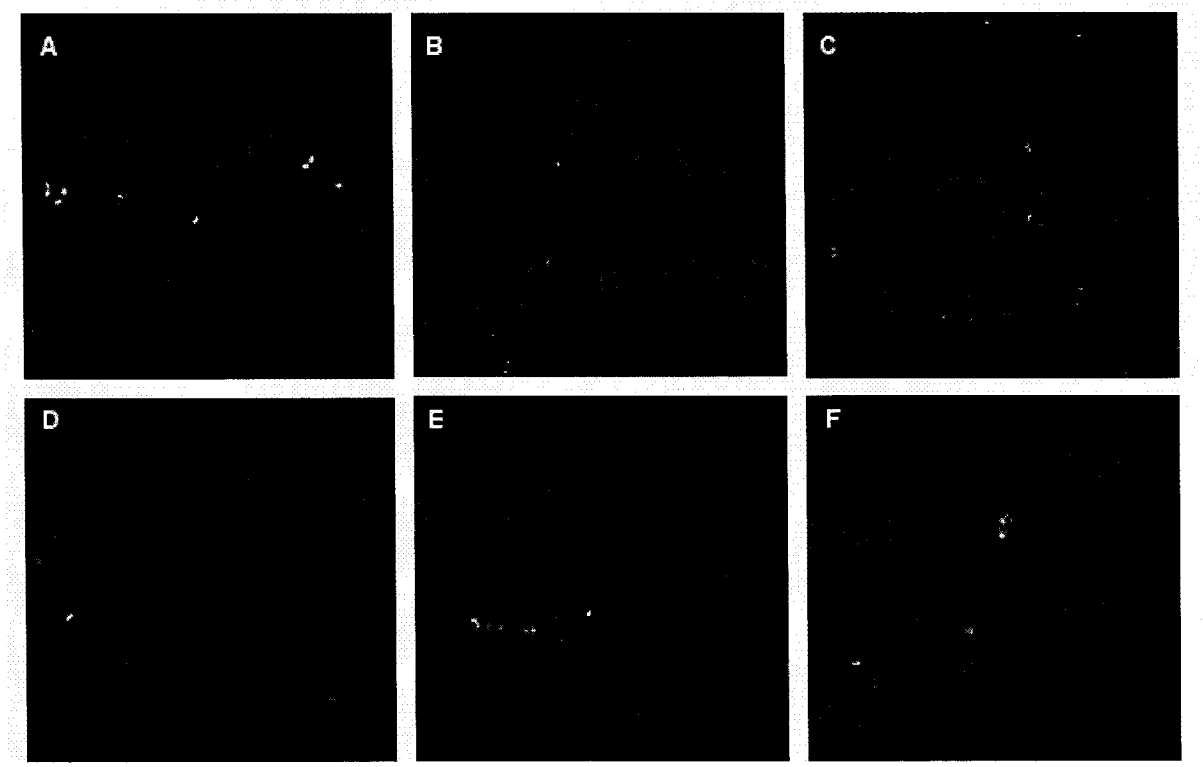


Figure 2: Morphology of BrdU-labelled cells in the early DG of animals treated with (A) saline vehicle alone (0.2 mL i.p.) or (B) TNF-α (1.0 μg, i.p. in 0.2mL vehicle). Consistent with previous reports, BrdU-labelled cells appeared oval in shape with a stippled pattern. Variations in the size of some of cells may reflect the birth of distinct neuronal or glial cohorts. Fluorescent microscopy, 40X magnification.

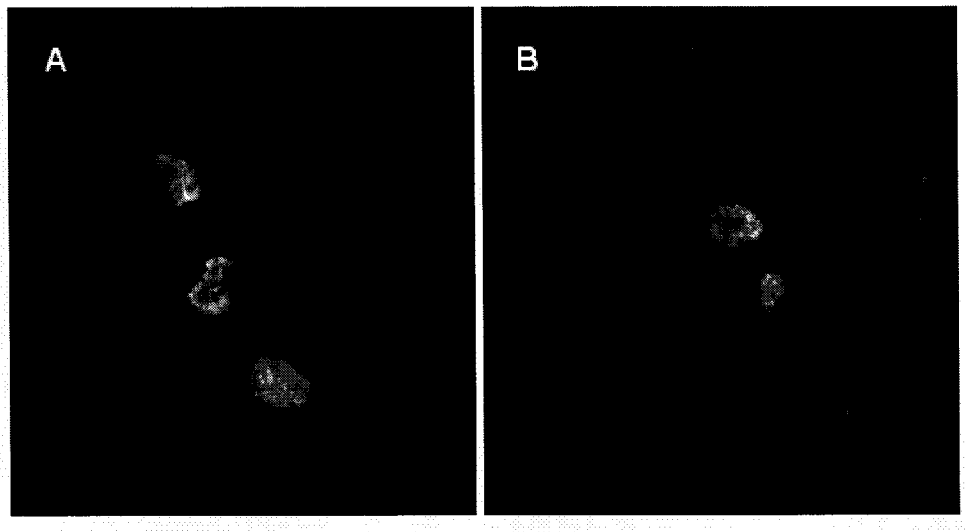


Figure 3: Mean (± SEM) number of BrdU-positive cells per 20μm coronal section in the early (bregma -1.22 to -1.82) DG following intra-peritoneal injection of either saline, IL-1β (0.1 μg), IL-6 (1.0 μg), or TNF-α (1.0 μg), or exposure to a neurogenic stressor [tube restraint (10 minutes) & foot shock (0.3mA shocks administered at 30 second intervals for ten minutes)] or psychosocial stressor (placed in large divided cage with 3 conspecifics for 24 hours until sacrifice). As can be seen, acute systemic injection of TNF-α significantly reduced BrdU labelling in the DG and exposure to a neurogenic stressor caused a modest but yet significant reduction in BrdU staining. $*p < .05$ vs. saline-treated animals.

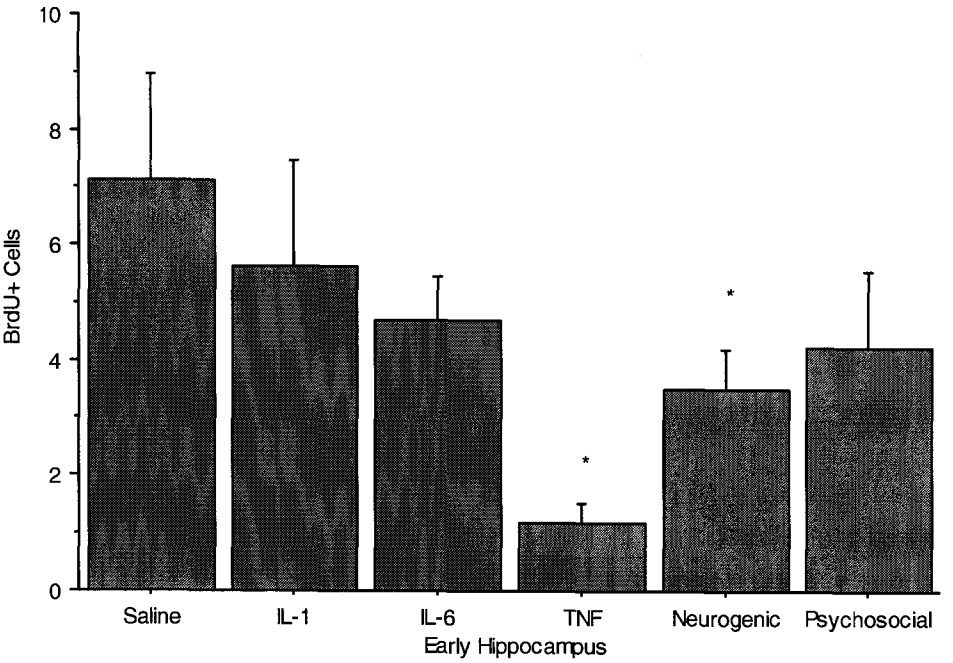


Figure 4: Mean (± SEM) number of BrdU-positive cells per 20μm coronal section in the middle (bregma -1.82 to -2.46) portion of the DG following intra-peritoneal injection of either saline, IL-1β (0.1 μg), IL-6 (1.0 μg), or TNF-α (1.0 μg), exposure to the neurogenic stressor [tube restraint (10 minutes) & foot shock (0.3mA shocks administered at 30 second intervals for ten minutes)] or psychosocial stressor (placed in large divided cage with 3 conspecifics for 24 hours until sacrifice).

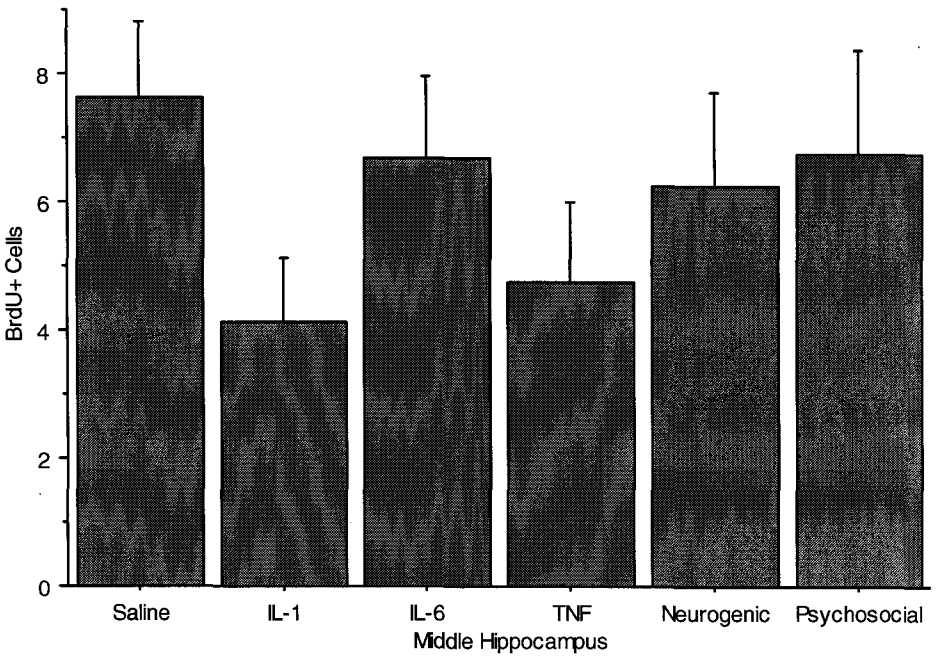


Figure 5: Mean (± SEM) number of BrdU-positive cells per 20μm coronal section in the late or most caudal portion of the DG (bregma -2.46 to -2.92) following intra-peritoneal injection of either saline, IL-1β (0.1 μg), IL-6 (1.0 μg), or TNF-α (1.0 μg), exposure to neurogenic stressor [tube restraint (10 minutes) & foot shock (0.3mA shocks administered at 30 second intervals for ten minutes)] or psychosocial stressor (placed in large divided cage with 3 conspecifics for 24 hours until sacrifice).

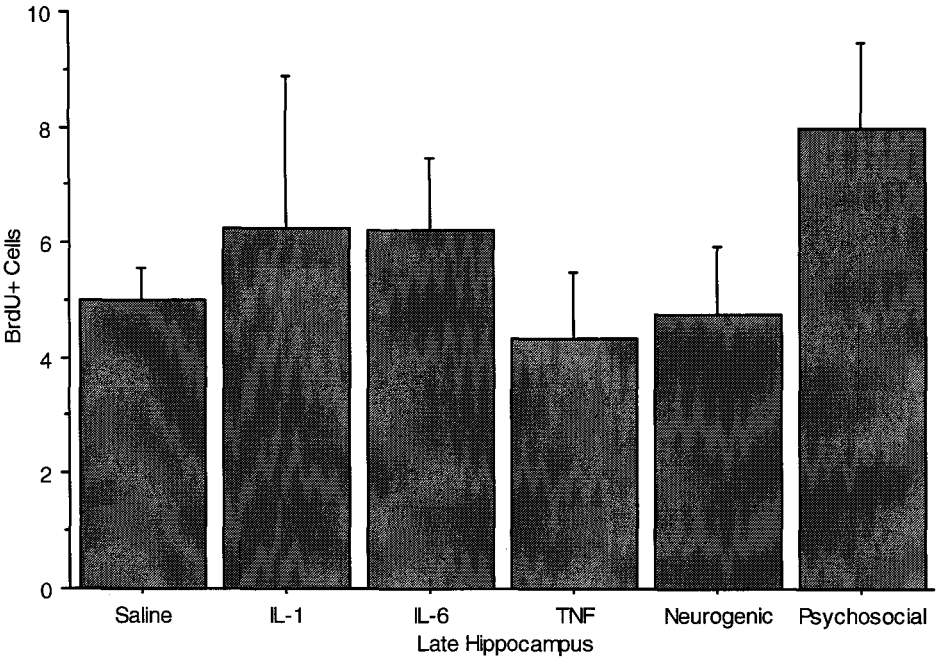
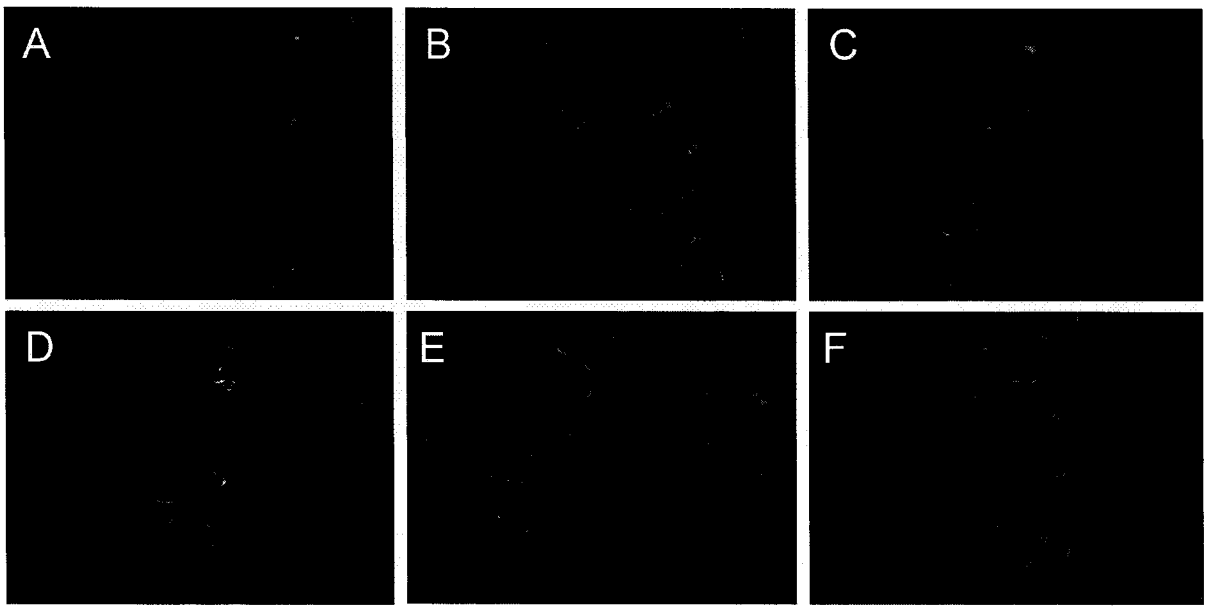


Figure 6: DCX-positive cells in the early DG (bregma -1.22 to -1.82) of animals subjected to (A) saline i.p. injection, (B) neurogenic stressor exposure [tube restraint (10 minutes) & foot shock (0.3mA shocks administered at 30 second intervals for ten minutes)], (C) psychosocial stressor exposure (placed in large divided cage with 3 conspecifics for 24 hours until sacrifice), (D) TNF-α (1.0 μg, i.p.), (E) IL-1β (0.1 μg, i.p.) or (F) IL-6 (1.0 μg, i.p.); Fluorescent microscopy; 20X magnification. As can be seen from panels C and D, there appears to be a reduction in DCX staining in animals subjected to psychosocial stressor challenge andor TNF-α injection.



Experiment 2

cd11b: As can be seen in Figure 7, the cd11b staining revealed that cannula placement was directly above the hippocampus. Very intense cd11b staining was observed around the cannula tract, however, a modest elevation of cd11b labelling was also evident within the corpus callosum and CA1 region of the hippocampus, directly above the granule cell layer of the dentate gyrus. These photomicrographs (Figure 7) also confirm that there was no physical damage to the hippocampus itself following cannulation.

BrdU staining: A three-way repeated measures analysis of variance (intra-hippocampal cytokine infusion X chronicity X hippocampal level) failed to reveal a significant interaction between these treatments with respect to BrdU cell counts. Similarly, the main effect of hippocampal level failed to reach significance ($F(2, 38) = 2.308$, $p = .1064$); hence, the data were collapsed across the three levels of the hippocampus. Thereafter, a two-way analysis of variance revealed a significant interaction between intra-hippocampal cytokine infusion and chronicity of administration $F(3, 38) = 4.466$, $p = .0088$. The follow up post hoc Tukey tests revealed that chronic, but not acute, intra-hippocampal IL-6 infusion significantly increased the hippocampal BrdU labelling, relative to saline infused mice ($p < .05$). Indeed, as shown in Figures 8 and 9, although acute IL-6 infusion did not influence BrdU staining, there was a dramatic rise in the proliferative marker associated with chronic infusion of the cytokine. Importantly, BrdU levels were identical in animals that received either chronic or acute intra-hippocampal saline infusion, suggesting that repeated liquid infusion alone did not affect cellular proliferation. In addition to IL-6, chronic, but not acute, IL-1β infusion

provoked a more modest but yet significant elevation of BrdU staining, relative to saline treated controls (~40 % rise; $p < .05$). Surprisingly, intra-hippocampal infusion of TNF-α failed to influence BrdU labelling, suggesting that the BrdU reduction observed in mice that received i.p. TNF-α in Experiment 1 may stem from actions of the cytokine at peripheral sites. As in Experiment 1, the BrdU-positive cells were located mainly in small clusters in the subgranular zone and the hilus regions of the dentate gyrus (Figure 9) and were oval-shaped with a stippled pattern, however, in this experiment, robust BrdU labelling was also observed in the corpus callosum, and around the injection tract. This could possibly reflect glial proliferation given that neural injury has been shown to increase proliferation of microglia and astrocytes (Rola et al., 2006). Interestingly, as can be seen in Figure 9, BrdU staining in animals chronically infused with IL-1β and IL-6 was also present around the DG. These BrdU-positive cells had the same oval shape and stippled pattern as the ones described earlier.

DCX staining: A three-way repeated measures analysis of variance (intra-hippocampal cytokine infusion X chronicity X hippocampal level) of DCX-positive cell counts showed no significant interaction or main effect for cytokine infusion or chronicity of administration. However, a significant main effect was observed for hippocampal level ($F(2, 31) = 15.394$, $p < .0001$). As depicted in Figure 10, the number of DCX-labelled cells was lower in the early part of the hippocampus compared to the middle and later levels of this brain region.

Figure 7: Photomicrographs of cd11b labelling in animals subjected to intra-hippocampal infusions of (A) saline (acute), (B) saline (chronic), (C) IL-6 (acute), or (D) IL-6 (chronic). Animals were cannulated directly above the hippocampus (placement coordinates in respect to bregma: A/P = -1.82mm, D/V = -1.25mm, M/L = ± 1.00mm). Cd11b immunoreactivity was evident in the corpus callosum and CA1 regions directly above the granule cell layer (GCL) of the dentate gyrus. Importantly, the hippocampus was not physically injured by cannulation. Fluorescent microscopy; 4X magnification.

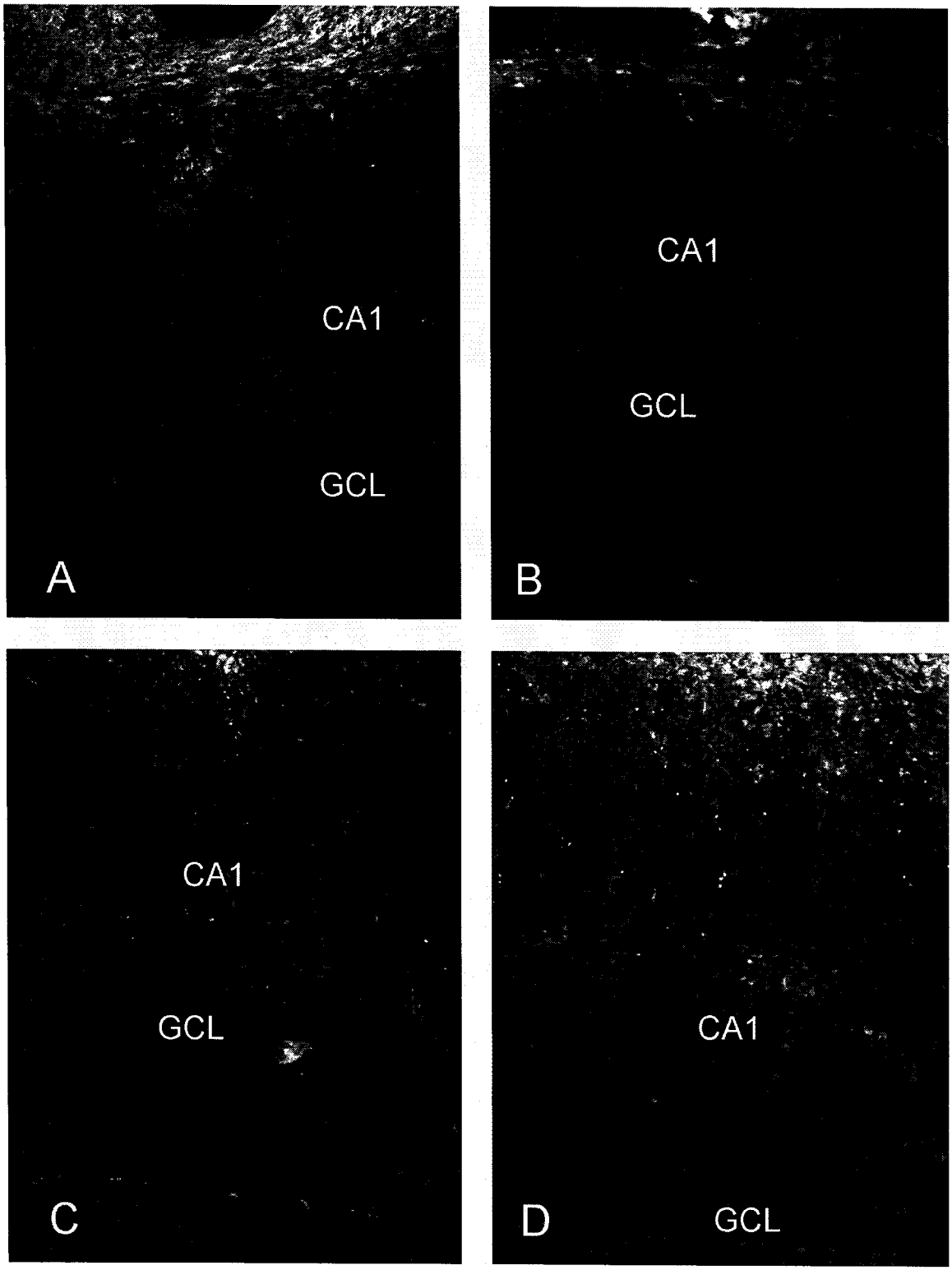


Figure 8: Mean (± SEM) number of BrdU-positive cells per 20μm coronal section of the DG following acute or chronic intra-hippocampal infusion of saline, IL-1β (0.01μg), IL-6 (0.05μg) or TNF-α (0.05μg). Chronic (grey bars) but not acute (white bars) hippocampal infusion with IL-1β and IL-6 significantly increased BrdU staining in the DG relative to animals that received infusion of saline. $*p < .05$ vs. saline-treated animals.

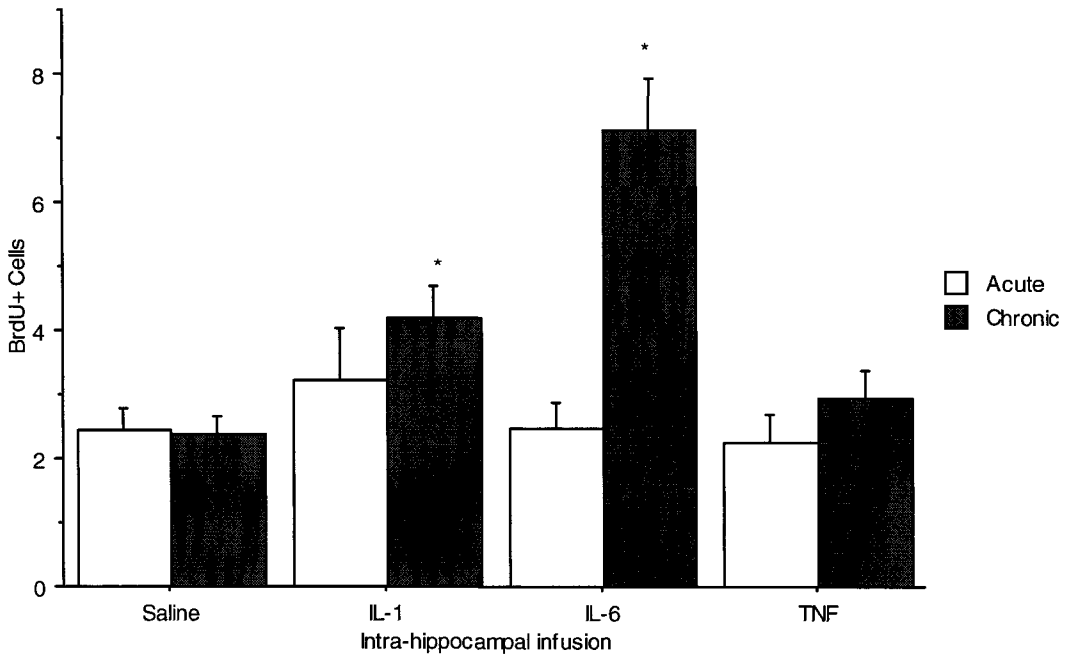


Figure 9: Photomicrographs of BrdU-positive cells within the DG following intra-hippocampal cytokine infusion (Fluorescent microscopy; 10X magnification). Consistent with our quantifications, elevated BrdU staining can be seen in the DG of animals subjected to the chronic IL-1β and IL-6 infusions. Furthermore, although the majority of BrdU cells were found within the granule cell layer (GCL) of the DG, BrdU labelling is also visible outside of the GCL of animals chronically infused with IL-1β and IL-6.

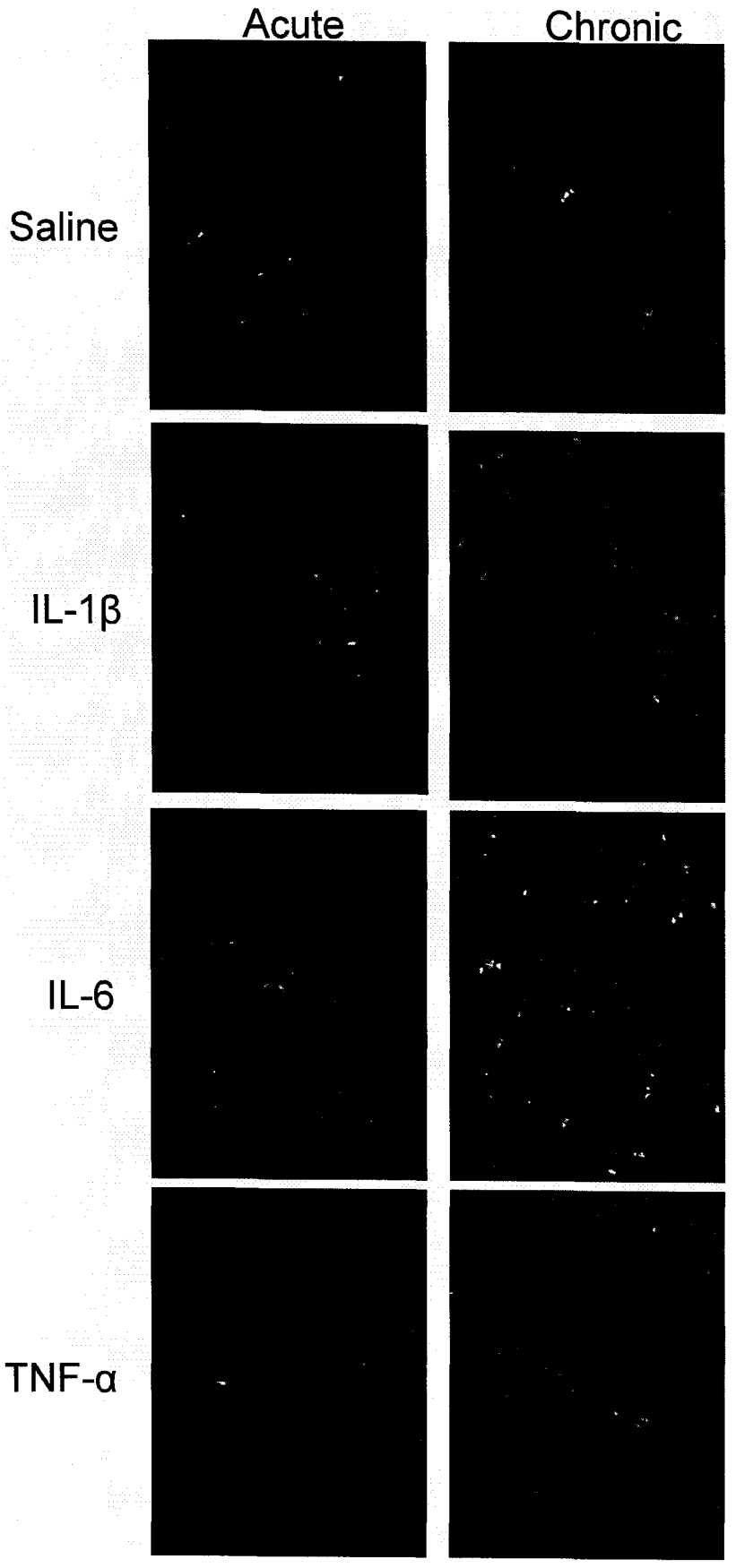
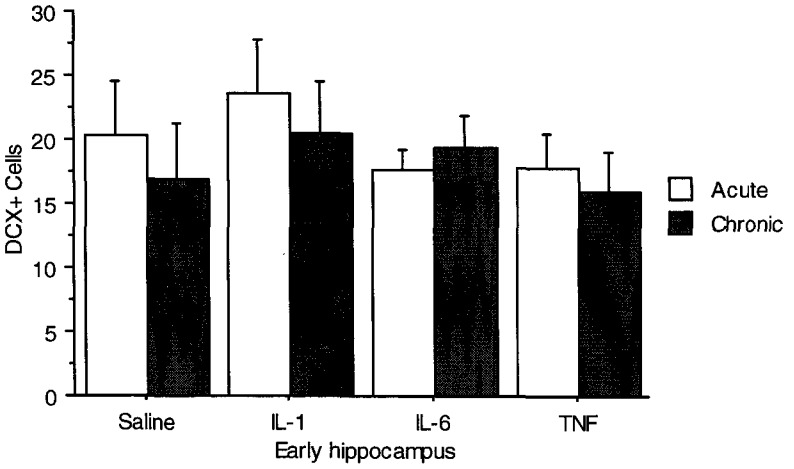


Figure 10: Mean (± SEM) number of DCX-positive cells per 20μm coronal section of the DG of mice that received acute or chronic intra-hippocampal infusion of either saline, IL-1β (0.01μg), IL-6 (0.05μg) or TNF-α (0.05μg). Significantly fewer DCX-labelled cells were identified in the early portion of the DG than in the late and middle areas yet there were no effects of treatment within each level. These treatments did not significantly affect DG DCX labelling at any level of the hippocampus.



Discussion

Acute and chronic stressful challenges are known to elicit neurochemical and behavioural changes that are believed to be reminiscent of those observed in depressed individuals (Anisman & Merali, 1999; McEwen, 2000a). Moreover, it has recently been demonstrated that these same stressors reduced levels of cell proliferation and more specifically, neurogenesis, in the dentate gyrus (DG) region of the hippocampus of rodents (Czeh et al., 2001; Gould et al., 1998; Kim & Diamond, 2002; McEwen, 1999; McEwen, 2000a; Tanapat et al., 1998; Tanapat, Hastings, Rydel, Galea, & Gould, 2001). We and others have posited that the reduction of hippocampal volume that has recently been reported in chronic depression may stem from disturbances of cellular proliferation, as well as other morphological abnormalities, such as reduced dendritic branching (D'Sa & Duman, 2002; Hayley & Anisman, 2005; Jacobs et al., 2000; Jacobs, 2002; Kempermann, 2002). In any case, it seems that impairment of hippocampal neurogenesis may have important functional implications for depression given recent evidence indicating that the efficacy of antidepressants was linked to the state of hippocampal neurogenesis (Santarelli et al., 2003). Yet, surprisingly little is known of the potential mechanisms through which stressors may impact upon hippocampal cellular proliferation. Although glucocorticoids are undoubtedly important in this respect, inflammatory factors may also be important given the accumulating evidence suggesting a role for proinflammatory cytokines in stressor-related pathology. In fact, IL-1β, IL-6 and TNF-α were all reported to provoke depressive-like pathology in rodents and their levels are altered in clinically depressed patients (Anisman et al., 2002; Bluthé et al., 1997; Hayley et al., 1999; Hayley & Anisman, 2005). Accordingly, we assessed the

impact of central and systemic administration of these cytokines upon hippocampal cell proliferation.

Stressor-induced impaired neurogenesis: Implications for depression

Animal models of depression have revealed that rodents exposed to a chronic stressor regimen or corticosterone treatment display impaired hippocampal neurogenesis (Coyle & Duman, 2003). Importantly, this impairment was reversed by chronic antidepressant treatment or by a single session of electroconvulsive shock, providing predictive validity for the view that neurogenesis may be relevant to depression (Jacobs, 2002). Similarly, more recent reports indicate that stressor-provoked learned helplessness (which has been taken to model depressive illness) was associated with a reduction of hippocampal cellular proliferation and this effect was preventable by treatment with the antidepressants fluoxetine or desiprimine (Chen, Pandey, & Dwivedi, 2006). However, antidepressant administration alone in naïve mice increased hippocampal cellular proliferation (Lee et al., 2001; Malberg et al., 2000; Santarelli et al., 2003), suggesting a pro-mitotic effect was associated with the drug irrespective of any depressive-like pathology.

The fact that more controversial methods of alleviating depressive symptoms, including increased exercise and temporary sleep deprivation, have also been shown to stimulate hippocampal neurogenesis (Ernst, Olson, Pinel, Lam, & Christie, 2006) provides support for the notion that generally any treatments which positively affect neurochemical processes linked to affective state generally impact on neurogenesis. However, it is also possible that newly born hippocampal cells are exquisitely sensitive to

a range of challenges that happen to impinge upon affective processes but that these hippocampal changes are of secondary importance in depressive pathology. Yet, Santarelli et al., (2003) recently demonstrated that selective irradiation of the hippocampus (thereby disrupting neurogenesis) prevented the antidepressant behavioural actions of fluoxetine, suggesting that neurogenesis may play a primary role in mediating the functional outcome of antidepressant treatment.

In the present study, exposure to a combination of restraint and footshock applied in the 24 hours before sacrifice reduced hippocampal BrdU labelling. In contrast, the social stressor of placing mice in a novel cage in proximity to four new conspecifics during this time caused a modest but non-significant reduction of BrdU. Interestingly, the stressor-induced BrdU reduction was only evident in the earliest portion of the hippocampal DG, suggesting an enhanced vulnerability of certain subtypes of DG cells. Hence, the particular anatomical level of the hippocampus had a profound effect upon the impact of these challenges on cellular proliferation. This is an important point to bear in mind given that the majority of published studies have simply reported changes in overall BrdU staining within the entire hippocampus.

Since BrdU incorporates into the dividing DNA of both neurons and glia, it is possible that the reduction of cellular proliferation observed may reflect a disturbance of neurogenesis or gliogenesis or some combination therein. However, as shown in Figures 1 and 2, the location and morphological characteristics of the majority of BrdU-stained cells (e.g. size and location within the subgranular zone of the DG) suggest that they are indeed dividing neuroblasts that will eventually migrate to the granule cell layer (GCL) and begin to express a neuronal phenotype (Christie & Cameron, 2006). Labelling with

the neuron-specific marker, doublecortin (DCX), indicated a diminution of DCX immunoreactivity in mice that were exposed to either of the disturbed housing or footshock + restraint treatment (see Figure 6), suggesting that a genuine reduction of neurogenesis was provoked by the stressors. The fact that the psychosocial stressor provoked a more robust reduction of DCX labelling whereas the neurogenic stressor more potently affected BrdU incorporation suggests that these different types of stressors may be influencing different cellular populations. Indeed, as will be described in the ensuing sections, DCX and BrdU label populations of neurons that may have been born at very different times, such that cells at the earliest stages of proliferation that have incorporated BrdU may not yet express DCX. Moreover, it may also be the case that certain BrdU-positive cells were non-neuronal in origin.

The severity of the stressors likely had an important influence upon whether of not neurogenesis was disturbed. In this respect Anisman et al. (2001) demonstrated that neurogenic (e.g. footshock and restraint), psychogenic (e.g. predator odour) and systemic (e.g. cytokine administration) stressors differentially affect levels of plasma corticosterone. Thus, it is possible that elevated corticosterone levels may have contributed to the present stressor-provoked BrdU reduction, particularly as ample evidence indicates that corticoids are potent inhibitors of neurogenesis (Bremner, 2006; Kanagawa et al., 2006; Mirescu & Gould, 2006; Wong & Herbert, 2006). Indeed, exposure to an acute stressor with ethological relevance, fox odor trimethyl thiazoline (TMT), was shown to inhibit cell proliferation in the hippocampus of adult rats and this effect was dependent on a stress-induced rise of corticosterone (Tanapat et al., 2001). Accordingly, prevention of this rise in corticosterone by removal of adrenal glands

(adrenalectomy) and low corticosterone replacement completely eliminated the suppression of cell proliferation (Tanapat et al., 2001). In contrast, Thomas, Urban, & Peterson (2006) reported that acute exposure to TMT elevated coticosterone levels and impaired exploratory behaviour of rats without having any immediate effects on hippocampal cellular proliferation. While the stressor exposure protocol was remarkably similar for both experiments, Tanapat et al. (2001) administered a single, saturating dose of BrdU (200 mg/kg) while Thomas et al. administered 50 mg/kg, a dose shown to only label 45% of dividing cells (Cameron & McKay, 2001). In this regard, unless the stressor treatment reduced cell proliferation by more than 55%, it is unlikely that Thomas et al. would detect any reduction in cell proliferation.

Several studies have demonstrated that non-corticoid mechanisms can also impact upon hippocampal proliferative processes. For instance, chronic electroconvulsive shock increased neurogenesis, even in the presence of elevated corticoid levels (Hellsten et al., 2002). It was similarly reported that the antidepressant fluoxetine normalized hippocampal neurogenesis in animals exposed to inescapable electric shock, independent of any actions upon corticosterone (Malberg & Duman, 2003). Given that inhibition of CRH or AVP receptors prevented the reduction of neurogenesis associated with a chronic stressor (Alonso et al., 2004), it is likely that these neuropeptides are important in the hippocampal changes evident in affective illness, just as they have been implicated in many of the other neuronal deficiencies apparent in the disorder. Other studies indicate that thyroid hormones also have the ability to regulate neurogenesis and that hypothyroidism may elicit mood disorders by impairing neurogenesis (Montero-Pedrazuela et al., 2006). Taken together, these data suggest that although corticoids

appear to be one of the most potent mechanisms of stressor induced alterations of neurogenesis, alternate processes may be induced in a stressor specific manner involving a host of peptides, growth factors and/or inflammatory messengers.

Cytokines affect neurogenesis: Implications for depression

It may be that cytokines influence behavioural processes important for depression through their impact upon neurogenesis. In fact, it was shown that IFN-α, when administered chronically (in a schedule mimicking an immunotherapeutic protocol) elicited a marked reduction of hippocampal neurogenesis (Kaneko et al., 2006). Interestingly, the impact of IFN-α upon neurogenesis was ameliorated by central treatment with the cytokine antagonist, IL-1Ra, suggesting that endogenous IL-β was the important factor inhibiting neurogenesis (Kaneko et al., 2006). Furthermore, attenuation of the inflammatory response using indomethacin normalized the reduction of neurogenesis associated with endotoxin treatment, as well as that provoked by irradiation (Monje et al., 2003). Yet, this does not necessarily imply a causal relation between the inflammatory cytokine induced reduction of neurogenesis and depressive pathology. However, it is significant that chronic stressor treatment was associated with impaired neurogenesis, whereas antidepressant treatment had the opposite effect (Duman, Nakagawa, & Malberg, 2001; Duman & Monteggia, 2006). As well, it was observed that the positive behavioural effects elicited by antidepressant administration were prevented by irradiation induced impairment of hippocampal neurogenesis (Santarelli et al., 2003). Thus, there is reason to believe that disturbances of neurogenesis may have important functional implications for depression and that inflammatory cytokines may be involved

in regulating such neuroplastic changes.

Systemic cytokine administration and hippocampal cellular proliferation: In the present investigation, we report a reduction of cell proliferation within the early portion of the hippocampal DG following systemic administration of TNF-α, and a modest but non-significant reduction associated with IL-6 treatment. In contrast, intra-hippocampal TNF-α infusion did not significantly affect hippocampal cellular proliferation, thus, the peripheral rather than central actions of TNF-α appear to be most important in altering the birth of new DG cells. Given the previously mentioned effects of glucocorticoids upon cell proliferation, these findings are in line with evidence demonstrating that systemic administration of TNF-α induced marked elevations of circulating corticosterone, yet central infusion of the cytokine had only modest effects upon the hormone (Hayley et al., 1999; Hayley et al., 2001; Hayley, Merali, & Anisman, 2002; Hayley, Merali, & Anisman, 2003; Webster et al., 1998). The fact that TNF-α was much more potent than IL-1β in reducing cellular proliferation was somewhat surprising given that IL-1β typically has much more profound actions upon HPA, central monoamine and behavioural processes than TNF-α (Brebner et al., 2000; Mefford, Masters, Heyes, & Eskay, 1991; Zalcman et al., 1994). Yet, TNF-α has a more primary role than IL-1β in a number of inflammatory immune processes, including acute phase reactions and T and NK cell cytotoxicity (Cerami, 1992).

In this regard, TNF-α may have influenced hippocampal cellular proliferation by activating apoptotic cascades (Varfolomeev & Ashkenazi, 2004). Indeed, TNF-α can trigger apoptosis through caspase-8 and this effect is mediated by TNF receptors 1 and 2

(TNFR1 and TNFR2; Varfolomeev & Ashkenazi, 2004) which were recently found to have contrasting actions upon neurogenesis. TNFR1-deficient mice showed enhanced hippocampal neurogenesis under both basal conditions and following status epilepticus, whereas TNFR2-deficient mice displayed a reduction of neurogenesis to the epileptic insult, suggesting divergent roles for these cytokine receptors (Iosif et al., 2006). Importantly, both receptors were found to be expressed on the progenitor cells themselves (Iosif et al., 2006) and both were upregulated by injury (Fontaine et al., 2002). Given that apoptotic effects generally do not target cells until they are a few days old (Dayer, Ford, Cleaver, Yassaee, & Cameron, 2003) TNF-α-induced apoptosis is more likely to underlie the observed reduction in DCX labelling than the BrdU reduction.

Central cytokine infusion and hippocampal cellular proliferation: Surprisingly, chronic intra-hippocampal infusion of IL-6 and to a lesser degree IL-1β actually increased levels of BrdU-positive cells in the DG, suggesting possible pro-mitotic effects of these cytokines. It is unclear as to why direct intra-hippocampal cytokine infusion had consequences so remarkably different from that observed with systemic administration. It may be that sufficiently high concentrations of the cytokines did not reach the hippocampus following systemic administration. Moreover, the fact that chronic but not acute intra-hippocampal IL-1β and IL-6 infusion markedly elevated BrdU staining indicates that the passage of time following introduction of the cytokines may be the critical aspect responsible for affecting hippocampal cellular proliferation or that a single infusion alone was simply not a potent enough challenge. The fact that chronic infusion of saline alone had no affect upon cellular proliferation relative to mice that received

acute exposure to the vehicle, argues against a possible influence of the repeated mechanical or psychological distress associated with the procedure.

Our findings demonstrating that chronic intra-hippocampal IL-1β increased cell proliferation are in clear conflict with findings of Kaneko et al. (2006), which reported that endogenous IL-1β levels may have suppressive effects upon cell proliferation. However, methodological differences may explain this divergence. Indeed, Kaneko et al. chose a low, non-saturating dose of BrdU and administered four injections (one every six hours) before sacrifice, in contrast to the single saturating dose used in the present study. It may be that Kaneko et al. failed to detect any initial elevations of cell proliferation immediately following cytokine administration. Furthermore, their findings of central IL-1β-induced suppression in cell proliferation may be attributable to cell cohorts labelled by their later BrdU injections. However, perhaps the most important difference between these studies is the fact that the present investigation involved direct infusion of IL-1β into the brain, whereas that of Kaneko et al. utilized central infusion of the IL-1β antagonist, IL-1Ra, to block the impact of peripheral interferon injection on hippocampal neurogenesis. Thus, differences associated with altering endogenous levels of IL-1β compared to exogenous application of supra-physiological levels of the cytokine may account for the divergent findings. Overall, the possibility should be considered that biphasic changes in the proliferation of distinct cell cohorts may occur following central IL-1β infusion.

Consistent with our IL-6-induced changes in cell proliferation, numerous studies illustrate that IL-6 can exert both beneficial and destructive effects on cells of the CNS by impacting upon their survival, proliferation and differentiation (Gadient & Otten, 1997;

Hirota, Kiyama, Kishimoto, & Taga, 1996; Loddick, Turnbull, & Rothwell, 1998; Marz et al., 1998). These divergent actions appear to be cell-specific and dependent upon the particular experimental preparation. In fact, (Vallières et al., 2002) reported a suppression of hippocampal neurogenesis in adult transgenic mice overexpressing IL-6. Yet, IL-6 was also shown to promote both astrogliogenesis and oligodendrogenesis (Monje et al., 2003). This raises the possibility that the elevated BrdU staining we observed following chronic IL-6 infusion may actually reflect augmented gliogenesis rather than neurogenesis. In line with this proposition, our failure to detect any changes in doublecortin staining for these same animals argues against any change in the recent birth of new neurons. Yet, as will be discussed in the ensuing section, BrdU and doublecortin can label entirely different populations of neurons, depending upon timing of their birth.

Identifying Cell phenotype: BrdU labelling

Several studies have established that under basal conditions, the majority (nearly 95%) of all BrdU-labelled cells in the granule cell layer of the DG progress on to express neuronal phenotype (Brown et al., 2003; Cameron & McKay, 2001). However, accumulating data indicates that psychological stressors, as well neuronal injury, can reduce the number of immature DG cells that progress on to become mature neurons (Hayley et al., 2005; Rola et al., 2006). In contrast, these same insults have been associated with an elevation in the number of hippocampal cells that progress on to display a glial phenotype (Parent, von dem Bussche, & Lowenstein, 2006; Pforte, Henrich-Noack, Baldauf, & Reymann, 2005; Rola, et al., 2006). Indeed, enhanced gliogenesis occurs in numerous brain regions following a host of insults, such as stroke,

neurodegenerative diseases and toxins (Mazurova, Latr, Osterreicher, & Cerman, 2004; Pforte et al., 2005), presumably to limit the extent of neuronal damage. Accordingly, it is highly probable that the cytokine and stressor treatments used in the present investigation had marked effects upon gliogenesis, as well as neurogenesis. Indeed, it is important to underscore that BrdU (as a thymidine analogue) is incorporated in all dividing DNA, hence, both neurons and glial cells may be labelled by this S-phase marker.

The location of BrdU-positive cells does provide an important clue as to the phenotype of these cells. It has been long held that neurogenesis is restricted to the hippocampus (DG), around the ventricles (SVZ) and olfactory bulbs, whereas gliogenesis can occur through the brain parenchyma. Accordingly, new cells generated outside of these neurogenic areas are believed to be glial cells. However, it is notable that a few recent reports suggest the presence of neurogenesis within the substantia nigra (Zhao et al., 2003). The present study documented robust BrdU labelling within the DG, however, as shown in Figure 9, staining in animals that received chronic IL-1β and IL-6 was also present outside of the DG. Indeed, in chronic IL-1β and IL-6 infused animals, BrdU labelling was observed within the corpus callosum and around the injection tract at the site of infusion, suggesting that chronic infusion of these cytokines likely promoted gliogenesis. To firmly establish phenotype, double-labelling immunohistochemical procedures are often used to assess whether new cells are expressing either neuronal (e.g. DCX) or glial [e.g. glial fibrillary acidic protein (GFAP)] markers but, as will be discussed, recent evidence warns of potential conflicting results when using glial markers to double-label young BrdU-positive cells.

BrdU provides a snap-shot of proliferative activity happening during or shortly after its administration. Following a saturating i.p. injection as used in the present investigation, BrdU will label all proliferating cells of the S-phase for approximately 2 hours (Hayes & Nowakowski, 2000; Packard, Menzies, & Skalko, 1973). After BrdU incorporation into DNA, some daughter cells of dividing precursors undergo re-division while others become post-mitotic (Dayer et al., 2003) but no new cells become labelled. Hence, depending on the length of time between BrdU injection and animal sacrifice, the niche of BrdU-labelled cells in the DG can consist of neural stem cells, immature neurons, mature neurons as well as glial cells (Cameron et al., 1993). Accordingly, important considerations must be acknowledged when choosing the lapse of time between BrdU injection and animal sacrifice.

With each cell cycle, the amount of BrdU incorporated into daughter cells is further diluted to the extent that BrdU-labelling may only be visible in cells having undergone a maximum of four divisions since initial incorporation (Dayer et al., 2003). For this reason, Dayer and colleagues (2003) described that, in rats, the birth of a BrdU-positive cell can only be determined to have occurred within a 4 day range (rats undergo 1 cell cycle/day) and by extension given that mice have a 13h cell cycle, the age of a mouse BrdU-positive cell can only be specified to have occurred within a two-day range. For survival times greater than four cell cycles, dilution of BrdU may render some previously labelled cells invisible and thus levels of BrdU labelling might underestimate cellular proliferation (Dayer et al., 2003). Moreover, by allowing for longer survival times, newly generated cell have the chance to undergo programmed cell death, further adding to the underestimation of BrdU labelling (Dayer et al., 2003) and in such cases, BrdU levels

may be better indicators of cell survival rather than cell proliferation. Given that the current study assessed BrdU incorporation 24 hours following cytokine or stressor challenge, any BrdU-positive cells would have been no older than two cell cycles and thus, no cells generated during or following treatment become invisible due to BrdU dilution.

Despite their many advantages, short BrdU labelling times require special considerations when assessing cell phenotype since newborn cells can transiently express conflicting phenotypic markers. Indeed, it was recently established that GFAP, a marker most often used to identify astrocytes, can also label neural progenitor cells (Garcia, Doan, Imura, Bush, & Sofroniew, 2004). In fact, the majority of neural progenitors in the adult forebrain were reported to transiently express GFAP under basal conditions (Garcia et al., 2004). Similarly, S100β, a marker of astrocytes and oligodendrocytes, was also shown to label some immature neurons (Hachem et al., 2005; Vives, Alonso, Solal, Joubert, & Legraverend, 2003). Thus, S100B and GFAP can be used as specific glial markers of post-mitotic cells but would not be useful for this purpose in cells at early differentiation stages. Accordingly, in the present investigation it was not possible to use these markers to definitively determine if some of our early labelled BrdU cells might be destined to become glia.

In addition to issues related to how the timing of BrdU administration may influence proliferative labelling, it is important to consider other factors that may affect BrdU incorporation. Firstly, there have been suggestions that BrdU is incorporated into cells undergoing DNA repair, thereby amplifying the amount of BrdU labelling beyond what is due to proliferative activity (Rakic, 2002a; Rakic, 2002b). However, because

DNA repair in the normal brain usually involves only 3-100 nucleotides per lesion, while the entire genome (containing approximately 2.5 billion nucleotides) is synthesized during the S-phase, it is unlikely that cells having incorporated BrdU strictly through DNA repair would be detectable through immunohistochemical processes. To quell concerns of increased BrdU labelling in the presence of elevated amounts of DNA repair, studies compared the location and amount of BrdU labelling under regular and irregular pathological conditions (Christie & Cameron, 2006). As a result, BrdU immunostaining was shown to be specific to dividing cells even in the presence of elevated amounts of DNA repair due to irradiation (Palmer, Willhoite, & Gage, 2000; Parent, 2002) and this specificity remains even when BrdU was given at high doses (Cameron & McKay, 2001; Eadie, Redila, & Christie, 2005).

Secondly, concerns have been raised about BrdU's accessibility to the brain. BrdU is transported across the blood-brain barrier (BBB) by the same active and facilitative nucleoside transport system as thymidine, the nucleoside it replaces (Lynch, Cass, & Paterson, 1977; Spector & Berlinger, 1982; Spector & Huntoon, 1984). Because proinflammatory cytokines such as IL-1β and TNF-α have been reported to disrupt the integrity of BBB (Fiala et al., 1997; Martiney, Litwak, Berman, Arezzo, & Brosnan, 1990), more BrdU might be able to access the brain of cytokine-treated animals, thereby increasing the number of cell having access to BrdU. Yet, as shown by Eadie et al., (2005), the use of a high, saturating dose of BrdU, as in the present investigation, ensures that all dividing cells will be labelled to such an extent that any enhanced penetration of BrdU through a leaky BBB will have no opportunity to bind (Cameron & McKay, 2001).

Identifying Cell phenotype: DCX labelling

Recent studies have begun to identify proliferating neurons by means of the microtubule-associated protein, doublecortin (DCX), which is transiently expressed only in cells committed to a neuronal phenotype (Couillard-Despres, Winkler, Uyanik, & Aigner, 2001; des Portes et al., 1998; Gleeson et al., 1998). Although first used to double-label BrdU-positive cells, this marker of immature neurons is now recognized as a reliable, stand-alone measure of ongoing neurogenesis (Brown et al., 2003). Cells begin expressing DCX while in the neuroblast stage and continue to express the microtubule associated protein until the appearance of the mature neuronal marker NeuN. This occurs at about two weeks, after which time DCX levels quickly decline (Brown et al., 2003; Cooper-Kuhn & Kuhn, 2002; Rao & Shetty, 2004). Brown et al. (2003) established that DCX can be expressed on BrdU-positive cells very rapidly (within hours) after injection of the exogenous thymidine analog. Although it has been observed that two hours following BrdU administration, 60% of labelled cells also expressed DCX, co-labelling only reached its maximum between the fourth and seventh days following BrdU administration, when >90% of all BrdU-positive cells expressed DCX (Brown et al., 2003). Accordingly, BrdU and DCX labelling in the present study represent cells of slightly different ages: BrdU-positive cells are no older than 24h and DCX-positive neurons range anywhere between two hours and one month of age. Based on this disparity we can assess the temporal effect of cytokine and stressor treatments by comparing their impact upon BrdU and DCX staining. For example, systemic TNF-α reduced BrdU labelling in the DG and appeared to also reduce DCX staining, suggesting that this treatment impacted upon the generation of young neurons. In the case of the

neurogenic stressor challenge, where we saw a reduction in BrdU labelling but no change in DG DCX staining, the treatment likely targeted young dividing cells that had yet to differentiate. Finally, in the case of chronic intra-hippocampal administration of IL-1β and IL-6, the augmented BrdU labelling observed in the absence of changes in DCX labelling indicates that these treatments promoted cell proliferation without specifically influencing neurogenesis. In this regard, it is likely that the BrdU-positive cells are glia.

Conclusions and future directions:

In summary, our findings suggest that central and systemic proinflammatory cytokines influence DG cellular proliferation and that these effects were dependent upon chronicity as well as route of administration. Furthermore, as evidenced by differences in BrdU and DCX staining, central and systemic cytokines appeared to target different cellular populations. Indeed, systemic but not central TNF-α reduced both BrdU and DCX labelling, supporting the notion that this cytokine reduced neurogenesis by influencing peripheral targets. Conversely, chronic central infusion of IL-1β and IL-6 increased BrdU but not DCX, suggesting that repeatedly elevated levels of these cytokines promoted an increased proliferation of non-neuronal cells, likely of glial origin.

Our ongoing analyses are aimed at determining the exact phenotype of the proliferating BrdU positive cells that were affected by the stressor and cytokine treatments. To this end, we are refining our double-labelling procedure, such that it will be assessed if BrdU was co-localized with neuron (DCX) or glial (Cd11b) specific markers. As well, it is of interest to determine if inhibiting specific cytokines may attenuate the stressor-induced BrdU alterations. To this end, since systemic TNF-α had

such marked inhibitory effects upon BrdU staining, applying soluble TNF-α neutralizing antibodies may antagonize the anti-proliferative actions of the stressors. Similarly, TNF-α deficient knockout mice would be expected to display altered BrdU staining patterns in response to stressful treatments if endogenous levels of the cytokine influence hippocampal cellular proliferation. The fact that chronic intra-hippocampal IL-6 had such a potent stimulatory effect upon BrdU labelling, suggests that this cytokine may have growth factor-like properties. Indeed, this is in keeping with previous reports that IL-6 can impact upon the survival, proliferation and differentiation of DG cells (Gadient & Otten, 1997; Hirota et al., 1996; Loddick et al., 1998; Marz et al., 1998). Accordingly, it would be worthwhile to evaluate whether IL-6 may act in a protective capacity in animals subjected to stressful or traumatic insults. We are presently testing this hypothesis in mice exposed to the dopaminergic toxin paraquat. Thus, the present findings may have implications for a range of conditions in which cytokine variations are expected and these may involve disturbances in neuroplasticity.

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