The role of neuroinflammation in regulating the age-related decline in neurogenesis

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The Role of Neuroinflammation in Regulating the Age-Related Decline in Neurogenesis

by

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ABSTRACT

Adult neurogenesis, is a lifelong process by which relatively few cells are added into two restricted regions of the brain. Integration of the cells into the existing neuronal circulatory, with the unique properties involved in the maturation of these cells, is possibly critical to the acquisition and retrieval of new memories. With the chronological aging of the organism a process of cellular senescence occurs throughout the body; a portion of which is independent of primary alterations to the stem cells; instead, it appears to be dependent on the environment where the cells reside, and is in part regulated by inflammation. Microglia, the resident immune cells in the brain, are neuroprotective but chronic activation of the microglia, such as the chronic activation that occurs with advanced age, can promote neurotoxic inflammation. However, it not clear if the aged-related increase in neuroinflammation is at least partly responsible for the aged related decrease in neurogenesis. To address the involvement in neuroinflammation in regulating neurogenesis we used 3 different potential therapeutically relevant manipulations. The first was a targeted approach directed at disrupting the synthesis of Interleukin-1β (IL-1β), which is a proinflammatory cytokine that is consistently found elevated in the aged brain. The second was a cell therapy approach in which human umbilical cord blood cells were injected into the systemic circulation. The final approach was directed at a chemokine system, fractalkine/CX3CR1, which has been shown as an important paracrine signal, from neurons that regulates the activation state of microglia. While the three approaches used to manipulate, aging-rodent model system were different, a consistent finding was reached in all three studies. In the aged brain, microglia which are the predominate produces of IL-1β, negatively regulate neurogenesis. When IL-1β is decreased or microglia activation is decreased, neurogenesis can be partially restored in the aged brain. The results of these studies, demonstrate a key role for microglia in regulating the neurogenic niche, which are amendable to therapeutic manipulations.
Introduction

Aging is the backdrop in which many neurodegenerative disease occur, including Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). As our population ages, there is a pressing need to develop therapeutic interventions for age-related neurodegenerative diseases. Not only is this true for the patient, but also for the sake of those who will care for those suffering from a neurodegenerative disease.

Alterations in neural plasticity are associated with aging; however, recent studies have demonstrated that the age-related alterations in neural plasticity are due to regional synaptic alterations and not neuron loss (for review see: (Burke and Barnes, 2006)). One aspect of neural plasticity is adult neurogenesis. Adult neurogenesis is the term used for a process, first described in 1965, which results in the addition of new neurons in the adult brain (Altman and Das, 1965a). The addition of new neurons is complementary to synaptogenesis, and adds another form of neural plasticity. Neurogenesis is one of the possible mechanisms that could be utilized, as a potential ‘reservoir’ of neural plasticity, to increase the quality of life of our aging population. However, it is an important to consider when designing therapeutic strategies for our aging population, with and without neurodegenerative diseases, that alterations occur to neural plasticity as a result of age, and therefore the response to a therapeutic may be less efficacious in the elderly.

Adult neurogenesis has been found in humans as old as 72 years of age (Eriksson et al., 1998). However, considerably less is known about adult neurogenesis in humans compare to rodents. In rodents, adult neurogenesis has now been extensively studied, and it is now well accepted that neurogenesis occurs in at least two germinal centers in the brain, the subventricular zone (SVZ) and the subgranlar zone (SGZ) of the hippocampal dentate gyrus (DG) (for review see: (Ming and Song, 2005; Zhao et al., 2008)).

The process of Adult Neurogenesis

There are five phases of hippocampal neurogenesis: (1) The first phase is proliferation of neural stem/progenitor cells (NPCs) which occurs in a region called SGZ, which is defined as a two cell diameter band occurring on the hilus side of the granule cell layer (GCL). (2) The second phase is the survival of the proliferating NPCs. During this phase the number of surviving neurons can vary greatly depending on the strain of animals used and can be as great as ~ 75% or a few as 25% of the amount of proliferating cells (Kempermann et al., 1997a). (3) The third phase, occurring in concert with the second phase, is the differentiation of the newly born cells. In this phase the majority of cells do become neurons, with a smaller percentage becoming astrocytes.
and oligodendrocytes. (4) The fourth phase involves migration of the neurons into the GCL; with most of the migration occurring around the first week (Kempermann et al., 2003a). (5) Finally, the fifth phase involves the functional maturation of the neurons in the granule cell layer which occurs around four weeks of age, but some cells may take weeks or even months longer to fully mature (van Praag et al., 2002). It has also been demonstrated that those adult born neurons that survive after 4 weeks will likely be present at least 11 months later (Kempermann et al., 2003a). The majority of the decrease in neurogenesis with age appears to occur mostly in the first phase through a decrease in proliferation (Drapeau and Nora Abrous, 2008). Survival of the newly born cells appears to be unaffected by age. Maturation of the new born cells, particularly in developing a mature neuronal phenotype and migrating into the GCL, does seem to be affected by age (Drapeau and Nora Abrous, 2008).

Non-Cell-Autonomous Changes Regulating Neurogenesis

In many pathological conditions, including AD and PD, neurogenesis is dramatically affected by the pathology (Hoglinger et al., 2004; Verret et al., 2007; Zhang et al., 2007; Nuber et al., 2008). Neurogenesis is also significantly decreased with age (Kuhn et al., 1996; Kempermann et al., 2002; Kronenberg et al., 2006a). The majority of the suppression of neurogenesis with age appears to be a function of the microenvironment(niche) and is not NPC autonomous (reviewed in: Zhao et al., 2008). Moreover, the pool of NPCs appears to be intact with respect to the total numbers of available NPCs (Hattiangady and Shetty, 2008), providing more evidence that that the neurogenic niche is at least partly responsible for the decrease in neurogenesis with age.

Cellular senescence has been known to occur with age since the 1960s (Hayflick and Moorhead, 1961), but the importance of the cellular senescence within the aged stem cell niche has only recently become an area of active interest. A clear example of the importance of the extrinsic or systemic influences on the stem cell niche was demonstrated in the stem cells that are found in the muscle, called satellite cells. Like the neural stem cells, the satellite cells in the muscle lose the potential to regenerate damaged tissue with age. In an elegant experiment, when aged rats were exposed to the systemic environment of a young rat by parabiosis the satellite cells were rejuvenated in the aged rats as demonstrated by an increase in the proliferation rate. Conversely, in young rats the exposure to the circulation of the aged rats caused a decrease in the regenerative potential of the satellite cells (Conboy et al., 2005), again supportive of an extrinsic/circulating factor that is influencing the proliferation of the stem cells in the aged animals. It is not clear whether the mechanism involved in the effect in the muscle would hold true in the brain, but the implication is that the aged environment is detrimental to stem cell function. This also holds true for even the most pluripotent of stem cells: the embryonic stem cells. When embryonic stem cells are transplanted into aged tissue they are not able to repair damaged tissue as well as when transplanted into young tissue (Carlson and Conboy, 2007).
Many changes occur to the aged organisms which are known to negatively impact neurogenesis (for review see (Drapeau and Nora Abrous, 2008)). However, there is little known about the role of neuroinflammation in regulating the age-related decline in neurogenesis. Neuroinflammation is known to negatively impact neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). It is known that neuroinflammation is elevated in aging and AD (Akiyama et al., 2000; Krabbe et al., 2004). The following chapters will begin to clarify the role of neuroinflammation in the age-related decline in neurogenesis.

**Inflammation in Aging and Disease**

Inflammation is an active process with the purpose of removing or inactivating potentially damaging agents or damaged tissue. The inflammatory response is typified acutely by pain, heat, redness, swelling, and loss of function. Following removal of the ‘danger signal’, a second pathway is initiated with the role of tissue remodeling. In the central nervous system (CNS) the inflammatory process must be well controlled. Since the majority of the CNS lacks the potential to replace lost cells, an inflammatory response could be devastating resulting in neural tissue loss. In general, inflammation is a beneficial with the primary result of removing the noxious agent and remodeling the adjacent tissue. When inflammation is not well regulated following response to ‘danger signals’ a chronic pathology will result. It has been clearly shown that in the CNS there is a marked increase in inflammatory activity associated with aging (Bodles and Barger, 2004; Joseph et al., 2005; Mrak and Griffin, 2005). It is less clear what causes this inflammatory state.

**Cytokines**

Cytokines are low molecular weight proteins, with diverse biological activity due to the various target cells and multiple response. Levels of specific cytokines expressed in the brain increase as a function of age, even in the absence of a pathologic stimulus. For example, there is a progressive increase in the expression of interleukin (IL)-1 and microglia activation with aging in patients without a neurological disease (Roubenoff et al., 1998; Wilson et al., 2002), but to a lesser extend then in patients with AD (Griffin et al., 1989). IL-6 levels also increase in the mouse brain with advancing age (Godbout and Johnson, 2004). In the cerebellum of aged rats, tumor necrosis factor-α (TNF-α) gene expression is dramatically increased compared to young rats (Gemma et al., 2002). Immune response-related molecules and their receptors are expressed throughout the brain, and recent research suggests that brain-derived immune factors disrupt normal physiology and contribute to cognitive and behavioral dysfunction in neurologic disease (Lynch, 1998; Pugh et al., 1999; Rachal Pugh et al., 2001; Barrientos et al., 2002).

**IL-1β**

IL-1β is one of the main inflammatory cytokines found in the CNS involved in neuroinflammation (Shaftel et al., 2008). IL-1β is constitutively expressed in the brain, synthesized by neuronal and/or glial cells, and released in response to a variety of stimuli, including immune system activation (Benveniste, 1992; Rothwell et al., 1997) and in AD (Mrak
IL-1β is a proinflammatory cytokine initially synthesized as an inactive precursor that is cleaved by caspase-1 to generate the biologically mature 17-kDa form. In rodents the enzymatic activity of caspase-1 is increased with normal aging (Gemma et al., 2005), but mRNA expression is not altered (Sheng et al., 2001).

Virtually every cell type is affected by binding of IL-1β to the high affinity receptor, IL-1RI (Rothwell et al., 1997; Dinarello, 1998). The IL-1β receptor expression is high in the hippocampus as indicated by binding studies (Farrar et al., 1987; Takao et al., 1990). The IL-1 family comprises three known ligands: IL-1α, IL-1β and IL-1ra. The biologic activity of IL-1β is dependent on its interaction with IL-1RI and recruitment of the IL-1 receptor accessory protein (IL-1Racp) (Sims, 2002). IL-1ra binds to IL-1RI but fails to associate with IL-1Racp, thereby acting as a highly selective competitive receptor inhibitor. The only known function of IL-1ra is to antagonize the biologic activity of IL-1.

IL-1β is a potent suppressor of neurogenesis but it is not completely clear how IL-1β achieves this effect. IL-1β has been shown in vitro to be able to induce apoptosis in NPCs via phosphorylation of the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) pathway (Wang et al., 2007), thereby having a direct effect on the survival of the new born neurons. The NF-κB/IKK pathway also seems to be critical in regulating the effects of IL-1β on proliferation, possibly by decreasing Cyclin D1 expression (Koo and Duman, 2008). IL-1β, in NPCs cultures also seemed to slightly favor differentiation into an astrocyte lineage as determined by the marker GFAP (Wang et al., 2007).

IL-1β is not alone in being able to directly affect NPC. Recently, Iosif et al. (2006) has shown that the inflammatory cytokine TNF-α can also assert a direct effect on NPC proliferation. TNF receptor 1 (TNF-R1) appears to have a regulatory function by blocking proliferation during inflammation. This action occurs directly at the progenitor cells which express both TNF-R1 and TNF-R2. TNF-R2 appears to play a neuroprotective role; although, its function is a little less clear than that of TNF-R1 (Iosif et al., 2006).

Chemokines

Chemokines are small molecule (8-14 kDa) proteins that are classically defined as chemotactic cytokines initially studied because of their role in leukocyte trafficking. Chemokines are classified into 4 subfamilies – C; CC; CXC and CX3C – by the position of the conserved cysteine residues near the NH2 terminus region of the protein (Bacon et al., 2002). The receptors, by which chemokines exert their effects, are of the seven-transmembrane-domain G-protein-coupled receptor (GPCR) family. Chemokine GPCRs belongs to the class A rhodopsin-like family, with the majority of the receptors being Gαi as indicated by the ability of pertussis toxin (PTX) to inhibit the response of the receptors (Murphy, 1996). The extracellular NH2 terminus and the three extracellular loops act to bind the chemokine ligand. The three intracellular loops and C-terminus act in signal transduction (Mellado et al., 2001).
Fractalkine/CX3CR1

In contrast to many other chemokines, fractalkine (FKN; CX3CL1; neurotactin) binds and activates a single receptor, CX3CR1. Interactions between FKN and CX3CR1 contribute to maintaining microglia in a resting phase, and partially controlling microglia induced neurotoxicity (Cardona et al., 2006). FKN acts in vitro as an anti-inflammatory molecule by down-regulating IL-1β, TNF-α, and IL-6 production (Zujovic et al., 2000; Zujovic et al., 2001). FKN can therefore, act as a bridge between neurons and microglia, to regulate cytokine production and neuroinflammation.

FKN is the only known member of the CX3C chemokine family (Bacon et al., 2002). FKN is unique among chemokines; with cell surface presentation of the N-terminal chemokine domain by a glycolated mucin-like stalk, that is anchored to membrane with a transmembrane and C-terminal cytoplasmic domain (Bazan et al., 1997; Pan et al., 1997). The chemokine domain can be shed from the mucin stalk by a constitutive expressed metalloproteinase 10 (ADAM10) (Hundhausen et al., 2003), or by an inducible cleavage by TNF-a converting enzyme (TACE / ADAM17) (Garton et al., 2001). FKN is not expressed on peripheral blood leukocytes (Bazan et al., 1997; Pan et al., 1997). FKN is expressed on endothelial cells (Bazan et al., 1997), epithelial cells (Lucas et al., 2001), smooth muscle cells (Ludwig et al., 2002), dendritic cells (Papadopoulos et al., 1999) and neurons (Pan et al., 1997; Harrison et al., 1998). FKN is most robustly expressed in the CNS (Harrison et al., 1998). In isolated primary cultures neurons RT-PCR showed FKN mRNA to be highest in neurons; however, low levels were detected in microglia and astrocytes (Nishiyori et al., 1998).

In the CNS, FKN is predominantly expressed on neurons and is one of only two chemokines that is constitutively expressed by neurons, the other being stromal cell-derived factor-1 (SDF-1; CXCL12). SDF-1 is monomeric like FKN (Crump et al., 1997; Mizoue et al., 1999). Recently it has been shown that SDF-1 has an important role in directly regulating adult neurogenesis (Bhattacharyya et al., 2008; Kolodziej et al., 2008). It is not currently know if FKN might have a similar role in regulating neurogenesis.

FKN also has a higher affinity for its receptor (CX3CR1) than other chemokine receptor ligand pairs (Haskell et al., 2001). The exclusive receptor for FKN is a PTX sensitive GPCR, named CX3CR1. Predominate expression of CX3CR1 in the CNS is found on microglia, which suggest a paracrine regulation of microglia by neurons (Harrison et al., 1998). However, expression of the receptor, is not sufficient to determine activity following ligation. In CD14+ monocytes less then 2% of cells that express CX3CR1 migrated to soluble FKN (Imai et al., 1997). Moreover, of all the CX3CR1+ cells only 10% of the cells became activated after exposure to soluble FKN (Imai et al., 1997). The adhesion properties of FKN appear to be independent of signaling via the GPCR as PTX was no effect on adhesion (Imai et al., 1997).

The expression of CX3CR1 includes a subset of dendritic cells and natural killer cells,
peripheral blood monocytes and tissue macrophages including microglia (Jung et al., 2000). In the CNS, CX3CR1 expression *in vivo* is limited to immune cells, namely microglia and not astrocytes or neurons (Cardona et al., 2006; Combadiere et al., 2007). However, CX3CR1 expression has been shown in cultured neurons. Moreover, *in vitro*, FKN via CX3CR1 was able to reduce excitotoxic cell death induced by glutamate and neurotoxicity induced by gp120	extsubscript{IIIB}+, an HIV envelope glycoprotein (Meucci et al., 2000; Tong et al., 2000; Limatola et al., 2005).

Recent evidence has shown a role for CX3CR1 polymorphisms in the pathogenesis of age-related macular degeneration (Tuo et al., 2004; Combadiere et al., 2007). There are at least two common coding polymorphisms, V249I and T280M, in the CX3CR1 gene (Faure et al., 2000; McDermott et al., 2001; Moatti et al., 2001), as well as, a single nucleotide polymorphism in the promoter region of CX3CR1 (DeVries et al., 2003). The two SNPs CX3CR1 with a strong linkage disequilibrium include: (1) A SNP at codon 249 that causes a switch from valine to isoleucine (V249I). The other SNP results in a switch from threonine to methionine at codon 280 (T280M). The M280/I249 variant caused reduced function of the receptor, including decreased adhesion, signaling and chemotaxis (McDermott et al., 2003). The M280/I249 variant is also associated with reduced risk for atherosclerosis. Moreover, in aged CX3CR1	extsuperscript{−/−} but not in aged CX3CR1	extsuperscript{+/-} or young CX3CR1	extsuperscript{+/-} there was an age-related accumulation of microglia that led to retinal neurodegeneration in the CX3CR1	extsuperscript{−/−} (Combadiere et al., 2007).

FKN/CX3CR1 signaling has been shown to be neuroprotective in a number of brain injury models (Cardona et al., 2006). The neuroprotective role of CX3CR1 has recently been questioned in a model of focal cerebral ischemia where CX3CR1	extsuperscript{−/−} mice had a worse outcome following the transient middle cerebral artery occlusion (MCAO) whereas, FKN	extsuperscript{−/−} mice had improved outcome following MCAO (Soriano et al., 2002; Denes et al., 2008). The results of this study may not disprove the role of CX3CR1 in neuroprotection, but instead provide more supporting evidence of the diverse role of microglia and macrophages. Following a short dramatic neuronal insult, such as an MCAO, a more robust early immune response may be protective to the insult. This is in comparison to a prolonged neurodegenerative condition such as AD or PD. Moreover, as the blood brain barrier (BBB) is broken by the MCAO a different complement of immune cells may be recruited to the injury compared to a neurodegenerative condition with an intact BBB.

**Microglia**

Microglia are always surveying the microenvironment, and once they sense the appropriate signals such as neuronal damage the cells will home to the site of damage (Davalos et al., 2005; Nimmerjahn et al., 2005). As the resident innate immune cells in the CNS, microglia constitutively express surface receptors that trigger or amplify the innate immune response, including toll-like receptors, complement receptors, cytokine receptors, chemokine receptors, major histocompatibility complex (MHC) II, and others (Aloisi, 2001). The main cell type in the
CNS that is responsible for immunity is the microglia. Lipopolysaccharides (LPS) is an endotoxin that is part of the outer membrane of gram-negative bacteria. LPS up-regulates the production of IL-1β by microglia (Lee et al., 1993). Cytokines such as IL-1β, and TNF-α can also act in an autocrine or paracrine manner to increase the production of IL-1β by microglia (Lee et al., 1993). Astrocytes can also be stimulated by IL-1β, but not by LPS, to produce TNF-α and IL-6. However, the production of cytokines by astrocytes is less than that of microglia (Lee et al., 1993). Therefore, while astrocytes and neurons can make inflammatory mediators, the microglia are the main source of inflammatory cytokines (Aloisi, 2001).

However, the role of microglia is not destructive. Upon detection of homeostatic disturbance, microglia rapidly respond by inducing a protective immune response. The protective immune response begins with a transient upregulation of inflammatory molecules, including proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 and IL-12 (Gao et al., 2002; Mantovani et al., 2004). This is followed by a protective phase that is immunomodulatory and neuroprotective. The protective phase includes neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and insulin-like growth factor 1 (IGF-1) (Miwa et al., 1997; Batchelor et al., 1999; Nakajima and Kohsaka, 2004). Thus, microglia remove cells damaged from acute injury and protect CNS functions.

Microglia are neither pro-neurogenic or anti-neurogenic but their influence on neurogenesis is dependent on their activation state of either ‘classically’ activated or ‘alternatively’ activated (Battista et al., 2006; Butovsky et al., 2006b) (figure 1). Much like what has been proposed for peripheral macrophages, microglia are very pleiotropic. Microglia, can become ‘classically’ activated as defined by the release of pro-inflammatory cytokines (e.g. TNF-α and IL-1β) (Giulian et al., 1986; Griffin et al., 1989). Once in this ‘classically’ activated, pro-inflammatory state, microglia are associated with further production of these pro-inflammatory cytokines, ROS, chemokines, and matrix metalloproteases, resulting in cell death of invading cells and tissue destruction, and type-I inflammation (Mantovani et al., 2007). A second type of microglia are those that are activated by such things as IL-4 and TGFβ, and are called ‘alternatively’ activated or in an ‘M2’ state (following the TH1/TH2 classification of T-helper cells) (Mantovani et al., 2007). Compare to the ‘M1’ or ‘classically’ activated state, microglia in the ‘M2’ state can be protective (Mantovani et al., 2004). When microglia are in this ‘M2’ state there is little release of pro-inflammatory cytokines and they are resistant to activation by agents such as LPS (Mantovani et al., 2007). Alternatively activated macrophages promote extracellular matrix formation and angiogenesis (Mantovani et al., 2007). A third type of microglia is one that becomes dysfunctional with age. The senescent microglia are not able to respond appropriately to stimuli and may exacerbate neuroinflammation (Schwartz et al., 2006a; Streit, 2006a). Isolated microglia from aged mice have elevated basal expression of TNF-α, IL-1β, IL-6, and TGFβ1 (Sierra et al., 2007). When stimulated the mice were stimulated with LPS, the isolated microglia from aged mice
produced more of the proinflammatory cytokines TNF-α, IL-1β, IL-6, then the young LPS stimulated mice, but the LPS fold change in cytokine production in aged mice was equal to that in young mice. Therefore, microglia from aged animals can respond to an inflammatory challenge the same as young mice, but the baseline of cytokine expression is elevated (Sierra et al., 2007).

Age Related Alterations in Adaptive Immunity

There is also a decrease in immunological function in the periphery (Miller, 1996; Goronzy and Weyand, 2005). It appears that the age of the T-cell when it first encounters an antigen will determine if a memory response is achieved. For example, T-cells from aged rats were able to maintain memory function acquired when they were young, but were not able to acquire new memory formation; this was most likely due to a lack of proliferative ability (Haynes et al., 2003). Furthermore, aged naive CD4 T-cells could be stimulated by exogenous IL-2 to induce memory, but once the IL-2 was removed the memory was lost (Haynes et al., 2003). Even as most T-cell function declines with age, the CD4⁺CD25⁻ T regulatory (Treg) cells are still able to suppress the activity of young CD4⁺CD25⁻ T helper (Teff/Th) (Nishioka et al., 2006). Even more important, the CD4⁺CD25⁻ T-cells not only become unresponsive with age they also become suppressive limiting the immune response (Shimizu and Morizumi, 2003). The CNS does have a reservoir to drain antigen in the cervical lymph nodes (Cserr and Knopf, 1992). The recruitment of leukocytes can also take place in postcapillary venules (Hickey, 2001). It has been shown that there are changes to structure and function of the blood-brain-barrier (BBB) as a result of age, which causes an increase in permeability (Mooradian, 1988, 1994; Morita et al., 2005). Normally, in a young adult in the absence of disease, there is a clear demarcation between the peripheral immune system, innate and adaptive, and the CNS immune system with its 'lone sentinel,' the microglia.

Following an acute stimulation such as a traumatic injury or ischemia, the protective status of the CNS is revoked and peripheral immune cells (dendritic cells and T-cells) can enter into the CNS. The consequences of the invading peripheral immune cells are to either, clear debris and rebuild damaged tissue or exacerbate the injury. T-cells can be involved in governing the immune response (Kim et al., 2007). T-cell response may act by altering the activity of the microglia and this response is important to maintain neurogenesis (Kipnis et al., 2004; Butovsky et al., 2006a; Butovsky et al., 2006b; Schwartz et al., 2006a)

Injury is not the only time that this immune privilege is revoked, in aging you can also find invasion of T-cells and dendritic cells (Stichel and Luebbert, 2007). Occurring as early as 12 months of age in rats, dendritic cells and T-cells can be found in the brain. These peripheral immune cells, which are absent in young adult rats, are found widely distributed throughout the aged rat brain, and are particularly associated with white matter tracks (Stichel and Luebbert, ; Bulloch et al., 2008). What causes this invasion of peripheral immune cells is not entirely known.
The increase of microglia presenting antigen, as shown by expression of major histocompatibility complex Class II (MHC II), as well as, an increase in the peripheral immune systems professional antigen presenting cells (dendritic cells) in the aged brain, may be a useless response as the T-cells don’t appear able to respond to the antigen. The presentation of antigen to T-cells by APCs is futile, as the T-cells do not appear able to respond to the antigen. The lack of an immune response appears to lead to tolerance of T-cells and continued activation of APC, and this futile response could be producing a positive feedback loop of chronic inflammation.

**Human Umbilical Cord Blood Cells as a Potential Therapeutic**

The mononuclear fraction of human umbilical cord blood (HUCB) contains a number of cell types including, B-Cells, and T-Cells, monocytes, as well as, mesenchymal and endothelial progenitor cells. HUCB is also a source of CD34⁺ hematopoietic stem cells (Bender et al., 1991; Ho et al., 1996; Wu et al., 1999). HUCB cells, particularly the CD34⁺ stem cells, have in animal models of stroke provided multiple benefits adding in the function recovery after the infarct (Chen et al., 2001; Saporta et al., 2003; Willing et al., 2003; Taguchi et al., 2004; Vendrame et al., 2004; Vendrame et al., 2005; Vendrame et al., 2006b) While HUCB CD34⁺ stem cell have the potential to differentiate into neurons *in vitro*, when these cells are transplanted into the brain few survive. Previously immunohistochemical analysis directed towards a human nuclear antigen (HuNu) has found few HuNu positive cells surviving after the transplant and the small number of surviving HUCB cells in the brain rules out a direct replacement of the lost cells as a mechanism for the beneficial effects of HUCB cells (Chen et al., 2001; Vendrame et al., 2004). Taguchi et al. (2004) showed that some of the functional recovery after stroke provided by CD34⁺ cells was due to enhancement of both neurogenesis and angiogenesis (Taguchi et al., 2004). The article by Taguchi et al. (2004) is concerned with the neurogenesis occurring post-stroke in regions of the brain considered non-neurogenic (Taguchi et al., 2004).

The effects of HUCB seem to be due to a regulation of the microenvironment of the brain, through releasing tropic factors or reducing inflammation and not by direct replacement of cells. It was recently demonstrated that a systemic injection of HUCB cells could suppress inflammation in the brain inflammatory response after stroke. Moreover, the effects of HUCB cells seemed to shift the cytokine expression from a Th1 response to a Th2 response (Vendrame et al., 2004; Vendrame et al., 2005; Vendrame et al., 2006b). Besides the immune modulatory effects, HUCB cells also produce a number of trophic factors including but not limited to, vascular endothelial growth factor, nerve growth factor, and cytokine colony stimulating factor-1, thrombopoietin, and IL-11 (Suen et al., 1994; Taguchi et al., 2004; Vendrame et al., 2004).

**Conclusion**

Neurogenesis is an important means of neural plasticity that is diminished with increasing age. Neurogenesis from endogenous NPC may provide an alternative to transplantation of stem cells as a means to replace damaged neural tissue after brain injury, such as stroke, or as a
result of a neurodegenerative condition. A growing body of research shows that neurogenesis may occur in ‘non-neurogenic’ regions as a result of a stroke or neurodegenerative disease (Kokaia and Lindvall, 2003). There is also the hope of recruiting neural stem cells, from the neurogenic regions of the brain to replace damaged cells after injury (Bernal and Peterson, 2004). Before either strategy of using neurogenesis as a therapeutic source of new cell can be implemented, a better understanding of the regulation of neurogenesis is necessary.

During aging and exacerbated in AD, a state of chronic inflammation occurs (Bodles and Barger, 2004; Joseph et al., 2005; Mrak and Griffin, 2005). Inflammation, while not the only cause for the decrease in neurogenesis with age, is very important in regulating neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). To harness the potential of neurogenesis to help in brain repair a better understanding of the mechanism of how inflammation is regulating neurogenesis is necessary. While a loss of trophic factors and increased corticosteroids are important contributors to the aged niche and have been extensively reviewed (Drapeau and Nora Abrous, 2008) the focus of following chapters of this dissertation are limited to the role of inflammation in regulating stem cell function in the aged neurogenic niche.
Fig. 1. Microglia: protective or harmful? Microglia are normally in a resting state in which they are actively surveying the microenvironment of the brain. The microglia are resting in the sense that they are not performing effector functions such as producing inflammatory mediators like IL-1β and TNFα. When microglia are producing inflammatory mediators the microglia would be considered in a ‘classically activated state or ‘TH1’ state. Microglia can also become 'alternatively activated' in such a way that they produce growth factors, such as IGF-1 and TGFβ. The ‘alternatively activated’ microglia can support tissue remodeling and repair. Beyond releasing signaling molecules, microglia also have an important role in phagocytosis. The role of microglia, as protective or harmful, depends upon the ability of the microglia to switch from the different activation states at the appropriate time. Understanding how and when to turn microglia ‘on’ or ‘off’ is an important future direction of research. This is especially the case with aging where microglia are most need for remodel and repair and to remove damaged cells and misfolded proteins. With age microglia may lose the ability to perform these important effector functions making the aged brain more susceptible to injury and insult.
Blockade of Caspase-1 Increases Neurogenesis in the Aged Hippocampus

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Abstract

Adult hippocampal neurogenesis dramatically decreases with increasing age, and it has been proposed that this decline contributes to age-related memory deficits. Central inflammation contributes significantly to the decrease in neurogenesis associated with aging. Interleukin-1β is a proinflammatory cytokine initially synthesized as an inactive precursor that is cleaved by caspase-1 to generate the biologically active mature form. Whether IL-1β affects neurogenesis in the aged hippocampus is unknown. Here we analyzed cells positive for 5`-bromo-deoxy-uridine (BrdU, 50 mg/kg) in animals in which cleavage of IL-1β is inhibited by the caspase-1 inhibitor Ac-YVAD-CMK (10 pmol). Aged (22 mo) and young (4 mo) rats received Ac-YVAD-CMK for 28 d intracerebroventricularly through a brain infusion cannula connected to an osmotic minipump. Starting on day 14, animals received a daily injection of BrdU for 5 consecutive days. Unbiased stereology analyses performed 10 d after the last injection of BrdU revealed that the total number of newborn cells generated over a 5-d period was higher in young rats compared to aged rats. In addition, there was a 53% increase in the number of BrdU-labeled cells of the aged Ac-YVAD-CMK- treated rats compared to aged controls. Immunofluorescence studies were performed to identify the cellular phenotype of BrdU labeled cells. The increase in BrdU-positive cells was not due to a change in the proportion of cells expressing neuronal or glial phenotypes in the subgranular zone. These findings demonstrate that the intracerebroventricular administration of Ac-YVAD-CMK reverses the decrease in hippocampal neurogenesis associated with aging.

Introduction

Neurogenesis occurs throughout life, predominantly in the subgranular zone (SGZ) of the dentate gyrus in the hippocampal formation and in the subventricular zone (SVZ) (Altman and Das, 1965b; Cameron et al., 1993; Eriksson et al., 1998; Gage, 2002; Lie et al., 2004; Mackowiak et al., 2004; Christie and Cameron, 2006). Neural stem cells in the SGZ give rise to progenitor
cells that migrate into the granule cell layer and differentiate into neuronal or glial phenotypes. Newly generated hippocampal granule cells acquire the morphologic and biochemical properties of neurons, develop synapses on their cell bodies and dendrites, and extend axonal projections along mossy fibers into the hippocampal CA3 region (Hastings and Gould, 1999; Markakis and Gage, 1999; Carlen et al., 2002). These newly generated granule neurons are electrically active, fire action potentials, and receive synaptic input (Cameron and McKay, 2001; van Praag et al., 2002; Schmidt-Hieber et al., 2004; Overstreet-Wadiche et al., 2006; Overstreet-Wadiche and Westbrook, 2006) and survive for an extended period of time (Altman and Das, 1965b).

A multitude of factors have been shown to regulate the production of new neurons in the adult hippocampus. For example, the rate of neurogenesis decreases with increasing age (Seki and Arai, 1995; Kuhn et al., 1996; Lichtenwalner et al., 2001; Bizon and Gallagher, 2003). Several lines of evidence suggest a correlation between adult neurogenesis and learning, and it has been proposed that a decline in hippocampal neurogenesis contributes to a physiologic decline in brain function (Gould et al., 1999b; Ambrogini et al., 2000; Kempermann and Gage, 2002; Bizon and Gallagher, 2003; Drapeau et al., 2003; Leuner et al., 2004; Bruel-Jungerman et al., 2005). New and important insights as to the production of new neurons may affect hippocampal-dependent memory ability have been provided by behavioral experiments performed using the Morris water-maze task, which is a hippocampal-dependent memory task used to analyze spatial memory. The learning performance in the water-maze task is characterized by two consecutive phases: an early phase during which the learning performance reaches 80% and a late phase during which the learning performance decreases to 20% after which a stable baseline is reached. Importantly, it has recently been shown that spatial learning in both young and aged rats increases the survival of cells generated before the learning (Gould et al., 1999b) but decreases the survival of cells born during the early phase of learning (Lemaire et al., 2000; Shors et al., 2001; Dobrossy et al., 2003; Drapeau et al., 2007). Thus, it seems that the new born cells generated before training may be at the critical period of sensitivity to be rescued by learning. On the other hand, cells generated during the early phase of learning may be too young to be rescued.

In rats, numerous conditions that increase adult hippocampal neurogenesis are associated with an increase in learning performance (Luine et al., 1994; de Quervain et al., 1998; Bizon and Gallagher, 2003; Drapeau et al., 2003; Mirescu and Gould, 2006). For example, decreases in glucocorticoid levels, which are elevated in aged rats, increases neurogenesis (Gould et al., 1998) and spatial memory in the water-maze (Drapeau et al., 2003). Furthermore, injection of N-methyl-D-aspartate receptor antagonists into aged rats significantly improves memory deficits and increases neurogenesis (Cameron et al., 1995; Nacher et al., 2001; Nacher et al., 2003; Nacher and McEwen, 2006; Nacher et al., 2007). Finally, insulin-like growth factor, fibroblast- growth factor -2, and endothelial-growth factor decrease with aging, and administration
of these trophic factors increases neurogenesis in the hippocampus (Beck et al., 1995; O’Kusky et al., 2000; Cheng et al., 2001; Zaman and Shetty, 2002). Likewise, an enriched environment, exercise, and caloric restriction or dietary supplementation with blueberries improve age-related memory deficits and increase neurogenesis (Paylor et al., 1992; Kempermann et al., 1997b, 1998; van Praag et al., 1999; Kempermann et al., 2002; Casadesus et al., 2004; Olson et al., 2006). These observations suggest that the age-related decrease in neurogenesis is due to a global, age-related alteration in the microenvironment of the brain. Recent reports indicate that brain inflammation negatively influences adult hippocampal neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). Intracortical infusion of lipopolysaccharides (LPS), a potent activator of the inflammatory response, induces an 85% reduction in the number of new neurons in the dentate gyrus SGZ (Ekdahl et al., 2003; Monje et al., 2003). Systemic administration of minocycline, which inhibits microglial activation, restores LPS-induced decreases in neurogenesis. In addition, tumor necrosis factor \( \alpha \) (TNF\( \alpha \)) is involved in the regulation of adult hippocampal neurogenesis (Iosif et al., 2006). Interleukin-1\( \beta \) (IL-1\( \beta \)) is a proinflammatory cytokine involved in the pathogenesis of several acute and chronic neurodegenerative diseases. There is extensive evidence that IL-1\( \beta \) is constitutively expressed in the brain, synthesized by neuronal and/or glial cells, and released in response to a variety of stimuli, including immune system activation (Benveniste, 1992; Rothwell et al., 1997). High brain levels of IL-1\( \beta \) are also correlated with natural aging and the development of cognitive dysfunction (Rachal Pugh et al., 2001; Lynch, 2002; Yirmiya et al., 2002). For example, contextual fear conditioning, a hippocampal-dependent memory task, is impaired in aged rats, and administration of a nonsteroidal anti-inflammatory drug, sulindac, reversed the impairment and decreased IL-1\( \beta \) levels (Mesches et al., 2004).

Almost every cell type is affected by IL-1\( \beta \) acting through its high-affinity receptor, IL-1RI (Rothwell et al., 1997; Dinarello, 1998). Binding studies indicate that the hippocampus contains the highest density of IL-1RI binding sites (Farrar et al., 1987; Takao et al., 1990). Interleukin-1\( \beta \) is synthesized as an inactive precursor that is cleaved by the protease caspase-1 to generate the mature 17kDa form. Caspases are a family of proteases that have a critical role in apoptosis and inflammation and are also implicated in cognition. The caspase family includes inflammatory caspases (caspase-1, 4, 5, 11, and 12), initiator caspases (2, 8, 9, and 10), and executioner caspases (caspase-3, 6, and 7). Caspase-1 (IL-1\( \beta \) converting enzyme) is constitutively expressed in macrophages and microglia and is involved in the activation of both apoptosis and inflammation through the production of IL-1\( \beta \). Caspase-1 inhibition has shown extraordinary promise in multiple disease models. For example, in cerebral ischemia, caspase-1 is neuroprotective by reducing apoptosis and decreasing the production of proinflammatory cytokines (Rabuffetti et al., 2000). Recently, we demonstrated that caspase-1 inhibition significantly improves hippocampal-dependent contextual memory function by inhibiting IL-1\( \beta \),
TNFα, and caspase-3 activity in the hippocampus (Gould et al., 1999b). Here we demonstrate that inhibition of caspase-1 activity increased hippocampal neurogenesis in the aged brain.

**Material and Methods**

**Animals.** All experiments were performed in accordance with the National Institutes of Health Guide and Use of Laboratory Animals.

**Surgical procedure and treatments.** The study used male Fischer 344 rats (NIA contract colony, Harlan Sprague Dawley, Indianapolis, IN). Rats were housed in pairs and maintained in environmentally controlled chambers on a 12:12-h light-dark cycle at 21 ± 1 °C. Food and water were provided *ad libitum*. We used groups of 8 aged (20 mo) or 8 young (4 mo) rats (4 subgroups = old caspase -1 inhibitor (Ac-YVAD-CMK), old controls, young Ac-YVAD-CMK, young controls). The rats were implanted intracerebroventricularly (icv) with a brain infusion cannula in the left lateral ventricle and an osmotic minipump (Alzet, Model 2004 pumping rate, 0.25 µl/h; total volume 200 µl) for 28 d. Before implantation, the pumps were incubated in sterile saline for at least 48 h at 37°C to prime the pumps. For implantation, rats were anesthetized with isofluorane and placed into a stereotaxic frame. A guide cannula was stereotaxically implanted in the left ventricle (AP = -1.0 mm; ML = 1.6 mm, DV = -3.5) and connected to the osmotic minipump, which was inserted subcutaneously. Pumps were weighed before implantation and at the end of the experiment to ensure complete delivery of their content. The caspase-1 inhibitor, Ac-YVAD-CMK (10 pmol/200 µl; Calbiochem, La Jolla, CA), was injected (0.25 µl/h) through the cannula which was connected to the filled minipump. The infusion started the day of the surgery and continued for 28 d. Control animals received the same volume of 0.6% DMSO (Sigma Aldrich, St. Louis, MO) in saline. Rats received a daily injection of BrdU (Sigma; 50 mg/kg) once a day for 5 d beginning on 14 d after surgery. Rats were sacrificed 10 d following the last injection of BrdU (28d post-surgery). Rats were then perfused transcardially with saline followed by 4% paraformaldehyde in PBS. The brains were removed and postfixed overnight and then moved to 30% sucrose in PBS prior to sectioning on a cryostat.

**BrdU staining procedure:** Cryostat sections were cut in the sagittal plane (40µm), collected in a cryoprotectant solution (30% ethylene glycol, 30% glycerol in 0.01 M PBS), and stored at −20 °C until processed. In preparation for an unbiased stereologic estimate of neuronal numbers, an initial tissue section was selected randomly at one anatomic border of the brain region to be examined. Thereafter, every 3rd section throughout the anatomic region of interest was used for each staining series. For the immunohistochemistry staining procedure, selected sections were floated individually in plastic multiwell carriers with nylon net bottoms. Free-floating sections (40 µm) were pretreated with 50% formamide/2X SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 h, rinsed in 2X SSC, incubated in 2 N HCL for 30 min at 37°C, and washed with borate buffer (pH 8.5). After washing with PBS three times, endogenous peroxidase activity
was quenched by incubating in 0.3% H₂O₂ solution, followed by a 1-h incubation in blocking solution (0.1 M PBS supplemented with 3% normal horse serum and 0.25% Triton X-100). Sections were then incubated overnight with mouse-anti-rat-BrdU (1:50; Roche) in PBS supplemented with 3% normal horse serum and 0.1% Triton X-100. Sections were washed and biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) in PBS supplemented with 3% normal horse serum and 0.1% Triton X-100 was applied for 1 h. The sections were incubated for 60 min in avidin-biotin substrate (ABC kit, Vector Laboratories). All sections were incubated for 3 min in DAB solution (Vector Laboratories). Sections were mounted onto glass slides and coverslipped with mounting medium.

**OX-6 staining procedure:** Immunohistochemistry was performed on every six section throughout the dentate gyrus. Free-floating sections were processed individually for immunoreactivity to the MHC Class IIa antibody OX-6 in plastic 24-well plates (Corning, Inc., Corning, NY). Sections were rinsed in 0.1 M PBS, pH 7.4. Three 5-min washes were conducted between steps except where indicated. Control sections not exposed to primary or secondary antibodies were prepared simultaneously. After equilibrating tissue sections in washing solution, endogenous peroxidase activity was quenched by incubating the sections in 0.1M PBS containing 0.3% H₂O₂ for 15 min. Background labeling was blocked by 1 h incubation in PBS containing 10% normal horse serum and 0.05% TX-100. Sections were then incubated overnight with mouse-anti-rat- OX-6 (Pharmingen, San Diego, CA; 1:750) in PBS containing 3% horse serum and 0.025% TX-100. After incubation in primary antibody, the sections were washed and incubated for 60 min in biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA; 1:300) diluted in washing solution containing 3% normal horse serum and 0.3% TX-100. Sections were then incubated for 90 min in avidin-biotin “ABC Elite” substrate (Vector Laboratories, Burlingame, CA). The sections were then rinsed three times for 10 min each in PBS. Color development was performed in a standardized manner such that all sections were developed in the same reagents for identical periods of time to allow for comparisons of fiber staining intensity. All sections were incubated for 3 min in diaminobenzidine solution (Vector Laboratories). Sections were mounted onto glass slides and cover-slipped with aqueous mounting medium.

**Stereology.** BrdU cells were examined with a Nikon Eclipse 600 microscope and quantified using Stereo Investigator software, Version 6 (MicroBrightField, Colchester, VT). Cells were counted within two cell diameters below the SGZ using the optical fractionator method of unbiased stereologic cell counting (West et al., 1991). The sampling was optimized to count at least 250 cells per animal with error coefficients of less than 0.07. Each counting frame (75 x 75 μm) was placed at an intersection of the lines forming a virtual grid (100 x 100 μm), which was randomly generated and placed by the software within the outlined structure.
**Stereologic estimation of the total number of OX-6 immunolabeled cells:** The dentate gyrus was outlined and analyzed. OX-6 positive cells in the dentate gyrus were counted using the optical fractionator method of unbiased stereologic cell counting techniques. Optical disectors were 100µm x 100µm. The sampling was optimized to sample at least 150 counted cells per animal. Each counting frame was placed at an intersection of the lines forming a virtual grid (200µm x 200 µm) that was randomly generated and randomly placed by the software within the outlined structure. OX-6 - labeled cells were counted using a 60X oil lens (NA 1.4) and were included in the measurement only when they came into focus within the disector (disector height of 20 µm and the average thickness of mounted sections was 30 µm; thickness was measured at random intervals throughout every section and estimated by the software program).

**Immunofluorescence procedure:** Immunofluorescence double labeling was performed to determine the phenotype of BrdU positive cells. Tissues were pretreated with 2 N HCL for 2 h at room temperature, washed, and incubated in blocking solution (0.1 M PBS containing 10% goat serum and 0.3% Triton X-100) for 1 h at room temperature. Tissues were then incubated in rat anti-BrdU (1:400; Accurate Chemical, Westbury, NY) overnight at 4°C. Tissues were rinsed three times in PBS and goat anti-rat secondary antibody conjugated to Alexa 594 (1:800; Molecular Probes, Eugene, OR) was applied for 1 h. Following 6 washes, one of the following primary antibodies was applied overnight at 4°C: rabbit anti-GFAP (1:500; Dako, Carpinteria, CA), mouse anti-NeuN (1:100; Chemicon, Temecula, CA), TuJ1 (1:800; Covance), rabbit cleaved caspase -3 (1:100; Cell Signaling, Danvers MA). Tissues were washed three times with PBS and species-appropriate secondary antibody conjugated to Alexa 488 (Molecular Probes) was applied for 2 h at a 1:200 dilution (anti-GFAP secondary was applied for 1 h at a dilution of 1:800). Following six washes in PBS, tissues were mounted on slides and coverslipped using Vectashield (Vector Labs). To calculate the percentage of each phenotype with respect to the number of BrdU-labeled cells, the total number of BrdU-labeled cells and their respective phenotypes for each animal were scored in at least six or more sections of a one-in-six series. The results from each section were summed, and the percentage for each marker was calculated. These percentages were then expressed as the average of 4 to 6 animals. Colocalization of BrdU-positive cells with GFAP, TuJ1, NeuN, and caspase-3 was validated using a DMI6000 inverted Leica TCS SP5 tandem scanning confocal microscope with a 63x/1.40NA oil immersion objective. 405 diode, 488 Argon and 546 laser lines were applied to excite the samples using AOBS line switching to minimize crosstalk between fluorochrome. Images and Z-stacks were produced with three cooled photomultiplier detectors and the LAS AF version 1.5.1.889 software suite.

**Statistical analysis:** Data are expressed as the mean ± standard error of the mean (SEM). Analyses were performed using a two-way ANOVA followed by Fisher’s LSD post-hoc analysis to identify significant effects. Differences were considered significant at *p ≤ 0.05; **p ≤
A two tail unpaired t-test was performed to identify significant differences in the number of OX-6 immunolabeled cells between aged treated and aged control rats.

Results

Chronic administration of Ac-YVAD-CMK increases the number of BrdU-positive cells in the subgranule cell layer of the hippocampus. We previously demonstrated that infusion of the caspase-1 inhibitor Ac-YVAD-CMK significantly ameliorates impairment of hippocampal-dependent memory by decreasing caspase-1 and caspase-3 activity, as well as protein levels of IL-1β and TNFα in the aged rat hippocampus compared to controls (Gemma et al., 2005). The aim of the present study was to investigate whether the age-dependent increase in hippocampal IL-1β affects the age-related decrease in hippocampal neurogenesis. The effects of a 28-d infusion of Ac-YVAD-CMK (10 pmol) on cell differentiation in the SGZ in young (4 mo) and aged (20 mo) F344 rats was determined by analyzing 5’-bromo-deoxy-uridine (BrdU) staining 10 days after the last BrdU injection. Figure 2 shows that BrdU-labeled cells in both young and aged rats were primarily located on the border of the granule cell layer. Unbiased stereologic quantification of total BrdU-labeled cells in the SGZ revealed that the number of newborn cells generated over a period of 5 d was much higher in young than in aged rats. The results were subjected to a 2 (age: young, aged) X 2 (treatment: saline, caspase-1 inhibitor) factorial ANOVA (Fig. 3). There was a significant effect of age (F (1, 13) = 76.84, p < .001), but no significant effect of treatment (F (1, 13) = 1.13, p = .306). Further, the age X treatment interaction was not statistically significant (F (1, 13) = 2.37, p = .148). Although the interaction was not statistically significant, the means clearly indicate that the response of the aged animals was different from that of the young animals to caspase-1 inhibition. The lack of interaction was likely due to the fact there was no treatment effect in the young animals and therefore the interaction itself was not that strong. Despite the lack of a significant interaction, we compared the treatment effects for the young and aged animals separately, but corrected for multiple comparisons because the original interaction was not statistically significant. The results confirmed that there was no treatment effect in the young animals (F (1, 6) = .06, P< .822), but the effect in the aged group was statistically significant (F (1, 7) = 19.13, P = .003). There was an increase of 53% in the number of BrdU-labeled cells in the SGZ of aged rats compared to aged controls. This was a substantial increase in neurogenesis, however, it was only 39% of the neurogenesis observed in young control rats.

Determination of neuronal and glial phenotypes of BrdU-labeled cells in the subgranule cell layer of the hippocampus. We then determined the phenotype of the newly born cells using the neural nuclear protein NeuN and immature neural marker TuJ1. We found that there was no difference in the proportion of BrdU-labeled cells expressing neuronal or glial phenotype in the SGZ between aged treated and aged control rats (Fig. 4). Our analysis
revealed that 54% of BrdU-labeled cells in the aged control animals expressed NeuN, while the percentage of BrdU/NeuN-positive cells in the aged treated rats increased to 73% (Tab. 1). The immature neural marker TuJ1 was expressed in 43% of BrdU-labeled cells in aged treated rats and 38% of cells in aged control rats. We then examined if there was a change in the glial phenotypes. We found that very few BrdU-labeled cells were positive for glial fibrillary acidic protein (GFAP; 4% control rats and 3% treated rats). Because BrdU is a marker of DNA synthesis, there is a possibility that BrdU labeled cells are undergoing apoptotic cell death; thus, to investigate this possibility, we double-labeled the BrdU cells with caspase-3. We found that only 6.5% and 5% of BrdU positive cells expressed caspase-3 in the control and treated rats respectively. When the percentage of each phenotype was summed, it revealed a number greater than 100%, indicating that cells express more than one phenotype.

Ac-YVAD-CMK decrease microglia activation. Because IL-1β activates microglia and activated microglia produce IL-1β, we explored whether the Ac-YVAD-CMK-induced increase in neurogenesis was accompanied by changes in microglial activation. Microglia were identified by OX-6 a marker for the major histocompatibility complex class II antigen expressed by activated microglia. Stereological counting was performed on OX-6-labeled cells expressed in the dentate gyrus. The stereologic cell count of OX-6-positive cells revealed that caspase-1 inhibition caused a significant decrease in the number of OX-6-positive cells in the aged treated dentate gyrus compared to controls (Fig. 5; p<0.01). Interestingly, as shown in Figure 5B and 5C, a clear morphological difference in the OX-6 positive cells phenotype was observed between treated and non-treated animals. In the non-treated animals microglia were characterized by highly ramified branching, indicating an activated state. In the treated animals, microglia were characterized by long thin branches which indicate a more quiescent and resting state. We were unable to detect enough OX-6-positive cells in the hippocampus of young animals to perform a stereologic study.

Discussion

The present study explored whether the age-related increase in IL-1β contributes to the decrease in hippocampal neurogenesis associated with aging. To examine this hypothesis, we used the irreversible caspase-1 inhibitor Ac-YVAD-CMK to block caspase-1 activity, which is known to be responsible for the production of the active mature form of IL-1β. Consistent with the results of previous studies (Seki and Arai, 1995; Kuhn et al., 1996; Gould et al., 1998; Lichtenwalner et al., 2001; Bizon and Gallagher, 2003), we show that neurogenesis is dramatically reduced in aged animals. In addition, we provide new evidence that chronic inhibition of caspase-1 activity increases the number of newborn cells in aged SGZ. The percentage of the newly generated cells in the aged treated SGZ that differentiated into neural phenotypes, such as NeuN and TuJ1, however, remains similar to that observed in aged control animals. This finding is in agreement with previous data showing that newly born cells in the aging dentate gyrus have a normal neuronal fate (Rao et al., 2006; Hattiangady and Shetty,
Whether the increase in neurogenesis observed in the present study is due to an increase in cell proliferation or to protection and survival of the newly generated neurons cannot be explained by the present data. Future studies looking at cell proliferation will help us to clarify this point. Consistent with the detrimental role of inflammation on adult neurogenesis, Monje et al. (2003) reported that systemic administration of LPS in adult female rats inhibits hippocampal cell proliferation and this effect is completely blocked by the administration of a non-steroidal anti-inflammatory drug.

Upregulation of IL-1β and activation of microglia occur during normal aging and accompany many neurodegenerative diseases. Concomitant with an increase in neurogenesis in aged treated rats, we observed a decrease in the number of activated microglia in the dentate gyrus. A negative correlation between the degree of inflammation, indicated by the activation of microglia, and the number of newborn neurons was previously reported (Ekdahl et al., 2003; Monje et al., 2003). Thus, a decrease in microglial activation could also account for the restorative effect of caspase-1 inhibition on neurogenesis. We observed a dramatic increase in the number of BrdU-labeled cell in the SGZ of aged treated rats compared to controls. Even with this substantial increase, however, neurogenesis remained substantially lower (39%) than in young control rats. This finding raises the question of whether neurogenesis is functionally important. Interesting, the same dose of Ac-YVAD-CMK completely reverses the impaired cognition associated with aging (Gemma et al., 2005). Recently Rao et al. (2006) demonstrated that the largest decrease in hippocampal neurogenesis occurs between 7.5 months and 12 months of age, which corresponds to the period of adult-to-middle age, and there is a small decline from 12 to 24 months of age (Rao et al., 2006). The authors suggest that there may be a threshold of minimal levels of neurogenesis that occurs in the adult brain below which declines in neurogenesis become detrimental, such as that observed at 24 months of age. If this hypothesis is correct the increase in neurogenesis observed in this report, although not back to that observed in very young animals, may be sufficient to cross above the threshold of minimal neurogenesis allowing for proper function of the adult brain. In light of the results presented in this study, we postulate that the increase in newborn neurons induced by caspase-1 inhibition might stimulate endogenous mechanisms responsible for behavioral improvement.

Multiple mechanisms might underlie the effect of caspase-1 activity on hippocampal neurogenesis in the aged brain. In a previous report, we have shown that the administration of Ac-YVAD-CMK at the same dose used in the present study induced a 70% decrease of caspase-1 activity and a 50% reduction of IL-1β protein in the hippocampus of aged rats (Gemma et al., 2005). One possibility is that the increased number of newborn neurons observed here is due to the inhibition of IL-1β. The effect of IL-1β most likely depends on several factors. For example, IL-1β activation triggers a cascade of molecular events that culminates in the alteration of other
proinflammatory cytokines, activation of prostaglandins, increases in glucocorticoids, and activation of apoptosis. All these factors have been shown to decrease neurogenesis. Thus, we cannot rule out the possibility that more than one mechanism is responsible for the beneficial effects of caspase-1 inhibition on neurogenesis.

Caspase-1 is involved in the activation of both apoptosis and inflammation. One important issue is whether the neuroprotective effects of caspase-1 inhibition on neurogenesis proceed exclusively via a cytokine-dependent pathway or whether its involvement in apoptosis is more critical. We previously demonstrated that the inhibition of caspase-1 decreases hippocampal IL-1β levels and caspase-3 activity (Gemma et al., 2005). Whether the decrease in caspase-3 activity is a downstream effect of IL-1β inhibition or whether caspase-1 directly regulates caspase-3 activity cannot be determined in the present experiments. Thus, the possibility that the neuroprotective effects of caspase-1 inhibition are a net consequence of a decrease in apoptosis cannot be ruled out. However, the observation that caspase-1 knockout mice develop normally, with no apparent physiologic or morphologic aberrations, however, suggests that caspase-1 has no major role in programmed cell death during development.

Although the results presented here show a clear effect of the caspase-1 inhibitor Ac-YVAD-CMK on hippocampal neurogenesis, it is important to point out that in the present study we did not measure the hippocampal activity of caspase-1 or the hippocampal protein levels of IL-1β after the administration of Ac-YVAD-CMK; thus, we can only assume that the administration of Ac-YVAD-CMK led to a decrease in the caspase-1 activity which led to a decrease in IL-1β protein levels. Thus, additional effects of Ac-YVAD-CMK in the brain may be responsible for the increase in hippocampal neurogenesis. Indeed, it has been shown Ac-YVAD-CM can exert neuroprotective effects without affecting caspase-1 activity and IL-1β levels. For example, the administration of Ac-YVAD-CMK counteract the gp-120-induced cytosolic cytochrome c elevation without affecting IL-1β levels (Corasaniti et al., 2005). Furthermore, Ac-YVAD-CMK has been shown to protect against oxygen and glucose deprivation-induced cell death in organotypic cultures of rat hippocampal slices independently from IL-1β inhibition (Ray et al., 2000). In conclusion, the findings in the present study demonstrated that the chronic intracerebroventricular administration of Ac-YVAD-CMK reverses the age-dependent decrease in hippocampal neurogenesis. Further studies need to be conducted in order to investigate in more detail the molecular and cellular mechanism underlying the effect of Ac-YVAD-CMK on hippocampal neurogenesis in the age rats.

Acknowledgements

This work was supported by the National Institutes of Health (AG024165A, AG04418) and the VA Medical Research Service. We wish to thank Dr. Small Brent for his assistance in the statistical analyses of the results presented in this work. This work was supported in part by the
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Table 1: To calculate the percentage of each phenotype with respect to the number of BrdU-labeled cells, the total number of BrdU-labeled cells and their respective phenotypes were scored in six or more sections of a one-in six series were scored. The percentages were then expressed as an average of 4 to 6 animals.

* Expressed as percentage of BrdU

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cell type marked</th>
<th>BrdU-labelled cells (%)*</th>
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<tr>
<td></td>
<td></td>
<td>Aged control rats treated with saline</td>
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<td>TUJ1</td>
<td>Immature neuron</td>
<td>38</td>
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<tr>
<td>NeuN</td>
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<td>54</td>
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<td>GFAP</td>
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<td>4</td>
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Figure 2. Inflammation decreases basal hippocampal neurogenesis.
Immunohistochemical staining for BrdU - positive cells (20X) in the dentate gyrus after 28 d intracerebroventricular infusion of vehicle or Ac-YVAD-CMK in young control rats and aged rats. A. Young control rats; B. Aged control rats; C. Aged treated rats. BrdU was injected intraperitoneally once a day for 5 consecutive days, and the rats were killed 10 d after the last injection of BrdU. Note the presence of BrdU - immunoreactive nuclei located in the subgranular zone. The number of BrdU-positive cells was dramatically lower in aged rats.
Figure 3. Inhibition of caspase-1 attenuates the decrease in hippocampal neurogenesis associated with aging. Stereologic quantification using an optical fractionator of BrdU-positive cells in young and aged rats following 28 d intracerebroventricular infusion of vehicle or Ac-YVAD-CMK. The results were subjected to a 2 (age: young, old) X 2 (treatment: saline, caspase-1 inhibitor) factorial ANOVA. The results indicate a significant effect of age (F (1, 13) = 76.84, p < .001), but no significant effect of treatment (F (1, 13) = 1.13, p = .306). Further, the age X treatment interaction was not statistically significant (F (1, 13) = 2.37, p = .148). Multiple comparison of Ac-YVAD-CMK effect for the young and aged rats revealed that there was no treatment effect in the young animals (F (1, 6) = .06, p = .822), but the effect in the old group was statistically significant (F (1, 7) = 19.13, p = .003).
Figure 4. Representative confocal micrograph of BrdU-labeled newborn cells (BrdU, red) and their respective phenotypes (green) in the subgranular zone. A, B and C. Overview of GFAP, NeuN and TuJ1 expression in the SGZ. D and E. Single confonfocal plane of the same cell, showing co-localization of NeuN+ (green), BrdU+ (red) and BrdU+/NeuN+ (orange) cells and TUJ1+ (green), BrdU+ (red) and BrdU+/TuJ1+ (orange), in the SGZ of Ac-YVAD-CMK treated rats. Newly formed double-labeled neurons are visualized by using confocal microscopy in an orthogonal projection composed of 25 optical z-planes (0.5mm thick).
Figure 5. Inhibition of caspase-1 significantly reduced microglia activation. A

Stereologic quantification using an optical fractionator of the number of OX-6 - positive cells in the dentate gyrus of aged control and aged AC-YDAV-CMK treated rats. B and C are representative photomicrograph of OX-6 positive staining (10X) in the dentate gyrus of aged control rats (B) and AC-YVAD-CMK treated rats (C). Note the morphological difference of OX-6 positive cells between treated and non treated animals.
Peripheral injection of human umbilical cord blood stimulates neurogenesis in the aged rat brain

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Abstract

Neurogenesis continues to occur throughout life but dramatically decreases with increasing age. This decrease is mostly related to a decline in proliferative activity as a result of an impoverishment of the microenvironment of the aged brain, including a reduction in trophic factors and increased inflammation. We determined that human umbilical cord blood mononuclear cells (UCBMC) given peripherally, by an intravenous injection, could rejuvenate the proliferative activity of the aged neural stem/progenitor cells. This increase in proliferation lasted for at least 15 days after the delivery of the UCBMC. Along with the increase in proliferation following UCBMC treatment, an increase in neurogenesis was also found in the aged animals. The increase in neurogenesis as a result of UCBMC treatment seemed to be due to a decrease in inflammation, as a decrease in the number of activated microglia was found and this decrease correlated with the increase in neurogenesis. The results demonstrate that a single intravenous injection of UCBMC in aged rats can significantly improve the microenvironment of the aged hippocampus and rejuvenate the aged neural stem/progenitor cells. Our results raise the possibility of a peripherally administered cell therapy as an effective approach to improve the microenvironment of the aged brain.

Introduction

Aging is accompanied by a process of cellular senescence that occurs throughout the body, resulting in a decrease in the regenerative potential of the stem cell pools (Collado et al., 2007). In the brain there are two stem cell pools, one residing in the subventricular zone (SVZ), and the other in the subgranular zone (SGZ) of the dentate gyrus of the hippocampus. As in other
stem cell pools such as the hemapoietic pool in the bone marrow or the satellite stem cells in the muscle, the stem cells in the brain lose there capacity to generate new cells with age (Kuhn et al., 1996; Cameron and McKay, 1999; Kronenberg et al., 2006b). In the brain it appears that the decrease in neurogenesis is a result of a decrease in proliferation of the stem cells and not due to a loss of the cells (Hattiangady and Shetty, 2006). In the muscle it has been shown that the stem cells can be rejuvenated by exposure of the cells to the systemic environment of a young animal through parabiosis (Conboy et al., 2005). Even though it has been known since the 1960s that a cellular senescence occurs with age (Hayflick and Moorhead, 1961), it is less clear if this cellular senescence leads to an aging phenotype, particularly to the age related cognitive decline.

However, it is clear that the process of cellular senescence that occurs with age is an important mechanism to protect against cancer. There are a number of tumor-supressor genes, including p53 and p16$^{ink4A}$, which respond to cellular stressors to induce senescence (Campisi, 2005). It has recently been shown that knocking out p16$^{ink4A}$ can restore the proliferative potential of the aged neural stem cells (Molofsky et al., 2006), but the animals have decreased longevity due to tumor formation (Beausejour and Campisi, 2006). This demonstrates the important balance that oncogenes play in protecting organisms from cancer, but with the negative consequence of inducing an aging state of cellular senescence. An effective target to lessen the amount of senescence might be the cellular stressors that accumulate with age which include telomere shorting (Collado et al., 2007), oxidative stress (Harman, 1956; Ames and Shigenaga, 1992; Ames et al., 1993), inflammation (Blalock et al., 2003), increased corticosteroid levels (Sapolsky, 1992), and a decrease in a number of trophic factors including brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), Insulin-like Growth Factor-1 (IGF-1) and fibroblast growth factor 2 (FGF-2) (Hattiangady et al., 2005; Shetty et al., 2005).

A potent cellular stressor that is increased with age is inflammation. Recently, our laboratory has shown that reducing neuroinflammation in aged rats by blocking the conversion of pro-IL-1β to IL-1β through inhibition of the converting enzyme caspase-1 rescued some of the age-related decrease in neurogenesis (Gemma et al., 2007a) and resulted in an improvement in cognitive function (Gemma et al., 2005). We believed that human umbilical cord blood mononuclear cells (UCBMC) may have a similar potential to restore some of the loss in capacity of the neural stem/progenitor cells ability to proliferate and differentiate into neurons.

In an animal model of stroke, UCBMC administered intravenously have reduced infarct volume and improved functional recovery on behavioral measures (Vendrame et al., 2004). The effects of UCBMC have been attributed to changes in the microenvironment of the brain, through the release of trophic factors or by reducing inflammation, and not by a direct replacement of cells (Borlongan et al., 2004; Vendrame et al., 2005; Newman et al., 2006). UCBMC contains a number of cell types including B-Cells and T-Cells, as well as, mesenchymal and endothelial
progenitor cells. UCBMC is also a rich source of CD34^+ hematopoietic stem cells (Bender et al., 1991; Ho et al., 1996; Wu et al., 1999). It was recently demonstrated that a systemic injection of UCBMC cells could suppress inflammation in the brain following stroke. Moreover, the effects of UCBMC cells seemed to shift the cytokine expression from a Th1 response to a Th2 response (Vendrame et al., 2004; Vendrame et al., 2005; Vendrame et al., 2006a). In addition to the immune modulatory effects, UCBMC cells also produce a number of trophic factors including, but not limited to, VEGF, nerve growth factor, and cytokine colony stimulating factor-1, thrombopoietin, and IL11 (Suen et al., 1994; Taguchi et al., 2004; Vendrame et al., 2004).

The goal of the present study was to determine if UCBMC could stimulate the endogenous stem/progenitor cells to regenerate new cells. To this end, young and aged rats were intravenously administered a single dose of UCBMC to determine if UCBMC could increase proliferation of the neural stem/progenitor cells as well as to determine if there would be an effect on neurogenesis in the aged rats. This study provides insight into how the aged stem cell niche could be rejuvenated. Furthermore, as the UCBMC are administered minimally invasively this study raises the possibility of a clinically applicable therapeutic for the aged brain.

Material and Methods

Cell preparation: Cryopreserved human umbilical cord blood mononuclear cells (UCBMCs) were obtained from Saneron CCEL Therapeutics, Inc. (Tampa, FL, USA). Cryopreserved Human peripheral blood cells (PBMC) (mononuclear fraction) were obtained from AllCells, LLC (Emeryville, CA, USA). Just prior to intravenous (i.v.) injection, the UCBMC or PBMC were thawed into media (Hanks' balanced salt solution, HBSS, Gibco) at 37°C, washed, and the number of viable cells was determined using the trypan blue exclusion method (Vendrame et al., 2005). Cell viability ranged from 85 to 88%. Cell concentration was adjusted to 10^6 viable cells/500 μl. Rats were then anesthetized with 3% isofluorane and randomly chosen to receive a single i.v. injection via the penile vein of UCBMC at a dose 10^6 cells shown most effective in a stroke model (Newman et al., 2006), 10^6 PBMC, or media for both the aged and young rats.

Animals: All experiments were conducted in accordance with the National Institute of Health Guide and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use committee of the University of South Florida, College of Medicine. Male Fisher 344 (F344) rats (NIA contract colony, Harlan Sprague Dawley, Indianapolis, IN), were pair-housed in environmentally controlled conditions (12:12h light:dark cycle at 21±1°C) and provided food and water ad lib. Two age groups of animals young (3 months old) and aged (20 months old) were used in this study. The mean life span of the F344 rats is approximately 29 months with a maximal life span of 36 months (Coleman et al., 1977).

Rats were then divided in three groups. Group 1 received 50 mg/kg of bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU; Sigma, St. Louis, MO, USA), intraperitoneal (i.p.) twice a day
beginning 24 hours the injection of UCBMC, and were sacrificed the subsequent day. Rats in group 2 received BrdU (50 mg/kg, i.p.) twice a day, beginning fourteen days after the administration of UCBMC and were sacrificed on the following day. Rats in Group 3 received BrdU (50 mg/kg, i.p.) for five consecutive days, beginning the day after the administration of UCBMC and were sacrificed day fifteen.

**Tissue collection and processing:** The rats were anesthetized with pentobarbital (50 mg/kg, i.p.). Blood was collected by cardiac puncture and smears were made of the blood to look for the presence of the transplanted cells. The rats were transcardiac perfusion with phosphate-buffered (PB), followed by 4% paraformaldehyde in PB. The brains were postfixed in 4% paraformaldehyde for 12 h, after which they were transferred into 30% sucrose in phosphate-buffered saline (PBS) for at least 16 h, and stored at 4°C. Exhaustive caudal sections of the left hemisphere were made, at 40μm using a Microm cryostat (Richard-Allan Scientific, Kalamazoo Michigan) and stored in cryoprotectant at 4°C.

**BrdU Immunohistochemistry:** All immunohistochemical staining was conducted on free-floating sections for every sixth section for the entire hippocampus beginning with a random start and including sections before and after the hippocampus to ensure that the entire structure was sampled; with one exception, in the aged animals from group 3 a one in three series was stained to allow for sampling of an adequate number of BrdU+ cells. For BrdU staining, sections were pretreated with 50% formamide/2X SSC (0.3 M NaCl, 0.03M sodium citrate) at 65°C for 2 hours, rinsed in 2X SSC, incubated in 2N HCL for 30 minutes at 37°C, washed with borate buffer (pH 8.5), then PBS. This was followed by quenching endogenous peroxidase activity in 0.3% H₂O₂ solution in 30% methanol; then one hour in blocking solution (0.1M PBS supplemented with 3% normal horse serum and 0.25% Triton X-100: PBS-TS); followed by incubation overnight with mouse-anti-rat-BrdU (1:100; Roche) in PBS-TS. The following day the sections were washed and then incubated for one hour in a biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) in PBS-TS; then washed before one hour incubation in avidin-biotin substrate (ABC kit, Vector Laboratories, Burlingame, CA); and then washed before 10 minutes incubation in DAB solution (Vector Laboratories, Burlingame, CA). Sections were then mounted onto glass slides and coverslipped with mounting medium.

**Doublecortin and OX-6 Immunohistochemistry:** Doublecortin (DCX) is a marker of migrating neurons that is expressed for approximately three weeks after the cell is born and has been shown to be a reliable indicator of neurogenesis (Rao and Shetty, 2004; Couillard-Despres et al., 2005). For DCX immunohistochemistry a polyclonal goat antibody raised against human DCX (1:200; SC-8066, Santa Cruz biotechnology, Santa Cruz, CA, USA) was used following a similar protocol to BrdU except the antigen retrieval steps were omitted and Goat serum (Vector Laboratories, Burlingame, CA) was used instead of horse serum. For OX-6 immunohistochemistry a monoclonal antibody directed against the rat major histocompatibility II
(MHCII) (RT1B, Becton, Dickinson Pharmingen, San Diego, CA, USA) was used at a concentration of 1:750 in place of the other primary antibodies all other steps were the same.

**Immunofluorescence:** Tissues were pretreated with 2N HCL for 2 hours at room temperature, washed, and incubated in blocking solution (0.1M PBS containing 10% goat serum and 0.3% Triton X-100) for 1 hour at room temperature. Tissues were then incubated in rat anti-BrdU (1:400; Accurate Chemical, Westbury, NY) and additional primary antibodies [anti-GFAP (1:500; Dako, Carpinteria, CA), mouse anti-NeuN (1:100; Chemicon, Temecula, CA), mouse anti-TUJ1 (1:800; Convance, Berkeley, CA)], overnight at 4°C. Tissues were then rinsed 3 times in PBS and the appropriate secondary antibody conjugated to an Alexafluor probe (Molecular Probes, Eugene, OR) was applied for 2 hour. Following 6 washes in PBS, tissues were mounted on slides and coverslipped using Vectashield (Vector Labs, Burlingame, CA).

**Human Nuclei immunofluorescence:** To detect for the presence of the transplanted cells, blood smears and tissue sections were stained with a mouse monoclonal antibody that recognizes Human Nuclei antigen (HuNu) (MAB 1281; 1:50; Chemicon, Temecula, CA), and does not react with rat nuclei. Prior to incubation overnight at 4°C in the HuNu antibody, the samples were washed in PBS and incubated in blocking solution (0.1M PBS containing 10% goat serum and 0.3% Triton X-100) for 1 hour at room temperature. The HuNu antibody was visualized by secondary antibody conjugated to an Alexafluor probe (Molecular Probes, Eugene, OR).

**Quantification and imaging:** To determine cell numbers the optical fractionator method of unbiased stereological cell counting techniques (West et al., 1991) was used with a Nikon Eclipse 600 microscope and quantified using Stereo Investigator software (MicroBrightField, Colchester, VT). For the proliferation study, because of the low number of BrdU+ cell in the aged animals the virtual grid and counting frame were both 125μm x 125μm in order to count all the cells that were present in the section. For all other counts sampling was optimized to count at least 200 cells per animal with error coefficients less than 0.07. Outlines of the anatomical structures were done using a 10x/0.45 objective and cell quantification was conducted using a 60x/1.40 objective. OX-6+ cells were counted in the entire dentate gyrus including the subgranular zone (SGZ: defined as a two cell diameter band on both sides of the granular cell layer (GCL)). All other cell counts were done in the SGZ/GCL. The phenotype of the BrdU+ cells were analyzed using an inverted Zeiss LSM 510 confocal microscope with a 40x/1.30NA oil immersion objective. Argon and HeNe laser lines in conjunction with 488 and 555 band pass filters were applied to excite the samples using line switching to minimize crosstalk between fluorochromes. Images and Z-stacks were produced with dual photomultiplier detectors and the LSM 5 version 3,2,0,115 software suite, and optical Z stacks where created at 2μm intervals throughout the 40μm of the sections with a guard region of 2μm excluded from top and bottom of the Z stack. The Z stacks were rotated in all planes to verify double labeling.
**Statistical analyses:** Data are presented as mean cell number ± SEM. Statistical analysis was performed using an unpaired, two-side $t$-test, or a one-way ANOVA followed by a Tukeys post-hoc test. $p<0.05$ was considered to be significant.

**Results**

**Human umbilical cord blood mononuclear cells (UCBMC) stimulate proliferation of the senescent hippocampal neural stem cell.** In the first of a series of experiments we wanted to determine if UCBMC given intravenously could stimulate the proliferation of the endogenous stem/progenitor cells in the hippocampus. We chose to study proliferation as a slowing of the cell cycle and a decrease in proliferation seems to be most affected with age when compared to the ability of the cells to survive and differentiate into neurons which appears to occur at relatively the same rate in young animals (Rao et al., 2005). The effect of a single intravenous injection of UCBMC on cell proliferation in the granule cell layer in young (3-months old) or aged (20-months old) F344 rats was determined by analyzing BrdU staining 24 hours after the BrdU injections (48 hours following UCBMC injection). Using the optical fractionator method of design based stereology (West et al., 1991), we found that in the aged animals there was a significant increase ($t(9)= 4.256; p < 0.005$) in the number of BrdU$^+$ cells in the UCBMC group ($2504 \pm 227.3 n=5$) compared to the animals that received media alone ($1549 \pm 82.07 n=6$) (Figure 6A). In young animals there was no significant effect of the UCBMC treatment (data not shown).

To determine if there might be a prolonged effect on proliferation in the aged F344 rats BrdU injections were given 14 days after the UCBMC treatment. Figure (6E) shows the effect of a single intravenous injection of UCBMC on the number of cells that incorporated BrdU on day 14. Stereological analysis revealed that in the aged UCBMC-treated rats there was a significant increase in the number of BrdU$^+$ cells ($t(12)= 3.468; p < 0.01$) ($2357 \pm 149.4 n=6$) compared to the media-treated group ($1548 \pm 176.9 n=4$).

**Neurogenesis is stimulated in the aged hippocampus following UCBMC treatment.** To determine if UCBMC would also stimulate neurogenesis in the aged rats, doublecortin (DCX) immunostaining was examined. Counting the number of DCX$^+$ cells in the SGZ/GCL, we found a significant increase ($t(16)= 2.188; p < 0.05$) in the number of DCX$^+$ cells in the aged rats 15 days after a single i.v. injection of the UCBMC ($2619 \pm 212.6 n=9$) compared to animals that received media alone ($1843 \pm 283.9 n=9$) (Figure 7A). To confirm the results obtained by DCX, and to determine if there was any change in the ability of the proliferating cells to differentiate following a UCBMC treatment, we injected the animals with BrdU (50mg/kg) for five days beginning 24 hours after our i.v. treatment. Quantifying the number of BrdU$^+$ cells in the SGZ/GCL using the optical fractionator method of design based stereology we found a similar increase in the number of BrdU$^+$ cells in the aged UCBMC treated group as was found using the neurogenic marker DCX (Figure 7A-D). In aged rats, there was a significant increase ($F(2, 14) = 10.94, p < 0.005$) in the number of BrdU$^+$ cells generated over a period of five days following a single i.v. injection of
UCBMC (2772 ± 263.3 n=5) compared to the rats that received media alone (1498 ± 206.1 n=5); as determined by the Tukey’s Multiple Comparison Test (p<0.01). In this experiment, we also included a group that was injected with adult human peripheral blood mononuclear cells (PBMC) as a control for the effect of delivering cells. The PBMC group was determined to have significantly fewer BrdU+ cells (1712 ± 171.2 n=5) than the UCBMC treated group (p<0.01), but this was not significantly different from the group that received media alone (Figure 7E-H). As with the results of the proliferation study, young rats showed no significant effect of UCBMC treatment (data not shown). To determine if the treatment with UCBMC might alter the phenotype of the newborn cells, we double labeled with the antibodies to Tuj1, NeuN and GFAP. While exhaustive sampling was not conducted, 50 BrdU+ cells were analyzed from each rat (4 rats per group) using confocal microscopy for each marker and there did not appear to be any change in phenotype due to the treatment (Figure 7I-J). To confirm that the increase in neurogenesis was from the endogenous stem/progenitor cells, sections were stained for HuNu to look for the presence of the transplanted cells in the brain. Cells positive for the HuNu were found in the blood smears of the rats that were treated with UCBMC, but no HuNu immunoreactive cells were found in the hippocampus of the rats (data not shown).

**A decrease in microglia activation following UCBMC correlates with the increase in neurogenesis.** Using the optical fractionator method of design based stereology, we counted the number of OX-6+ cells in the dentate gyrus 15 days after a single UCBMC injection; this was at the same time point that we observed an increase in DCX+ cells and BrdU+ cells. OX-6 is a marker for MHCII and presumably stains for microglia in an activated, proinflammatory state. In aged rats, we found that 15 days after the UCBMC treatment there was a significant decrease (t(12)= 2.699; p < 0.05) in the total number of activated OX-6+ microglia in the UCBMC group (678.7 ± 155.3 n=7) compared to the media control (1217 ± 128.0 n=8) (Figure 8A). The decrease in OX-6+ microglia negatively correlated with the number of DCX+ cells (Spearman r(15)= −0.6429; p<0.01) (Figure 3E).

Morphologically the OX-6+ cells expressed two main phenotypes (see Figure 8F). Type 1 microglia appear to be in a more quiescent state based on morphology Type 2 microglia were thought to represent a more activated state. The type 1 microglia make up the majority of the OX-6+ cells in the dentate gyrus and were found to be significantly decreased (unpaired t(12)= 2.791; p < 0.05) in aged rats following UCBMC treatment (426.6 ± 117.0 n=7) compared to controls (842.4 ± 94.67 n=8) (Figure 8G). The type 2 microglia, while representing a smaller percentage of the total OX-6+ microglia, were significantly reduced (t(12)= 3.281; p < 0.01) to a greater extent by the UCBMC treatment (53.30 ± 13.27 n=7) compared to controls (212.9 ± 43.82 n=8) than the total microglia (Figure 8H). Fifteen days after the UCBMC treatment, there was a 4 fold change in the number of type 2 OX-6+ microglia, whereas there was only a 1.8 fold change in the total
number of OX-6+ microglia. It appears that the highly activated microglia are being reduced to a greater extent by the UCBMC treatment, although all OX-6+ microglia are affected.

Discussion

The present study explored whether human umbilical cord blood mononuclear cells (UCBMC) could improve the neurogenic niche of the aged brain and stimulate the endogenous stem/progenitor cells to generate new neurons. As determined by stereological analysis of both DCX and BrdU, a single peripherally administered injection of UCBMC appeared to stimulate neurogenesis. The finding that the administration of UCBMC also increased the number of proliferative cells generated within 24 hours following the treatment, suggests that the increase in neurogenesis observed in this study may be a consequence of an increase in proliferation rather than changes in differentiation or survival of newly generated cells. To support this hypothesis, it will be important to allow more time for the cells to fully mature and then determine if there is still no change in the phenotype of the BrdU+ cells. It will also be important to determine what effect UCBMC have on the survival of the BrdU+ cells.

In addition, it was determined that UCBMC were able to increase cell proliferation for at least fifteen days in the aged rats. This suggests that the UCBMC may have a beneficial effect on the microenvironment of the aged brain. In support of this hypothesis we show that coinciding with an increase in neurogenesis in the aged treated rats, there was a decrease in the number of activated microglia in the dentate gyrus. A negative correlation between the degree of inflammation as indicated by the activation of microglia and the number of newborn neurons has been previously described (Ekdahl et al., 2003). Consistent with previous studies showing that UCBMC have the potential to reduce neuroinflammation (Vendrame et al., 2004; Rao et al., 2005; Vendrame et al., 2005; Vendrame et al., 2006a) in the aged brain, we did find that neurogenesis correlated with the number of activated microglia, suggesting that UCBMC were stimulating neurogenesis by decreasing microglia activation. Although other possibilities cannot be excluded, since UCBMC could be having multiple effects including increasing trophic support as previously published (Suen et al., 1994; Taguchi et al., 2004; Vendrame et al., 2004).

UCBMC have been shown to reduce neuroinflammation (Vendrame et al., 2004; Rao et al., 2005; Vendrame et al., 2005; Vendrame et al., 2006a) and, consistent with previous studies, we show here that the peripherally administered UCBMC do have anti-inflammatory properties. It appears that one of the factors that leads to the negative regulation of neural stem cells is inflammation (Ekdahl et al., 2003; Monje et al., 2003; Battista et al., 2006). A primary source of inflammation in the CNS is from the macrophages/microglia which can produce a wide array of cytotoxic factors, including proinflammatory cytokines such as tumor necrosis factor (TNF), IL-1, IL-6 and IL-12 (Gao et al., 2002; Mantovani et al., 2004). With age, microglia shift from a quiescent state into an active proinflammatory state. It is not clear if this change in activation state is in response to injury, infection, or debris or if it is due to dysregulated cytokine levels. Another
possibility recently proposed, is that microglia becoming senescent and this leads to them becoming dysfunctional (Schwartz et al., 2006b; Streit, 2006b). It has previously been demonstrated in models of induced inflammation through the use of LPS or radiation, a dramatic decrease in proliferation and neurogenesis occur, and when the inflammation is alleviated the replicative potential of the stem cells returns (Ekdahl et al., 2003; Monje et al., 2003). This effect is likely a protective mechanism so that the DNA is not exposed to the noxious inflammatory environment which could damage the replicating DNA. This correlation also imparts support to the hypothesis that UCBMC stimulate neurogenesis by decreasing inflammation, particularly the activation state of microglia. However, it does not rule out the possibility that UCBMC may be acting on multiple targets, with microglia only representing one part of the total mechanism.

While UCBMC do seem to have an effect on microglia, it is not clear how this occurs. A number of studies have shown that T-cells appear to act on macrophages/microglia to cause them to adopt a phenotype that is ‘pro-repair’ (i.e. the macrophages/microglia: clear debris, buffer toxic compounds, and produce growth factors), without being pro-inflammatory (i.e. producing TNF-α, NO, or COX-2) and this effect can promote neurogenesis and be neuroprotective (Shaked et al., 2004; Butovsky et al., 2006c; Ziv et al., 2006a; Ziv et al., 2006b; Ziv et al., 2007). As T-cells are a major fraction of UCBMC, it is possible that the naïve T-cells in the UCBMC are able to induce a protective T-cell mediated response in the aged rats, since adult PBMC did not have an effect. Alternatively, the CD34+ stem cells in the UCBMC may be involved. Taguchi et al. (Taguchi et al., 2004) has shown that CD34+ stem cells can increase both angiogenesis and neurogenesis as part of the protective mechanism against stroke. From the results of the current study it can not be determined if the effects of the UCBMC are a result of direct action on the brain or though peripheral effect. However, the fact that we did not detect any immunoreactivity for human nuclei in the brains of the UCBMC-treated rats raises the possibility that the UCBMC may be acting through a peripheral mechanism. Moreover, the observation that the adult PBMC did not alter hippocampal neurogenesis ruled out the possibility of a non-specific effect due to an influx of cells, supporting our belief that the increase in neurogenesis, which occurred following treatment with UCBMC was not due to an influx of cells but was specific to UCBMC.

The present study did not attempt to determine if decreasing senescence of the neural stem cells could reverse the cognitive decline with age. There is still much debate surrounding the role of neurogenesis in learning and memory (Gould et al., 1999a; Shors et al., 2001; Shors et al., 2002; Merrill et al., 2003; Leuner et al., 2006; Kee et al., 2007) and whether cellular senescence of the stem cell pool with age leads to an aging phenotype. While not a goal of the current study, it will be important to determine if the rejuvenation of the aged stem/progenitor cell pool can reverse the age-related cognitive decline.

In summary, this study demonstrates that a single peripheral injection of UCBMC could stimulate the endogenous neural stem/progenitor cells to increase proliferation. We also
determined that the UCBMC were able to improve the microenvironment of the aged brain by reducing the number of activated microglia, and this reduction is correlated with an increase in neurogenesis. Further work will be important to determine the mechanism of action of UCBMC in the aged rats, including the possible role of the immune system in a T-cell mediated response, as well as the affects of angiogenesis via the CD34+ stem cells. It will also be important in future experiments to determine the duration that a single injection of UCBMC will elevated proliferation in aged rats. Not only do the results of this study provide novel insight into the state of the aged stem cell niche, the ability of the UCBMC to exert their effects while being administered minimally invasively may make translation to the clinical setting more likely. For this reason it will be important in future studies to determine the most efficacious dose and dosing regimen. Nevertheless, this is the first time that a systemic injection of hematopoietic cells has been shown to restore the regenerative potential of the aged brain, providing a novel insight into how the regenerative potential of the aged stem cell niches could be restored.

Conclusions

The results demonstrate that a single intravenous injection of UCBMC in aged rats can significantly improve the microenvironment of the aged hippocampus and rejuvenate the aged neural stem/progenitor cells. Our results raise the possibility of a peripherally administered cell therapy as an effective approach to improve the microenvironment of the aged brain.

Abbreviations

GCL (granular cell layer), PBMC (peripheral blood mononuclear cells), UCBMC (umbilical cord blood mononuclear cells), SGZ (subgranular zone)

Competing interests

PCB, AEW are consultants to Saneron CCE L Therapeutics Inc (SCTI). PRS is a co-founder of SCTI. AEW & PRS are inventors of UCBMC related patents applications.

Authors' contributions

ADB, PRS, AEW, PCB, CG designed research. ADB, MMP, MJC, CEH, CG performed research. ADB wrote paper. All authors read and approved the final manuscript.

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Figure 6: Proliferation is increased in aged rats following UCBMC treatment. To determine if UCBMC could stimulate proliferation of the hippocampal neural progenitor/stem cells, rats received two i.p. injections of BrdU (50 mg/kg) and were sacrificed the following day. (A) Quantification of the BrdU immunoreactive cell in the SGZ/GCL in aged rats 2 days after the UCBMC treatment showed that there was a significant (p<0.005) increase in the number of BrdU immunoreactive cells. (B,C) Photomicrographs of the dentate gyrus of a media-treated rat (B) and a UCBMC-treated rat (C) shows the BrdU staining in those animals sacrificed 2 days after the treatment. (D) The arrow in C points to a cluster of BrdU immunoreactive cells from the UCBMC-treated rat shown in D at higher magnification. (E) To determine how long proliferation might remain elevated injections of BrdU (50 mg/kg) began 14 days after the treatment. Quantification of the BrdU immunoreactive cells determine that the UCBMC-treated group had significantly (p<0.01) more cells in the SGZ/GCL then the animals that received media alone. (F,G) BrdU staining of the media-treated (F) and the UCBMC-treated (G) animals in the dentate gyrus of the hippocampus 15 days after the treatment. (H) Arrow in G points to cells shown at higher magnification in H. (scale bar for B,C,F,G is 100µm; scale bar for D,H is 25µm)
Figure 7: 15 days after a UCBMC treatment neurogenesis is increase in aged rats.

To determine if UCBMC treatment could stimulate neurogenesis aged F344 rats were sacrificed and immunohistochemical stained for DCX and BrdU. (A) A significant increase (p<0.05) in the number of DCX+ cells, quantified in the SGZ/GCL, was found in the UCBMC treated rats. (B,C) Photomicrographs show the dentate gyrus demonstrating the DCX immunohistochemistry in the media-treated (B) and UCBMC-treated (C) rats. (D) A higher magnification photomicrograph of area indicated in C shows a number of DCX+ cells showing the different morphologies of the cells. (E) The results obtained with DCX were confirmed by BrdU. BrdU was injected i.p. for five consecutive days after the single injection of UCBMC. 10 days after the last injection of BrdU the animals were sacrificed. Compare to both a media control as well as an human adult peripheral blood (PBMC) control the UCBMC treated animals had significantly more BrdU+ cells (p<0.01). (F,G,H) Photomicrographs of dentate gyrus shows BrdU immunohistochemistry in the media-treated (F), PBMC-treated (G) and UCBMC-treated (H) rats. (I,J) Immunofluorescence was conducted to determine the phenotype of the BrdU+ cells. (I) An example of the cells double labeled with BrdU+/NeuN+ (I; shown in orthogonal projection) and BrdU+/TUJ1+ (J; shown using maximum projection). (scale bar for B,C,F,G,H is 100µm; scale bar for D is 25µm)
Figure 8: The decrease in microglia activation correlates with neurogenesis. 15 days after the UCBMC treatment a significant reduction (p<0.05) was found in the number of OX-6+ cells in the dentate gyrus of the aged rats (A). (B,C) Photomicrographs are shown of the hippocampus of media-treated (C) and UCBMC-treated (C) rats. (D) A higher magnification photomicrograph of area indicated by arrow in B. (E) A significant negative correlation (p<0.01) was found between the number of OX-6+ cells and the amount of neurogenesis as determine by the number of DCX+ cells. (F) The OX-6+ were further characterized based on morphology. The cell on the left represents a typical ‘Type 1’ cell the cell on the right represents a typical ‘Type 2’ cell. Both ‘Type 1’ (p<0.05; G) and ‘Type 2’ (p<0.01; H) OX-6+ cells were significantly reduced in the aged animals following UCBMC treatment, but there was a greater reduction in ‘Type 2’ cells amounting to a four fold change. (scale bar for B,C is 200µm; scale bar for D is 25µm).
Fractalkine and CX3CR1 regulate hippocampal neurogenesis in adult and aged rats.

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Abstract

Cellular senescence occurs throughout the body during chronological aging of an organism. A portion of senescence is independent of primary alterations to the stem cells, and is dependent on the environment where the cells reside. While microglia have neuroprotective capacities, their chronic activation can promote neurotoxic inflammation, thereby contributing detrimental effects to the neural stem cell niche. The causes of age-related increases in microglial activation and neuroinflammation are currently not well understood. Neuronally-expressed fractalkine (FKN), acting via interaction with its receptor CX₃CR1, can suppress excessive microglia activation. To address the role of this chemokine system in hippocampal neurogenesis, we examined the impact of interfering with FKN/CX₃CR1 interactions in young and old rodents. Disruption of FKN/CX₃CR1 signaling in young adult rodents decreased survival and proliferation of neural progenitor cells. These anti-neurogenic effects, resulting from loss of CX₃CR1 function, were reversed by IL-1β antagonism. Aged rats had decreased levels of hippocampal FKN protein although interruption of CX₃CR1 function in these animals did not affect neurogenesis. Moreover, delivery of exogenous FKN reversed the age-related decrease in hippocampal neurogenesis in aged rats but did not produce any effects in young animals. The results suggest that FKN/CX₃CR1 signaling has a regulatory role in modulating hippocampal neurogenesis via mechanisms that involve indirect modification of the niche environment. As elevated neuroinflammation is associated with many age-related neurodegenerative diseases, enhancing...
FKN/CX3CR1 interactions could provide an alternative therapeutic approach to slow neurodegeneration, while also minimizing non-specific immunosuppressive responses.

Introduction

Adult neurogenesis is a lifelong process, continuing even in elderly humans (Eriksson et al., 1998). However, studies in rodents have demonstrated a continual age-related decline in neurogenesis (Rao et al., 2006; Ben Abdallah et al., 2008). An extensive list of neurogenic regulators has been identified, many of which change as a result of aging (Drapeau and Nora Abrous, 2008) making a unified theory to account for the age-related decrease in neurogenesis unlikely. Yet, the potential importance of neurogenesis in some affective (Sahay and Hen, 2007) and cognitive behaviors (Drapeau and Nora Abrous, 2008), as well as endogenous tissue repair mechanisms, makes further investigation of neurogenic regulators warranted. Seminal studies demonstrated that microglia can be detrimental to neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). Proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, have been shown to act directly on neural stem/progenitor cells (NPC)(Monje et al., 2003; Iosif et al., 2006; Koo and Duman, 2008). Microglia are pleiotropic, and can also support neurogenesis through the production of growth factors (Ziv and Schwartz, 2008). Therefore the involvement of microglia in the neurogenic niche is not clear, as microglia can both increase and decrease neurogenesis. Until recently, neurons were believed to be submissive to the effects of microglia; however, a number of neuronal signals were found that can regulate microglia activation (Biber et al., 2007), suggesting a neuron-microglia dialog.

One neuronally derived signal that has been shown to be important in regulating the neurotoxic affects of microglia is the chemokine fractalkine (FKN; CX3CL1; neurotactin). In contrast to many other chemokines, FKN binds and activates a single receptor, CX3CR1. Although there is some debate concerning the cell types expressing these two molecules, in vivo FKN is principally expressed on neurons while CX3CR1 is found on microglia (Harrison et al., 1998; Cardona et al., 2006; Lauro et al., 2008). Previous reports establish that interactions between FKN and CX3CR1 contribute to maintaining microglia in a resting phase, partially controlling their neurotoxicity. FKN acts in vitro as an anti-inflammatory molecule by down-regulating IL-1β, TNFα, and IL-6 production (Zujovic et al., 2000; Zujovic et al., 2001). FKN can also elicit neuroprotective effects on pure neuronal cultures (Meucci et al., 1998; Meucci et al., 2000; Tong et al., 2000). Moreover, mRNA and protein expression of CX3CR1 were found in isolated NPCs (Ji et al., 2004; Krathwohl and Kaiser, 2004).

With age there is an increase in the number of activated microglia, which can suppress neurogenesis (Gemma et al., 2007b; Bachstetter et al., 2008). We hypothesized that, as a consequence of aging, FKN signaling becomes disregulated, which leads to increased microglial activation and decreased neurogenesis. Our findings demonstrate for the first time that FKN/CX3CR1 signaling is critical for the regulation of hippocampal neurogenesis.
Materials and Methods

Animals. All experiments were conducted in accordance with the National Institute of Health Guide and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use committee of the University of South Florida, College of Medicine or the University of Florida as appropriate. CX3CR1-deficient (CX3CR1GFP/GFP) mice, backcrossed to the C57BL/6 background for greater than 10 generations were obtained from JAX Laboratories (Bar Harbor, Maine). Colonies of the CX3CR1+/GFP and CX3CR1GFP/GFP mice were maintained at the University of Florida. Four-month-old male CX3CR1+/GFP and CX3CR1GFP/GFP littermates were used in the experiments. Male Fisher 344 (F344) rats (NIA contract colony, Harlan Sprague Dawley, Indianapolis, IN), were pair-housed in environmentally controlled conditions (12:12 h light:dark cycle at 21 ± 1°C) and provided food and water ad libitum. Three age groups of rats used in this study included: young (3 months old), middle aged (12 months old) and aged (22 months old). Animals were excluded from the study if they became jaundiced, had pituitary tumors, or developed post-surgery infections.

Surgical procedure. For all surgical procedures, rats were anaesthetized with isofluorane. For intracerebroventricular infusion, a guide cannula was stereotaxically implanted in the left ventricle (AP, −1.0; ML, 1.6; DV, −3.5 mm) and connected to an osmotic minipump, which was inserted subcutaneously. For the first 7 days all rats received an osmotic minipump (Alzet Model, 2001: pumping rate, 1.0 µL/h; total volume, 200 µL) filled with sterile saline, to allow time for the rats to heal before drug treatment was started. After the first 7 days, a mid-scapular incision was made and the saline pump was switched for the treatment pump for either an additional 7 days (Alzet Model, 2001: pumping rate, 1.0 µL/h; total volume, 200 µL), 14 days (Alzet Model, 2002: pumping rate, 0.5 µL/h; total volume, 200 µL), or 28 days (Alzet Model, 2004: pumping rate, 0.25 µL/h; total volume, 200 µL). The treatments used in this study included: (1) rabbit-anti rat CX3CR1 blocking antibody (α-CX3CR1) (10µg per day; Torrey Pines Biolabs, San Diego, CA; Cat no. TP 501)(Milligan et al., 2004); (2) rabbit non-immune IgG (10µg per day; Sigma-Adrich; Cat no. I-5006); (3) recombinant rat FKN (aa 22-100) chemokine domain (30ng per day R & D systems, Inc.; Cat no. 568-FR/CF)(Milligan et al., 2004). (4) r-metHu IL-1Ra (10µg per day; Amgen, Thousand Oaks CA). For controls, the proteins were heat-inactivated for 45 minutes in a water bath at 90°C.

Thymidine analog labeling. Following the time line in Figure 2A animals received two intraperitoneal (i.p.) injections of one or more thymidine analogs with a 12 hour interval. Bromodeoxyuridine (BrdU) (5-bromo-2-deoxyuridine; Sigma, St. Louis, MO) was injected at dose of 50 mg/kg. Equimolar solutions, to be equivalent to the 50 mg/kg of BrdU, were prepared from chlorodeoxyuridine (CldU) (42.5 mg/kg; Sigma, St. Louis, MO) and iododeoxyuridine (IdU) (57.5 mg/kg; MP Biomedicals) as previously described(Vega and Peterson, 2005).
**Tissue collection and processing.** For immunohistochemistry studies animals were anaesthetized with pentobarbital (50 mg/kg, i.p.). The rats were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in phosphate-buffered. The brains were postfixed in 4% paraformaldehyde for 12 h, after which they were transferred into 30% sucrose in PBS for at least 16 h at 4°C. Exhaustive sagittal sections of the left hemisphere were made at 40μm using a Microm cryostat (Richard-Allan Scientific, Kalamazoo Michigan) and stored in cryoprotectant at 4°C. For biochemical experiments, animals were deeply anaesthetized with isofluorane before decapitation. The brain was quickly removed and the brain regions were dissected. Hippocampal tissues were dissected from both hemispheres and collected separately. In animals that received treatment, only the hemisphere that received the treatment was used. In naïve animals, both hemispheres were included. Homogenization of tissues was performed using an ultrasonic cell disrupter, in a 1:10 weight/volume of ice cold cell lysis buffer (Cell Signaling Technology, Inc.; Danvers, MA; Cat no. 9803) and phenylmethylsulphonyl fluoride, 1 mm (Sigma, St. Louis, MO). Samples were centrifuged at 21,000 g at 4 °C for 15 min and supernatant was collected. Determination of total protein, using a Bradford protein assay (BIO-RAD Laboratories, Hercules, CA, USA) and an enzyme-linked immunosorbent assay (ELISA) were performed on the same day to avoid repetitive thawing of samples. The ELISA for both rat IL-1β (eBioscience, Inc.; San Diego, CA; cat no. 88-6010-22) and rat FKN (RayBiotech, Inc.; Norcross GA; Cat no. ELR-Fractalkine-001C) were performed using a commercially available kit following the manufacturer’s protocol.

**Real-Time RT-PCR.** Dissected tissues stored at -80°C were used for RNA isolation using RNeasy mini-columns (Qiagen; Cat no. 74104) with on-column DNase treatment (Qiagen; Cat no. 79254) according to the manufacturer’s protocol. RNA quantity was determined and normalized using Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen; Cat no. R11490). Integrity of the RNA was confirmed on an agarose gel assessing the 18s and 28s rRNA bands. Reverse transcription (RT) was done following the manufacturer’s protocol using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Cat no. 4368814). A no template and a no RT control were conducted to control for contamination. qPCR reaction was performed using SYBR® Green PCR Master Mix (Applied Biosystems; Cat no. 4309155) following the manufacturer’s protocol, with the exception of variable annealing temperatures (55°C-65°C) as determined most optimal during primer validation. A melt curve beginning at 55°C and increasing by 0.5°C to 95°C every 10 sec with fluorescence measured at each interval. A single peak in the melt curve was used to check for a single product. A standard curve that covers 3 logs was made of pooled cDNA from all the rats and used each plate to check the efficiency of the reaction as determined by the slope of the standard curve and to assess plate to plate variations. All samples were run in triplicate. The primers that were used included; Rpl19 (NM_031103; sense: AATCGCCAATGCGAACTC; antisense: CACTTCCTCCTTCCCTATGC) as the reference gene, to normalize the expression of
FKN (NM_134455; sense: CGAGTTCTGCTCTACCAATCTG; antisense: GAAGTGGTGACGCTTGAGTAG). Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

**Immunohistochemistry and Immunofluorescence.** Except where specifically indicated, standard staining procedures were conducted on free-floating sections using every sixth section for the entire hippocampus beginning with a random start and including sections before and after the hippocampus to ensure that the entire structure was sampled. The standard staining procedures used began with 0.3% H$_2$O$_2$ solution in 30% methanol to block endogenous peroxidase activity (this step was omitted for immunofluorescence). Sections were blocked in 10% normal serum from the species that secondary antibody was raised in, with the addition of 0.1% Triton X-100. Sections were incubated, with primary antibody diluted in 3% normal serum with 0.1% Triton X-100, overnight at 4°C. For immunohistochemistry biotinylated secondary antibodies were diluted in 3% normal serum with 0.1% Triton X-100 and were incubated for 2 hours at room temperature. For immunofluorescence appropriate secondary antibody conjugated to an Alexafluor probe (Molecular Probes, Eugene, OR) was applied for 2 hours. For immunohistochemistry, enzyme detection was done using avidin-biotin substrate (ABC kit, Vector Laboratories, Burlingame, CA) followed by color development in diaminobenzidine solution (Sigma, St. Louis, MO). For BrdU, sections were pretreated with 50% formamide/2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C for 2 hours, rinsed in 2× SSC, incubated in 2N HCL for 30 minutes at 37°C, and washed with borate buffer (pH 8.5). For IdU and CldU, sections were pretreated 2N HCL for 20 minutes at 37°C followed by a wash in borate buffer (pH 8.5). BrdU was detected using a mouse anti-BrdU (1:100; Roche; Indianapolis IN; Cat no.11 170 376 001, clone BMC 9318). CldU was detected with rat anti-BrdU (Accurate Chemicals, Westbury, NY Cat no OBT003 clone: BU1/75 (ICR1)). For IdU, mouse anti-BrdU (Becton Dickinson Bioscience, San Jose, CA; Cat no.347580, clone B44), was used at a dilution of 1:500. Doublecortin (DCX) is a marker of migrating neurons that is expressed for approximately three weeks after the cell is born and has been shown to be a reliable indicator of neurogenesis(Rao and Shetty, 2004; Couillard-Despres et al., 2005). For DCX immuno-detection, incubation in primary antibody was done for 36 hours at 4°C using a polyclonal goat antibody C-terminus of human DCX (1:200; SC-8066, Santa Cruz biotechnology, Santa Cruz, CA, USA). Ki67 is expressed in cells G1 through M phase of the cell cycle (Scholzen and Gerdes, 2000). For detection of Ki67, a rabbit anti-human Ki67 antibody (NCL-Ki67p; Novocastra Laboratories/Vision BioSystems, Newcastle upon Tyne, UK) was used at a dilution of 1:500. Mature neurons were stained with the marker NeuN (1:100; Chemicon, Temecula, CA). For OX-6 immuno detection a monoclonal antibody directed against the rat major histocompatibility II (MHCII) (RT1B, Becton, Dickinson Pharmingen, San Diego, CA, USA) was used at a concentration of 1:750. For microgliar analysis in mice, two antibodies were used; mouse major histocompatibility II(I-A/I-E, Becton, Dickinson Pharmingen, San Diego, CA, USA;
Cat no. 556999 Clone: M5/114.15.2); and rat anti-mouse CD45 (AbD Serotec; Raleigh, NC; 1:10,000; Clone: YW62.3; Cat no. MCA1031G). Detection of FKN was accomplished using a goat anti-rat polyclonal antibody that recognizes the chemokine domain of FKN at a concentration of 1:100 (R&D Systems; Minneapolis, MN; Cat no. AF537).

**Quantification and imaging:** To determine cell numbers, the optical fractionator method of unbiased stereological cell counting techniques (West et al., 1991) was used with a Nikon Eclipse 600 microscope and quantified using Stereo Investigator software (MicroBrightField, Colchester, VT). Due to the low number of BrdU+, CldU+, Idu+, and DCX+ cells in the aged animals, the virtual grid and counting frame were both 125 μm x 125 μm in order to count all the cells that were present in the section. For all other counts, sampling was optimized to count at least 200 cells per animal with error coefficients less than 0.07. Outlines of the anatomical structures were done using a 10x/0.45 objective and cell quantification was conducted using a 60x/1.40 objective. An Olympus FluoView FV1000 confocal microscope was used for all Immunofluorescence photomicrographs, only linear adjustments (brightness and contrast) were made to the figures. When quantification of percentage of positive cells was determined, Z stacks were created at 1 μm intervals throughout the 40 μm of the sections with a guard region of 2 μm excluded from top and bottom of the Z stack. The Z stacks were rotated in all planes to verify double labeling.

**Statistical analyses:** Data are presented as mean±SEM. Statistical analysis was performed using an unpaired, two-side t-test, or a one-way ANOVA followed by unpaired t-test. Correlations were tested using a Pearson product-moment correlation coefficient. A value of $p<0.05$ was considered to be significant.

**Results**

**CX3CR1-deficient mice have decreased hippocampal NPC proliferation and neurogenesis.** Previous studies suggest that CX3CR1 suppresses the neurotoxic effects of activated microglia (Cardona et al., 2006). However, in the absence of a neurotoxic insult, CX3CR1<sup>GFP/GFP</sup> mice lack any obvious phenotype, and appear to have normal brain development (Jung et al., 2000; Cook et al., 2001; Haskell et al., 2001). To date, no studies have investigated whether loss of CX3CR1 signaling results in changes in adult neurogenesis. To begin to address this issue, the number of DCX+ cells in the subgranular zone (SGZ) and granular cell layer (GCL) of the dentate gyrus in CX3CR1-expressing and deficient mice were quantified using the optical fractionator method of design based stereology. A significant decrease ($t_{(9)}=3.857; p=0.0062$) in the number of DCX+ cells in the CX3CR1<sup>GFP/GFP</sup> mice was found as compared to the CX3CR1<sup>+/GFP</sup> mice (Fig. 10A). To determine if this decrease might be due to reduced proliferation, mice were injected twice (8 hours interval) with BrdU (50mg/kg) and the mice were euthanized on the following day. Quantification of the number of BrdU+ cells revealed a significant decrease ($t_{(13)}=2.513; p=0.026$) in the number of BrdU+ cells in the CX3CR1<sup>GFP/GFP</sup> mice compared to the...
CX3CR1+/GFP mice (Fig. 10B). Figure 1C shows that CX3CR1+ cells (green) were widely distributed in the dentate gyrus, with a typical appearance of ramified microglia. The CX3CR1+ cells in the SGZ had a morphological appearance of microglia and were located in close proximity to the NPC. To determine whether neurons or astrocytes also expressed CX3CR1, sections from CX3CR1+/GFP mice and CX3CR1GFP/GFP mice were stained for GFAP (blue), BrdU (red), and NeuN (Magenta) (Fig. 10D) and for DCX (red) (Fig. 10E-F). Colocalization of GFP with GFAP, NeuN, or DCX was not observed. Colocalization of BrdU and GFP was rarely seen, and most likely represented proliferating microglia and not expression of CX3CR1 on a NPC. Figure 10F-G shows CX3CR1+ cells adjacent to the DCX+ cells, with the CX3CR1+ cell processes interdigitating with the DCX+ cells (Fig. 10F) suggesting an important cell-to-cell regulation of the NPCs and the maturation and survival of the adult born neurons.

**Proliferation of NPCs is decreased by α-CX3CR1 treatment in young but not middle aged or old rats.** To develop a pharmacological model of decreased FKN signaling, a blocking antibody to CX3CR1 was employed. This model allowed for transient loss of signaling as compared to the mouse model that, as a result of the permanent developmental loss of FKN signaling, might evoke compensatory mechanisms. To determine if blocking of CX3CR1 would cause a decreased NPC proliferation and neurogenesis as seen in the mouse model, we infused blocking antibody to CX3CR1 for 7 days via an osmotic minipump to the left lateral ventricle. Young adult rats (3 months old), middle aged rats (12 months old) and old aged rats (22 months old) were injected with BrdU on the 6th day of treatment. The animals were euthanized on the following day and sections of the hippocampus were evaluated for proliferation and short-term survival of the NPCs. Quantification of the number of BrdU+ cells in the SGZ showed a significant decrease ($t(10)=4.688; p=0.0009$) in the number of BrdU+ cells in the α-CX3CR1-treated rats compared to the non-immune IgG-treated animals (Fig. 11A). To confirm the BrdU results, the number of Ki-67+ cells were quantified in the SGZ. A significant decrease ($t(5)=3.596; p=0.0156$) was found in the number of Ki-67+ cells in the α-CX3CR1-treated rats compared to the non-immune IgG-treated animals (Fig. 11B). This was also confirmed by quantification of the number of DCX+ cells. In the young adult rats a significant decrease in the number of DCX+ cells was found following treatment for 7 days with α-CX3CR1 ($t(5)=2.629; p=0.0466$) (Fig. 11C). However no significant differences in the number of BrdU+ cells (Fig. 11A), Ki-67+ cells (Fig. 11B), or in the number of DCX+ cells (Fig. 11C), were found in the middle aged rats or old aged rats following treatment with the blocking antibody.

**FKN reversed the age-related decrease in neurogenesis, but had no effect in young or middle aged rats.** To further investigate whether FKN/CX3CR1 signaling could modulate hippocampal neurogenesis, we treated the three different age groups of rats with 30ng/d of recombinant rat FKN. Following the same protocol used for the α-CX3CR1 study, FKN was continuously infused into the left ventricle by an osmotic minipump for 7 days. In the first
experiment, BrdU was injected 6 days following the beginning of the FKN treatment and the animals were euthanized on day 7 (Fig. 12A). A significant increase ($t_{(10)}=2.639; p=0.0248$; Fig. 12B) in the number of BrdU$^+$ cells in the aged rats treated with FKN was found as compared to the aged rats treated with heat-inactivated (HI)-FKN (Fig. 12A). Quantification of the number of DCX$^+$ cells in the aged rats after 7 days of FKN treatment indicated no significant difference between the two groups (Fig 12C). In the young adult and middle aged rats, FKN treatment did not produce any measurable changes in the number BrdU$^+$ cells (Fig. 13A), or in the number of DCX$^+$ cells (Fig. 13B).

**FKN treatment in aged rats mainly affects proliferation.** To determine whether the disruption in FKN/CX3CR1 signaling induced changes in proliferation or survival of the newly born cells, we used a multiple thymidine analog approach. Both CldU and IdU were used to date new born cells at 2 different time points (see timeline Fig. 12A). FKN was delivered via an osmotic minipump to the left lateral ventricle for 15 days. One day prior to the beginning of the treatment aged rats were injected with CldU (b.i.d.; 42.5 mg/kg; equimolar to BrdU). IdU was injected on day 6 (b.i.d.; 57.5 mg/kg; equimolar to BrdU), allowing an additional 7 days of treatment to measure the effect of treatment on the survival of IdU$^+$ cells. Stereological quantification using an optical fractionator demonstrated no effect of FKN treatment in the number of CldU$^+$ cells (Fig 12D). In contrast, there was a significant increase ($t_{(7)}=3.831; p=0.0065$; Fig. 12E) in the number of IdU$^+$ cells in the FKN-treated rats compared to the HI-FKN-treated rats. By comparing the number of BrdU$^+$ cells in the first experiment to the number of IdU$^+$ cells in the second experiment it is possible to determine if there were combined effects on survival and proliferation following treatment, or if these effects were limited to proliferation. In the aged rats, FKN increased the number of IdU$^+$ cells. Comparison of the number of IdU$^+$ cells to the number of BrdU$^+$ cells, demonstrated that FKN treatment increased the survival by about 18%, but these changes were within 95% confidence interval (FKN 82.7%±16.45%; HI-FKN 64.7%±21.0%; mean±SD). The number of DCX$^+$ cells was quantified to determine if the increased number of BrdU$^+$ and IdU$^+$ cells translated to an increase in DCX$^+$ cells. After 14 days of treatment there was a significant increase ($t_{(8)}=2.945; p=0.0116$; Fig. 12H) in the number of DCX$^+$ cells in the FKN group compared to the HI-FKN group. The increase in the number of IdU$^+$ cells was found to significantly correlate with the number of DCX$^+$ cells (Pearson $r=0.933; p=0.0002$; Fig 12I).

**Expression of FKN in the rat hippocampus.** As we were able to reverse the age-related decline in neurogenesis through the addition of recombinant FKN in the aged rats, we hypothesized that FKN might be altered with age. FKN levels in hippocampal tissue homogenates from young and aged rats were analyzed by ELISA. A significant decrease ($t_{(16)}=4.374; p=0.0005$; Fig. 12A) in the level of FKN protein in the aged rats, compared to the young rats, was evident. However, mRNA levels of FKN were unaltered in the aged rats compared to the young rats (Fig. 13B). We further investigated the protein localization of FKN in the dentate gyrus. Figure 13C
shows a photomicrograph of staining with DAPI (blue), FKN (green), and Tuj1 (red). While FKN was abundantly expressed on the cell bodies of presumable neurons in the GCL, FKN staining was not found on Tuj1+ cells. Moreover, photomicrographs of Ki-67 (red) and FKN (green) (Fig.13D,E) also demonstrated a lack of FKN expression on the proliferating cells. These data indicated that FKN is not expressed on immature neuronal cells; however, it is not clear exactly when FKN begins to be expressed on neurons.

**CX3CR1 blocking antibody increased IL-1β.** To determine whether inhibition of CX3CR1 activity would lead to an increase in IL-1β protein levels, the CX3CR1 blocking antibody was infused in young rats for 28 days via an osmotic minipump. The 28 day time point was chosen to ensure that if there was a difference in IL-1β levels, the difference would be large enough to be easily detected by a standard ELISA. In Figure 15, we found that there was a significant effect in the amount of IL-1β following treatment for 28 days with the CX3CR1 blocking antibody ($F_{(2,16)}=16.89; p=0.0002$). Compared to either the saline treated rats ($t_{(11)}=5.179; p=0.0003$) or the non-immune IgG treated rats ($t_{(7)}=2.630; p=0.0339$) the α-CX3CR1 treated rats had a significant elevation in IL-1β. The saline treated rats and the non-immune IgG treated rats were also significantly different from each other ($t_{(10)}=4.064; p=0.0023$). Nevertheless, the data indicates that the pharmacological antagonism of CX3CR1 leads to increased production of IL-1β.

**IL-1β mediates the effects of α-CX3CR1 treatment.** To determine if the anti-proliferative effects of the CX3CR1 blocking antibody were dependent on IL-1β, young rats were infused with IL-1 receptor antagonist (IL-1Ra) along with either the α-CX3CR1 or non-immune IgG, following the same 7 day protocol as described earlier. A One-Way ANOVA revealed a significant effect ($F_{(3,17)}=5.476; p=0.0081$) in the number of BrdU+ cells (Fig.16A). To determine if the addition of IL-1Ra could have an effect on NPC proliferation a comparison was made between the non-immune IgG groups that received an active IL-1Ra or a heat-inactivated (HI)-IL1Ra. No difference was found as a result of IL-1Ra, suggesting that antagonizing the effects of IL-1β in the control rats that received the non-immune IgG did not alter proliferation (IgG+IL-1Ra n=6; IgG+HI-IL1Ra n=4). The next comparison was made between the rats that received a heat-inactivated IL-1Ra along with the either the α-CX3CR1 or IgG. A significant decrease ($t_{(7)}=3.499; p=0.010$) was found in the number of BrdU+ cells in the rats that received the α-CX3CR1 compared to the IgG group (α-CX3CR1+HI-IL-1Ra 4236±422.6 n=5). The results of this comparison replicate the findings in Fig.11B, demonstrating that without, an active IL-1Ra, treatment with α-CX3CR1 decreases proliferation of NPCs. A significant decrease was also found between the α-CX3CR1+HI-IL1Ra treated group and the non-immune IgG treated group that received the active IL-1Ra ($t_{(9)}=3.373; p=0.0082$). Finally, to determine if the effects of the blocking antibody were mediated through IL-1β we compared the rats that received the blocking antibody along with the active or inactive IL-1Ra. In the α-CX3CR1 treated rats the addition of IL-1Ra was able to prevent the decrease in proliferation that occurred following treatment with the α-CX3CR1. There was a significant
The difference in the number of BrdU+ cells ($t(9)=4.220; p=0.0022$); such that, the rats which received the blocking antibody with the inactive IL-1Ra had fewer cells than the rats that received the blocking antibody and an active IL-1Ra ($\alpha$-CX3CR1+IL1Ra n=6; $\alpha$-CX3CR1 HI-IL-1Ra n=5). The effects on proliferation following treatment with $\alpha$-CX3CR1 appear to be mediated through IL-1$\beta$ as the IL-1Ra was able to completely attenuate the decrease in proliferation following treatment with $\alpha$-CX3CR1.

To determine if IL-1$\beta$ also mediated the decrease in neurogenesis found after treatment with $\alpha$-CX3CR1, the number of DCX$^+$ cells was quantified in the rats that received IL-1Ra along with $\alpha$-CX3CR1 or non-immune IgG. After seven days of treatment a significant effect was found ($F(3,15)=7.615; p=0.0025$)(Fig.16B). To confirm the results from the two earlier experiments in the, CX3CR1$^{GFP/GFP}$ mice, and the initial experiment in rats, that demonstrated that loss of CX3CR1 function caused a decrease in the number of DCX$^+$ cells, a comparison was made between the rats that received the inactive IL-1Ra along with the $\alpha$-CX3CR1 or non-immune IgG. A significant ($t(7)=2.441; p=0.0447$) reduction in the number of DCX$^+$ cells was found in the rats that received the heat-inactivated IL-1Ra+ $\alpha$-CX3CR1 group compared to the matching non-immune IgG group (HI-IL-1Ra+ $\alpha$-CX3CR1 n=5; HI-IL-1Ra+IgG n=4). Rats that received the active IL-1Ra along with $\alpha$-CX3CR1 showed the significant ($t(8)=2.441; p=0.0358$) decrease in the number of DCX$^+$ cells induced by $\alpha$-CX3CR1 was prevented by the addition of IL-1Ra (IL-1Ra+ $\alpha$-CX3CR1 n=5). Rats that received the IL-1Ra with $\alpha$-CX3CR1 were not significantly different than the IgG+HI-IL-1Ra group with respect to the number of DCX$^+$ cells. The results demonstrate that the decrease in DCX$^+$ cells following blocking antibody treatment was mediated through IL-1$\beta$.

An unexpected finding was the physiological role of IL-1$\beta$ in regulating neurogenesis. When the IL-Ra was given to the non-immune IgG group (IL-1Ra+IgG n=5) a significant reduction in the number of DCX$^+$ cells was found compared to the non-immune IgG group that received the heat-inactivated IL-1Ra ($t(7)=6.573; p=0.0003$). As there was not a significant decrease in proliferation in the IL-1Ra+IgG group the DCX data suggests that a physiological level of IL-1$\beta$ is important for the survival of the DCX$^+$ cells, a similar finding was previously reported (Spulber et al., 2008). The number of BrdU$^+$ cells (green) that were also DCX$^+$ (red) were quantified (Fig.16C), and no differences in the number of BrdU$^+/DCX^+$ cells was found between any of the groups in the percentage of double-labeled cells (81.75%±6.3%).

**CX3CR1 blocking antibody decreased survival of cells born prior to treatment.** After seven days of $\alpha$-CX3CR1 treatment, the number of DCX$^+$ cells was significantly decreased in the young adult rats (Fig.16B). The decrease in DCX$^+$ cells could be due to decreased proliferation, as measured at day six (Fig.16A). Alternatively, treatment with $\alpha$-CX3CR1 could also decrease the survival of the immature neurons leading to a decrease in DCX$^+$ cells. To determine if $\alpha$-CX3CR1 treatment affected survival of cells born prior to treatment, CldU was injected one day before the beginning of the 14 days of infusion of non-immune IgG or $\alpha$-CX3CR1. Figure 2A
shows the different time points that were compared. Quantification of the number of CldU+ cells demonstrated a significant decrease ($t_{(7)}=3.566; p=0.0091$; Fig. 16D) in the number of CldU+ cells. After 14 days of treatment, in the rats that received α-CX3CR1 fewer of the cells that were born the day before treatment began survived compared to the non-immune IgG group. Figure 17 shows CldU+/NeuN+ cells in the non-immune IgG group (Fig.17A) and α-CX3CR1 group (Fig.17B).

The preceding experiments demonstrated that α-CX3CR1 treatment decreased proliferation of NPCs. To determine if α-CX3CR1 treatment could alter the survival of cells born during the treatment, IdU was injected on day six of a 14 day of treatment. Day six was chosen so that a comparison could be made between the number of IdU+ cells and BrdU+ cells quantification from the earlier experiment (Fig.16A). In the α-CX3CR1 treatment group significantly ($t_{(7)}=2.506; p=0.0406$; Fig. 16E) fewer IdU+ cells were found compared to the non-immune IgG. To determine if there was an effect on survival or if the effects were limited to proliferation, the number of BrdU+ cells were compared to the number of IdU+ cells. Comparisons of BrdU+ to IdU+ revealed no difference between the treatment groups (α-CX3CR1 49.6%±10.11%; IgG 50.7%±7.9%; mean ± SD), demonstrating that the decrease in the number of IdU+ cells was due to a decrease in proliferation and not survival. A decrease in survival was seen in the cells born prior to treatment. These results suggest, that following treatment with α-CX3CR1, the niche environment becomes unfavorable for the survival of the new born cells, and the cells born before the change in environment die. After the change in environment, there is a decrease in proliferation but not survival as the number of cells born is limited to what the environment can support. The changes in the niche environment translated to a decrease in neurogenesis in the α-CX3CR1 group. In rats that were treated for 14 days, a significant decrease ($t_{(7)}=2.690; p=0.0311$; Fig. 16F) was found in the number of DCX+ cells in the α-CX3CR1 group compared to the non-immune IgG treated group.

**FKN is necessary to maintain microglia in an unactivated state.** FKN/CX3CR1 has been proposed to maintain microglia in a quiescent resting state. The expression of MHC Class II on microglia is induced when the cell becomes activated. To determine if microglia became activated following the CX3CR1 blocking antibody treatment, the number of cells expressing MHC Class II was quantified using the marker OX-6. In the young adult rats the entire hippocampus was used as the region of interest in order to sample a large enough population of cells, due to the few OX-6+ cells in young adult control rats. Following 7 days ($F_{(3,18)}=3.644; p=0.0326$; Fig.18A) or 15 days ($t_{(8)}=2.653; p=0.0291$; Fig.18B) of treatment there was a significant effect in the number of OX-6+ cells. At 7 days, the group of rats that were treated with the α-CX3CR1 and the heat-inactivated IL-1Ra (HI-IL-1Ra+ α-CX3CR1; n=5) had significantly more OX-6+ cells compared to the non-immune IgG treated rats with either the inactive IL-1Ra (HI-IL-1Ra+IgG; n=5) ($t_{(8)}=2.591; p=0.0321$) or active IL-1Ra (IL-1Ra+IgG; n=5) ($t_{(8)}=2.412; p=0.0423$). The group
that received the α-CX3CR1 blocking antibody along with the active IL-1Ra was not significantly different from any of the other groups (IL-1Ra+ α-CX3CR1; n=6). At 15 days a similar significant increase in the number OX-6+ cells was found in the α-CX3CR1 treated group compared to the non-immune IgG treated group.

Aging is associated with increased activation of microglia. We hypothesized that a portion of the microglia activation might be a result of a decreased inhibitory signaling by FKN. To test this hypothesis, we quantified the number of OX-6+ cells in SGZ/GCL only. In the aged rats there was a large enough population of OX-6+ cells in this region of interest to sample adequate number of cells following treatment with FKN or HI-FKN. After 14 days (Exp.2) of FKN treatment, a significant decrease in the number of activated OX-6+ cells was found ($t(8)=3.030; p=0.0163$; Fig.18C) in the FKN group compared to the HI-FKN group. Previous studies have shown a negative correlation between the number of activated microglia and the amount of new cells that are born (Ekdahl et al., 2003; Bachstetter et al., 2008); a correlation analysis was conducted to determine if similar effect was observed following FKN treatment. In the aged rats, after 14 days of treatment with FKN, the number of OX-6+ cells was found to significantly negatively correlate ($r(9)=-0.7425; p=0.0219$; Fig.18D) with the number of IdU+ cells (Exp.2; Fig.12E). We did not find a significant correlation between the number of CldU+ cells or DCX+ cells and the number of OX-6+ cells (data not shown). Furthermore, in aged rats treated for 7 days with FKN we did not find any differences in the number of OX-6+ cells (data not shown).

**Discussion**

Two questions were addressed by the present study: Is CX3CR1/FKN signaling important for maintaining adult hippocampal neurogenesis? Could a disruption in CX3CR1/FKN signaling contribute to the age-related decline in neurogenesis? We demonstrated three main findings: first, loss of function of CX3CR1 in young adult rodents, mice and rats, resulted in a significant decrease in hippocampal neurogenesis; second, administration of exogenous FKN reversed the decline in neurogenesis associated with aging; third, IL-1Ra protected against the decrease in hippocampal neurogenesis induced by blocking CX3CR1 function.

Our results suggest that neurons which are the major producers of FKN, and microglia which express CX3CR1 are actively involved in a cross-talk to regulate the production of new neurons. During development the expression of FKN in the brain has been shown to increase nearly 10 fold in four week old mice compared to one day old mice (Labrada et al., 2002). In our study, we found that FKN expression was absent on immature neurons, suggesting that FKN might be important in protecting mature neurons from the consequences of overactive microglia. It is also possible that mature neurons, through an indirect mechanism, could communicate with microglia to regulate the addition of new neurons into the mature circuit. This could occur via IL-1β decreasing the proliferation of the NPCs. Furthermore, FKN might also be involved in the removal of apoptotic cells, as FKN has been shown to enhance phagocytosis of apoptotic cells (Fuller and
Van Eldik, 2008). Removal of apoptotic cells is an important mechanism to make room for new cells to be added, and to prevent secondary necrosis of the apoptotic cell which occurs if dead cells are not quickly removed.

FKN is anchored to the cell membrane, but can be cleaved off the membrane by metalloproteinase 10 (ADAM10) (Hundhausen et al., 2003) or by TNF-a converting enzyme (TACE / ADAM17)(Garton et al., 2001). FKN signaling, when decreased beyond a physiological level, as in the case of young rats treated with α-CX3CR1, was observed to decrease neurogenesis. On the other hand, when FKN signaling was already decreased, as we observed in the aged control rats, a further loss did not affect neurogenesis. Similarly in the young control rats, in which FKN levels are normal, addition of exogenous FKN did not alter neurogenesis. However, our current study doesn’t address if the membrane or shed form of FKN might have unique roles in regulating neurogenesis. Therefore, future studies are warranted to discern if there are different mechanisms of actions produced by the different forms of FKN.

CX3CR1/FKN signaling is proposed to keep microglia in a non-proinflammatory state (Cardona et al., 2006), as inhibition of FKN/CX3CR1 function has been shown to increase microglial activation and increase production of TNFα and IL-1β (Zujovic et al., 2000; Mizuno et al., 2003; Cardona et al., 2006). We found that inhibition of FKN/CX3CR1 function increased microglia activation. This finding is in agreement with our hypothesis that disruption of CX3CR1 function leads to an increase in microglia activation, which could be ultimately responsible in negatively regulating neurogenesis. However, we did not find increased microglia activation as measured by MHC-II expression, in the CX3CR1<sup>GFP/GFP</sup> mice compared to the heterozygote littermates (data not shown). A possible explanation could be that permanent loss of CX3CR1 since birth results in compensatory changes in the expression of cell surface markers of microglia activation. An additional discrepancy was found in the aged rats treated with FKN for 7 days, where we saw an increase in proliferation but no changes in the number of MHC-II<sup>+</sup> cells, or DCX<sup>+</sup> cells. Thus, it is possible that FKN does not exert its effect directly through microglia. Alternatively, alterations in MHC-II expression may not be the best indicator of the activation state of microglia induced by alteration in FKN/CX3CR1 axis. This may be because MHC-II expression can occur in activated microglia which can be classically activated to produce pro-inflammatory cytokines, as well as in microglia that are alternatively activated to produce growth factor and anti-inflammatory cytokines. However after 14 days of FKN treatment there was a significant reduction in the number of MHC class II<sup>+</sup> cells. Furthermore, we found a negative correlation between the number of MHC-II<sup>+</sup> cells with the number of IdU<sup>+</sup> cells. While we cannot rule out the possibility that other pathways are involved in the effects observed in our study, our data in aged rats strongly indicate that FKN/CX3CR1 suppression of microglia activation, at least in part, modulates hippocampal neurogenesis in aged rats.

Aging is associated with chronically elevated levels of IL-1β in the hippocampus (Gemma
IL-1β has been shown to act directly at the NPC via the IL-1R1 to block cell cycle progression and thereby decrease proliferation (Koo and Duman, 2008). Moreover, we have recently shown that reducing the levels of IL-1β in aged rats is able to reverse some of the age-related decreases in neurogenesis (Gemma et al., 2007b). CX3CR1 regulates the PI3K pathway in microglia resulting in inhibiting the production of IL-1β (Re and Przedborski, 2006). We found disruption of FKN signaling by a blocking antibody to the FKN receptor CX3CR1 caused an increased production of IL-1β. In the young adult rats we found that IL-1Ra completely reversed the decrease in proliferation and neurogenesis that resulted after blocking CX3CR1, suggesting that the effects of FKN on NPC proliferation are mediated through inhibition of IL-1β.

Several studies have shown that FKN can have direct effects on neurons in vitro (Meucci et al., 1998; Meucci et al., 2000; Tong et al., 2000). Using the GFP expression in the CX3CR1+/GFP mice we found that the receptor for CX3CR1 was not found on neurons in the GCL, which confirmed earlier findings in vivo that found CX3CR1 expression only in microglia (Harrison et al., 1998; Jung et al., 2000; Cardona et al., 2006). Moreover, it has been recently shown that the survival effects of FKN on primary neuron al culture was dependent on microglia (Lauro et al., 2008). These results suggest that FKN acts via the microglia expressed CX3CR1 to regulate IL-1β which then acts on the NPCs and neurons.

The second major finding of this study is an age-related disruption of FKN/CX3CR1 signaling. Cardona et al. (06) demonstrated in a number of models of neurodegeneration that loss of neuron-microglia interactions by disruption of FKN/CX3CR1 signaling results in increased microglia neurotoxicity and an associated worsening in disease pathology. It is unclear if the disregulation of FKN signaling observed in our study is a cause or consequence of the increased activation of microglia and neuroinflammation that occurs as a result of normal aging. Levels of FKN in aged control rats were decreased in the hippocampus compared to young adult rats. The lack of alteration in FKN mRNA suggests that post-translational changes are responsible for the age-related decrease in FKN. Therefore, as a result of normal aging, the alterations in FKN/CX3CR1 signaling are mostly likely a result of changes in ligand levels or post-translational processing and not alterations to the receptor as administration of exogenous FKN restored the age-related loss in neurogenesis. Age-related changes in the FKN/CX3CR1 axis have been characterized in other scenarios. There are at least two common single nucleotide polymorphisms in the promoter region of CX3CR1 which cause reduced CX3CR1 function, including decreased adhesion, signaling, and chemotaxis of CX3CR1+ cells. These polymorphisms have been associated with reduced risk for atherosclerosis (McDermott et al., 2003) and increased risk of age-related macular degeneration (Tuo et al., 2004; Combadiere et al., 2007). Moreover, our results are in agreement with data obtained in Alzheimer’s disease patients in which lower levels of soluble plasma FKN were correlated with lower mini-mental
status examination score (Kim et al., 2008). Additionally, APP transgenic mice showed a decrease in neuronal levels of FKN at 9 months of age (Duan et al., 2008).

Conclusion

Microglia have been demonstrated to be both pro and anti-neurogenic depending upon their activation state. This study demonstrates that neurons may actively regulate microglia in the neurogenic niche, and are not necessarily passive actors to the effects of microglia. However with age, the dialog between neuron and microglia via FKN appears to be disrupted, but can be re-established through the addition of recombinant FKN. Inflammation is believed to be a contributing factor to the pathogenesis of a number of neurodegenerative diseases, many of which are age-related, including: Alzheimer's disease, Parkinson's disease, and age-related macular degeneration. Understanding the mechanism by which age-related alterations in the inflammatory response contribute to the progression of the aforementioned neurodegenerative diseases is key to developing therapeutic interventions for the age-related neurodegenerative condition.
Figure 9: CX3CR1\textsuperscript{GFP/GFP} mice have diminished hippocampal neurogenesis. (A) Unbiased stereology revealed a significant decrease (p=0.0062) in the number of DCX\textsuperscript{+} cells in the hippocampus of adult male CX3CR1\textsuperscript{GFP/GFP} mice (n=5) compared to heterozygote control (n=4). (B) Quantification of the number of cells that were proliferating during the proceeding 24 hours, as determined by the incorporation of BrdU, was significantly fewer (p=0.026) in the CX3CR1\textsuperscript{GFP/GFP} mice (n=9) compared to control (n=6). (C) CX3CR1 (GFP) and dapi (blue), demonstrate the localization of the CX3CR1 cells in the dentate gyrus. (D) Localization of CX3CR1 (GFP) cells was not found in NeuN\textsuperscript{+} cells (magenta) or in GFAP\textsuperscript{+} cells (blue), and only rarely in BrdU\textsuperscript{+} cells (red). (E). Low power photomicrograph of DCX\textsuperscript{+} cells (red) and CX3CR1 (GFP) cells are also shown in higher power in maximum projection of confocal z-stack (F) Arrow indicated CX3CR1 (GFP) cells that are in close proximity to the DCX cells.
Figure 10: Proliferation is decreased by α-CX₃CR1. Three ages of male F344 rats were treated with α-CX₃CR1 or the IgG control for 7 days via an osmotic minipump to the left lateral ventricle. On day 6 of treatment BrdU was injected twice 8 hours apart. The animals were sacrificed on day 7. (A) Quantification of the number of BrdU+ cells in the subgranular zone, in the young adult rats revealed a significant decrease (**p=0.0009) in the number of adult young rats following α-CX₃CR1 (n=6) compared to IgG control (n=6). In the middle aged rats and the aged rats there was no significant differences in the number of BrdU+ cells in the IgG control group compared to the α-CX₃CR1 treated group. (B) The decrease in BrdU+ cells in the young rats treated with α-CX₃CR1 (n=4) compared to IgG control (n=3) was confirmed by Ki-67, as a significant decrease was also found in the number of Ki-67+ cells (*p=0.0156). Confirming the BrdU data, in the aged rats no difference in the number of Ki-67+ cells was found. (C) The number of DCX+ cells was also found to be significantly decreased (*p=0.0466) in the young adult rats following α-CX₃CR1 treatment (α-CX₃CR1 n=3; IgG n=4), but no effect was found in the middle aged rats or old aged.
Figure 11: FKN reverses the age-related decrease in neurogenesis.

(A) Timeline: In experiment 1 treatment lasted for 7 days, with injections of BrdU occurring at day 6. In experiment 2, treatment lasted for 14 days, with injections of CldU occurring at day -1 and injections of IdU occurring at day 6. BrdU was used to study the affects of the treatment on proliferation of hippocampal NPC. CldU was used to study the affects of treatment on the cells born prior to the treatment. IdU was used to study the affects of survival of the cells born after treatment and to make direct comparisons with BrdU data. (B) A significant increase (p=0.0248) in proliferation (FKN n=6; HI-FKN n=6) but not in the number of DCX+ cells (FKN n=4; HI-FKN n=6) (C) was found in the aged treated with FKN for 7 days. (D) In the second experiment, in the cells born before treatment began (labeled with CldU) and lived for 15 days no difference was found between groups (FKN n=5; HI-FKN n=4). When the cells were labeled at the same time point as BrdU (B) with IdU a significant increase (p=0.0065; E) in number of IDU+ cells was found in the FKN treated group (FKN n=5; HI-FKN n=4). (H) which appeared to translated to the significant increase (p=0.0116) in the DCX+ as the two makers were strongly correlated (r=0.933; p=0.0002) (I).
Figure 12: FKN exerts proliferative effects in aged rats. Rats were injected with BrdU on day 6 of a 7 day treated of FKN (30ng/d) or a heat-inactivated (HI)-FKN control. (A). No differences were found in the number of BrdU+ cells in the young or middle aged animals between treatment groups. As shown in figure 2B, in the aged rats a significant increase in the number of Brdu+ cells was found (p=0.0248) in the FKN treated rats compare to the control rats. (B) Quantification of DCX+ cells demonstrated an absence of a treatment effect in all three ages after 7 days of treatment. BrdU and DCX data from the 22 mo old rats was also presented in Fig 2B and 2C respectively, and was re-presented in the supplementary data for comparison purposes.
Figure 13: Expression of FKN in the hippocampus. Quantification of protein (A) and mRNA (B) levels of FKN in the hippocampus of young adult and aged rat, demonstrated a significant decrease in protein levels of FKN (p=0.0005) (n=9 per group), but not in mRNA levels (n=6 per group). (C) FKN expression (green) was not found on Tuj1+ (red) cells, but was expressed on the majority of the cells (dapi: blue) in the GCL. (D) and (E), shows low and high magnification respectively, of Ki-67 staining (red) and FKN staining (green), with dapi (blue). Similar to the Tuj1+ cells, the Ki-67+ cells also lack FKN expression.
Figure 14: CX3CR1 blocking antibody increases hippocampal IL-1β levels. In 3 month old male rats treated for 28 days with the blocking antibody we found a significant increase in IL-1β protein levels compared to non-immune IgG or Saline control animals. (††p=0.0023 Saline vs. IgG) (***p=0.0003 saline vs. α-CX3CR1) (*p=0.039 IgG vs. α-CX3CR1) (Saline n=8; IgG n=4; a-CX3CR1 n=5).
Figure 15: IL-1Ra reverses the effects of α-CX3CR1. To determine if the decrease neurogenesis caused by blocking antibody to α-CX3CR1 was mediated by IL-1β we infused IL-1Ra along with the blocking antibody for 7 days of treatment. On day 6, young adults rat were injected with BrdU. Gray bars are the groups that received heat-inactivated IL-1Ra. White bars are groups that received active IL-1Ra. (A) In the α-CX3CR1/HI-IL-1ra group, there was significantly fewer BrdU+ cells than the three other groups which were not different from each other. (B) IL-1ra blocked the decrease in DCX+ cells caused by α-CX3CR1. In the non-immune IgG group IL-1Ra (white) caused a significant decrease in the number of DCX+ cells compared to the IL-1Ra inactive control (grey). (C) A representative photomicrograph of the BrdU (green) and DCX (red) double labeling. Following the 14 day infusion paradigm (see Fig.3A), (D) we found a significant ($p=0.0091$) decrease in the number of CldU+ cells which were born the day before we started infusion in the α-CX3CR1 (n=4) compared to the non-immune IgG group (n=5). (E) In the 14 day experiment, a significant decrease ($p=0.0406$) was also found in the number of IdU+ cells in the α-CX3CR1 (n=4) compared to the non-immune IgG group (n=5). (F) Quantification of the number of DCX+ cells also demonstrated a significant ($p=0.0311$) decrease following treatment in the α-CX3CR1 (n=4) compared to the non-immune IgG group (n=5).
Figure 16: CldU colocalizes with NeuN. CldU⁺ cells (red) were found to co-localize with NeuN⁺ cells (green) in the IgG treated group (A) and α-CX₃CR1 treated group (B).
Figure 17: FKN signaling regulates microglia activation. Quantification of the number of OX-6+ cell, which is a marker for MHC class II, (A) we found in the rats that received the blocking antibody with an inactive IL-1Ra (gray) a significant increase in the number of OX-6+ cells compared to the two non-immune IgG groups. (B) After 15 days of blocking antibody treatment in the young rats a similar significant increase in the number of OX-6+ cells was found in the young α-CX3CR1 treated rats (n=5) compared to the non-immune IgG treated rats (n=5). (C) In the aged rats after 14 days of treatment with FKN a significant decreased (p=0.0163) the number of OX-6+ cells was found in the α-CX3CR1 treated rats (n=6) compared to the non-immune IgG treated rats (n=4). (D) The number of OX-6+ cells was also found to significantly correlated with the number of IDU+ cells (r(9)=-0.7425; p=0.0219)
Conclusions: The Role of neuroinflammation in regulating the age-related decline in neurogenesis: could restoring the balance rescue neural plasticity

Introduction

Deteriorations in cognitive function occur as a consequence of growing old in the absence of clear neurological damage or disease. Aging is the background in which many neurodegenerative diseases manifest; therefore, in understanding of the age-related alterations that occur in the central nervous system (CNS) are an important consideration when developing therapeutic interventions for age-related neurodegenerative diseases. Age-associated memory impairments, should not be considered unavoidable. On it’s on merit, strategies for ‘successful’ aging’ are worthy of scientific investigation.

The hippocampus is critical structure for normal memory functions. Alterations in neural plasticity, not cell loss, appear to be responsible for the age-related alterations in hippocampal dependent cognitive function. There are numerous subtle age-related alterations in neural plasticity (for review see: (Burke and Barnes, 2006)). Adult neurogenesis is one form of neural plasticity that is dramatically limited with age.

Adult neurogenesis

The majority of the neurons in the adult central nervous system (CNS) are postmitotic. In the absence of pathology, neurons are believed to remain postmitotic, and survive thought the lifespan of the organism. However, an ongoing neurogenesis continues to occur in two germinal centers of the CNS: the subventricular zone (SVZ), and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus. Adult neurogenesis is a process that involves a continuum of developmental stages. The final result of the process is that a few newly born cells are added to the existing neuronal circuitry. Adult hippocampal neurogenesis occurs in a region called the SGZ, which is roughly defined as a two cell diameter band on the hilus side of the granule cell layer (GCL). Proliferation of the neural stem/progenitor cells (NPCs) produces a pool of immature cells, the majority of which will become neurons. Following the generation of the daughter cell, the postmitotic cell goes through an early survival phase. During this phase the number of surviving neurons can vary greatly depending on the strain of animals used and can be
as great as ~ 75% or as few as 25% of the amount of proliferating cells (Kempermann et al., 1997c). Moreover, in the young adult rodent, most of the regulation of neurogenesis occurs in the early survival phase and not in the proliferative phase (Kempermann et al., 2006). The newly born cells the majority of which do become neurons, go through a migratory phase (Kempermann et al., 2003b), which occurs while the cells are becoming functionally mature. The adult-born neurons become morphologically and physiologically fully mature and indistinguishable by 6 to 8 weeks after birth in the young adult rodent (van Praag et al., 2002; Esposito et al., 2005; Zhao et al., 2006; Toni et al., 2008). Finally, an additional phase of neurogenesis appears to be the eventual death of the cell, as a continual turnover of cells both mature and immature is necessary to ‘make room’ for the addition of more cells.

**Adult neurogenesis and memory are they related?**

The addition of new neurons into the hippocampus, a region of the brain important for learning and memory, has elicited interest in understanding if and how the addition of new neurons would contribute to cognitive function (Leuner et al., 2006; Aimone et al., 2009). However, the involvement of neurogenesis, in cognitive function is complex. Correlations, between genetic and environmental alterations, were initially used to justify the importance of neurogenesis in cognitive function. For example environmental enrichment (EE) has been shown to enhance memory and increase neurogenesis (Kempermann et al., 1997b). However, following irradiation to block neurogenesis, the behavior improvements of EE on spatial learning in the Morris water maze (MWM) was found to be independent of neurogenesis (Meshi et al., 2006). In a subsequent experiment that used irradiation to block neurogenesis without EE, blocking neurogenesis was found to impair contextual fear conditioning with no effect on MWM (Saxe et al., 2006). Using an inducible mouse model in which NPC are selectively eliminated by over-expressing the pro-apoptotic protein Bax only in nestin expressing cells found that neurogenesis was required for MWM but nor for contextual fear conditioning (Dupret et al., 2008).

Often the contradictions in fact support the role of neurogenesis in cognition. A recent study by Dupret et al (2007) demonstrated that not only is the addition of new neurons into the hippocampal circuit important for cognition but so is the removal of granule cells important for cognition (Dupret et al., 2007). Like the hippocampus, several thousand newly generated neurons are added to the olfactory bulb (OB) (Lledo and Lagier, 2006). In the OB, learning of olfactory information also appears to be coupled to the survival of some newly generated neurons and the removal of others (Mouret et al., 2008).

Despite the often contradictory findings, numerous studies have shown that the de novo production of neurons in the hippocampus is physiologically relevant for cognitive function (For review see: (Leuner et al., 2006; Drapeau and Nora Abrous, 2008)). From the numerous studies that have been done the involvement of neurogenesis in cognitive function can be classified into 3 groups: 1) effects on proliferation, 2) effects on survival, and 3) effects on cell death. It appears
that alterations in one or more of these 3 aspects of neurogenesis can impact cognition.

**Neurogenesis and aging: is neurogenesis involved in age-related cognitive decline.**

Neurogenesis appears to continue throughout the lifespan, however studies in rodents describe a nearly linear decline in neurogenesis as a function of age. Despite the thousands of new neurons that are born every day in the young adult hippocampus, with age a greater then 40 fold decrease can be seen in the number of new neurons that are added into the aged hippocampus. A peak in neurogenesis occurs during adolescents, after which point, there is a continuous decline in neurogenesis until senescence when very few new cells are added to the aged brain (Ben Abdallah et al., 2008). The decline in neurogenesis has led to speculation concerning the contribution of neurogenesis to the impairments in cognitive function that occur during normal aging? A troubling issue arises in that the decline in neurogenesis well precedes any age-related cognitive decline. The largest decrease in neurogenesis occurs between 7.5 months and 12 months of age in rats (Rao et al., 2006). A threshold hypothesis has been presented to account for this discrepancy. The basic concept of the hypothesis is that the decrease in neurogenesis with age does not impact cognition until the level of neurogenesis reaches some physiologically minimum number of cells.

Drapeau et al. (2003) addressed this threshold hypothesis by using aged rats that are ‘good’ or ‘bad’ learners. Not all aged rats have cognitive-impairment, at least as measured by the spatial learning task of the Morris water maze (Gallagher et al., 1993). Numerous studies over the years have used the natural variation in cognitive function in aged rats to investigate if there are differences in between the rats that demonstrate impaired cognition compare to the rats whose cognitive performance is equal to that of a young rat. Drapeau et al (2003) exploited these natural variations in cognitive function to ask the question if neurogenesis could be involved in age induced cognitive impairments. The results of their study demonstrated that rats that were defined as being cognitively un-impaired had increased proliferation of the NPC and more neurogenesis then cognitively impaired rats. However, while the cognitively intact rats had more neurogenesis than the impaired rats, the cognitively intact rats still had great deal less neurogenesis then the young rats. Moreover, in the young rats then amount of neurogenesis did not correlated with cognitive performance (Drapeau et al., 2003). The results of Drapeau et al. (2003) were directly contradicted by Bizon et al. (2004) who showed that the aged cognitively impaired rats had more neurogenesis (Bizon et al., 2004). Moreover, other reports have showed that neurogenesis does not correlate with spatial learning (Bizon and Gallagher, 2003; Merrill et al., 2003).

**Where do we put all these new neurons?**

An estimated 9000 new neurons are added each day to the hippocampus (cammeron and Mckay 2001), but the total number of neurons in the hippocampus has been shown to be stable over the entire lifespan, including in elderly. From the first month of life, to one year of age the number of granule cells in the dentate gyrus does increase in rodents (Bayer et al., 1982).
However the increase in size is no where near the amount that would be expected, based upon the number of cells born in the SGZ. Moreover, despite the decrease in neurogenesis with age the total number of granule cells does not decrease in the aged rat. Therefore it appears that tissue homeostasis (total number of granule cells) in the dentate gyrus appears to be tightly regulated. To maintain tissue homeostasis neurogenesis must be coupled to a process of programmed cell death (PCD). This process occurs in tissues thoughtout the body, but until discovery of adult neurogenesis, the central nervous system (CNS) was believed to be exempt from this process. In fact, excluding disease or injury, the neurons we are born with are presumed to be the same neurons that we will die with. However, in the hippocampus and in the SVZ neurons are born every day, requiring the homeostatic death and removal or older neurons in these two neurogenic regions (Biebl et al., 2000).

BCL-2 (B-Cell Leukemia 2) is the prototypical member of the Bcl-2 family of apoptotic regulatory proteins. BCL-2 is an anti-apoptotic protein. The balance between the pro vs anti-apoptotic BCL-2 family members following an apoptotic stimuli determines if the cell lives or dies (Oltvai et al., 1993). Bax and Bak are two of the many members of the pro-apoptotic of the BCL-2 family. Using neuronal cell cultures from Bak\textsuperscript{-/-}Bax\textsuperscript{+/-} mice it was found that NPC require Bak and Bid to induce excitotoxic PCD; whereas, mature neurons do not require Bak and Bid to induce PCD (Lindsten et al., 2003). Sun et al. (04) was the first to investigate the biochemical pathways that regulate the PCD in adult-born neurons, through the use of Bax deficient mice. The Bax deficiency resulted in an absence of apoptosis in the adult DG, without any alterations in the production of new neurons. In the wild type mice the number of neurons in the DG remained stable from 2 months to 12 months of age. In comparison, there was an age-dependent increase in the number of cells in the DG in the Bax deficient mice (Sun et al., 2004). Kuhn et al. (05) replicated the findings of Sun et al. (04) through the overexpression of BCL-2 in transgenic (Tg) mice. The overexpression of BCL-2 reduced by did not completely block apoptosis. The increase in neurogenesis was also not as dramatic as that seen following Bax deficiency. The total number of cells in the GCL was also increased in the BCL-2 Tg mice (Kuhn et al., 2005). The BCL-2 Tg mice have also been shown to have cognitive deficits (Rondi-Reig et al., 2001). These results support the notion that the loss of neurons is important for cognition (Dupret et al., 2007).

Microglia Regulation of cell death in developmental and adult neurogenesis

Microglia are immune cellular component and the professional phagocyte of the brains. Recognition of pathogen associated molecular pattern (PAMPs) and is recognized by microglia pattern-recognition receptors (PRR). The most common PAMP used experimentally, particularly in neuroscience research, is lipopolysaccharide (LPS). LPS a major component of the outer membrane of gram-negative bacteria and is used to induce inflammation in the CNS to understand the involvement of the immunity in a particular condition. LPS can be very useful, as a
way to induce a replicable and specific immune response. However, LPS induces a microbial defense immunity, resulting in the activation of pro-inflammatory pathways, producing inflammatory mediators including cytokines and chemokines. The response to eliminate the 'danger signals' does not discriminate and will also cause damage to the uninfected tissue. Besides the PRRs, microglia also express a number of scavenger receptors that are involved in the recognition and removal of dead cells and other cellular debris; such as Aβ.

In many ways the process of adult neurogenesis recapitulates events during embryonic development. During development of the nervous systems, in excess of neurons are born then ultimately survive into adulthood. (Oppenheim, 1991) It has been hypothesized that during development the excess of cells are produced to allow for a competition between the newly born neurons for trophic support from other neurons and glia (Oppenheim, 1991). The competition for trophic support, results in a proportion of the cells being eliminated. The 'pruning' of the excess cells, presumably by some form of PCD, leaves the cell which received the adequate trophic support to survive and be integrated in the neuronal circuit (Oppenheim, 1991). A similar process is believed to occur during adult neurogenesis as well.

During development the dead cells are recognized and removed phagocytes. In the brain the dead cells are recognized by microglia and are removed (Mallat et al., 2005). The primary 'eat-me' signal expressed by dying cells is phosphatidylycerine (PS) on the outer leaflet of the plasma membrane of the apoptotic cells (Fadok et al., 1992). Severe developmental brain malformations occur if microglia are not able to recognize PS due to genetic deletion of PS receptor (Li et al., 2003). Some the 'eat-me' signals expressed by dying cells are shared with PAMP, and have therefore been called, apoptotic cell associated molecular pattern (ACAMPs)(Savill et al., 2002). While both PAMPs and ACAMPs are recognized by the macrophage through the PRR, the response by the macrophage is distinct. Recognition of a PAMPs by a macrophage promote an inflammatory response; whereas, recognition of an ACAMP inhibits inflammation (Stuart and Ezekowitz, 2005). This is important for the 'silent' removal of the apoptotic cells (Medzhitov, 2008).

To ensure healthy cells are not the victims of over active microglia, healthy cells present an additional class of inhibitory signals which have been called 'don’t eat me' signals or self associated molecular patterns (SAMP). The SAMPs expressed by neurons include CD200, CD47, and fractalkine among others.

Microglia are not only involved the phagocytosis of the apoptotic cells, but also contribute to demise of the apoptotic cells. If the dying cell does not present the 'kill-me' signals, PCD can be stopped and the cells will survive (Hoepnner et al., 2001; Reddien et al., 2001). During development two models have shown that microglia responded to the 'kill-me' signal presented by neurons with a respiratory release of ROS to kill the cells. This has been shown in the cerebellum and the hippocampus (Marin-Teva et al., 2004; Wakselman et al., 2008). In the hippocampus the
process was CD11b and DAP12 mediated (Wakselman et al., 2008). As described earlier, the removal of excess neurons is important to cognitive function as the addition of neurons. Microglia have an important role in the removal (pruning) of excess neurons.

Inflammation and adult neurogenesis

Two seminal studies, published simultaneously, a number of years ago showed that inflammation tightly regulates neurogenesis (Ekdahl et al., 2003; Monje et al., 2003). Ekdahl et al. (03) used LPS that they delivered into the cortex continuously by an osmotic mini pump. After 28 days of LPS there was a dramatic activation of ED-1+ cells. ED-1 (CD68) is a member of the scavenger receptors, which is highly expressed on monocytes and some tissue macrophages. The expression of ED-1 on microglia is normally absent, but can be induced following an inflammatory insult, which is what was found after 28 days of intracortical infusion of LPS. In the young adult rat, LPS-induced inflammation resulted in an 85% reduction in the number of new neurons born during the inflammatory insult (Ekdahl et al., 2003). Monje et al. (03) also found that LPS given systemically also cause in increase in microglia activation and a decrease in neurogenesis, which could be prevented by the nonsteroidal anti-inflammatory drug (NSAID) indomethacin. Toll-like receptor (TLR)-4 is the specific receptor for LPS. Expression of TLR-4 in CNS glial cells was found to be limited to microglia (Lehnardt et al., 2002). NPC and mature neurons have been shown to express TLR-4 (Rolls et al., 2007; Tang et al., 2007); thus, it is possible that the anti-neurogenic effects of LPS could be independent of microglia.

While TLRs (particularly TLR-4) often acts a co-receptor in many immune responses, the immune response to bacteria, virus, or parasite, is quite different then that started from tissue damage. Monje et al (03) found that inflammation was also at least in part responsible for the decrease in neurogenesis after irradiation. Following irradiation there was an increase in microglia activation that could be prevented by treatment with NSAID. The treatment with the NSAID was also able to prevent anti-neurogeneic effects of irradiation. Microglia besides being responsible for protecting the CNS from invading pathogens, are also important for removing cellular debris. Microglia are the professional phagocytes of the brain.

Microglia would appear to be the optimal cell to regulate neurogenesis. Microglia perform 3 key functions that would make them key in regulating neurogenesis. First microglia have the potential to be neurotoxic though the production of reactive oxygen species thereby being involved in the removal of mature or immature cells(Marin-Teva et al., 2004; Wakselman et al., 2008). Microglia are also important for the phagocytosis of the dead cells (Mallat et al., 2005). The second way that microglia can be involved in regulating neurogenesis is through the production of cytokines. This is particularly true for the key innate cytokines IL-1β and TNF-α, with activated microglia being the major source of TNF-α and IL-1β in the CNS (Gebicke-Haerter, 2001)(Shaftel et al., 2008). At low concentrations TNF-α, induced proliferation of NPC, but at higher concentrations TNF-α induced PCD (Bernardino et al., 2008). NPC cultures have been
shown to have constitutively express the TNF-α RI and TNFRII (R. E. Iosif et al., 2006). TNF-α induced apoptosis in the NPC is dependent on TNFRI (Sheng et al., 2005). IL-1β can also directly suppress neurogenesis by blocking the production of cyclic dependent kinesis (Iosif et al., 2006; Koo and Duman, 2008). Inflammation also alters the way the new neurons integrate into the existing neuronal circuit (Jakubs et al., 2008). The third way in which microglia can regulate neurogenesis is by a pro-repair/pro-neurogenic mechanism. Microglia are able to produce a number of growth factors including IGF-1 and BDNF which have been shown to promote neurogenesis (For review see:(Ziv and Schwartz, 2008)).

**Age-related cognitive impairment: is it a numbers game?**

The dramatic decrease in neurogenesis with age has led to much speculation concerning the extent in which the decline in neurogenesis contributes to age-related cognitive impairments. During the 1990’s, when adult neurogenesis was accepted by the scientific community the commonly held view was the total number of newly formed mature neurons would correlate with cognitive performance. To this end, the age-related decline in the total number of new neurons was believe to, at least impart contribute to the cognitive decline that occurs during the course of normal aging. As discussed earlier, the result of Drapeau et al. (2003) suggest the total number of new neurons may indeed be critical for cognitive performance. However, it has become increasingly clear that in the young adult where the addition of new neurons is in abundance, it is not so much that total number of cells that is important, but the experience-dependent regulation of neurogenesis is that is important for certain types of cognitive function. The same dynamic holds true in the aged brain, where rats that are aged-unimpaired demonstrate experiences dependent addition and removal of new neurons (Drapeau et al., 2007).

The primary cause for the decrease in neurogenesis in aging is due to a decrease in proliferation of the NPC (Hattiangady and Shetty, 2008). During development an excess of cells are born with the ‘fittest’ cell surviving. A similar process occurs in young adult, where the majority of the experience-dependent regulation of neurogenesis occurs as a result of survival of the ‘fittest’ cell. If an ‘unfit’ cell is forced to survive by inhibiting PCD memory has been shown to be impaired (Dupret et al., 2007). What about with age where the majority of the regulation of the number of new neurons occurs at the proliferation stage and not at the survival phase? Could the change at which point neurogenesis is regulated be responsible for the age-related cognitive decline? With fewer cells, there is less competition, which could allow the survival of an ‘unfit’ cell. There is no direct experimental evidence to support or reject either hypothesis. However, Bizon et al. (2004) found that aged-impaired rats had more new neurons. The results of Bizon et al. (2004) has led to the suggestion that neurogenesis is not involved in neurogenesis. However, an alternative hypothesis of the findings of Bizon et al. (2004) could be that in the aged-impaired rats ‘unfit’ cells survive. The survival of ‘unfit’ cells could be a compensatory mechanism in the aged impaired rats to overcome for losses in other forms of synaptic plasticity. Alternatively, the aged-
impaired rats may be deficient in the removal of ‘unfit’ cells resulting in abnormal connections that impair memory.

**Conclusion**

It is known that with aging and age-related neurodegenerative diseases, there is a state of chronic inflammation. The cause of the chronic inflammation in aging is not clear. However, the age-related increase inflammation has been shown to impair neurogenesis by decreasing proliferation, survival, and integration of new born neurons. The experience dependent, integration and removal of neurons, appears to be important for cognitive attributes of neurogenesis (Aimone et al., 2009). It is plausible, that with age inflammation alters the ability of the new neurons to properly integrate into the existing neuronal circuit in an experience-dependent manner. The total number of new neurons may not be the best predictor of the involvement of neurogenesis in age-related cognitive impairment. Alternatively, the ability of the new neuron to be regulated in an experience-dependent manner appears most important for the neural plasticity afforded by neurogenesis. The previous three chapters, demonstrated, that inflammation does contribute to the age-related decline in neurogenesis. Figure 19 demonstrates a cartoon summary of the findings of the previous three chapters. In the future, it would be informative to determine if restoring the inflammatory balance in humans could forestall age-related cognitive impairments.
Figure 18: How neuroinflammation can modulate adult hippocampal neurogenesis.
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