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Effects of G-CSF on Monocytes and Neurons: in vitro and in vivo studies in a Mouse Model of Alzheimer's Disease

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Effects of G-CSF on Monocytes and Neurons: *in vitro* and *in vivo* studies

in a Mouse Model of Alzheimer's Disease

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of
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Concentration Neuroscience
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**Abstract**

G-CSF is routinely used to treat neutropenia/leukopenia or to increase hematopoietic stem cell generation in bone marrow donors. G-CSF and its receptor, G-CSFR, are produced by various cell types both in the peripheral circulation and within brain. As a consequence, exogenous administration of G-CSF results in a broad spectrum of effects involving hematopoietic, immune and central nervous systems.

G-CSF administration in a mouse model of Alzheimer’s disease (AD) has revealed both cognitive benefits and disease modifying effects: a) decreased Aβ plaque burden, b) increased microgliosis, c) increased neurogenesis and d) improved performance in radial arm water maze (RAWM). In clinical studies, G-CSF plasma levels were found to be lower in patients with early AD in comparison to healthy age matched controls. A course of G-CSF administration in humans is known to increase levels of circulating hematopoietic stem cells (CD34 cells), monocytes and neutrophils in patients with neutropenia and when administered to patients with AD, there is also a similar increase in absolute monocyte count, CD34 cells and total neutrophils. The extent to which the beneficial effects of G-CSF in AD depend on monocyte infiltration into CNS, compared to direct neurotrophic actions of G-CSF on the CNS, is not known.

The overall goal of this study was to investigate and understand the effects of G-CSF in an AD mouse model, but more specifically to distinguish the
actions of G-CSF that affect the peripheral monocyte population from the direct actions on CNS. The first approach was to examine in vitro effects of G-CSF within a monocytic cell line (THP-1) and a neuronal cell line (SH-SY5Y). The second approach was to study effects of G-CSF on infiltration of bone marrow-derived cells into the brain by utilizing a chimeric GFP+ APP/PS1 AD mouse model. The third approach was to assess the effects of G-CSF on hippocampal neurogenesis in both a wild-type and AD mouse model.

Comparison of the monocytic and neuronal cell lines showed a) G-CSF interacts with its cognate receptor with different binding kinetics and with a greater affinity for the monocyte G-CSFR, b) the number of G-CSF receptors in neurons is greater than in monocytes, and c) the anti-apoptotic response in neurons occurs at lower concentrations of G-CSF than in monocytes. Various concentrations of G-CSF increased proliferation of both the monocytic and neuronal cell line in vitro. G-CSF did not improve migratory properties of the monocytic cell line, either adhesiveness or migration through a membrane.

In vivo G-CSF treatment (250μg/kg s.c. qd for 2 ½ weeks) in both the AD chimeric and non-chimeric AD mice resulted in increased microgliosis and decreased amyloid plaque burden in the hippocampus. In the chimeric AD mice, G-CSF treatment did not increase infiltration of GFP+ bone marrow derived cells (BMDC) into brain parenchyma and did not increase adhesion to microvasculature. In the non-chimeric AD mice there was improvement of neurogenesis to non-transgenic levels after G-CSF treatment and an increase in synaptogenesis in the CA1 region of the hippocampus.
The effects of G-CSF on the endogeneous microglial population are most likely responsible for the increase in microgliosis, as no significant increase of BMDC infiltration into the brain parenchyma was found in vivo. The enhanced proliferation and improved viability of the neuronal cell line after G-CSF treatment may explain the improvement in neurogenesis and significant increase in synaptogenesis seen in the AD mouse model. The actions of G-CSF on neural stem/progenitor cells to stimulate hippocampal neurogenesis and to enhance resident microglial capacity to decrease amyloid burden are the most likely mechanisms responsible for the behavioral improvement seen in the AD mouse model.
Chapter 1: Introduction

Note to Reader

Portions of this introduction have been previously published (Rowe et al., 2009) and are utilized with permission of the publisher.

Alzheimer’s Disease (AD) is a progressive neurodegenerative disease causing problems with memory, thinking and behavior severe enough to affect work, lifelong hobbies or social life. Patients with AD lose the ability to encode new memories. AD is also characterized by synaptic loss in the hippocampus and association cortices, which more strongly correlates to the overall cognitive decline, than β-amyloid plaque and neurofibrillary tangle development (Selkoe, 2002).

AD is the sixth-leading cause of death in the United States and the fifth leading cause in death of those over the age of sixty-five years old (Alzheimers-Association, 2011). AD gets worse over time, it is fatal and there is currently no cure. Without the emergence of new treatments, AD threatens to become a pandemic. According to a 2011 study, Alzheimer’s Disease Facts and Figures there are now more than five million people in the United States living with AD. One in eight people aged 65 and older have AD. Most people survive an average of four to eight years after an Alzheimer’s diagnosis, but some live as long as twenty years with the disease. Without a cure or effective treatments to delay the
onset or progression of Alzheimer’s disease, the prevalence would grow to 7.7 million people with the disease by 2030 and is expected to soar to approximately 16 million by mid-century. AD is the only cause of death among the top 10 in America without a way to prevent, cure, or even slow its progression (Alzheimers-Assocation, 2011).

**Neuropathology**

The pathological hallmarks of AD are extracellular β-amyloid plaques, intracellular neurofibrillary tangles, and neurodegeneration in areas of the brain important for learning and memory, such as the hippocampus, the entorhinal cortex and other associated cortices (DeKosky et al., 1996). Major risk factors include age and Apo-E-epsilon 4 (APOE4) (Swaab et al., 2002). APOE4 reduces age of onset of AD most likely due to dysfunctional clearance of β-amyloid; as those with APOE4 have less overall apolipoprotein activity than those with APOE2 or APOE3 allele (Corder et al., 1993, Rebeck et al., 1993, Rebeck et al., 1994, Kim et al., 2009).

In the dysregulation of the metabolism of β-amyloid precursor protein (APP) in AD; APP is broken down by two proteases or enzymes, β and γ secretase (Li et al., 2000), into β-amyloid peptides, Aβ 1-40 and Aβ 1-42 respectively (Hardy and Selkoe, 2002), and then form extracellular fibrils and β-pleated sheets which eventually result in aggregation into plaques within the brain (Selkoe, 2000). The overproduction and eventual deposition of Aβ currently is regarded as the crucial step for the development of AD (Selkoe, 2000). Aβ is considered toxic both *in vitro* and *in vivo*, resulting in DNA damage, apoptosis,
and axonal damage, which lead eventually to neuronal death or neurodegeneration (Paradis et al., 1996, Naslund et al., 2000, Vickers et al., 2000). β-amyloid deposition begins ten to twenty years prior to the appearance of clinical dementia (Naslund et al., 2000, Blasko and Grubeck-Loebenstein, 2003). It is not only important to inhibit the production of toxic Aβ, but also to clear the β-amyloid deposits to mitigate the neurodegeneration and cognitive deficits found in AD.

Intraneuronal neurofibrillary tangles (NFT) are composed of abnormally folded and hyperphosphorylated tau, a protein involved in microtubule formation (Blasko and Grubeck-Loebenstein, 2003). The presence of NFT in AD has been shown to be induced by Aβ plaques. This is thought to be carried out by the hyperphosphorylation of the tau protein by the enzyme glycogen synthase kinase-3 beta (GSK-3β) which causes destabilization of the cytoskeleton resulting in the cell death seen in AD (Ishizawa et al., 2003, Jamsa et al., 2004).

The presence of NFT is another mandatory pathology for the diagnosis of AD (Braak et al., 1993, Jellinger, 1998, Hardy and Selkoe, 2002, Blasko and Grubeck-Loebenstein, 2003). The appearance of NFT in the AD brain occurs after β-amyloid deposition has already begun (Hardy and Selkoe, 2002).

**Neuropathological Staging**

AD pathology can be separated into different stages of propagation dependent on the location of neurofibrillary changes (tangle-bearing neurons) and the severity of the changes within the brain. The first two stages (Stage 1 & 2) are considered transentorhinal, which are the clinically silent cases. The
middle two stages (Stage 3 & 4) are the limbic stages, which include the hippocampus and temporal lobe, early AD. The final two stages (Stage 5 & 6) are called the isocortical or neocortical stages, which include the cingulate and angular cortices, and are consistent with fully developed AD (Braak and Braak, 1991, 1995, Nagy et al., 1998).

**Clinical stages of Alzheimer’s Disease**

AD symptoms may vary between patient, and an individual patient may switch between various stages of AD throughout the course of the disease, but the stages provide a general idea of how abilities change during the course of the disease. In stage 1, there is no noticeable impairment. In stage 2, there is a very mild cognitive decline, which may be noticeable to the individual, but not to the clinician or the friends and family. In stage 3, there is a mild cognitive decline that becomes noticeable to others and early stage AD may be diagnosed at this point. In stage 4, there is a moderate cognitive decline, which can be diagnosed on clinical exam and mild or early-stage AD can be diagnosed at this point. In stage 5, there is a moderately severe decline, cognitive impairment is apparent and the patient probably will need assistance with activities of daily living and therefore the diagnosis is moderate or mid-stage AD. In stage 6, there is a severe cognitive decline and personality changes may arise, moderately severe or mid-stage AD. Finally in stage 7, there is a very severe cognitive decline in which patient’s no longer respond to environmental stimuli and are dependent on others for all activities of daily living, severe or late-stage AD (Alzheimers-Association, 2007).
FDA approved treatments for Alzheimer’s Disease

There are no effective treatments that reverse or delay the neurodegeneration or decrease in cognition seen in AD patients. As neurons in the AD brain are assaulted by toxic A\(\beta\) as well as inflammatory cytokines from activated glial cells, they result in diminished neuronal metabolism and disturbances of multiple transmitter systems, such as loss of cholinergic neurons in the basal forebrain. The current FDA approved AD treatment regimens temporarily assist in alleviating this deficit of neuronal transmitters.

The current treatment of acetylcholinesterase inhibitors (AChE-I) increase levels of synaptic acetylcholine (ACh), such as Donepezil, Galantamine and Rivastigmine, and therefore temporarily improves memory function by increasing levels of acetylcholine in the brain; these are used in early to moderate stages of AD, although Donepezil can also be used in the severe stage of AD. Another drug class used is NMDA glutamate receptor antagonists, such as Memantine, which also acts by improving memory function, by protecting glutamatergic neurons from excess glutamate; this is used in moderate to severe stages of AD and can also be used in conjunction with Donepezil. These treatments help alleviate the early symptoms caused by the loss or dysfunction of cholinergic and glutamatergic neurons or synapses. Current standard treatments regimens delay the worsening of symptoms temporarily but do not alter the course of the disease.
Circuitry of the hippocampus

The hippocampus is important in encoding or the formation of long-term memories, while the parahippocampal region, including the entorhinal cortex, solidifies these memories (Eichenbaum, 2000). The entorhinal cortex (EC) projects to the dendrites of the granular zone (GZ) of the dentate gyrus (DG) in the hippocampus (HC) via the perforant pathway. The GZ projects to the pyramidal neurons in the CA3 region of the HC via mossy fibers. The CA3 region projects contra-laterally to the CA1 region of the HC via the associational commissural pathway and projects ipsilaterally to the CA1 region via the Shaffer collateral pathway. The CA1 region projects to the subiculum which projects to the EC. Long term potentiation (LTP) is comprised of the perforant pathway, mossy fibers and Schaffer collaterals, which may require simultaneous pre and post synaptic activity for LTP, or long-term memory storage, to occur (Bliss and Gardner-Medwin, 1973, Bliss and Lomo, 1973, Kandel and Pittenger, 1999). The circuitry between the HC and EC is important in encoding long-term memories, and LTP is impaired in AD, therefore the effects on this region of the brain are important when studying AD (Rowan et al., 2003).

Neurogenesis in the adult brain

Specific regions of adult brain retain the capacity to generate new neurons throughout life. These neuro-anatomic regions house neural stem cells in a micro-environment (neurogenic niche) appropriate for generation, of new neurons (McKay, 1997, Cameron and McKay, 1998, Eriksson et al., 1998, Kempermann et al., 1998, Gage, 2000). Neurogenic niches are localized both in
the subventricular zone (SVZ) of the anterior lateral ventricles and the
subgranular zone (SGZ) of the DG of the HC.

Not only can NSC be produced by the brain endogenously, but it has been
reported that bone marrow stem cells (BMSC) can differentiate into glial cells,
such as microglia (Simard and Rivest, 2004), or neuronal-like cells in the brain
(Brazelton et al., 2000). Our laboratory has been studying bone marrow derived
cells (BMDC) as a potential source of neurons for treatment of neurologic
diseases over the last 10 years. BMDC have been shown to differentiate into
neural cells in vitro and in vivo migrate to the brain where they can differentiate
into neurons or glial cells, such as microglia (Brazelton et al., 2000, Mezey et al.,
Both BMSC, that express nestin, and adult brain NSC may become cells with the
morphological, immunocytochemical (ICC), and functional characteristics of
neurons (Song et al., 2007). Although in comparison to the amount of microglia
derived from BMSC the amount of neuronal cells derived from BMSC is less,
only 0.2-0.3% of total neurons (Brazelton et al., 2000). The exact mechanism of
the transdifferentiation of BMDC to neural cells is unclear although may reflect
their ability to fuse with injured neurons (Weimann et al., 2003). In vivo studies
suggest that generation of new neurons from a BMDC, if it really occurs, is a rare
phenomenon.

**Bone marrow derived cells (BMDC) & microglia in AD brain**

Bone marrow (BM) supplies approximately ten percent of the normal adult
brains microglia, brain immune cells, and this amount increases in the injured
brain, such as in AD (Bowling et al., 1993, Ip and Yancopoulos, 1994, Hess et al., 2002, Sanchez-Ramos et al., 2008). In a study by Maler et al. the amount of hematopoietic stem cells (HSC) in the blood was decreased in AD patients as compared to age matched controls (Maler et al., 2006). Also there was a correlation between these low HSC counts and a decrease in cerebral spinal fluid (CSF) \( \text{A}\beta 1-42 \) peptide and \( \text{A}\beta \) ratio 42/40 which have been shown to be possible CSF markers for AD in patients with mild cognitive impairment (Wiltfang et al., 2005). Therefore the decrease in HSC may be contributing to the cognitive deficits seen in AD. Microglia are dysfunctional in their ability to produce the \( \text{A}\beta \)-binding receptor and \( \text{A}\beta \)-degrading enzymes in the Tg APP/PS1 AD mouse model (Hickman et al., 2008). Microglia also are attracted to \( \text{A}\beta \) plaques and can have both positive and negative effects on the AD brain (Kim et al., 2005, Walker and Lue, 2005).

The mechanism of microglial accumulation into AD brains is not completely understood. Circulating leukocytes in the blood express chemokine receptors such as CC-chemokine receptor 2 (CCR-2). The primary ligand of CCR-2 is monocyte chemoattractant protein (MCP-1) which mediates the recruitment of CCR-2 expressing monocytes into the brain as well as activation of macrophages (Matsushima et al., 1989, Yamamoto et al., 2005). MCP-1 expression occurs mainly on monocytes (Kitagawa et al., 2004). MCP-1 is mostly expressed in the perivascular space and brain parenchyma during neuroinflammation (Mastroianni et al., 1998, Losy and Zaremba, 2001, Sindern et al., 2001, Chen et al., 2003, Sorensen et al., 2004). Other monocyte
chemotactic pathways that are independent of MCP-1 allow a minimal level of monocyte attraction under non-disease conditions (Mantovani, 1999). CCR-2 is expressed in both neurons and astrocytes within the brain, specifically in the cerebral cortex, the striatum, the limbic system and the cerebellum (Banisadr et al., 2002, Gillard et al., 2002). CCR-2 is also expressed in microvascular endothelial cells in both mice and humans (Dzenko et al., 2001).

MCP-1 may also function as a regulator of brain endothelial permeability in vitro and induce blood-brain barrier (BBB) disruption in vivo (Tekstra et al., 1999, Stamatovic et al., 2003, Stamatovic et al., 2005). Aβ amyloid peptides can up-regulate the release of chemokines such as MCP-1 in both monocytes as well as several cells within the brain (Lue et al., 2001, Dawson et al., 2003). MCP-1 is up-regulated early in brains of transgenic mice that develop AD–like pathology (Ishizuka et al., 1997, Sly et al., 2001, Janelsins et al., 2005, Yamamoto et al., 2005, El Khoury et al., 2007). Elevated levels of MCP-1 have been observed in AD patients both in microglia associated with mature senile plaques and in microvessels isolated from brain cortices (Ishizuka et al., 1997, Grammas and Ovase, 2001).

Early microglial accumulation is CCR-2 dependent and begins before formation of AD senile plaques, which suggests that early neurotoxic species of Aβ are capable of inducing microglial accumulation, probably through the recruitment of mononuclear phagocytes from the bone marrow and blood (Walsh et al., 2002, Babcock et al., 2003, El Khoury et al., 2007). The strategy of blocking the MCP-1/CCR2 interaction, either genetically or pharmacologically,
has been proposed as being effective in preventing macrophage-induced tissue damage. Loss of MCP-1 function is enough to impair monocyte trafficking in several inflammatory models (Dawson et al., 2003). Mice bred to have the CCR-2 receptor knocked-out (CCR-2-/-) exhibited impairment in modulating recruitment of monocytes to areas of inflammation (Boring et al., 1997). It also was reported that cross breeding Tg2576 AD mice with Tg mice deficient in CCR-2 (TgCCR-2-/-) resulted in decreased infiltration of microglia, increased β-amyloid plaque load and accelerated disease progression (El Khoury et al., 2007). Antibodies to MCP-1 significantly inhibit the migration of microglia and monocytes in response to Aβ-stimulated macrophage supernatant in vitro (Meda et al., 1996, El Khoury et al., 2003).

**Hematopoietic growth factors as a treatment for neurogenerative diseases**

Hematopoietic cytokines or growth factors (HGF) stimulate cell growth and differentiation of HSC. Several cytokines known to act as growth factors on the brain include fibroblast growth factor-2 (FGF2), fibroblast growth factor-8 (FGF8), endothelial growth factor (EGF), sonic hedgehog (Shh), bone morphogenetic protein 4 (BMP4), brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), stem cell factor (SCF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), and erythropoietin (EPO). Of these HGF that act as growth factors on the brain, the following review will focus on those that have the greatest potential to act as treatments for neurodegenerative diseases,
including AD, such as SCF, GM-CSF, G-CSF, M-CSF, SDF-1 and EPO. HGF have several effects on the central nervous system (CNS) (Table 1). They can be neuroprotective, anti-apoptotic, stimulate stem cell neuronal plasticity, induce the proliferation and differentiation of NSC into new neurons or glial cells such as microglias.

**Erythropoietin (EPO)** is a 30 kDa hematopoietic cytokine consisting of 165 amino acids and is coded by a single copy on chromosome (Maiese et al., 2004, 2005). The biological activity of EPO appears to be dependent on its glycosylated chains as well as the disulfide bonds between the cysteine residues located at 7 and 160 and at 29 and 33 (Chong et al., 2002). EPO is ubiquitous throughout the body and primarily produced in the kidney through peri-tubular fibroblasts in the renal cortex (Jacobson et al., 1957, Fliser and Haller, 2007). The main actions of EPO include to promote red blood cells (RBC) survival through the prevention of apoptosis in threatened RBC and to stimulate colony forming erythroid cells to proliferate into new RBC (Lasne and de Ceaurriz, 2000, Sathyanarayana et al., 2007). The production of EPO has also been indicated to occur in the liver through Kupffer cells and hepatocytes as well as in the uterus (Schmeding et al., 2007). Surprisingly EPO production and the presentation of the erythropoietin receptor (EPOR) have been found to exist in both cells of the nervous and cardiac/vascular system inferring an extension of its cellular protective abilities to these systems (Maiese et al., 2004).

Expression of EPO and EPOR have been shown to correlate with decreased levels of oxygen, or increased metabolic stress, and have been
shown to be under control of hypoxia-inducible factor 1 (HIF-1). HIF-1 is constitutively produced in cells, but undergoes elimination through ubiquitination and proteasome degradation in the presence of oxygen. In hypoxic events, however, degradation of HIF-1 is inhibited, allowing it to up-regulate the expression of both EPO and EPOR. The timely appearance of EPO and EPOR in the event of a decrease in oxygen allows EPO to protect cells from apoptosis (Maiese et al., 2004, 2005). Currently EPO is used to treat anemia in various clinical scenarios (Fisher, 2003).

The presence of EPOR on cellular membranes and the synthesis of EPO have been found to occur in neurons and astrocytes (Marti et al., 1996, Morishita et al., 1997, Juul et al., 1999a, Brines et al., 2000). EPO is able to cross the BBB through receptor mediated transport, allowing EPO produced in peripheral tissues to enter the brain (Brines et al., 2000). This event, however, only accounts for 6-8% of the total EPO concentration found in the CSF indicating that most of the EPO in the CSF is produced in the brain (Maiese et al., 2004). Various regions of the brain have been attributed to production of EPO such as the hippocampus, cortex, and mid-brain (Maiese et al., 2004, 2005). The presence of EPO and EPOR are relatively low in the normal brain and only after metabolic stress or hypoxia does the concentration increase. This has been indicated by increases in the expression of EPO in the CSF as well as increase of EPO in postmortem brains that have undergone injury, hemorrhage or stroke (Juul et al., 1999b, Siren et al., 2001, Springborg et al., 2003, Sairanen et al., 2006). EPOR has also been indicated to increase in concentration in chronic
brain diseases, such as AD, possibly inferring constant hypoxic or metabolic stress (Assaraf et al., 2007). It is thought that Aβ plaques in the brain, as well as the characteristic tangle, are strongly correlated to cause of AD (Hardy and Allsop, 1991, Iwatsubo, 2000). Aβ plaques are believed to increase the amount of reactive oxygen species in the brain thus increasing the oxidative stress and Aβ-induced apoptosis associated with AD (Barkats et al., 2000, Sultana et al., 2004, Boldogh and Kruzel, 2008, Moon et al., 2008).

It has been revealed that EPO is able to attenuate the apoptosis induced by reactive oxygen species (ROS) which is induced by Aβ. Aβ was shown to decrease the ratio of Bcl-2/Bax thus leading to the activation of caspase 3 and eventual apoptosis. However EPO was able to increase this ratio by increasing the expression of Bcl-2, an anti-apoptotic gene, and by decreasing the expression of Bax, a pro-apoptotic gene, thus leading to the protection of PC12 cells, a cell line used as a model for neuronal differentiation, from Aβ induced oxidative stress. This was linked to the activation of the intracellular Jak2/PI3K/Akt pathways which was indicated by the increase in phosphorylated Akt when PC12 cells were treated with EPO (Li et al., 2008, Ma et al., 2009). The fragmentation of DNA through caspases, apoptotic enzymes, is a relatively late occurring phenomenon as many earlier stimuli also lead to phagocytosis or apoptosis of cells in the CNS. Aβ plaques are able to induce microglial secretion of TNF-α as well as other inflammatory cytokines (Bornemann et al., 2001, Chong et al., 2005a). Also Aβ plaques cause the early expression of externalized phosphatidyl-serine (PS) signaling microglia to phagocytize the compromised
neurons (Kang et al., 2003a, 2003b, Chong et al., 2005b). EPO has been shown to provide long term survival through Akt activation to cause the translocation and activation of NF-kB to the nucleus leading to expression of anti-apoptotic genes thus preventing TNF-α induced apoptosis and the activation of caspase pathways as well as preventing the externalization of PS that would normally lead to microglial activation and phagocytosis of a damaged neuron (Wang et al., 1998, Reed, 2001, Chong et al., 2005c). EPO was indicated to induce the dephosphorylation and deactivation of GSK-3β by EPO binding its receptor and up-regulating Akt activity through the Jak2/PI3K pathway thus preventing the hyperphosphorylation of tau protein and subsequent tangle formation. This provided a possible avenue in preventing Aβ induced cellular death in the brain (Sun et al., 2008, Ma et al., 2009). In addition to its neuroprotective abilities, EPO has the capacity to provide the necessary signaling to cause mesenchymal stem cells (MSC) to differentiate into neurons during simultaneous hypoxia (Zwezdaryk et al., 2007, Danielyan et al., 2009). In the presence of EPO, MSC have been shown to increase the expression of neural markers, such as choline acetyltransferase (ChAT), neuronal nuclei (NeuN), and muscarinic receptors and to increase functional markers, such as synaptophysin, alutamate aspartate transporter (GLAST) and neprilysin (Danielyan et al., 2009). EPO has also been shown to up-regulate the production of ACh and, along with the increase in muscarinic receptors, it appears these MSC can be used to replace ACh neurons that are often associated with cognitive loss in AD (Tsang et al., 2007, Danielyan et al., 2009). It has also been observed that EPO not only increases the
differentiation of MSC into neurons, but also inhibits the differentiation into glial-like cells due to the decrease in glial fibrillary acidic protein (GFAP) positive cells under EPO administration. EPO treated MSC are able to produce glutamine synthase (GS), which is normally produced by astrocytes to combat glutamate toxicity, but GS activity is decreased in AD, which causes neurons to synthesize it (Robinson, 2000, Danielyan et al., 2009). Along with EPO induction of GLAST and GLT-1 production, it has been suggested that EPO treated MSC can take over the task of metabolizing glutamate that is normally done by astrocytes (de Hemptinne et al., 2004, Danielyan et al., 2009). It is therefore hypothesized that co-administration of transplanted MSC and EPO could offer possible therapeutic potential in treating AD (Danielyan et al., 2009). Intravenous EPO therapy also has been shown to have cognitive improvement in other neurocognitive disorders, such as schizophrenia (Ehrenreich et al., 2007). Although EPO therapy comes with potential cardiovascular risks due to its potential to increase blood viscosity as well as blood pressure and therefore EPO derivatives should be further investigated as a method for AD treatment (Maiese et al., 2009).

**Stem Cell Factor (SCF)** is an 18.5kDa glycoprotein that acts a HGF and therefore plays a key role in hematopoiesis. It is also known as c-kit ligand, steel factor or mast cell growth factor. Its structure is similar to M-CSF. SCF exists in both a soluble (sSCF) and membrane-bound (mSCF) form. sSCF activates the c-kit (SCF) receptor more transiently and the down-regulation is induced more rapidly than in mSCF. mSCF may help in guiding the HSC to their final destinations in the body during embryogenesis and be biologically more active
than sSCF (Broudy, 1997). SCF and its receptor are found in areas throughout the body, but of particular interest, both are expressed in the CNS, such as in the neuroproliferative zones in the brain (Matsui et al., 1990, Hirota et al., 1992, Morii et al., 1992, Hirata et al., 1993).

SCF has several biological functions that have been observed either in vitro or in vivo. In vitro SCF has been shown to increase the survival of HSC, generate direct colony-forming cells, to potentiate the ability of epinephrine & ADP to stimulate platelet aggregation, to assist in serotonin secretion, to promote melanogenesis and gametogenesis, to have synergistic effects on megakaryocytes, and to support the growth and function of mast cells (Broudy, 1997). In vivo SCF has been shown to help in stem cell (SC) self-renewal, have a synergistic effect with other HGF such as G-CSF, have chemotactic effects for SC, to assist in the development of early T cells, and to help in the mobilization of HSC from the bone marrow into the blood (Broudy, 1997). Its specific functions in the CNS include acting as a survival factor and chemokine for NSC, neurogenesis, neuroprotective effects, and modulation of microglia (Zhang and Fedoroff, 1999, Jin et al., 2002, Erlandsson et al., 2004, Dhandapani et al., 2005). SCF also has an increased expression in the injured brain, mobilization of BMSC into the brain through systemic administration of SCF in the normal brain and mobilization of neuron-like BMSC & resident brain NSC to areas of brain injury such as areas of ischemia (Corti et al., 2002, Sun et al., 2004, Kawada et al., 2006, Zhao et al., 2007). SCF is primarily produced by neurons not the glial
cells in the brain, as intense staining for SCF can be observed in the neutropil (Sun et al., 2004).

It has been shown by Leyhe et al. that current AD treatments such as donepezil (AChE-I) may not only act by increasing synaptic connectivity through the increase of ACh, but also through the increase of HGF, such as BDNF and SCF (Leyhe et al., 2009). Those AD patients with higher levels of SCF had a higher level of cognitive function, or a smaller decrease in loss of cognitive function, than those with lower levels of SCF (Leyhe et al., 2009). AChE-I may be acting by activating protein kinase C (PKC) which cleaves mSCF releasing it from the cell surface. This increase in SCF may be responsible for the increase of BDNF and decrease in neuroinflammation through the suppression of microglia (Zhang and Fedoroff, 1998).

In c-kit mutant rats a decrease in escape latency with repeated trials of the water maze was not shown as in the wild type, which displayed impairment in spatial memory. These c-kit mutant rats also showed impairment in hippocampal synaptic potentiation in comparison to wild type rats in studies with paired-pulse facilitation (PPF) in the mossy fiber-CA3 (cornu ammonis 3) hippocampal pathway and long term potentiation (LTP) (Katafuchi et al., 2000). Therefore a lack of SCF impairs both spatial memory and hippocampal synaptic potentiation or long term memory.

Lower levels of SCF have been observed in plasma levels of AD patients in comparison to age matched healthy controls. Also lower levels of plasma SCF
have been significantly correlated to an increase in the severity of dementia. Patients with AD in comparison to those with non-inflammatory neurological disease (NIND) had significantly lower CSF levels of SCF. No correlation exists between the levels of SCF found in the CSF versus the plasma, so the SCF in these different locations may have different sources (Katafuchi et al., 2000). Therefore, a decrease in HGF, such as SCF, could be contributing to a decrease in HSC in patients with AD. Although due to the negative feedback loop between sSCF and mSCF it is possible sSCF is decreasing while mSCF is increasing (Miyazawa et al., 1995).

Both the HGF SCF and FGF2 protein and mRNA levels are increased with hypoxia in cell culture. Also SCF is increased in immature neurons in both SGZ and SVZ in the rat brain and administration of SCF increases bromodeoxyuridine (BrdU) labeling of immature neurons, showing the effect of SCF on areas of neurogenesis (Jin et al., 2002). In those cells adjacent to the areas of brain injury higher levels of SCF are expressed then those that are a further distance from the injury and SCF mRNA is over-expressed in the injured area of the brain (Sun et al., 2004).

In subacute brain ischemia the administration of SCF plus G-CSF decreased the infarction size and resulted in functional improvement (Kawada et al., 2006). In a study by Piao et al. SCF plus G-CSF treatment in mice with a model of chronic stroke increased BMSC mobilization and differentiation resulting in an increase in neurogenesis and angiogenesis and therefore enhancement of
brain repair in ischemia (Piao et al., 2009). Both SCF and G-CSF pass through the intact BBB (Zhao et al., 2007).

Although SCF may be a potential treatment for neurological diseases, such as AD and stroke, it has also been shown to have side effects in early clinical trials. At sites of injection it can cause wheals due to activation of mast cells and darkening in pigmentation due to activation of melanocytes, although if given at lower doses in conjunction with a synergistic factor such as G-CSF these side effects may be eliminated (Broudy, 1997). Phase 1 clinical trials have been completed in use of SCF as a treatment for aplastic anemia, myelodysplasia and AIDS/AIDS-related malignancies.

**Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)** is a 23 kDa glycoprotein with a four helical bundle structure (Nicola, 2001a). It is a HGF that assists in the maturation and differentiation of HSC into the lineage of the granulocytes & macrophages. HGF, such as GM-CSF, stimulate the progression of the cell cycle through the up-regulation of cyclins and therefore stimulate the proliferation of microglia and their transformation into the ameboid (active) form (Koguchi et al., 2003). According to Malipiero et al. GM-CSF is made from astrocytes in the brain, although in a more recent study by Schabitz et al. GM-CSF is found in similar cells in the brain as its receptor, therefore predominantly in neurons and to a lesser extent in oligodendrocytes (Malipiero et al., 1990, Schabitz et al., 2008).

Its receptor (GM-CSFR) is heterodimeric and it contains an α chain, the GM-CSF ligand binder, and a β chain, the signal transducer which is coupled to
STAT pathway (Nicola, 2001b, Maurer et al., 2008). GM-CSFR is found both on hematopoietic cells as well as in the CNS (Sawada et al., 1993). In neurons in the uninjured brain the GM-CSFR is found throughout the brain in areas such as hippocampus CA2 and CA3, entorhinal cortex, pyramidal cells in cortex, as well as several other areas. Also GM-CSFR can be detected in some oligodendrocytes, but not microglia or astrocytes (Schabitz et al., 2008). But according to Guillemin et al. GM-CSFR can be found on astrocytes (Guillemin et al., 1996). GM-CSFR has been found on NSC stained with nestin in the hippocampus (Kruger et al., 2007). In areas of the brain with ischemia and the penumbra, an area where neurons are at risk of dying due to nearby infarct, up-regulation of the receptor in large pyramidal neurons may be an adaptive response to the neuronal damage (Schabitz et al., 2008).

In the peripheral nervous system GM-CSF stimulates phagocytosis of myelin during wallerian degeneration (WD), although it is inhibited in a later stage of WD by a binding protein (Reichert et al., 1994, Mirski et al., 2003). In the CNS GM-CSF causes proliferation, induction and differentiation of microglia and has a strong anti-apoptotic effect on recruited neutrophils and neurons (Suzumura et al., 1996, Coxon et al., 1999, Schermer and Humpel, 2002, Schabitz et al., 2008). GM-CSF asserts this anti-apoptotic effect through increase in the anti-apoptotic proteins Bcl-XI and Bcl-2 and partially through activation of the Akt pathway; this anti-apoptotic effect can be activated by such stimuli as ischemic insult. GM-CSF has been identified as a neuronal growth factor in the brain which is both anti-apoptotic and diminishes infraction size in vivo in rats (Schabitz et al., 2008).
GM-CSF treatment in a disease model of spinal contusion decreased apoptosis within the spinal cord (Ha et al., 2005). NSC treated with GM-CSF \textit{in vitro} had a significant increase in staining for β III-tubulin and MAP-2 or an increase in differentiation of NSC into neurons (Kruger et al., 2007).

In a study by Manczak et al. AD transgenic (Tg) 2576 mice were treated with anti-GM-CSF antibody and a significant decrease was seen in Aβ plaque deposits, soluble Aβ 1-42 production, and microglial activation (Manczak et al., 2009). When levels of cytokines, such as GM-CSF, are decreased the level of microglia also decreased, therefore elevated GM-CSF levels in AD may contribute to the increase of microglia in the brain (Tarkowski et al., 2001). Administration of GM-CSF and Aβ through adenovirus vectors intranasally in Tg2576 mice resulted in lower Aβ levels in the brain in comparison to control (Kim et al., 2004). In Tg APP mice treated with a combination of Aβ/GM-CSF/Interleukin-4 (IL-4) antibodies significantly decreased the Aβ plaque load (DaSilva et al., 2006).

An increase in GM-CSF levels have been observed in the CSF and plasma in patients with stroke, AD, and vascular dementia, although patients with stroke only had an elevation of GM-CSF in CSF. Elevation in GM-CSF may be due to an increase in neuronal damage and cell death, as a positive correlation was shown between GM-CSF and markers for neuronal damage, such as Tau and Fas/APO-1 gene, (Tarkowski et al., 1997, Tarkowski et al., 2001, Tarkowski et al., 2003). In an observational study both GM-CSF and G-CSF were overproduced by AD-derived peripheral blood mononuclear cells (PBMC)
activated by recombinant Aβ 1-42 (Pellicano et al., 2010). In another study, M-CSF, G-CSF or GM-CSF was administered unilaterally into the hippocampi of aged cognitively impaired AD mice via 5 μg bolus injections G-CSF and GM-CSF both significantly reduced amyloidosis throughout the treated brain hemisphere one week following bolus administration, although there was a greater reduction of amyloid with GM-CSF treatment. Subcutaneous GM-CSF (5 μg daily injections x 20) administration in AD mice significantly reduced brain amyloidosis and reversed the cognitive impairment, while increasing hippocampal synaptic area and microglial density (Boyd et al., 2010).

Clinically GM-CSF, or Leukine a recombinant human GM-CSF, has been used for the treatment of neutropenia. GM-CSF, as well as G-CSF, is in clinical trials for use as a treatment for stroke and AD. And therefore may possess the potential to be a future treatment for neurodegenerative diseases like AD.

**Macrophage-Colony Stimulating Factor (M-CSF)** (aka CSF-1) is another HGF that is 80-100 kDa glycoprotein or 130-160 kDa chondroitin sulfate-containing proteoglycan, or expressed as membrane bound 68-86 kDa glycoprotein on M-CSF producing cells. In all its active forms it is dimeric (Stanley et al., 1994). M-CSF is produced by astrocytes (Giulian and Ingeman, 1988). M-CSF has also been shown to reduce cognitive deficiency and Aβ plaque load in APP/PS1 Tg mice, receiving M-CSF treatment intraperitoneally (ip). This was observed by decreased escape latencies in repeated T-water maze trials in M-CSF treated Tg mice and wild type mice versus saline-treated Tg mice which shows an improvement in spatial memory with M-CSF treatment.
M-CSF treated 6mo old Tg mice had an improved quality of nest building which reflects diminished social withdrawal and apathy as compared to saline-treated Tg mice. Also M-CSF treated Tg mice had a higher ratio of microglia per plaque than saline-treated Tg. Although the increase in microglia per plaque could potentially be related to the decreased density, size, and overall Aβ burden in the plaques seen in the M-CSF treated Tg mice (Boissonneault et al., 2009). It has also been shown that M-CSF can promote the proliferation, activation and development of microglia; and therefore mice with decreased levels of M-CSF have a lower amount of microglia found in the brain (Giulian and Ingeman, 1988, Raivich et al., 1994, Davoust et al., 2006). M-CSF causes proliferation of microglia in culture without affecting its response to lipopolysaccharide (LPS) or Aβ, therefore M-CSF increases amount of microglia without affecting microglial activation responses (Vidyadaran et al., 2009). Low levels of M-CSF in pre-symptomatic AD in combination with low levels of other HGF displays a progression towards a decline of cognitive function to dementia (Ray et al., 2007).

In a study by Laske et al., 50 patients with AD had significantly increased M-CSF plasma levels in comparison to 22 patients with mild cognitive impairment (MCI) and 35 age-matched healthy controls. In contrast, MCI patients showed significantly decreased M-CSF levels in CSF compared to AD patients and 20 patients with other non-inflammatory neurological disease (NIND). M-CSF, but not Aβ 1-42 and tau-protein, was a significant parameter for distinction between
MCI and NIND patients. This supports that M-CSF in CSF could be a putative biomarker for MCI (Laske et al., 2010).

**Stromal cell-derived factor-1 (SDF-1)**, also known as Chemokine (C-X-C motif) ligand 2 (CXCL2), is a member of the C-X-C subfamily of chemokines and therefore is a potent chemoattractant for HSC that are expressed in the BM (Bleul et al., 1996, Aiuti et al., 1997, Lapidot et al., 2005, Zhou et al., 2007). CXCL2 and its receptor CXCR4 may play a role in blood vessel growth and development as well as attraction of HSC to injured vessel walls (Carr et al., 2006, Langer et al., 2006, Stellos et al., 2008). It plays an important role in the mobilization, differentiation and adhesion of HSC and has neuroprotective effects in the CNS (Robin et al., 2006, Stumm and Holtt, 2007, Stellos et al., 2008). Decreased plasma levels of SDF-1 have been seen in patients with AD (Laske et al., 2008). Therefore low levels of SDF-1 may further contribute to the deficient HSC support in the AD brain.

In a study by Stellos et al., peripheral blood concentrations of circulating CD34+/CD133+ and CD34+ progenitor cells were measured in 45 AD patients and in 30 healthy elderly controls by flow cytometry. AD patients with moderate to severe dementia showed significantly increased circulating CD34+/CD133+ and CD34+ progenitor cells compared to healthy elderly controls independent of cardiovascular risk factors and medication. In addition, the number of circulating CD34+/CD133+ progenitor cells in AD patients was significantly inversely correlated with cognitive function, age, and plasma levels of SDF-1, a potent chemokine for progenitor cells (Stellos et al., 2010).
In another study, memory deficits in an AD mouse model were significantly improved by intraperitoneal injection of G-CSF/AMD3100 (CXCR4 antagonist) and simultaneous intracerebral injection with SDF-1α (to help induce migration of BM-HPC into the brain), but Aβ deposition was unchanged (Shin et al., 2011). Microglial activation was increased with alternative activation of microglia to a neuroprotective phenotype. Also by utilizing an amyloid precursor protein/presenilin 1 (APP/PS1)-green fluorescent protein (GFP) chimeric mouse, BM-derived GFP positive microglia were identified in the brain. Additionally, increased hippocampal neurogenesis and improved memory was observed in mice receiving combined G-CSF/AMD3100 and SDF-1α treatment, but not in controls or in animals receiving each treatment alone. These results suggest that SDF-1α is an effective adjuvant in inducing migration into brain of the endogenous BM-HPC, mobilized by G-CSF/AMD3100, and that the two can act synergistically to produce a therapeutic effect. This approach also shows potential as a therapeutic option for the treatment of AD patients in the future (Shin et al., 2011).

Of greatest importance to our laboratory, **granulocyte colony stimulating factor (G-CSF)** is a 19 kDa glycoprotein formed into 4 antiparallel alpha helices (Wells and de Vos, 1996). G-CSF is a HGF that stimulates the proliferation and differentiation of HSC and exists in both a 174 and a 180 amino acid forms, although the 174 form is more abundant and active and has been used in three proprietary recombinant G-CSF proteins including lenograstim (Granulocyte®), filgrastim (Neupogen®), and pegylated filgrastim (Neulasta®).
which has a longer duration of action (Sanchez-Ramos et al., 2008). G-CSF has been shown to be synthesized by many cell types including bone marrow stromal cells, fibroblasts, and macrophages (Malipiero et al., 1990, Demetri and Griffin, 1991, Wells and de Vos, 1996) and its receptor G-CSFR has also been indicated to be just as ubiquitous as it can be found on the cell surfaces of endothelial cells, lymphocytes, platelets, and neutrophils (Bussolino et al., 1989, Hanazono et al., 1990, Shimoda et al., 1993, Morikawa et al., 2002). G-CSF and G-CSFR are also both expressed by neurons in several areas of the brain including pyramidal cells in cortical layers (specifically II and V), Purkinje cells of the cerebellum, SVZ, and in cerebellar nuclei in rats (Schneider et al., 2005). Also the G-CSF ligand has been identified in cells within the CA3 region of the HC, SGZ and the hilus of the DG, entorhinal cortex, and the olfactory bulb (Schneider et al., 2005). In postmortem studies G-CSFR has been found in the human frontal cortex (Schneider et al., 2005).

G-CSFR is composed of six functional domains which are broken up into an immunoglobulin-like domain, a cytokine receptor-homologous domain (CRH) and three additional fibronectin type III domains within its extracellular region. By G-CSF binding to G-CSFR various signaling cascades can be ignited. G-CSF induces the Janus kinase (JAK)/ signal transducer and activator transcription (STAT) pathway, the Ras/Mitogen activated protein (MAP) kinase, and Phosphatidyl inositol 3-kinase (PI3K)/ protein kinase B (aka Akt) pathways. These various pathways have been shown to induce cellular proliferation, apoptotic processes, and anti-inflammatory processes (Tian et al., 1994,
Shimoda et al., 1997, Hunter and Avalos, 1998, Dong and Larner, 2000, Ward et al., 2000). These signaling pathways induced by G-CSF are a major objective of current studies (Sampson et al., 2007).

In a rat stroke model G-CSF and G-CSFR are up-regulated two hours post-occlusion and reperfusion of the middle cerebral artery (MCA) in the ipsilateral forebrain (Schneider et al., 2005). In another study looking at ischemia, G-CSF mRNA levels were greatly elevated in the ischemic brain versus the normal brain although returned to normal after two days. This elevation in G-CSF mRNA extended to non-ischemic areas of the frontal cortex after photothrombosis in a rat model of focal cerebral ischemia (Kleinschnitz et al., 2004). G-CSF in combination with SCF administered 10 days after ischemia resulted in improved motor performance, induced transition of BM-derived neuronal cells into the penumbra, and stimulated proliferation of brain-derived NSC in the zones of neuroproliferation (Kawada et al., 2006). G-CSF may have a protective autocrine signaling mechanism, similar to that seen in other HGF like EPO in response to brain injury (Solaroglu et al., 2007). It is known that G-CSF is able to mobilize CD34+ HSC from the BM into the blood allowing them to circulate and migrate to areas of ischemic damage (Bodine et al., 1994, Grigg et al., 1995, Orlic et al., 2001a, Orlic et al., 2001b, Shyu et al., 2004).

In a study by Tsai et al. two AD models were used to evaluate the effect of G-CSF on AD mice (Tsai et al., 2007). In the acute model aggregated Aβ was injected into the hippocampal-cortical junction of the mouse brain and the chronic model used the Tg2576 AD mouse. After a 5 day dosing of G-CSF in the acute
AD mice learning and memory function in the Morris water maze was restored to
levels similar to that of the control mice. It was found that white blood cell levels
were elevated in the G-CSF treated mice. BrdU+/NeuN double-labeling of cells
was increased in both the acute and chronic AD mice models treated with G-
CSF, although this double-labeling was not necessarily found in the neurogenic
niches therefore may not be a result of improved neurogenesis, but of cellular
repair or death. 20% of the BrdU+ cells in the acute AD mice were CD34+ which
could indicate that HSC were mobilized by G-CSF and directed to the Aβ
injection site in the brain. After a 5 day dosing of G-CSF Tg2576 mice an
improvement in memory and learning was seen due to the reduced latency in the
Morris water maze similar to the improvement seen in the acute AD mice treated
with G-CSF. Three months after treatment with G-CSF Tg2576 mice were
retested and found to still have significant learning and memory abilities over the
untreated mice indicating a long lasting effect of G-CSF. The amount of
acetylcholine (ACh) was increased by 20% in G-CSF treated Tg2576 mice,
showing a potential improvement in synaptic potential. Although learning and
memory were improved no reduction in the Aβ plaques were seen. Therefore,
overall the treatment with G-CSF in both the acute and chronic mouse model for
AD showed an induction in the release of HSC from the bone marrow, potential
stimulation of neurogenesis, an increase in ACh production and an overall
improvement in neurological function, specifically improvement in learning and
memory (Tsai et al., 2007).
In a study previously done in this laboratory Tg APP/PS1 AD mice were treated with s.c. G-CSF injections every other day for two weeks (Sanchez-Ramos et al., 2009). A reduction in Aβ deposits in both the hippocampus and entorhinal cortex was seen as well as a reduction in soluble Aβ within the hippocampus, which differs from what was seen in the study done by Tsai et al (Tsai et al., 2007). A correlation was observed between a decrease in Aβ deposition and improvement in cognitive function. In other studies it has been shown that an increase in Aβ decreases cognitive function (Arendash et al., 2001, Ethell et al., 2006). Improved cognitive function also was seen with the removal of Aβ in the case of long term caffeine administration (Arendash et al., 2006) and in the case of administration of Leuprolide acetate (a gonadotropin releasing hormone (GRH) analogue) (Casadesus et al., 2006). It was proposed that G-CSF reduces the deposited Aβ, which then causes the soluble Aβ to become deposited and then it to be removed by G-CSF induced pathways. This mechanism could prove to be important as the soluble form of Aβ has been indicated to be more toxic than the deposited form of Aβ and can also inhibit LTP (Walsh et al., 2002, Walsh and Selkoe, 2004). Despite the increase in neutrophil count with G-CSF treatment due to its neuroprotective effects the release of pro-inflammatory mediators is inhibited (Hartung, 1998, Boneberg et al., 2000). In G-CSF treated APP/PS1 mice synaptophysin density also was increased in the hippocampus indicating an increase in synapses. Increased levels of Aβ have been linked to decreased levels of synapses, but with decreased levels of Aβ the loss of synapses is self-correcting leading to an improvement in LTP (Hsia et al.,
G-CSF was also shown to increase neurogenesis, but it is unclear whether or not neurogenesis is directly influential on the cognitive improvement of Tg AD mice (Kuhn et al., 2007).

In a previous study done by Laske et al. a significant decrease G-CSF plasma levels in patients with early AD was seen in comparison to healthy age matched controls (Laske et al., 2009). Also a significant inverse correlation was shown between G-CSF plasma levels and Aβ 1-42 levels in the CSF in AD patients. A significant inverse correlation in G-CSF levels and age was seen in both AD patients and healthy controls, therefore G-CSF levels decrease with age, it also would be interesting to assess if G-CSF levels decreased as the severity of AD progresses, although unfortunately it was not assessed in this study. The decrease in G-CSF in early AD may contribute to the deficient HSC support in the AD brain (Laske et al., 2009).

Currently G-CSF is being used therapeutically to induce proliferation of neutrophils in patients suffering from neutropenia after anti-cancer drug therapy as well as in patients with congenital neutropenia (Welte et al., 1996, Kocherlakota and La Gamma, 1997, Carlsson et al., 2004). Because G-CSF is already in clinical use, it has been suggested that it could be used to treat various neurological disorder without compromising the patient. Clinical trials to examine the ability of G-CSF to treat ischemic strokes are currently being assessed (Schabitz and Schneider, 2007). Additionally G-CSF has been used to treat myocardial infarctions (Takano et al., 2003, Kuethe et al., 2004, Nienaber et al., 2009).
2006, Ripa et al., 2006, Suzuki et al., 2006). Due to the AD brain lack of hematopoietic support it could be promising to further evaluate HGF such as G-CSF as potential treatments for AD.

**Specific Aims**

G-CSF, known generically as filgrastim, has been reported to have disease-modifying effects in a transgenic mouse model of AD. G-CSF is known to stimulate hematopoiesis in bone marrow, and to increase circulating levels of stem/progenitor cells and neutrophils. G-CSF also has direct actions on CNS neural cells and may rescue dying or dysfunctional neurons and promote hippocampal neurogenesis. The overall objective of this dissertation is to determine the extent to which the actions of G-CSF on blood stem/progenitor cells and inflammatory cells are responsible for its disease-modifying effects in AD in comparison to direct actions on CNS neurons.

**Specific Aim 1.** To test the hypothesis that G-CSF promotes trafficking of blood stem/progenitor cells from marrow to blood and from blood to brain where marrow-derived monocytes (or pro-monocytes) differentiate into fresh microglia, a set of *in vitro* and *in vivo* experiments will be conducted. In the *in vitro* studies, various parameters will be assessed including binding kinetics, anti-apoptotic proteins, proliferation, viability, adhesion, and migration in order to further describe the mechanism of action of G-CSF on marrow-derived monocytes. For the *in vivo* studies, a chimeric AD mouse model with green fluorescent bone marrow cells will be treated with G-CSF in order to determine the proportion of...
total microglia that derive from the periphery (GFP+ cells). Secondary endpoints in the in vivo studies will be total amyloid burden and microgliosis in the hippocampus and entorhinal cortex.

**Specific Aim 2.** To test the hypothesis that MCP-1 mediates the mobilization of BMDC triggered by G-CSF administration, in vitro experiments will be conducted. Using a cell migration system, the effects of an antagonist of the MCP-1 receptor expressed on monocytes (also known as the CCR-2 receptor) on monocyte migration will be evaluated. In addition, the effects of G-CSF, with and without the CCR-2 receptor antagonist on monocyte migration will be assessed.

**Specific Aim 3.** To test the hypothesis that G-CSF has direct actions on hippocampal neurons and may promote neurogenesis, thereby ameliorating or mitigating the behavioral deficits observed in Tg AD mice as they age; both in vitro and in vivo experiments will be utilized. In the in vitro studies, various parameters will be assessed including binding kinetics, anti-apoptotic proteins, proliferation, and viability in order to further describe the mechanism of action of G-CSF on CNS neurons. For the in vivo studies, an AD mouse model will be treated with G-CSF in order to determine the effect of G-CSF on hippocampal neurogenesis through analysis of incorporation of BrdU in the SGZ of the HC and immature neuronal markers. Secondary endpoints in the in vivo studies will be total amyloid burden and microgliosis in the hippocampus and entorhinal cortex.
Successful completion of this study will increase understanding of the primary mechanism(s) responsible for the beneficial effects of G-CSF on the AD disease process, which may result in new therapeutic targets for the treatment of AD.
Table 1. Summary of effects of hematopoietic growth factors (HGFs) in brain.

<table>
<thead>
<tr>
<th>SCF</th>
<th>M-CSF</th>
<th>GM-CSF</th>
<th>SDF-1</th>
<th>G-CSF</th>
<th>G-CSF +SCF</th>
<th>EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival factor &amp; chemokine of NSCs</td>
<td>Reduced cognitive deficiency and Aβ plaque load in APP/PS1 Tg mice</td>
<td>Uprogulation of its receptor in areas of ischemia in the brain</td>
<td>Plays role in angiogenesis &amp; attraction of HSCs to injured vessel walls</td>
<td>Upregulated in ischemic brain</td>
<td>Improved neurogenesis, angiogenesis, &amp; enhancement of brain repair in ischemia</td>
<td>Its receptor is found in both the nervous &amp; cardiovascular system</td>
</tr>
<tr>
<td>Neurogenesis</td>
<td>Decreased Aβ plaque deposition in AD mice</td>
<td>Receptor found on NSC in hippocampus</td>
<td>Potent chemotactrant for HSCs</td>
<td>Neurogenesis in AD mice</td>
<td>Increased levels of it and its receptor in the brain with hypoxia or ischemia</td>
<td></td>
</tr>
<tr>
<td>Neuroprotective effects</td>
<td>Improvement in spatial memory in AD Tg mice</td>
<td>Anti-apoptotic effect on neurons &amp; effect induced by ischemia</td>
<td>Neuroprotective effects</td>
<td>Protective autocrine signaling in response to brain injury</td>
<td>Anti-apoptotic effects for NSCs against Aβ induced oxidative stress</td>
<td></td>
</tr>
<tr>
<td>Modulation of microglia &amp; possible suppression of microglia to decrease neuroinflammation</td>
<td>Promotion of the proliferation, activation and development of microglia</td>
<td>Increased neurogenesis, angiogenesis, &amp; enhancement of brain repair in ischemia</td>
<td>Increased levels of it and its receptor in the brain with hypoxia or ischemia</td>
<td>Prevention of hyperphosphorylation of tau and formation of neurofibrillary tangles</td>
<td></td>
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<tr>
<td>Mobilization of BMSC into the brain</td>
<td>Low levels in pre-symptomatic AD in combination with low levels of other HGF's displays a progression towards a decrease of cognitive function to dementia</td>
<td>Proliferation &amp; differentiation of microglia</td>
<td>Decreased plasma levels in AD pts</td>
<td>Improvement in learning, cognitive function and memory in AD mice</td>
<td>Reduced Aβ plaques in hippocampus &amp; entorhinal cortex as well as decreased soluble Aβ in the hippocampus</td>
<td></td>
</tr>
<tr>
<td>In brain primarily produced by neurons</td>
<td>In brain primarily produced by astrocytes or neurons (conflicting data)</td>
<td>In brain primarily produced by astrocytes or neurons (conflicting data)</td>
<td>In brain primarily produced by astrocytes</td>
<td>In brain produced in neurons &amp; astrocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobilization of neuron-like BMSC &amp; resident brain NSC to areas of brain injury such as ischemia</td>
<td>Antibodies to GM-CSF causes decreased Aβ plaque deposits, decreased soluble Aβ 1-42 production, and microglial activation in AD pts</td>
<td>Reduced Aβ plaques in hippocampus &amp; entorhinal cortex as well as decreased soluble Aβ in the hippocampus</td>
<td>Reduced Aβ plaques in hippocampus &amp; entorhinal cortex as well as decreased soluble Aβ in the hippocampus</td>
<td>Expression of anti-apoptotic genes preventing the activation of caspases and microglia as well as prevention of phagocytosis of damaged neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AChE-I increases SCF in AD pts</td>
<td>Combination of Aβ/GM-CSF/IL-4 antibodies decreased Aβ plaque load</td>
<td>Increased synaptophysin in AD mice</td>
<td>Increased detection of MSCs to neurons and decreased differentiation to glial-like cells in the presence of hypoxia</td>
<td></td>
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</tr>
<tr>
<td>Increased SCF levels is correlated with increased cognitive function in AD pts</td>
<td>Increased synaptophysin in AD mice</td>
<td>Increased production of glutamine synthase in neuronal-like cells</td>
<td></td>
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<tr>
<td>May increase BDNF</td>
<td>Increased differentiation of MSCs to neurons and decreased differentiation to glial-like cells in the presence of hypoxia</td>
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<tr>
<td>Modulate spatial memory and hippocampal synaptic potentiation</td>
<td>Increased production of glutamine synthase in neuronal-like cells</td>
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<td>Increased levels in areas of brain injury</td>
<td>Increased production of glutamine synthase in neuronal-like cells</td>
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<tr>
<td>Lower plasma levels of SCF in AD pts</td>
<td>Increased production of glutamine synthase in neuronal-like cells</td>
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</table>

NSCs, neural stem cells; BMSCs, bone marrow stem cells; AChE-I, acetylcholinesterase inhibitors; SCF, stem cell factor; AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; MSCs, mesenchymal stem cells.
Figure 1. Granulocyte colony-stimulating factor (G-CSF) has direct actions on bone marrow hematopoietic stem cells (HSCs), as well as direct and indirect effects on brain.

References


Simard AR, Rivest S (2004) Bone marrow stem cells have the ability to populate the entire central nervous system into fully differentiated parenchymal microglia. FASEB J 18:998-1000.


Chapter 2: Comparison of the Cellular Effects of G-CSF on Neurons and Monocytes

Abstract

An expanding body of animal and human research attests to the neurotrophic potential of granulocyte colony stimulating factor (G-CSF) in the treatment of stroke and neurodegenerative diseases. G-CSF has been reported to improve hippocampal-dependent learning, increase microgliosis, decrease amyloid burden and stimulate hippocampal neurogenesis in a mouse model of AD. The objective of the present study was to elucidate differences and similarities between brain cells and bone marrow-derived cells exposed directly to G-CSF. Method: Cell culture models of monocytes (THP-1) and neurons (SH-SY5Y) were incubated with G-CSF. The following parameters were measured: G-CSF receptor binding kinetics using [125I]-G-CSF; DNA synthesis using [3H]-thymidine incorporation; viability using a live-dead assay; signal transduction, in particular expression of alternatively spliced protein kinase C (PKCδVIII) and anti-apoptotic protein Bcl2; changes in adhesiveness and migratory properties induced by G-CSF in the monocytes. Results: G-CSF receptor binding kinetics in neurons differed from that in monocytes, with Kd in neurons being significantly higher than that of monocytes. Addition of G-CSF to THP-1 cells and to SH-SY5Y resulted in a concentration-dependent increase in DNA synthesis that was similar in both cell lines. Despite higher affinity of G-CSF for receptors on
monocytes, G-CSF treatment increased Bcl2 expression in neurons at lower concentrations than that required in monocytes. G-CSF treatment improved viability in neurons, but not in monocytes. G-CSF did not increase either cellular adhesiveness or migration through a semi-permeable membrane, whereas monocyte chemotactic protein (MCP-1) significantly improved migration. Conclusions: The cellular and molecular responses to G-CSF treatment of monocytes suggest that neither changes in adhesiveness nor migratory capacity are responsible for the beneficial effects of G-CSF administration in a Tg mouse model of AD. It is likely that the increased microgliosis observed in Tg AD mice treated with G-CSF is a consequence of increased levels of circulating monocytes in the setting of Aβ-fueled neuroinflammation and MCP-1 release which facilitates monocyte infiltration.

Introduction

G-CSF (granulocyte colony stimulating factor or Filgrastim) is a therapeutic hematopoietic cytokine commonly used for treatment of neutropenia and to increase generation of hematopoietic stem/progenitor cells in bone marrow donors (Dale, 2003, Pusic and DiPersio, 2008). G-CSF is naturally synthesized by several cell types including endothelium, macrophages, bone marrow stromal cells, fibroblasts and a number of other immune cells (Malipiero et al., 1990, Demetri and Griffin, 1991, Wells and de Vos, 1996). Interestingly, G-CSF also exerts neurotrophic effects and has been identified, along with its receptor (G-CSFR), in neurogenic zones of the hippocampus (HC), the sub-ventricular zone and the olfactory bulb (Schneider et al., 2005). G-CSF and G-
CSFR are also both expressed by neurons in several other areas of the brain including pyramidal cells in cortical layers (specifically II and V), entorhinal cortex, Purkinje cells of the cerebellum, and in cerebellar nuclei in rats (Schneider et al., 2005). The G-CSF receptor is also fairly ubiquitous and can be found on the cell surfaces of endothelial cells, lymphocytes, platelets, and neutrophils (Bussolino et al., 1989, Hanazono et al., 1990, Shimoda et al., 1993, Morikawa et al., 2002).

Mice bred to have a deficiency in G-CSF exhibited defects in memory formation and the development of motor skills, as well as impairment in the induction of long term potentiation in the CA1 region, decrease in neuronal precursor cells in the dentate gyrus (DG) and decreased dendritic complexity in neurons in the DG and CA1 region of the HC (Diederich et al., 2009b). The defects seen in G-CSF deficient mice support the importance of G-CSF as an essential neurotrophic factor and its part in facilitating neural cells in hippocampal formation (Diederich et al., 2009b). In combination with cognitive training, G-CSF can also significantly improve spatial learning and new neuron survival in the hippocampus (Diederich et al., 2009a).

A significant decrease of G-CSF plasma levels was found in patients with early AD in comparison to healthy age matched controls (Laske et al., 2009). In addition, the authors reported a significant inverse correlation between G-CSF plasma levels and Aβ1-42 levels in the cerebrospinal fluid of AD patients. These observations, coupled with evidence that bone marrow-derived cells contribute to microgliosis in AD, led to studies of the disease-modifying impact of G-CSF in
animal models of AD (Malm et al., 2005, Tsai et al., 2007, Sanchez-Ramos et al., 2009).

Treatment of transgenic (Tg) APP/PS1 AD mice with subcutaneous G-CSF for two weeks resulted in significant reduction of total β-amyloid (A-β) deposits in both hippocampus (HC) and entorhinal cortex (EC) (Sanchez-Ramos et al., 2009). G-CSF treated Tg mice also exhibited a significant increase in microgliosis in both HC and the EC. A correlation was observed between decreased Aβ deposition and improvement in cognitive function as well as a suggestion of increased neurogenesis (Sanchez-Ramos et al., 2009). The mechanism of action of G-CSF responsible for the cognitive changes (performance in the radial arm water maze) in Tg mice is unknown. However, it is clear that G-CSF has the ability to act on cells in the peripheral immune system, such as on hematopoietic stem progenitor cells and monocytes (Kondo et al., 1991), as well directly upon CNS neurons (Schneider et al., 2005). It is not easy in animal studies to determine the extent to which the disease-modifying and beneficial effects on behavior are mediated by stimulation of hematopoiesis, with increased numbers of circulating monocytes and increased infiltration of these cells into brain, and/or from the direct actions of G-CSF on neurons and neural stem/progenitor cells in hippocampus.

The objective of the present study was to elucidate differences and similarities between brain cells and bone marrow-derived cells exposed directly to G-CSF. Understanding the molecular and cellular responses triggered by G-CSF will provide insights into the complex mechanisms of action of G-CSF.
administered in vivo. A series of experiments were undertaken to compare effects of G-CSF on monocytes and neurons in cell culture models. The parameters of cellular functions compared were a) G-CSF receptor binding kinetics, b) DNA synthesis, c) viability assay and d) signal transduction, in particular expression of alternatively spliced protein kinase C (PKC) and the anti-apoptotic protein Bcl2. Finally, migration and adhesiveness of monocytes were investigated in vitro to determine if these actions play a role in mobilization and infiltration of circulating monocytes into brain.

**Materials & Methods**

**Cells and cell lines.** Two cell lines have been chosen for the in vitro studies. One is a human monocyte cell line that is representative of the peripheral immune system, THP-1, which grows in suspension. THP-1 cells are derived from a human acute monocytic leukemia cell line often used as a model for human blood monocytes, but it is important to recognize that they are not fully differentiated monocytes, but promonocytes (Auwerx, 1991, Tompa et al., 2011). The other cell line is a human neuroblastoma cell line SH-SY5Y, which is a mixed cell line although only adherent cells were utilized (Xie et al., 2010). Both cell lines are known to express the G-CSFR and the THP-1 cell line is also known to express the receptor, CCR2 (Schneider et al., 2005, Chen et al., 2008). Both cell lines were purchased from ATCC (Manassas, VA). THP-1 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) plus 1% penicillin-streptomycin (ATCC, Manassas, VA) in a humidified atmosphere of 5% CO₂ at 37°C. SH-SY5Y cell line was cultured in 1:1
EMEM/F12 medium with 10% FBS plus 1% penicillin/streptomycin in a humidified atmosphere of 5% CO$_2$ at 37˚C.

**Proliferation assay.** THP-1 cells (or SH-SY5Y cells) were cultured in 24 well plates (3x10$^5$ cells/well, 6-8 replicates/concentration of G-CSF) for 24 hours then G-CSF was added to THP-1 cell line in basal media of RPMI-1640 (or EMEM/F12) + 10% FBS. 24 hours after G-CSF addition 37,000 Bq/ml (500pmol/ml) [$^3$H]-thymidine (Perkin-Elmer Life and Analytical Sciences, Inc., MA, USA) was added to the cultures for 4h, then the cells were harvested, and [$^3$H]-thymidine incorporated into DNA was determined. The specific activity of [$^3$H]-thymidine was 74 Bq/pmol. Uptake was normalized based on counts per minute (CPM) over total protein in µg and expressed as percent change from control.

**Viability assay for human monocytes and human neuronal cells in vitro.** THP-1 cells were cultured in 96 well plate in basal media of RPMI-1640 + 10% FBS for 24 hours then G-CSF was added directly to wells. SH-SY5Y cells were cultured in 24 well plates for 24h then G-CSF was added to SH-SY5Y cell line in basal media of EMEM/F12 +10% FBS. 24h after addition of G-CSF cells were incubated with 2µM Calcien AM (live cell dye) and 4µM Ethidium Homodimer-1 (EthD-1) (dead cell dye) for 30min (Invitrogen, Grand Island, NY) and analyzed using a Biotek plate reader fluorescence area scan. The mammalian live-dead assay (Invitrogen, Grand Island, NY) assesses the integrity of the cellular membrane (ethidium homodimer) as well as intracellular esterase activity (calcien AM) in order to assess overall viability.
**Adhesion assay.** THP-1 cells are a monocyte cell line which are not typically very adhesive; when PMA (phorbol myristate acetate) is added to these cells they become more macrophage-like and hence more adhesive (Shapira et al., 1993). THP-1 cells were cultured in a 24 well plate pre-coated with fibronectin (10ng/mL) and were treated with either basal media alone (RPMI-1640 medium + 2% FBS), G-CSF (250ng/ml) in basal media, PMA (200nM) in basal media, or G-CSF+ PMA in basal media for 24h. Media was then discarded and unattached cells were gently rinsed off with sterile 1X PBS. A total of 5 fields per well were counted at 20x magnification under bright field microscopy (IX2 inverted microscope; Olympus, Tokyo, Japan).

**Migration assay.** THP-1 cells were harvested then brought up in DMEM media + 10% FBS. CCR-2 antagonist, RS 504393, (Tocris Biosci, Inc, Minneapolis, MN) was used to inhibit the effect of recombinant human MCP-1 (R&D Systems, Inc., Minneapolis, MN, 279-MC) on the chemotaxis of human monocytes. Cells were pre-treated with CCR2 antagonist in DMEM + 10% FBS prior to start of migration assay. RS-504393 is a selective CCR-2 antagonist that shows modest inhibitory activity in a chemotaxis assay to MCP-1. It inhibits CCR-2 by occupying the CCR-2 binding site that includes acidic residue Glu291 and specifically inhibits MCP-1 and MCP-3 signaling. This compound is not a chemotaxis agonist and does not stimulate post receptor signaling of any kind (Mirzadegan et al., 2000). It also binds significantly to α-adrenergic receptors (Dawson et al., 2003). A reusable modified Boyden 48-well chamber (Neuroprobe, Gaithersburg, MD) with a 5μM polycarbonate fibronectin coated
filter was utilized. The top wells of chamber contained 55µL of THP-1 cell line in DMEM media + 10% FBS and the bottom wells contained 27µL of potential chemoattractant diluted in DMEM media +10% FBS. The plate was then incubated for 2 hours in humidified incubator @ 37°C in 5% CO₂. The number of monocytes, which migrated to the bottom side of the filter, were quantified using Vectashield Hardset mounting media (Vector Labs, Burlingame, CA) containing 40-6-diamidino-2-phenylindole (DAPI). All (20x) images of DAPI-labeled monocytes (5 images/well, 6 wells per concentration) were acquired using an Olympus BX60 microscope with an attached digital camera system (DP-70, Olympus, Tokyo, Japan), and the digital image was routed into a Windows PC for quantitative analysis using ImageJ (NIH, Bethesda, MD).

**G-CSF receptor parameters assessed with [¹²⁵I]-G-CSF.** [¹²⁵I]-G-CSF (specific activity 46.55 TBq/mmol) was purchased from Perkin Elmer (Waltham, Massachusetts). Binding of [¹²⁵I]-G-CSF was measured in previously described method with some modifications (Kondo et al., 1991). Cells were incubated in 180 µL of binding medium containing 0.2% BSA, 5 mM MgSO₄ and 50 mM Hepes, pH 7.2 at a concentration of 1.5×10⁷cells/ml and the appropriate concentration of [¹²⁵I]-G-CSF. Incubations were carried out at room temperature with periodic shaking to ensure continuous mixing of cells and radioactive ligand. The incubation time of 2 h was estimated from preliminary experiments as being found sufficient to reach equilibrium. Nonspecific binding of [¹²⁵I]-G-CSF was measured by incubations in the presence of a 100-fold molar excess of unlabeled G-CSF. At the end of the incubation, bound and free radio ligands were
discriminated by using separating oil according to previously published method (Dower et al., 1985). Namely, 80 µL aliquots were sampled from the incubation mixture, each aliquot was layered on 300 µL of separating oil placed in 500 µL polyethylene tubes and centrifuged for 5 min at 6000 rpm. The separating oil consisted of 1.5 parts dibutyl phthalate and 1 part bis(2-ethylhexyl)phthalate (Aldrich-Sigma, St. Louis, MO). Bound and free ligand activities were counted after cutting tubes in two pieces and placing top and tip into separate scintillation vials. Counting was performed on Beckman Coulter LS6500 scintillation counter. Glacial acetic acid was employed to solubilize the pellet (Jordan et al., 1974). The specific binding was determined from the amount of bound \[^{125}\text{I} \]-G-CSF blocked by competition with excess unlabeled G-CSF. The parameters of saturation binding experiments including dissociation constant (\(k_d\)), maximum binding capacity (\(B_{\text{max}}\)) and binding cooperativity (\(h\)) were calculated with GraphPad Prism 5 software (La Jolla, CA) by using nonlinear regression analysis.

**Signal transduction triggered by G-CSF (measurement of PKCδVIII and Bcl2 with Western blot).** THP-1 cells or SH-SY5Y cells 3x10⁶ cells in 5ml media in a 25 cm² flask were treated with or without 100ng/ml G-CSF for 24h. Whole cell lysates (60mg) were separated on 10% polyacrylamide gel electrophoresis-SDS (PAGE-SDS). Proteins were electrophoretically transferred to nitrocellulose membranes, blocked with Tris buffered saline- 0.1% Tween 20 containing 5% nonfat dried milk, washed, and incubated with a polyclonal antibody against either Bcl2 (Cell Signaling) or PKCδVIII-specific polyclonal
antibody (Patel laboratory). (Jiang et al., 2008). The house keeping gene GAPDH was used as an internal standard. Following incubation with anti-rabbit IgG-HRP, enhanced chemiluminesence (Pierce™) was used for detection and the gels were analyzed using UN-SCAN-IT™ software (Silk Scientific, Inc.).

**Statistical analysis.** In the case of western blot analysis, two-tailed unpaired t-test was utilized, and significance was determined after 3 or more experiments. The remaining in vitro data analyses were performed using one-way or two-way ANOVA. All data was presented as mean ± SD, except for western blot graphs which are presented as SEM. All statistical analysis of data was done via PRISM4 or PRISM5 statistical analysis software (GraphPad Prism, La Jolla, CA). All comparisons were considered significant at level of P<0.05.

**Results**

**G-CSF receptor binding kinetics in neurons differs from that in monocytes.** Increasing concentrations of radio-labeled G-CSF (alone or in the presence of 100 fold excess “cold” G-CSF) were added to the cell cultures to generate a saturation curve. Specific binding of \( ^{125}\text{I} \)-G-CSF to receptors expressed in monocytes (THP-1 cells) and neurons (SH-SY5Y) revealed significant differences in Kd, Bmax and number of receptors per cell (Figure 2.1). The Kd for monocytes was 10 fold lower (greater affinity) than that in neurons. The relationship between specific binding and concentration of the ligand was not linear in the neuronal cultures and exhibited a Hill coefficient of 2.29, indicating substantial cooperative binding of the ligand to receptors expressed in neurons compared to the monocytes with a Hill coefficient of 0.92. Although there is a
greater binding affinity in the monocytes in comparison to the neurons, there is a 5-fold greater receptor density in the neurons in comparison to the monocytes.

**G-CSF treatment increases expression of anti-apoptotic proteins**

**Bcl2 and PKCδVIII in neurons and monocytes.** G-CSF treatment activates multiple signal transduction pathways including anti-apoptotic pathways. To evaluate the biological response of G-CSF, the anti-apoptotic proteins Bcl2 and PKCδVIII were measured. Protein kinase C (PKC) δ is a serine/threonine kinase mediating cellular growth, differentiation and apoptosis. PKCδ is alternatively spliced to generate isoforms with distinct functions in apoptosis. Human PKCδVIII and its mouse homolog PKCδII function as anti-apoptotic proteins (Patel et al., 2006, Jiang et al., 2008). PKCδVIII has been demonstrated to be associated with neuronal differentiation induced by retinoic acid in human neuroteratocarinoma (NT2) cells and promotes neuronal survival (Jiang et al., 2008). PKCδII, the mouse homolog of PKCδVIII, is demonstrated to directly increase the expression of Bcl2. (Apostolatos et al., 2012).

Increasing concentrations of GCSF (5.3nM or 10.6 nM) were added to the cell culture and incubated for 24 hours. Whole cell lysates were immunoblotted for Bcl2 and PKCδVIII. Despite the much lower binding affinity of G-CSF for receptors borne on neurons compared to binding affinity in monocytic cells, the biological response i.e. expression of the anti-apoptotic protein Bcl2 and PKCδVIII, was much greater at equivalent concentrations (5.3 nM) (Figure 2.2) in the human neuronal cell line. G-CSF, however, also increased the expression of PKCδVIII and Bcl2 in the monocytic line at a higher concentration (10.6 nM).
G-CSF increases DNA synthesis in both human monocytes and neurons. G-CSF is known to stimulate proliferation of hematopoietic stem/progenitor cells and to increase proliferation of neural stem/progenitor cells. To compare the relative efficacy of G-CSF to stimulate cell proliferation, DNA synthesis was measured using 3H-thymidine uptake. Addition of G-CSF to THP-1 cells and to SH-SY5Y resulted in a concentration-dependent increase in DNA synthesis that was similar in both cell lines (Figure 2.3A, 2.3B).

G-CSF treatment increases viability in neurons but not monocytes. THP-1 cells treated with increasing concentrations of G-CSF showed no significant difference in viability (Fig 2.4A & 2.4B), either when assessing live cells (calcien AM positive) or dead cells (ethidium homodimer positive). G-CSF did not improve monocyte viability, although it did increase proliferation. The viability dye of the live-dead assay includes all healthy cells while the proliferation assay only assesses those cells which are actively dividing; this may explain the difference seen between the proliferative data versus the viability data in the monocyte cell line.

SH-SY5Y cells treated with increasing concentrations of G-CSF showed a concentration-dependent increase in viability (Fig 2.4C & 2.4D), although there was no change in the proportion of dead cells, there was a significant increase in the proportion of live cells at G-CSF concentrations 25 & 50ng/ml. The neuronal cell line also had an increase in anti-apoptotic proteins at lower concentrations of G-CSF, than the monocytic cell line, which also may explain why there is no
significant difference in the viability of the monocytic cell line at these lower concentrations of G-CSF.

**Effect of G-CSF and MCP-1 on migration of human monocytes.** To assess whether G-CSF effects on circulating monocytes is responsible for increased infiltration of monocytes into brain, where they contribute to the increase in total microglia observed in earlier studies (Sanchez-Ramos et al., 2009), an *in vitro* assessment of migratory capacity of the monocytes was undertaken. MCP-1 is a known monocyte chemoattractant and therefore was used as a standard control to compare to G-CSF to examine its potential as a chemoattractant. At MCP-1 concentrations 5, 10, & 20ng/mL there was a significant increase in migration of THP-1 monocytes (Figure 2.5B) across the fibronectin coated filter, although at various concentrations of G-CSF no chemoattractant effect was found (Figure 2.5A). Also G-CSF in combination with MCP-1 did not result in a synergistic effect on migration of THP-1 cells (Figure 2.7).

**Effect of CCR2 antagonist on migration of human monocytes.** Increased migration of monocytes triggered by MCP-1 was antagonized by pre-incubation with a CCR2 antagonist (20µg/ml) for 30min (Figure 2.6). Although G-CSF alone did not act as a chemoattractant it was of interest to see if G-CSF could overcome the antagonism of MCP-1 caused by CCR2 antagonist. As shown in Figure 2.7 there was a lack of interaction between G-CSF and MCP-1 in regards to migration.
Effect of G-CSF on adhesion on human monocytes. When PMA (phorbol myristate acetate) is added to THP-1 cells they become more macrophage-like and hence significantly more adhesive (Shapira et al., 1993). If G-CSF could improve adhesion this could lead to better diapedesis and therefore the potential for an increase migration into the brain. Unfortunately, G-CSF did not significantly improve adhesion of THP-1 cells at various concentrations and did not further improve adhesion caused by PMA (Figure 2.8A, 2.8B).

Discussion

Both monocytes and neurons express G-CSF receptors, but pharmacokinetic parameters of radio-labeled ligand binding and subsequent biological responses were markedly different. The Kd for binding of the ligand with the neuronal G-CSF receptors was 10 fold greater (i.e. 10 fold lesser affinity) than binding with monocyte receptors and the number of receptors per cell was 5 fold greater in the neurons. Moreover, neuronal binding exhibited significant cooperativity, indicated by Hill coefficient of 2.29 compared to 0.92 in the monocytic cell line. Cooperative binding refers to enhanced binding of a ligand to a macromolecule when there are already other ligands present on the same macromolecule. The relevance of cooperative binding to the differential effects of GCSF in neurons and monocytes is not clear, but may reflect the fact that G-CSF signaling in neuronal populations is predominantly of an autocrine nature. Both the GCSF ligand and its receptor are expressed in neurons, so autocrine signaling and cooperative binding may trigger a well-coordinated response in a population of neurons.
Another important distinction between the monocytes and neurons was found in signal transduction triggered by G-CSF. The expression of the anti-apoptotic proteins PKCδVIII and Bcl2 were substantially increased in the neuronal cells, but not in the monocytic cells treated with equal, relatively high concentrations (5.3nM). It is paradoxical that neurons exhibit a greater anti-apoptotic response to G-CSF than monocytes, despite the much greater affinity of monocyte G-CSF receptors for its ligand. Differences in G-CSF signal transduction observed here may be attributed to variations in the structure of G-CSF receptor or variations in the signaling portion of the receptor in neurons and monocytes. It is known that spliced transcript variants of this gene occur in the part of the G-CSF receptor gene (CSF3R) encoding the intracellular domain of the G-CSFR (Dong et al., 1995, Germeshausen et al., 2008). It is not known whether variations in the intracellular domain distinguish the neuronal from monocytic G-CSF receptors, a possibility that merits further investigation.

An explanation for the differences between monocytes and neurons also might be related to the capacity of monocytes to be replenished, in vivo, by progenitors in the bone marrow. Mature neurons can be replaced only in limited neurogenic niches such as the sub-ventricular zone of the hippocampus. Therefore the survival of differentiated, mature neurons may be more dependent on the capacity to overexpress anti-apoptotic proteins than monocytes. However, the neuronal line used here (SH-SY5Y) maintains mitotic competency and increases DNA synthesis in response to G-CSF treatment just as well as the monocytes.
Although G-CSF did not appear to directly alter migratory capacity or cellular adhesiveness to enhance infiltration into the CNS, a course of G-CSF administration in humans is known to increase absolute levels of circulating monocytes in addition to total neutrophil count and hematopoietic stem cells (CD34 cells) (Sanchez-Ramos J, 2012). This also corresponds to the in vitro data where proliferation in a human monocytic cell line was increased with G-CSF treatment. The mechanism of infiltration of a monocyte population from blood to brain, differentiation into microglia and peri-amyloid plaque accumulation in AD brains is not completely understood, but it is known that cell signaling by monocyte chemoattractant protein (MCP-1) plays a key role. Addition of MCP-1 to monocytes, but not G-CSF, enhanced their migration through a micropore in a cell culture migration system. G-CSF treatment did not result in an improvement in adhesive properties, which would be helpful to monocytes undergoing diapedesis through the endothelial cells of the BBB and into the brain. If G-CSF does in fact enhance monocyte infiltration into the brain it does not appear to do so by improved migratory properties, such as chemoattraction or adhesion, and therefore it would more likely be caused by overall increase in total peripheral monocytes or circulating hematopoietic stem cells, which may result in increased migration through “mass action.” In other words, more monocytes in the peripheral circulation may result in a greater extent of brain microgliosis in the setting of neuroinflammation.

During the inflammatory process, monocyte chemoattractant protein (MCP-1) mediates the recruitment of CCR-2 expressing monocytes into the brain.
(Yamamoto et al., 2005) as well as activation of macrophages (Matsushima et al., 1989). MCP-1 is mostly expressed in the perivascular space and brain parenchyma during neuroinflammation (Mastroianni et al., 1998, Losy and Zaremba, 2001, Sindern et al., 2001, Chen et al., 2003, Sorensen et al., 2004). CCR-2 is not only expressed by monocytes but also by both neurons and astrocytes within the brain (Banisadr et al., 2002, Gillard et al., 2002). CCR-2 is also expressed in microvascular endothelial cells in both mice and humans (Dzenko et al., 2001).

A potent trigger of MCP-1 release is the pro-inflammatory Aβ amyloid peptides, which can up-regulate the release of MCP-1 (and other chemokines) in both monocytes as well as several cells within the brain (Dawson et al., 2003). MCP-1 is up-regulated early in brains of transgenic mice that develop AD–like pathology (Ishizuka et al., 1997, Sly et al., 2001, Janelsins et al., 2005, Yamamoto et al., 2005, El Khoury et al., 2007). Elevated levels of MCP-1 have been observed in AD patients both in microglia associated with mature senile plaques (Ishizuka et al., 1997) and in microvessels isolated from brain cortices (Grammas and Ovase, 2001).

The strategy of blocking the MCP-1/CCR2 interaction has been proposed as being effective in preventing macrophage-induced tissue damage. Loss of MCP-1 function is enough to impair monocyte trafficking in several inflammatory models (Dawson et al., 2003). Mice bred to have the CCR-2 receptor knocked-out (CCR-2/-) exhibited impairment in modulating recruitment of monocytes to areas of inflammation (Boring et al., 1997). It also was reported that cross
breeding Tg2576 AD mice with Tg mice deficient in CCR-2 (TgCCR-2−/−) resulted in decreased infiltration of microglia, increased plaque load and accelerated disease progression (El Khoury et al., 2007). Another method to inhibit the MCP-1/CCR-2 interaction is through pharmacological methods such as inhibition of the CCR-2 receptor or through antibodies to MCP-1 directly. Anti-bodies to MCP-1 significantly inhibit the migration of microglia and monocytes in response to Aβ-stimulated macrophage supernatant *in vitro* (Meda et al., 1996, El Khoury et al., 2003).

A limitation of this study is its reliance on immortalized human cell lines that differ from mature neurons and monocytes *in vivo*. Some observations reported here result from the immortal nature of the cells. For example, the finding of increased DNA synthesis and proliferation induced by G-CSF treatment of the neuronal line is unlikely to occur in mature differentiated neurons in vivo. Nevertheless, there is evidence that G-CSF stimulates proliferation of neural stem/progenitor cells of the sub-granular zone of the hippocampus both *in vitro* and *in vivo* (Sanchez-Ramos et al., 2009). A major advantage to studying these two cellular phenotypes in a cell culture system is the ability to isolate and control the micro-environment for each cell population.

In summary, the cellular and molecular responses to G-CSF treatment of monocytes suggest that neither changes in adhesiveness nor migratory capacity are responsible for the beneficial effects of G-CSF administration in a Tg mouse model of AD. It is clear that G-CSF treatment increases absolute monocyte count in both mouse models of AD and human patients with AD. Therefore it is likely
that the increased microgliosis observed in Tg AD mice treated with G-CSF is a consequence of increased levels of circulating monocytes in the setting of Aβ-fueled neuroinflammation. If increased microgliosis is due to enhanced infiltration of monocytes, it is also possible that vigorous macrophagic activity results from the recruitment of fresh microglia to the plaques. The stimulation of DNA synthesis along with increased anti-apoptosis observed in the neuronal cell line might explain the previously reported improvement in hippocampal neurogenesis and hippocampal-dependent learning seen in the AD mouse model after treatment with G-CSF.

Figure 2.1. The saturation binding curves for radio-labeled G-CSF in neuronal and monocytic cell lines. The results were calculated using non-linear fit and one-site specific binding with Hill slope. (A) The neuronal cell line SH-SY5Y saturation curve reveals a Kd = 533.4 pM and exhibits cooperative binding with a Hill coefficient of 2.29. The neuronal cell line calculated results are based on the mean of 4 experiments with 3 replicates per concentration of 125I-G-CSF within each experiment. (B) The monocytic cell line (THP-1) saturation curve reveals a much lower Kd = 57.49 pM and a Hill coefficient of 0.92. The monocytic cell line calculated results are based on the mean of 2 experiments with 3 replicates per concentration of 125I-G-CSF within each experiment.
Figure 2.2. Western blot of PKCδVIII and Bcl2 expression in human neuronal (SH-SY5Y) and monocytic cell lines (THP-1) incubated with G-CSF for 24h. Both PKCδVIII and Bcl2 expression are increased in the neuronal cells (100ng/ml & 200ng/ml; 5.3nM & 10.6nM), and in the monocytic cells (200ng/ml; 10.6nM). The above graphs (B) & (D) show PKCδII or Bcl2 percent densitometric units normalized to GAPDH, and represent the results of three separate experiments. The results were analyzed with two-tailed Student’s t-test, *** = p<0.0001.
Figure 2.3. G-CSF increased DNA synthesis of human monocytes (THP-1) and human CNS neurons (SH-SY5Y). (A) THP-1 cells treated with concentrations of G-CSF 25, 50 & 100 ng/mL had a significant increase in thymidine incorporation (DNA synthesis) ($n=6$ replicates per G-CSF concentration, one-way ANOVA, $*=p<0.0001$). (B) SH-SY5Y cells treated with concentrations of G-CSF 25, 50, & 100ng/mL had a significant increase in thymidine incorporation (DNA synthesis) ($n=8$ replicates per G-CSF concentration, one-way ANOVA $*=p<0.0001$).

Figure 2.4. Effect of G-CSF on THP-1 and SH-SY5Y viability (A)&(B) There was no statistical significant difference in RFU emitted by dead cells or by live cells at any of the G-CSF treatment concentrations in THP-1 cell line ($n=6$ replicates per G-CSF concentration, one-way ANOVA, $p=0.6921$). Therefore G-CSF has no effect on the viability of this monocytic cell line (THP-1). (C)&(D) There was a significant difference in RFU emitted by live cells at 25 & 50 ng/mL of G-CSF treatment concentrations in SH-SY5Y cell line ($n=10$ replicates per G-CSF concentration, one-way ANOVA, $*=p<0.0001$). Therefore G-CSF has a significant effect on the viability of this neuronal cell line (SH-SY5Y).
Figure 2.5. MCP-1, but not G-CSF served as a chemoattractant for human monocytes (THP-1 cell line). (A) Migration of monocytes was not elicited by G-CSF at various concentrations (G-CSF added to lower wells of migration assay) (n=6 replicates per G-CSF concentration, one-way ANOVA, p=0.2430). (B) MCP-1 stimulated cell migration in a concentration-dependent manner through a 5µm fibronectin coated filter over a 2h period (n=6 replicates per MCP-1 concentration, one-way ANOVA, *=p<0.0001).

Figure 2.6. Increased migration of monocytes triggered by MCP-1 was antagonized by pre-incubation with a CCR-2 antagonist. Monocytes (THP-1 cells) were pre-incubated with CCR-2 antagonist (20µg/mL) for 30min (n=6 replicates per treatment, two-way ANOVA,*=p<0.0001).
Figure 2.7. Lack of interaction of G-CSF with MCP-1 in stimulating migration. (A) MCP-1, but not G-CSF significantly increased migration from control/media alone (n=6 replicates per treatment, one-way ANOVA, p<0.001). Addition of G-CSF to media containing MCP-1 had no effect on migration of THP-1 cells. (B) Pre-incubation with CCR-2 antagonist prevented the increase in migration caused by MCP-1 (20ng/mL) and addition of G-CSF did not prevent the inhibition of migration (n=6 replicates per treatment, one-way ANOVA, p>0.05).

Figure 2.8. Effect of G-CSF on cellular adhesiveness (THP-1). (A) THP-1 cells were treated with a range of G-CSF concentrations in a 24 well plate pre-coated with fibronectin (n=6 replicates per treatment, one-way ANOVA, *=p<0.001). G-CSF did not significantly improve adhesion of THP-1 cells. PMA was used as a positive control. (B) Addition of G-CSF to PMA did not increase or decrease cellular adhesiveness elicited by PMA (n=6 replicates per treatment, one-way ANOVA, *=p<0.0001).

References


Chapter 3: Effect of G-CSF on GFP+ BMDCs in a chimeric AD mouse model

Abstract

G-CSF has been reported to increase microgliosis and decrease amyloid burden in a mouse model of AD. The objective of the present study was to determine the extent to which effects of G-CSF on mouse bone marrow derived cells (BMDC) are responsible for the beneficial effects seen in the AD mouse model. Method: Transgenic (Tg) green fluorescence protein (GFP) mouse BM was harvested and incubated with or without G-CSF in vitro to determine if the cytokine changes the proportion of BMDC of monocytic lineage and that express GFP. To assess the effects of G-CSF in vivo and to study its influence on infiltration of cells of monocytic lineage into brain, non-transgenic (NT) and APP+PS1 Tg chimeric mice (with GFP+ BMDC) received subcutaneous (s.c) injections of G-CSF or vehicle every other day for a total of 8 injections. The following parameters were measured: percentage of GFP+ and GFP+CD11b+ doubly-labeled BM cells in vitro; the total number of GFP+ BMDC as well as the percentage of microglia and amyloid burden in the hippocampus (HC) and entorhinal cortex (EC) in chimeric NT & Tg AD mice in vivo. Results: G-CSF increased the percentage of both GFP+ cells and GFP+CD11b+ BM cells in vitro. Despite the increase in microgliosis and decrease in β-amyloid burden observed in the hippocampus after G-CSF treatment in the chimeric Tg mice, there was no difference in the migration of GFP+ BMDC into the capillaries of the brain in
either the HC or EC, and no GFP+ cells were found within the brain parenchyma.

Conclusions: The percentage of GFP BM cells of monocyte lineage are increased under in vitro conditions, although it is unclear if BMDC are responsible for the beneficial effects of G-CSF administration in a Tg mouse model of AD. It is likely that the increased microgliosis observed in chimeric Tg AD mice treated with G-CSF is a consequence of increased proliferation of endogenous perivascular or parenchymal microglia in the setting of Aβ-fueled neuroinflammation and the decrease in β-amyloid burden is due to improved clearance by newly generated microglia, although the direct effect of G-CSF on microglial cells will need to be further assessed.

Introduction

Embryological origins of microglia. The exact origins for the various subtypes of microglia are not yet completely understood. During embryogenesis microglial are derived from either “neuroectodermal” or neuroepithelial origins, similar to that of astrocytes (Richardson et al., 1993, Eglitis and Mezey, 1997, Fedoroff et al., 1997), or “myeloid-monocytic” origins, such as a myeloid/mesenchymal progenitors from yolk sac (Hickey et al., 1992, Ling and Wong, 1993, Chan et al., 2007, Soulet and Rivest, 2008). It is proposed that the parenchymal microglia are derived from the neuroectodermal matrix, while most likely perivascular microglia and macrophages in the central nervous system (CNS) are derived from myeloid progenitors from the yolk sac. However, greater
than 95% of microgliosis occurs postnatal or after the formation of the blood brain barrier (BBB) (Soulet and Rivest, 2008).

**Adult origins of microglia.** During adulthood there are several proposed theories of microglial turnover. One hypothesis postulates microglia self-replicate or proliferate in the CNS (Soulet and Rivest, 2008). Another hypothesis postulates that circulating monocytes, or precursor cells/bone marrow stem cells (BMSC), infiltrate across the BBB and the precursors become parenchymal or perivascular microglial or macrophages within the CNS (Lawson et al., 1992, Eglitis and Mezey, 1997, Priller et al., 2001, Hess et al., 2004, Simard and Rivest, 2004, Simard et al., 2006). This hypothesis may have some flaws because microglia have more immature cell markers than circulating monocytes, or are not as terminally differentiated as mature circulating monocytes. Therefore, it may be only circulating precursor cells, and not mature monocytes, that can become microglia (Alliot et al., 1991, Kennedy and Abkowitz, 1997, Carson et al., 1998, Kennedy and Abkowitz, 1998, Santambrogio et al., 2001, Simard and Rivest, 2004, Davoust et al., 2006).

**Function and characteristics of microglia.** Microglia are the immune monitoring cells of the CNS. Microglia are constantly scanning the brain for changes and rapidly respond to these changes, such as infection, inflammation, trauma, tumors, and neurodegeneration (Kreutzberg, 1996). Microglia are also known as the resident macrophages of the CNS and therefore have phagocytic properties (Perry and Gordon, 1988, Kreutzberg, 1996, Kempermann and Neumann, 2003). Morphological differences distinguish parenchymal microglia
which have a ramified morphology, and perivascular microglia, amoeboid cells which reside near blood vessels/capillaries. The grey matter in the brain has a greater proportion of microglia than the white matter (Lawson et al., 1990, Soulet and Rivest, 2008); also the microglia in the grey matter have a round cell body and branched processes, while the microglia in the white matter have an oval cell body and fewer processes (McKay et al., 2007).

Resting and activated microglia. Resting microglia have short spiny projections and little cytoplasm or are ramified in shape (Lawson et al., 1990, Barron, 1995). When microglia become activated, their shape becomes more amoeboid with abundant cytoplasm, and they express the MHC II marker (Barron, 1995). Activated microglia proliferate more rapidly, migrate to the area of pathogenesis or trauma, resemble a more macrophage-like morphology and may release cytokines, which may be protective (anti-inflammatory) or cytotoxic (inflammatory) (Kreutzberg, 1996).

Role of microglia in neurodegenerative diseases. Several hypotheses have been proposed to explain the behavior of microglia during neurodegenerative diseases. In the “neuro-inflammatory hypothesis”, β-amyloid peptides act directly on the microglia causing a release of inflammatory cytokines, reactive oxygen species (ROS), and nitrous oxide (NO) which can result in neurodegeneration (Oken, 1995, Kempermann and Neumann, 2003). Therefore, in this hypothesis, activation of microglia by β-amyloid results in deleterious effects on neurons in the CNS. In the “non-cell-autonomous hypothesis”, β-amyloid peptides act directly on astrocytes and neurons inflicting
damage, causing the release of cytokines and chemokines which act both on each other as well as activate microglia, which result in further inflammation and neurodegenerative effects (Lobsiger and Cleveland, 2007, Ilieva et al., 2009). In the “microglial dysfunction hypothesis”, it is not about the negative effects of microglia, but the lack of the positive effects of microglia, meaning β-amyloid peptides act on microglia overtime eventually causing them to become dystrophic and unable to release the necessary cytokines, enzymes and growth factors the neurons need to survive (Gebicke-Haerter et al., 1996, Simard and Rivest, 2007, Farfara et al., 2008). In reality not one hypothesis is entirely correct and it is probably a combination of the hypotheses that correctly explain how microglia react in a neurodegenerative situation, such as AD (Polazzi and Monti, 2010).

**Clearance of β-amyloid by microglia.** In the AD brain as well as in the aging brain there is an increase in reactive or activated microglia (Carpenter et al., 1993, Barron, 1995). These microglia help mediate the clearance of β-amyloid through phagocytosis (DiCarlo et al., 2001, Britschgi and Wyss-Coray, 2007, Herber et al., 2007). Although in aging and neurodegenerative diseases microglial phagocytosis becomes impaired (Marx et al., 1998, Rogers et al., 2002, Lucin and Wyss-Coray, 2009). Therefore although the amount of microglia is increasing, they become less effective at clearing β-amyloid as the disease progresses.

**Tracking of BMDCs from periphery to brain.** Proliferation of microglia within the brain occurs both through the self-renewal of parenchymal microglial as well as a contribution from circulating peripheral precursor cells (Davoust et
al., 2006, Soulet and Rivest, 2008). Circulating peripheral cells can undergo
diapedesis across the BBB into the brain parenchyma (Priller et al., 2001, Hess
et al., 2004, Simard and Rivest, 2004). BM supplies approximately ten percent of
the normal adult brains microglia, brain immune cells, and this amount increases
in the injured brain, such as in Alzheimer’s Disease (AD) (Bowling et al., 1993, Ip
and Yancopoulos, 1994, Hess et al., 2002, Sanchez-Ramos et al., 2008). The
importance of the migration of BMDC into the brain is their contribution to the
repair of neural cells in degenerative diseases, such as in AD as well as in stroke
and traumatic brain injury (Stalder et al., 2005, Simard et al., 2006). To
distinguish blood-derived microglia from resident microglia within the brain, GFP
chimeric mice are often utilized (Furuya et al., 2003). These GFP chimeric mouse
models have been useful in the study of neurodegenerative diseases, such as
Parkinson’s Disease (PD) and AD (Hess et al., 2004, Kokovay and Cunningham,
2005, Malm et al., 2005, Simard et al., 2006, Mildner et al., 2007, Rodriguez et
al., 2007, Mildner et al., 2011).

Research Rationale

In a previous study done by the Sanchez-Ramos laboratory, G-CSF
treatment in an AD mouse model resulted in increased microgliosis, decreased
β-amyloid burden, enhanced neurogenesis and improved behavior in the radial
arm water maze (RAWM) (Sanchez-Ramos et al., 2009). The mice utilized to
demonstrate these disease-modifying effects were not GFP chimeric and
therefore the contribution of BMDC to the increase in microgliosis or β-amyloid
plaque clearance could not be assessed. The purpose of the present research is
to elucidate the effects of G-CSF on the infiltration of peripherally circulating BMDC into the AD mouse brain *in vivo* and to assess the effect of G-CSF directly on GFP Tg mouse BM cells *in vitro*.

Materials & Methods

**Animals.** AD mice were generated from a cross between F6 generation mice heterozygous for the mutant APPK670N, M671L gene (i.e. the APPsw, Swedish mutation derived from Tg2576 mice), and littermate mutant PS1 (6.2 line) mice bearing the M146V mutation (all mice contained a mixed background of C57B6, B6D2F1, SJL, and SW). Mice were initially genotyped at the time of weaning and then had a confirmatory genotyping at 6 weeks of age. A total of 9 APP-PS1 double Tg mice (e.g. mice bearing both APPsw and PS1 mutations) and 6 non-Tg (NT) littermates were utilized to generate chimeric mice with GFP-expressing bone marrow. GFP mice were generated from a cross between Tg GFP mice (C57BL/6-Tg [ACTB-EGFP] 1Osb/J, 003291) and C57BL/6 mice (the Jackson Laboratory, Bar Harbor, ME, USA). GFP mice were confirmed with blood smears taken at time of weaning. Microscopic examination of blood smears from tail clippings were analyzed for the presence of green fluorescence (GFP expression) in leukocytes. All mice were housed and maintained in standard laboratory cages in a temperature and humidity controlled room in a specific pathogen free facility under a 12-h light/dark cycle with light onset at 6:30 AM, with ad libitum access to rodent chow and water. All experiments conformed to guidelines for the ethical use of animals as provided by the Association for the Assessment and Accreditation of Laboratory Animal Care, International
The protocol was approved by the IACUC of the University of South Florida.

**Generation of Chimeric Tg APP/PS1 mice.** The APP-PS1 and NT mice (4 months old) were lethally irradiated with 800 rads total body irradiation, delivered in two fractions four hours apart, each fraction 400 rads was given at dose rate of 106 rads/min in a Gammacell 40 Extractor (Nordion International, Inc., Ontario, Canada) (Furuya et al., 2003)). Approximately 2 h later, irradiated mice were rescued with a bone marrow transplant of $1 \times 10^7$ mononuclear cells in 0.2 ml from Tg GFP mice (C57BL/6-Tg [ACTB-EGFP] 1Os/J, 003291; the Jackson Laboratory, Bar Harbor, ME, USA) infused via tail vein. Bone marrow-derived cells in the rescued mice were readily tracked by virtue of their green fluorescence. Examination of blood smears from tail clippings for the presence of green monocytes confirmed successful engraftment. 100% of the irradiated mice had successful engraftment and exhibited GFP mononuclear cells in their peripheral blood.

**G-CSF treatment of mouse GFP BM cells.** BM was harvested and pooled from both the tibia and femurs of eight Tg GFP mice (C57BL/6-Tg [ACTB-EGFP] 1Os/J, 003291; the Jackson Laboratory, Bar Harbor, ME, USA). BM cells were cultured in 75cm$^2$ flasks in DMEM media + 10% FBS (with or without G-CSF 250ng/ml). After culturing for 24h cells were harvested from flasks, filtered through a 40 μm nylon cell strainer (BD Labware, Bedford, MA, USA) to remove particulate matter, red blood cells were lysed using 1X RBC lysis buffer
(Ebioscience, San Diego, CA), cells were rinsed in phosphate buffered saline (PBS) three times, then diluted in PBS and counted using a hemocytometer.

**Fluorescence activated cell sorting (FACS) of mouse GFP BM cells.**

Cells were incubated for 15min on ice with Fc blocking CD16/CD32 (BD Pharmingen, San Jose, CA, USA), washed with PBS, and incubated for 30min on ice with APC Rat Anti-Mouse CD11b (clone M1/70) antibody (BD Pharmingen, San Jose, CA). CD11b+ is a cell marker commonly expressed by monocytes. Cells were then washed twice in PBS. Cells were stained with propidium iodide (PI) to exclude dead cells. Cell samples were analyzed (in replicates of three) with Accuri Cytometer (Accuri Cytometers, Ann Arbor, MI) to quantify the percentage of live cells that are GFP+ and GFP+CD11b+. 100,000 events were run for each sample. Gating was used to exclude debris and dead cells.

**G-CSF dose and administration schedule in AD chimeric mice.**

G-CSF, a natural human glycoprotein, exists in two forms of a 174-and 180-amino-acid-long protein of molecular weight 19,600 g per mol. The more-abundant and more-active 174-amino acid form has been used in the development of pharmaceutical products by recombinant human DNA (rhDNA) technology. Filgrastim (Neupogen®, Amgen, Inc., Thousand Oaks, CA, USA), one of three proprietary G-CSF compounds, was utilized in the present study. The dose of G-CSF (250 μg/kg x 8 doses) administered in the present study was determined based on the dose used in the previous study published by the Sanchez-Ramos laboratory (Sanchez-Ramos et al., 2009), in which this dose significantly
decreased Aβ plaque formation, increased microgliosis and improved neurogenesis and behavior in radial arm water maze (RAWM). This dose is lower than that reported to be effective in mobilizing bone marrow in a rat model of stroke (300μg/kg x 10 days), but higher than that utilized by others in rodent models of stroke (Schabitz et al., 2003, Six et al., 2003, Solaroglu et al., 2006) and in a published report on the treatment of memory impairment in a mouse model of AD (50 μg/kg s.c. for 5 days) (Tsai et al., 2007).

The schedule of administration of G-CSF was one subcutaneous (s.c.) injection every other day over two and a half weeks. G-CSF was diluted in 5% dextrose at a 1/20 dilution as recommended by the manufacturer (Amgen, Inc.). Vehicle (5% dextrose) was administered to control groups for both Tg and NT mice.

**Immunohistochemistry.** At the conclusion of the treatment mice were euthanatized using an intraperitoneal (i.p.) injection of 0.05-0.1ml somnasol, then receiving a 100-200 unit injection of heparin into the heart, followed by transcardial perfusion of 0.9% saline (~40ml/mouse) followed by four percent paraformaldehyde (PFA) (~20ml/mouse). Brains were then removed from skull and fixed in 4% PFA for 24h; the right hemisphere of the brain then was transferred to 30% sucrose solution for 4 days at 4°C for immunofluorescence. After incubation in 30% sucrose solution the right hemisphere was frozen in isopentane on dry ice then transferred to -80°C freezer. The right hemispheres were then transferred to the cryostat (set at -20°C) (Leica, Germany) one hour prior to sectioning the brains to allow them to equilibrate in temperature, then
brains were sectioned coronally using the cryostat at 30μM in a 1:6 series and stored in 24-well plates in a cryoprotectant/antifreeze solution at (-20 to -18°C). Prior to immunofluorescence staining sections were washed three times in 1X PBS then placed in a blocking solution (PBS+ 10% normal donkey serum, 3% bovine serum albumin (BSA), & 10% Triton-X 10X) for one hour at 4°C on maxi rotator and incubated for 24 hours at 4°C in an primary antibody cocktail of chicken anti-GFP (1:500, Millipore, Billerica, MA) plus rabbit anti-Iba1 (1:1000, Wako, Osaka, Japan). Sections were washed in PBS and incubated for one hour at room temperature in a secondary antibody cocktail of donkey anti-chicken FITC (1:400, Millipore, Billerica, MA) plus donkey anti-rabbit IgG Alexa Fluor 594 (1:1000, Invitrogen, Grand Island, NY) and coated with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Labeled cells were visualized with fluorescence microscopy (IX2 inverted microscope; Olympus, Tokyo, Japan) using appropriate filters. Digital images were captured with the DP-70 digital camera system (Olympus).

The left hemisphere was routinely embedded in paraffin with 24 h processing. For paraffin sectioning, five coronal sections (per set) with a 150 μm interval were cut at a thickness of 5 μm in hippocampus (HC) and entorhinal cortex (EC), bregma -2.92 to -3.64 mm (Paxinos G, 2001). Four sets of five sections from H and EC were prepared for analyses of Aβ plaque and Iba1 (ionized calcium-binding adapter molecule 1). Immunohistochemical staining was performed following the manufacturer’s protocol using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) coupled with the diaminobenzidine
(DAB) reaction, except that the biotinylated secondary antibody step was omitted for Aβ immunohistochemical staining. The following primary antibodies were used for immunohistochemical staining: a biotinylated human amyloid-β monoclonal antibody (clone 4G8; 1:200, Covance Research Products, Emeryville, CA, USA) and an Iba-1 polyclonal antibody (1:1000, Wako, Osaka, Japan).

Quantification of Immunofluorescence. GFP+ labeled cells were estimated using every 6th section taken throughout the hippocampus and entorhinal cortex (every 180 microns). To avoid counting partial cells a modification to the optical dissector method was used so that cells on the upper and lower planes were not counted. The number of GFP+ cells counted in every 6th section was multiplied by 6 to get the total number of GFP+ cells in the hippocampus (HC) or the entorhinal cortex (EC) (Shors et al., 2002).

Quantification of Bright Field Immunohistochemistry. Images of DAB stained sections were acquired as digitized tagged-image format files to retain maximum resolution using an Olympus BX60 microscope with an attached digital camera system (DP-70, Olympus), and digital images were routed into a Windows PC for quantitative analyses using SimplePCI software (Compix, Inc. Imaging Systems, Cranberry Township, PA, USA). Images of five sections (each 5 μm thick and 150 μm apart) were captured from serially sectioned EC and HC and a threshold optical density was obtained that discriminated staining from background. Each anatomic region of interest was manually edited to eliminate artifacts. For Aβ and Iba1 (microgliosis) burden analyses, data was reported as
the percentage of labeled area captured (positive pixels) divided by the full area

captured (total pixels). Bias was eliminated by analyzing each entire region of

interest (HC and EC) represented by the sampling of five sections per region.

Stereological equipment was not used. Each analysis was done by a single

examiner blinded to sample identities.

**Statistical Analysis.** An unpaired two-tailed (or Student’s) t-test was used
to evaluate the effect of G-CSF on pooled GFP bone marrow cells *in vitro*. While

an unpaired one-tailed t-test was used to evaluate β-amyloid burden as well as

Aβ 1-40 and Aβ 1-42 plasma levels; a one-tailed t-test was able to be utilized due
to the expected result of G-CSF to decrease, and not increase, Aβ load based on

previous results (Sanchez-Ramos et al., 2009). Two-way analyses of variance

(ANOVA) were used to evaluate the effects of G-CSF (treatment) on microgliosis

(percent Iba1) as well as total GFP positive cells in the HC and EC of NT and Tg

AD mice (genotype). When appropriate, post hoc analyses, such as Bonferroni

Multiple Comparison test, were used to isolate drug effects from genotypic
effects. All statistical analyses were determined significant at the 0.05 alpha

levels. Data was presented as mean ± standard deviation (SD).

Results

**Effects of G-CSF on Tg GFP mouse bone marrow cells *in vitro.*

Examination of the BM harvested from Tg GFP mice revealed that less than half

of the nucleated cells in the BM were GFP. Approximately 43 percent of the

basal media-treated BM cells expressed GFP and this proportion was

significantly increased to approximately 52 percent with G-CSF treatment (Figure
Also of importance was the percentage of GFP+ cells that have the propensity to migrate into the brain, or those cells of monocyte/myeloid lineage, CD11b+. G-CSF treatment (250ng/ml) for 24 h significantly increased the percentage of GFP+CD11b+ BM cells from approximately 21 percent to 29 percent (Figure 3.1B).

**Effects of G-CSF on β-amyloid burden, β-amyloid plasma levels, and microgliosis in the chimeric AD mouse brain.** In order to assess the effect of G-CSF on β-amyloid burden, β-amyloid plasma levels and microgliosis in 8mo old chimeric AD mice; chimeric NT (n=4) and Tg AD mice (n=5) were treated with s.c. injections of G-CSF or vehicle every other day for a total of 8 injections. Treatment with G-CSF resulted in a significant decrease in the percentage of β-amyloid burden (4G8) in the HC, but not the EC, in the chimeric Tg AD mice (one-tailed unpaired t-test P<0.05) (Figure 3.2A), although there was no significant change in either Aβ 1-40 or Aβ 1-42 plasma levels with G-CSF treatment (Figure 3.2B). Total microgliosis was measured using the marker Iba1, which labels both resting and activated microglia, in both the HC and the EC. There was a significant genotypic effect on microgliosis, in which Tg AD mice had significantly increased levels of microgliosis in the HC and EC in comparison to the NT littermates (two-way ANOVA, P<0.0001). G-CSF treatment significantly increased microgliosis in Tg AD mice in the HC, but not the EC, although did not have a significant impact on microgliosis in NT AD mice (two-way ANOVA, P<0.01) (Figure 3.2C & 3.2D).
**Effects of G-CSF on migration on GFP+ cells into the chimeric AD mouse brain.** GFP+ cells migrated to the blood vessels (BV) of the HC and the EC as well as the choroid plexus of brain in chimeric NT & Tg AD mice, but did not migrate into the brain parenchyma in either the HC or the EC (Figure 3.3). G-CSF did not significantly increase migration of GFP+ cells into the BV of either the HC or the EC, also there was no significant genotypic effect on GFP+ migration between the Tg and NT AD mice (Figure 3.4).

**Discussion**

The proportion of GFP positive cells harvested from chimeric Tg GFP bone marrow was 43 percent of the total nucleated cells, and G-CSF treatment increased this proportion increased by almost 10 percent. This finding has several implications for studies using this similar chimeric mouse models for tracking and following the phenotypic fate of GFP-labeled BMDC. Without knowing the proportion of GFP+ cells that compose the BM of each chimeric mouse, results from these experiments might lead to incorrect conclusions. For example, if a difference in the numbers of GFP positive cells found in brain following a particular drug treatment is found, it would be unclear if the difference in GFP+ cells within the brain was due to the treatment administered, or to a difference in proportion of GFP+ BM cells in the bone marrow compartment of the treatment group of mice. Hence, studies showing significant differences in BMDC infiltration should be examined with this caveat in mind. To interpret this data correctly and to perform comparisons, one would need to normalize the number of infiltrated cells to the proportion of labeled cells. In this study we did
not normalize the proportion of GFP labeled cells in the brain to that within the BM, but this normalization will be done in future studies.

Unexpectedly, GFP+ cells were not found in the brain parenchyma of the chimeric GFP Tg APP/PS1 mice or NT mice regardless of the proportion of GFP positive cells in the bone marrow. Reviewing other studies of chimeric GFP mice, we found a report in which irradiated AD mice, with “shielded” heads, did not result in infiltration of GFP+ cells into brain parenchyma of the mice, but only into blood vessels within the brain (Mildner et al., 2011). Although the mice in this study did not have their heads “shielded” they received a lower dose of irradiation 800rads (split into two doses), while the “non-shielded” and “shielded” mice in the Mildner et al. study received 1100rads (in a single dose) as well as experienced a change in fur depigmentation that was not noted in the current study (Mildner et al., 2007, Mildner et al., 2011). The “unshielded” AD mice in the Milder et al. study resulted in GFP+Iba1+ cells within the brain parenchyma both in the cortex and HC; in other words GFP+ BMDC that differentiated into microglia. The higher dose of irradiation in the “unshielded” mice, in the Mildner et al. study, may have affected BBB permeability resulting in a greater influx of BMDC into the parenchyma (Mildner et al., 2011). However, GFP+CD11b+ circulatory cells have been shown to enter the CNS of APP+PS1 Tg AD mice without irradiation and also become GFP+Iba1+ cells within the brain parenchyma (Lebson et al., 2010). Therefore, the exact conditions that result in infiltration of BMDC into the brain in the AD mouse model are unclear. In addition, the present study did not reveal differences in GFP positive BMDC within capillaries in both the HC and EC of the
Tg and NT chimeric AD mice, regardless of treatment. Another point to mention is that BM cells migrate into other organs within the AD mice besides the brain, as \textit{in vivo} GFP BM cells also have been found to migrate into the blood, spleen and pancreas (data not shown). Therefore the combination of the GFP BM cells containing less than fifty percent GFP positive cells and the migration of these BM cells to various organs throughout the body may contribute to the lack of detection of GFP cells in the brain parenchyma. It will be important to select a transgenic GFP mouse with a higher percentage of GFP BM for future studies.

Even though there was no change in BMDC infiltration into the brain, a significant increase in microgliosis as well as a significant decrease in β-amyloid burden was found in the HC of chimeric Tg AD mice (Figure 3.2). As this increase in microgliosis could not be attributed to BMDC, the microgliosis is likely a result of direct stimulation of resident microglia by G-CSF. G-CSF is known to cross the BBB (Zhao et al., 2007) and microglia are known to express the G-CSFR (Hasselblatt et al., 2007). Unfortunately the pharmacokinetics of G-CSF ligand binding to receptors on microglia and the direct actions of G-CSF on microglia \textit{in vitro} were not determined here at this time.

Previous \textit{in vitro} studies of rat microglia treated with G-CSF over 48-72 h did not report effects on microglial proliferation measured by tritiated thymidine (Giulian and Ingeman, 1988). Ameboid microglial phagocytosis \textit{in vitro} and \textit{in vivo} was also not improved with G-CSF treatment (Giulian and Ingeman, 1988). In stroke-induced rats, G-CSF treatment increased microgliosis in the uninjured CNS (Iba1+BrdU+ cells) and bone marrow derived microglia.
(GFP+Iba1+ cells) were found both in the uninjured and injured CNS, but the extent of BMDC infiltration into the brain was not increased with G-CSF treatment (Bartolini et al., 2011). G-CSF treatment increases migration of SOD-1 mouse microglia \textit{in vitro} and a significant increase in microgliosis in the spinal cords of G-CSF treated ALS mouse model \textit{in vivo} (Yamasaki et al., 2010). The increase in microgliosis seen in various animal models of neuroinflammation or neurodegeneration with G-CSF treatment can potentially be caused by effects both on circulating BMDC and endogenous microglia. In the present study, it is difficult to determine which mechanism is the major contributor as less than half of the BMDC in Tg green mice express GFP and the percentage of GFP+ BM cells in the chimeric AD mice as well as the direct effects on microglia were not assessed. The decrease in β-amyloid burden could potentially be due to clearance by activated microglia engulfing the plaques as well as the clearance of β-amyloid by perivascular macrophages.

A weakness of this study is a very small number of both NT and Tg AD mice were used (n=1-3 mice per group), and therefore both a genotypic effect and a treatment effect of G-CSF on GFP+ cell migration may be seen with a larger number of mice. Due to the small N in this study a power analysis was performed to determine the sample size of animals that would be necessary in future studies to detect a difference based on the current studies variability. It was determined that an N of 8 animals per group will be necessary to detect a significant difference between the means with an alpha = 0.05 and beta= 0.80. Also in future chimeric mouse studies it will be of interest to assess the
proportion of GFP+ cells in the BM compartment in comparison to those GFP+ cells that migrate into the brain, to isolate the true drug effects on migration from those effects due to “mass action” of an increase in number of cells in the BM compartment.

In conclusion, the effect of G-CSF on BMDC in a mouse model of AD is still unclear. The ability of G-CSF to increase the percentage of GFP+ and GFP+CD11b+ BM cells \textit{in vitro} as well as the increase in microgliosis in the hippocampus of chimeric Tg AD mice may contribute to a more rapid turn-over of fresh perivascular and parenchymal microglia, which have not yet been exposed to chronic assault by β-amyloid peptides. This is important as Tg AD mice have been shown to exhibit reduced expression of Aβ-binding receptors and Aβ-degrading enzymes resulting in dysfunctional microglia (Hickman et al., 2008). The combined effect of G-CSF on both BMDC as well as endogenous microglia could result in improved clearance of β-amyloid, which would explain the significant decrease in hippocampal β-amyloid burden found in Tg chimeric AD mice.
Figure 3.1. G-CSF increases the percentage of GFP+ and GFP+CD11b+ mouse BM cells. BM cells were harvested and pooled from 8 GFP mice, then incubated for 24h with or without G-CSF 250ng/mL. (A) The percentage of total GFP+ cells significantly increased (paired t-test two-tailed, *p=0.003) after treatment with G-CSF. (B) The percentage of double-positive GFP+CD11b+ cells significantly increased (paired t-test two-tailed, *p=0.005) after treatment with G-CSF.
Figure 3.2. Effect of G-CSF on β-amyloid burden, Aβ plasma levels and microgliosis in chimeric NT & Tg AD mice. (A & D) G-CSF significantly decreased amyloid plaque burden in the HC, but not the EC (n=2-3 mice per treatment group, one-tailed unpaired T-test, *P=0.03). (B) G-CSF did not have a significant effect on plasma Aβ levels (C) Tg AD mice have significantly more microgliosis (Iba1 immunoreactivity) in both the HC & the EC than NT littermates. G-CSF increased Iba1 immunoreactivity in the HC (E), but not the EC (image not shown) in Tg AD mice (n=2-3 mice per treatment group, two-way ANOVA, *P<0.01). This data is comparable to that seen in non-irradiated 8mo NT and Tg AD mice used in the neurogenesis experiments (see Chapter 4).
Figure 3.3. Fluorescence captured images (20X) of GFP+ (green), Iba1+ (red) and DAPI (blue) cells in BVs of various regions of the brain. (A) Images of GFP+ cells in capillaries of the HC, (B) of the EC, and (C) of the choroid plexus.

Figure 3.4. The effect of G-CSF on migration of GFP+ cells from the BM to the BVs of the HC and the EC. There was no significant genotypic effect or significant G-CSF treatment effect on migration of GFP+ cells to either the BVs of the HC or the EC (n=1-3 mice per treatment group, two-way ANOVA, p=0.35 (HC), p=0.56 (EC).
References


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Chapter 4: Effect of G-CSF on Neurogenesis in an AD mouse model

Abstract

G-CSF has been reported to promote neurogenesis both in vitro and in vivo mouse model of AD. The objective of the present study was to further identify the effect of G-CSF on neural stem/progenitor cell proliferation and neurogenesis in a normal and AD mouse model. Methods: Flow cytometry was used to quantify numbers of BrdU+ and double-labeled (BrdU+/NeuN+) cells in the hippocampi of a wild-type and AD mouse model with and without G-CSF treatment. Brdu+ and DCX+ cells were also counted in the DG of the HC using fluorescence immunohistochemistry in G-CSF or vehicle treated AD and in NT mice. Synaptophysin in CA1 region of HC, amyloid and microglial burden in both HC and EC were determined using biotinylated immunohistochemistry. Results: In normal ICR mice G-CSF significantly increased proliferation of stem/progenitor cells and generation of new neurons in the SGZ of the HC as determined via FACS. Using immunohistochemistry for quantification, DCX+ counts in the SGZ of the HC of APP/PS1 mice treated with G-CSF were increased to NT mouse levels. Synaptophysin density in the CA1 region was significantly increased in both NT and APP/PS1 mice after G-CSF treatment. Amyloid burden (4G8) was significantly decreased while microgliosis (Iba1) was significantly increased in the HC of APP/PS1 G-CSF treated mice. Conclusions: G-CSF has direct effects on
the AD brain, 1) direct effects of G-CSF on microglia improve amyloid plaque clearance allowing for self-correction of neurogenesis and synaptogenesis, and 2) direct effects of G-CSF on neural stem/progenitor cells promote neurogenesis and synaptogenesis. A larger study using multiple proliferative and neurogenic markers and various time points is needed to more clearly explain the impact G-CSF has on the various stages of neurogenesis in the AD mouse brain.

Introduction

Neurogenesis occurs throughout life in the adult brain but differs from neurogenesis during embryological development. One key difference is that neurogenesis in the adult mammalian brain is restricted to neurogenic niches, the subventricular zone (SVZ) of the anterior lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (HC). The cells of the SVZ migrate by way of the rostral migratory stream to the olfactory bulb, while the cells of the SGZ migrate to the granular layer of the DG and integrate into the hippocampal circuitry.

The existence of adult neurogenesis was first reported by Joseph Altman (Altman, 1962, Altman and Das, 1965, Altman, 1969) using the tritiated thymidine method (Messier et al., 1958, Sidman et al., 1959, Messier and Leblond, 1960), although the idea of gliosis was accepted fairly readily by the scientific community it was not until later that formation of new neurons in adult brain was demonstrated experimentally. Using the bromodeoxyuridine (BrdU) double-labeling method, adult neurogenesis was documented in both mice (Cameron and McKay, 1998, Kempermann et al., 1998) and humans (Eriksson et al., 1998);
and after these results were published adult neurogenesis eventually became an accepted phenomenon.

Another key difference of adult neurogenesis is that it is heterogeneous; with all the stages of neurogenesis occurring simultaneously (Kempermann et al., 2004). It is important to note that most of the new born proliferating cells in the SGZ of the adult HC eventually undergo cell death, instead of differentiation into mature neurons, which is a rare event (Young et al., 1999, Biebl et al., 2000, Kempermann et al., 2003). The differentiated mature neurons integrate with neural circuits of the hippocampus (Dayer et al., 2003).

**Cell markers used to assess neurogenesis.** The first method used to assess neurogenesis in the adult brain was tritiated thymidine, which is taken up by DNA during the S phase of the cell cycle or during cell replication; although this has the limitation of a short biological/effective half-life *in vivo* (Messier et al., 1958). The second method to assess neurogenesis was BrdU uptake by dividing cells, in which labeling of DNA within the cell was permanent and was passed on to the progeny of the dividing cell (Nowakowski and Hayes, 2001, Taupin, 2007). The down side to assessing neurogenesis with BrdU is that it also can label dying cells, therefore it is important to also look at apoptotic markers (caspase-3 and TUNEL) or proliferative markers (Ki67), when using BrdU alone. Several cell markers can be used in conjunction with BrdU to better describe the phenotypic fate of new born cells in the HC, such as immature and mature neuronal markers (Table 4.1). The different stages of neurogenesis can be defined through the use of various cell markers; for example as neural progenitor cells begin to
differentiate into neurons they lose markers such as nestin, but they gain transient immature neuronal cell markers, such as doublecortin (DCX), and eventually gain more permanent neuronal cell markers, such as Neuronal Nuclei (NeuN) (Table 4.2). For this study the cell markers chosen to assess proliferation and neurogenesis in the adult mouse HC were BrdU and DCX, an immature neuronal marker.

Factors which influence neurogenesis and synaptogenesis in the hippocampus. A variety of stimuli can induce proliferation of neural progenitor cells and promote their differentiation into mature neurons in the adult HC. Stimuli such as brain injury, physical activity, enriched environment, chronic anti-depressant medication treatment, and cytokines can positively influence or increase hippocampal neurogenesis (Kempermann et al., 1997, van Praag et al., 1999, Aberg et al., 2000, Malberg et al., 2000, Kernie et al., 2001, Arvidsson et al., 2002, Jin et al., 2002, Lee et al., 2002, Brown et al., 2003a, Boldrini et al., 2009). A specific cytokine, G-CSF, has been shown to promote hippocampal neurogenesis (Schneider et al., 2005, Schabitz and Schneider, 2007, Sanchez-Ramos et al., 2009). Other stimuli such as chronic stress, aging, and depression can negatively influence or decrease hippocampal neurogenesis (Gould et al., 1992, Kuhn et al., 1996, Kempermann et al., 1998, Malberg et al., 2000). Aβ deposition in the brain has also been shown to decrease or impair hippocampal neurogenesis in AD mouse models (Haughey et al., 2002, Dong et al., 2004, Wang et al., 2004, Donovan et al., 2006).
Synatophysin is a pre-synaptic vesicle protein marker, which has been used to measure synaptogenesis in the hippocampus in both animal models and human patients (Li et al., 2002). In Tg AD mice, aberrant synaptic terminal staining has been associated with Aβ deposits (Cracchiolo et al., 2007). A decline in synaptogenesis or synapse pathology has been correlated with cognitive decline in both AD patients as well as other forms of dementia (DeKosky and Scheff, 1990, Terry et al., 1991, Dickson et al., 1995, Terry, 1996, Scheff and Price, 2003). Aβ deposition may cause a loss of synapses, although a decrease in synaptophysin and impaired synaptic function may occur prior to Aβ deposition in various Tg AD mouse models (Hsia et al., 1999, Mucke et al., 2000, Oddo et al., 2003). G-CSF has been shown to increase synaptophysin density in both the CA1 and CA3 regions of the HC in an AD mouse model (Sanchez-Ramos et al., 2009).

**FACS method as a tool to measure neurogenesis.** The current method to assess proliferation and neurogenesis in the hippocampus is through immunohistochemistry (IHC), in which BrdU uptake and double-labeling with a neuronal marker, such as NeuN, is quantified (Kempermann et al., 2003). The limitation of IHC is that it is laborious and has a low throughput as it can take several weeks to complete. To resolve the limitation of IHC, a flow cytometry, or fluorescence activated cell sorting (FACS), method has been used as a robust and rapid means of BrdU quantification, which has higher throughput screening for potential treatments that may be used to improve neurogenesis (Bilsland et al., 2006, Balu et al., 2009). The flow cytometry method of quantification of
hippocampal proliferation also has been used by assessment of an in vivo stable isotope and a proliferation marker, Ki67 (Shankaran et al., 2006, Henry et al., 2009). The limitation of the FACS method is that the positional information within DG as well as cell morphological information is lost, which is present in IHC. When assessing proliferation alone with FACS, it is important to note that proliferating cells in locations outside of the DG may be counted such as endothelial cells and oligodendrocytes, although this concern can be alleviated when double-labeling with a neuronal marker.

**Research Rationale**

In a previous study done by the Sanchez-Ramos laboratory, G-CSF treatment of adult mouse HC neural stem/progenitor cells *in vitro* increased proliferation measured by incorporation of tritiated thymidine. Moreover, *in vivo* studies with a Tg AD mouse model resulted in increased numbers of immature neurons, Calretinin (CRT) expressing cells in the SGZ of the HC (Sanchez-Ramos et al., 2009). There are other assessments of neurogenesis which were not performed in those studies, such as BrdU uptake in the DG of the HC and determination of subsequent differentiation of BrdU birth-dated cells into neurons expressing immature neuronal markers (DCX) and mature neuronal markers (NeuN). The use of a variety of proliferative and neuronal markers is important for a comprehensive understanding of the effect that G-CSF has on hippocampal neurogenesis. Therefore, the purpose of this study is to further investigate the effects of granulocyte colony stimulating factor (G-CSF) on hippocampal
neurogenesis in an AD mouse model as well as a wild-type mouse model, ICR, using both immunohistochemistry and FACS to assess neurogenesis.

Materials and Methods

**Animals for flow cytometry method.** ICR mice purchased from Jackson laboratories were utilized in the study looking at the effect of G-CSF on neurogenesis in wild-type mice using fluorescence activated cell sorting (FACS) method. ICR mice were used for each wild-type treatment group (6-8 mice per group). A total of 10 APP-PS1 double transgenic (Tg) mice (e.g. mice bearing both APPsw and PS1 mutations) and 15 non-Tg (NT) littermates, ranging from 10-14mo old were utilized in the FACS neurogenesis portion of this study. AD mice were generated from a cross between F6 generation mice heterozygous for the mutant APPK670N, M671L gene (i.e. the APPsw, Swedish mutation derived from Tg2576 mice), and littermate mutant PS1 (6.2 line) mice bearing the M146V mutation (all mice contained a mixed background of C57B6, B6D2F1, SJL, and SW). Mice were initially genotyped at the time of weaning and then had a confirmatory genotyping at 6 weeks of age.

**Animals for immunohistochemistry method.** Doubly transgenic amyloid precursor protein (APP) + presenilin-1 (PS1) mice (Holcomb et al., 1998) that were a cross between mice heterozygous for the mutant APPK670N, M671L gene (i.e. the APPsw, Swedish mutation derived from Tg2576 mice) (Hsiao et al., 1996) and littermate mutant PS1 (5.1 line) mice bearing the M146L mutation (Duff et al., 1996). Mice were initially genotyped at the time of weaning and then...
had a confirmatory genotyping at 6 weeks of age. A total of 11 APP-PS1 double Tg mice and 8 non-Tg (NT) littermates were utilized in the immunohistochemistry portion of this study.

All mice, for both methods, were housed and maintained in standard laboratory cages in a temperature and humidity controlled room in a specific pathogen free facility under a 12-h light/dark cycle with light onset at 6:30 AM, with ad libitum access to rodent chow and water. All experiments conformed to guidelines for the ethical use of animals as provided by the Association for the Assessment and Accreditation of Laboratory Animal Care, International (AAALAC #000434). The protocol was approved by the IACUC of the University of South Florida.

**G-CSF dose and administration schedule of IHC AD mouse group.** G-CSF, a natural human glycoprotein, exists in two forms of a 174-and 180-amino-acid-long protein of molecular weight 19,600 g per mol. The more-abundant and more-active 174-amino acid form has been used in the development of pharmaceutical products by recombinant human DNA (rhDNA) technology. Filgrastim (Neupogen®, Amgen, Inc., Thousand Oaks, CA, USA), one of three proprietary G-CSF compounds, was utilized in the present study. The dose of G-CSF (250 μg/kg x 8 doses) administered in the present study was determined based on the dose used in the previous study published by the Sanchez-Ramos laboratory (Sanchez-Ramos et al., 2009). In that study, G-CSF significantly decreased Aβ plaque formation, increased microgliosis and improved neurogenesis and behavior in radial arm water maze (RAWM). This dose is lower
than that reported to be effective in mobilizing bone marrow in a rat model of stroke (300μg/kg x 10 days), but higher than that utilized by others in rodent models of stroke (Schabitz et al., 2003, Six et al., 2003, Solaroglu et al., 2006) and in a published report on the treatment of memory impairment in a mouse model of AD (50 μg/kg s.c. for 5 days) (Tsai et al., 2007).

The APP/PS1 and NT were 8mo old at the time of G-CSF treatment. The schedule of administration of G-CSF was one subcutaneous (s.c.) injection every other day (qod) over two and a half weeks. G-CSF was diluted in 5% dextrose at a 1/20 dilution as recommended by the manufacturer (Amgen, Inc.). Vehicle (5% dextrose) was administered to control groups for both Tg and NT mice.

**G-CSF dose and administration schedule in AD & ICR mice for FACS neurogenesis method.** The dose of G-CSF (250 μg/kg x 6 doses) administered in FACS neurogenesis model was based on the dose used in the previous study published by the Sanchez-Ramos laboratory (Sanchez-Ramos et al., 2009). The schedule of administration of G-CSF was one subcutaneous (s.c.) injection every day over 6 days, which began when the ICR mice were 6 mo old and the APP/PS1 and NT mice were 10-14 mo old. G-CSF was diluted in 5% dextrose at a 1/20 dilution as recommended by the manufacturer (Amgen, Inc.). Vehicle (5% dextrose) was administered to control groups for Tg AD, NT and ICR mice.

**BrdU preparation and administration.** BrdU solution was prepared at 10mg/ml at pH 7.4 in 1X PBS. Mice received an intraperitoneal (i.p.) injection of 75 mg/kg BrdU twice daily, 6:30AM and 6:30PM, for the last 3 days of G-CSF treatment regimen in the AD mouse model IHC study. ICR mice and APP +PS1
or NT mice utilized for the FACS neurogenesis method received an i.p. injection of 100 mg/kg BrdU daily on the last two days of G-CSF treatment.

**FACS neurogenesis: preparation of cells and flow cytometry.** After the mice were sedated with Nembutal and perfused with 0.9% NaCl, their brains were removed and halved along the midline. The hippocampi of the halves to be analyzed by flow cytometry were dissected out and finely minced. Each hippocampus was dissociated into a single cell solution using MACS cell dissociation kit (Miltenyi Biotech) and fixed overnight in 75% ethanol. After fixation the cells were washed in PBS and the DNA denatured in 2N HCl/ 5% Triton-X and neutralized in 0.1M borate buffer. For fluorescence labeling, each single cell solution was incubated for 30 minutes at room temperature in a cocktail containing FITC-conjugated mouse anti-BrdU antibody (BD Bioscience, 1:5), fluorescently conjugated mouse anti-NeuN (Chemicon, 1:25), 1% BSA, and 1.5% Tween 20 (Sigma). Anti-NeuN was conjugated using Dylight 405 Microscale Antibody Labeling Kit (Thermo Scientific). Finally, the cells were washed, re-suspended in 1mL of 10μg/ml propidium iodide (PI) (Sigma), and analyzed using the BD Aria cell sorter. Only diploid (2N) cells of the cell cycle were gated out of the PI positive cells (Baisch et al., 1975). The diploid cells were then “backgated” to eliminate debris. To analyze cell proliferation by BrdU incorporation, a FITC fluorescence vs. PI-diploid cells scatter plot was generated. The FITC positive cluster was further gated and visualized in a FITC fluorescence vs. Alexa 405 fluorescence to determine the number of double positive (Brdu+/NeuN+) cells in the BrdU positive cluster. Gates were set using
negative controls obtained by incubating samples without the fluorochrome in question.

**Immunohistochemistry.** Mice were euthanatized two weeks after the last treatment of G-CSF using an intraperitoneal (i.p.) injection of 0.1ml somnasol, then received a 100-200 unit injection of heparin into the heart, followed by transcardial perfusion of 0.9% saline (~40ml/mouse) followed by four percent paraformaldehyde (PFA) (~20ml/mouse). Brains were then removed from skull and fixed in 4% PFA for 24h, the right hemisphere of the brain was transferred to 30% sucrose solution for 4 days for immunofluorescence. After the 25% sucrose solution the right hemisphere was frozen in isopentane on dry ice then transferred to -80°C freezer. The right hemisphere was then transferred to the cryostat (set at -20°C) (Leica, Germany) one hour prior to sectioning the brains to allow them to equilibrate in temperature, then brains were sectioned coronally using the cryostat at 30μM in a 1:6 series and stored in 24-well plates in a cryoprotectant/anti-freeze solution at (-20 to -18°C). For those sections undergoing immunohistochemistry for BrdU, sections were first denatured using 2N HCl for 1h at room temperature and neutralized in 0.15M borate buffer for 15min. Prior to immunofluorescence staining sections then were washed three times in 1X PBS then placed in a blocking solution (PBS+ 10% normal donkey serum, 3% bovine serum albumin (BSA), & 10% Triton-X 10X) for one hour at 4°C on maxi rotator. Sections were then incubated for 24 hours at 4°C in a primary antibody cocktail of rat anti-BrdU (1:75, AbD Serotec, Raleigh, NC) plus mouse anti-NeuN(1:100, Millipore, Billerica, MA), or a primary antibody cocktail
of rabbit anti-DCX (1:2000, abcam, Cambridge, MA). Sections were washed in PBS and incubated for one hour at room temperature in a secondary antibody cocktail of donkey anti-rat Alexa Fluor 594 (1:1000, Invitrogen, Grand Island, NY) plus donkey anti-mouse IgG Alexa Fluor 488 (1:1000, Invitrogen, Grand Island, NY), or a secondary antibody cocktail of donkey anti-rabbit Alexa Fluor 594 (1:1000, Invitrogen, Grand Island, NY), and coated with Vectashield mounting medium without or with DAPI (Vector Laboratories, Burlingame, CA). Labeled cells were visualized with fluorescence microscopy (IX2 inverted microscope; Olympus, Tokyo, Japan) using appropriate filters. Cells in the subgranular zone of the dentate gyrus were counted via microscope by a single blinded examiner. Digital images were captured with the DP-70 digital camera system (Olympus).

The left hemisphere was transferred from 4% PFA then routinely embedded in paraffin with 24 h processing. For paraffin sectioning, five coronal sections (per set) with a 150 μm interval were cut at a thickness of 5 μm in hippocampus (HC) and entorhinal cortex (EC), bregma -2.92 to -3.64 mm (Paxinos G, 2001). Four sets of five sections from HC and EC were prepared for analyses of Aβ plaque and Iba1 (ionized calcium-binding adapter molecule 1). Immunohistochemical staining was performed following the manufacturer’s protocol using a Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) coupled with the diaminobenzidine (DAB) reaction, except that the biotinylated secondary antibody step was omitted for Aβ immunohistochemical staining. The following primary antibodies were used for immunohistochemical
staining: a biotinylated human amyloid-β monoclonal antibody (clone 4G8; 1:200, Covance Research Products, Emeryville, CA, USA), an Iba-1 polyclonal antibody (1:1000, Wako, Osaka, Japan), and rabbit synaptophysin polyclonal antibody (undiluted, DAKO, Carpinteria, CA, USA).

**Quantification of Immunofluorescence.** BrdU+ or DCX+ labeled cells were estimated using every 6th section taken throughout the hippocampus (every 180 microns). To avoid counting partial cells a modification to the optical dissector method was used so that cells on the upper and lower planes were not counted. The number of BrdU+ or DCX+ cells counted in every 6th section was multiplied by 6 to get the total number of BrdU+ or DCX+ cells in the SGZ of the hippocampus (Shors et al., 2002).

**Quantification of biotinylated immunohistochemistry.** Images of DAB stained sections were acquired as digitized tagged-image format files to retain maximum resolution using an Olympus BX60 microscope with an attached digital camera system (DP-70, Olympus), and digital images were routed into a Windows PC for quantitative analyses using SimplePCI software (Compix, Inc. Imaging Systems, Cranberry Township, PA, USA). Images of five sections (each 5 μm thick and 150 μm apart) were captured from serially sectioned EC and HC and a threshold optical density was obtained that discriminated staining from background. Each anatomic region of interest was manually edited to eliminate artifacts. For Aβ and Iba1 (microgliosis) burden analyses, data are reported as the percentage of labeled area captured (positive pixels) divided by the full area captured (total pixels). Bias was eliminated by analyzing each entire region of
interest (HC and EC) represented by the sampling of five sections per region. Stereological equipment was not used. To evaluate synaptophysin immunostaining, after the mode of all images was converted to gray scale, the average optical density of positive signals from each image was quantified in the CA1 region of HC as a relative number from zero (white) to 255 (black) and expressed as mean intensity of synaptophysin immunoreactivity. Each analysis was done by a single examiner blinded to sample identities.

Statistical Analysis. An unpaired two-tailed t-test was used to evaluate $\beta$-amyloid burden, A$\beta$ 1-40 and 1-42 plasma levels, and FACS analysis in ICR mice. Two-way analyses of variance (ANOVA) was used to evaluate the effects of G-CSF (treatment) on immunohistochemical analyses and FACS analysis in APP/PS1 and NT mice. All statistical analyses were determined significant at the 0.05 alpha level. When appropriate, post hoc analysis was used, such as the Bonferroni, to isolate effects of G-CSF treatment. Data was presented as mean ± standard deviation (SD).

Results

Effect of G-CSF on neurogenesis in a wildtype (ICR) and AD Tg mouse model as measured via FACS. Traditionally neurogenesis is measured by analyzing either immature neuronal markers, such as DCX (Brown et al., 2003b), or new cells of neuronal lineage, i.e. cells birth-dated by BrdU that express NeuN (Kempermann et al., 2003), through histological analysis using immunohistochemistry (IHC) to identify these positive cells within the DG of the HC. Due to the time and labor expenditure needed to perform IHC, newer
methods to assess neurogenesis were of interest, such as FACS which can be completed in a matter of a few days (Bilsland et al., 2006, Spoelgen et al., 2011). Particularly of interest was the effect of G-CSF on neurogenesis in normal or wild-type mice (ICR), as well as in an AD mouse model (APP/PS1 and NT). Mice received 6 days of s.c. G-CSF followed by i.p. BrdU injections daily on the last two days of G-CSF treatment. Then, the animals were euthanatized two weeks following last injection of G-CSF to allow time for differentiation of new born cells within the DG of the HC. In the wild-type ICR mice there was a significant increase in both BrdU+ cells in the HC (p=0.03) and in BrdU+NeuN+ cells in the HC (p=0.004) (Figure 4.1). In the APP/PS1 and NT mice there was no significant increase in BrdU+ cells (data not shown) or in BrdU+NeuN+ cells in the HC (Figure 4.2). However, there was a slight increase in BrdU+NeuN+ cells in the HC of the G-CSF treated APP/PS1 mice that almost reached significance (p=0.052).

**Effect of G-CSF on proliferation and neurogenesis in AD Tg mouse model (as measured via IHC).** 8mo NT or APP/PS1 mice received G-CSF or vehicle s.c. injections (8 inj qod); these mice received BrdU injections twice daily during the last two days of G-CSF treatment in order to measure the amount of BrdU uptake, or proliferation, in the subgranular zone (SGZ) of the DG in the HC. G-CSF did not significantly increase BrdU+ counts in either the NT or Tg APP/PS1 mice in the SGZ of the HC (Figure 4.3). An immature neuronal marker, DCX, was also measured in the SGZ of the HC in this group of mice and it was found that APP/PS1 vehicle DCX counts were significantly lower than that of
either vehicle or G-CSF treated NT DCX counts, and treatment with G-CSF increased DCX counts in the SGZ of the HC of Tg mice to that of NT DCX levels (Figure 4.4).

**G-CSF increases synaptogenesis in the CA1 region of the hippocampus in a NT and Tg AD mouse model.** A decline in synaptogenesis is thought to be a robust correlate of AD-like cognitive decline. Therefore, synaptophysin density using optical density was measured in the CA1 region of the HC in both NT and Tg AD mice treated with G-CSF or vehicle (Li et al., 2002). G-CSF treatment in both the NT and Tg AD groups resulted in a significant increase of synaptogenesis in the CA1 region of the HC (Figure 4.5). This data is similar to the results found in a previous study by Dr. Sanchez-Ramos laboratory (Sanchez-Ramos et al., 2009).

**Effects of G-CSF on β-amyloid burden, β-amyloid plasma levels, and microgliosis in the AD mouse brain.** Since irradiation and bone marrow transplantation is a factor that may influence effects of G-CSF, it is important to compare G-CSF effects on amyloid burden and microgliosis in non-chimeric Tg APP/PS1 with those parameters in chimeric Tg APP/PS1 mice (Chapter 3). Hence, the effect of G-CSF on β-amyloid burden, β-amyloid plasma levels and microgliosis was also assessed in this study; NT (n=8) and Tg AD mice (n=11) were treated with s.c. injections of G-CSF or vehicle every other day for a total of 8 injections. Treatment with G-CSF resulted in a significant decrease in the percentage β-amyloid burden (4G8) in the HC, but not the EC, in the Tg AD mice (two-tailed unpaired t-test P<0.001) (Figure 4.6A). There was no significant
change in either Aβ 1-40 or Aβ 1-42 plasma levels with G-CSF treatment (Figure 4.6B). Total microgliosis was measured using the marker Iba1, which labels both resting and activated microglia, in both the HC and the EC. There was a significant genotypic effect on microgliosis, in which Tg AD mice had significantly increased levels of microgliosis in the HC and EC in comparison to the NT littermates (two-way ANOVA, P<0.0001). G-CSF treatment significantly increased microgliosis in Tg AD mice in the HC, but not the EC, although did not have an effect on NT AD mice in either the HC or the EC (two-way ANOVA, P<0.01) (Figure 4.6C).

Discussion

G-CSF has been shown to directly augment proliferation of neural stem cells (NSC) in hippocampal mouse cell cultures in vitro and increase formation of new neurons in the SGZ of the HC in vivo (Sanchez-Ramos et al., 2009). This previously unrecognized effect promoted the further investigation of the neurogenic effects of G-CSF in adult mouse models. Only one neuronal marker was assessed in the previous study, Calretinin, a transient immature neuronal marker, it was therefore of interest to see if G-CSF also positively impacted other proliferative and neurogenic markers in the HC. It was of interest to see if this increase in neurogenesis elicited by G-CSF occurred only in an AD mouse model, or whether it could also be observed in a normal, or wild-type, mouse model.
The FACS method is a rapid yet robust method for measuring hippocampal proliferation and neurogenesis (Bilsland et al., 2006, Spoelgen et al., 2011). Using this approach, a significant increase in proliferation (BrdU+) and neurogenesis (BrdU+NeuN+) was observed in the normal mouse (ICR), but there was only a slight increase of hippocampal neurogenesis (BrdU+NeuN+ cells) in the G-CSF treated APP/PS1 mice (p=0.052).

During hippocampal neurogenesis there is an initial production of neural progenitors that may or may not differentiate into mature neurons as many of these cells undergo apoptosis prior to differentiation. Therefore it may not be that G-CSF is increasing the number of neural stem/progenitor cells, but is directing a greater percentage of those progenitors to differentiate into a neuronal phenotype as opposed to choosing apoptosis or differentiation into a glial lineage. In the older AD mice, G-CSF did not increase proliferation unlike the stimulation of BrdU incorporation observed in younger normal mice. An increase in new neuron formation was noted in both normal and AD mice, but the increase was small in AD mice.

Another interesting observation is the increase in microgliosis as well as the decrease in amyloid plaque burden in the hippocampus of APP/PS1 mice after G-CSF treatment. Aβ is known to be toxic to neurons, therefore by decreasing the amount of Aβ burden in the hippocampus neurons are better able to proliferate, differentiate, and project from the dentate gyrus and form neural circuits through synaptogenesis. So not only may G-CSF have the ability to act directly on neurons, as they express the G-CSFR, but by assisting in amyloid
clearance, the micro-environment of the dentate gyrus of the hippocampus becomes more conducive for neurogenesis.

Due to the slight increase in neurogenesis in an AD mouse model using both the flow cytometry and immuhistochemical methods, it would be worth pursuing further studies on the effect of G-CSF on neurogenesis with a larger N in the same cohort of mice simultaneously with both methods, FACS and IHC. It will also be important to assess a broader range of neurogenic cell markers, both proliferative, immature neurogenic, and double-labeled mature neurogenic, at various time points (2 weeks and 4 weeks) after G-CSF treatment. One should be able to determine which stages in the determination of neuronal fate are most sensitive to the influence of G-CSF.

Due to the small N in this study a power analysis was performed to determine the sample size of animals that would be necessary in future studies to detect a difference in neurogenesis based on the current IHC studies variability. It was determined N of 14 animals per group is necessary to detect a difference between the means with an alpha=0.05 and beta=0.80.

Summary

We have generated evidence that G-CSF has effects on cells of the CNS that result in microgliosis which appears to improve amyloid plaque clearance allowing for self-correction of neurogenesis and synaptogenesis. G-CSF was also shown to directly promote neurogenesis and synaptogenesis. A larger study using multiple proliferative and neurogenic markers and various time points is
needed to more clearly explain the impact G-CSF has on the various stages of neurogenesis in the AD mouse brain. Taken together, with the *in vitro* and chimeric AD mouse experiments in this present study it is clear that G-CSF has beneficial effects in an AD mouse model although the exact mechanism of action remains unclear, but is most likely multifaceted.

Table 4.1. Cell markers used to assess phenotypic fate of neural progenitors.

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>Description/Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromodeoxyuridine (BrdU)</td>
<td>Cell division marker; thymidine analog; nuclear</td>
<td>(Nowakowski and Hayes, 2001, Taupin, 2007)</td>
</tr>
<tr>
<td>KI67</td>
<td>Protein expressed during the G1, S, G2 and M phases of the cell cycle; nuclear proliferation marker</td>
<td>(Scholzen and Gerdes, 2000)</td>
</tr>
<tr>
<td>Nestin</td>
<td>Multipotent progenitor; class IV intermediate filament protein; neural precursor cells</td>
<td>(Fukuda et al., 2003)</td>
</tr>
<tr>
<td>Gliarial Fibrillary Acidic Protein (GFAP)</td>
<td>Intermediate filament protein found in astrocytes; gliogenesis can also occur from HC progenitor cells</td>
<td>(Fuchs and Weber, 1994, Filippov et al., 2003, Steiner et al., 2004)</td>
</tr>
<tr>
<td>Doublecortin (DCX)</td>
<td>Immature neurons; microtubule associated protein found in migratory neuronal cells; dendritic</td>
<td>(Meyer et al., 2002, Brown et al., 2003b)</td>
</tr>
<tr>
<td>III β-tubulin (Tuj1)</td>
<td>Immature neurons; tubulin protein; soma &amp; processes</td>
<td>(Geisert and Frankfurter, 1989, Menezes and Luskin, 1994)</td>
</tr>
<tr>
<td>Calretinin (CRT)</td>
<td>Immature neurons; early transient post-mitotic; Ca+2 binding protein</td>
<td>(Brandt et al., 2003)</td>
</tr>
<tr>
<td>Calbindin (CLB)</td>
<td>Mature neurons; late post-mitotic; Ca+2 binding protein</td>
<td>(van Praag et al., 2002, Kempermann et al., 2004)</td>
</tr>
<tr>
<td>Neuronal Nuclei (NeuN)</td>
<td>Mature granule cell or mature neuron; post-mitotic neuronal marker; nuclear</td>
<td>(Mullen et al., 1992)</td>
</tr>
</tbody>
</table>
Table 4.2. Expression of various cell markers throughout hippocampal neuronal development (derived from Kempermann et al., 2004)

<table>
<thead>
<tr>
<th>Proposed cell types in adult hippocampal neurogenesis</th>
<th>Nestin</th>
<th>GFAP</th>
<th>DCX</th>
<th>CRT</th>
<th>CLB</th>
<th>NeuN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated unlimited proliferative progenitor</td>
<td></td>
<td>+/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slightly differentiated limited proliferative progenitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiated limited proliferative progenitor</td>
<td>+/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immature post-mitotic granule cell (begin to form network connections, long-term survival)</td>
<td>-</td>
<td></td>
<td>+/-</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Mature post-mitotic granule cell</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 4.1. Effect of G-CSF on neurogenesis in the hippocampus of normal mice (measured via FACS). ICR mice were treated with 250μg/kg s.c. for 6 days with daily injection of BrdU 100mg/kg on the last two days of G-CSF treatment. Mice were euthanatized two weeks later. (A) G-CSF significantly increased the number of BrdU+ (n=7 mice per group, unpaired two-tailed t-test, *P=0.03), as well as (B) BrdU+NeuN+ in the hippocampus of normal ICR mice (n=7 mice per group, unpaired two-tailed t-test, *P=0.004), which shows an improvement in both hippocampal proliferation and neurogenesis with G-CSF treatment.
Figure 4.2. Effect of G-CSF on hippocampal neurogenesis of NT and Tg APP/PS1 AD mice (measured via FACS). APP/PS1 and NT mice were treated with 250μg/kg s.c. for 6 days with daily injection of BrdU 100mg/kg on the last two days of G-CSF treatment. Mice were euthanatized two weeks later (error bar represents SEM). G-CSF did not cause a significant increase in BrdU+NeuN+ cells in the hippocampus, although there was an increase with G-CSF treatment in APP/PS1 with a trend towards significance (n=5-8 mice per group, one-way ANOVA).

Figure 4.3. Effect of G-CSF on proliferation in the dentate gyrus (DG) of NT or APP/PS1 Tg mice. 8mo NT or APP/PS1 mice received G-CSF or vehicle s.c. injections (8 inj qod). (A) G-CSF did not significantly increase BrdU+ counts in either the NT or Tg AD mice, although there was a significant genotypic effect in which there are greater BrdU counts in NT mice (n=3 mice per group, two-way ANOVA, genotype p=0.0119, treatment p=0.1278); (B) BrdU positive cells in the subgranular zone of the DG in Tg AD mice (20x image).
Figure 4.4. Effect of G-CSF on neurogenesis in the dentate gyrus (DG) of NT or APP/PS1 Tg mice. 8mo NT or APP/PS1 mice received G-CSF or vehicle s.c. injections (8 inj qod). (A) APP/PS1 vehicle DCX counts were significantly lower than that of vehicle or G-CSF treated NT mice (n=3 mice per group, two-way ANOVA, * genotype p=0.0243, treatment p=0.0163), and G-CSF treatment increased DCX counts in Tg mice to NT levels; (B) Doublecortin (DCX+) positive cells in the subgranular zone of the DG (20X).
Figure 4.5. Effect of G-CSF on synaptogenesis in the CA1 region of the hippocampus in NT or APP/PS1 Tg mice. 8mo NT or APP/PS1 mice received G-CSF or vehicle s.c. injections (8 inj qod). (A) G-CSF significantly increased synaptogenesis in the CA1 region in both NT & APP/PS1 mice (n=4 mice per group, two-way ANOVA, *=P<0.001) (B) Synaptophysin (SP) positive staining in the CA1 region of the hippocampus (40X).
Figure 4.6. Effect of G-CSF on β-amyloid burden, Aβ plasma levels and microgliosis in NT & Tg AD mice. (A) G-CSF significantly decreased amyloid plaque burden (4G8) in the hippocampus, but not the entorhinal cortex, in Tg AD mice (n=4 mice per group, unpaired two-tailed t-test, *=P=0.002); (B) G-CSF did not have an effect on plasma Aβ levels; and (C) G-CSF significantly increased microgliosis in the hippocampus, but not the entorhinal cortex, in Tg AD mice (n=4 mice per group, two-way ANOVA, *=P<0.01).

References


carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat Med 4:97-100.


Chapter 5: Final Discussion

In the preceding chapters, the effects of G-CSF on peripheral cells of bone marrow origin and cells of the central nervous system were investigated both in vitro and in vivo. The overall objective was to elucidate the mechanism(s) of action responsible for the previously reported beneficial actions of G-CSF in an AD mouse model (Tsai et al., 2007, Sanchez-Ramos et al., 2009).

For the in vitro studies, cell lines were chosen to model human monocytes (THP-1) and human neurons (SH-SY5Y). Studies of radio-labeled ligand (\(^{125}\text{I-}\)GCSF) binding to the cognate GCSF-receptor resulted in saturation curves with distinctly different shapes (hyperbolic in the monocytes; sigmoid in the neuronal line). The pharmacokinetic binding parameters were also distinctly different. The monocytic cell line exhibited a greater binding affinity (lower Kd) while the neuronal cell line revealed a greater Kd, Bmax and receptor density. The neuronal line exhibited cooperative binding kinetics (Hill coefficient ~2) while the monocytic line revealed linear binding (Hill coefficient ~1).

The marked differences in G-CSF binding kinetics to monocytic and neuronal cells observed in vitro do not easily fit the in vivo observation that levels of G-CSF in brain are significantly lower than those in blood in untreated Alzheimer’s Disease. Theoretically, it would be expected that the Kd in neurons of the brain would be much lower than that of monocytes so that the normally lower levels of brain G-CSF could interact with receptors
expressed on neurons. However, during severe inflammatory processes in brain (e.g. meningitis), levels of G-CSF are known to increase to concentrations higher than in the blood. In those situations, there would be high enough concentrations of endogenous G-CSF to activate neuronal G-CSF receptors. However, administration of exogenous G-CSF at high doses to patients with AD might result in sufficiently high levels of G-CSF in brain, at least transiently, to activate receptors borne on neurons and neural stem/progenitor cells.

Further comparison of the monocytic and neuronal cell lines showed anti-apoptotic proteins (PKCδVIII and Bcl2) in the neuronal cell line were up-regulated at lower concentrations (100ng/mL) of G-CSF than in the monocytic cell line (200ng/mL). This greater degree of up-regulation of anti-apoptotic proteins occurs in neuronal cells which have a greater number of receptors per cell despite lesser binding affinity for the ligand. It is likely that the neuronal cell line has different number or amount of the G-CSFR effector molecules, cytosolic tyrosine kinase, or that it has a greater proportion of spare receptors, which would allow a full biological response even when only a small proportion of the total receptors are bound.

It was of interest to assess if the beneficial effects of G-CSF on AD mice seen in previous studies (Tsai et al., 2007, Sanchez-Ramos et al., 2009) could be attributed to an increase in the number of circulating bone marrow derived monocytes (or pro-monocytes) infiltrating the brain parenchyma. To objectively study this potential mechanism, the migratory properties of a
monocyte cell line (THP-1) were examined in a migration chamber system. G-CSF did not change migratory properties of monocytes *in vitro*, neither through improved adhesion in cell culture, nor through migration through a micropore membrane.

However, we showed that G-CSF treatment increased the proportion of mouse GFP+BM cells that express a monocytic marker (CD11b) and significantly stimulated DNA synthesis in the human monocytic cell line. These results are consistent with the observation that G-CSF treatment (10µg/kg/day) of AD patients for five days increased the absolute number of circulating monocytes (and blood stem/progenitor cells) in subjects with AD (Sanchez-Ramos J, 2012). These observations in the transgenic GFP+ mouse and AD patients suggest that the total amount of bone marrow derived monocytes or pro-monocytes infiltrating the brain parenchyma *in vivo* might occur through “mass action” or an increase in total circulating monocytes, and not through improved migratory properties. A caveat to this statement is that absence of changes in migratory capacity of an immortalized monocytic/pro-monocytic cell line *in vitro* may not reflect what happens to authentic monocytes *in vivo*.

In the irradiated chimeric GFP and non-irradiated APP/PS1 AD mice there was a significant increase in microgliosis and reduction of amyloid burden in the hippocampus with G-CSF treatment, but the infiltration of monocytes did not contribute to this increase in microgliosis as there were no GFP labeled bone marrow derived cells found in the brain parenchyma of the chimeric AD
mice. Although GFP labeled cells were found in the microvasculature of the brain as well as in the choroid plexus, the proportion of GFP cells in the microvasculature of the hippocampus and entorhinal cortex was not increased with G-CSF treatment. This part of the research had several limitations that will require further investigation. Although inspection of a blood smear from chimeric mice transplanted with GFP+ bone marrow was performed to ascertain “successful” engraftment, the percentage of GFP+ cells in the chimeric mice was not quantified from blood samples before administration of G-CSF or after euthanasia. It is possible that the engraftment of the GFP+ donor cells was minimal and the host bone marrow stem cells that survived irradiation repopulated the marrow to a greater extent than the GFP+ cells. If the percentage of GFP+ monocytic cells in the circulation were to be very low, it would result in an underestimation or failure to detect monocytic cell infiltration.

Nevertheless, failure to observe GFP+ cells in the brains of AD mice in these experiments suggests that it is not the action of G-CSF on the BM or the circulating monocytes that result in an increase in microgliosis in both irradiated chimeric and non-irradiated APP/PS1 mice. It is most likely due to the direct action of G-CSF on endogenous microglia, which have also been known to express the G-CSFR. It will be important to examine the effects of G-CSF on microglial proliferation and phagocytosis of Aβ in vitro in future studies, as it is likely that the increase in microgliosis results in improved
phagocytosis of amyloid plaques resulting in the increase in amyloid burden clearance that was seen \textit{in vivo} with G-CSF treatment.

After observing the stimulatory effects of G-CSF on DNA synthesis and proliferation, enhancement of viability, and up-regulation of anti-apoptotic proteins in the human neuronal cell line, the next step was to investigate the \textit{in vivo} neurogenic effects of G-CSF in non-irradiated mice (both in normal and in a Tg AD mouse model). In normal mice (ICR) using fluorescence activated cell sorting (FACS), both hippocampal proliferation (BrdU) and hippocampal neurogenesis (BrdU/NeuN) were significantly improved with G-CSF treatment, although FACS analysis did not reveal similar increases in the AD mouse model. In a non-irradiated AD mouse model using immunohistochemistry, G-CSF increased doublecortin (DCX) expressing cells (or immature neurons) and increased synaptophysin-density in the CA1 region of the hippocampus indicating enhanced neurogenesis and synaptogenesis in this APP/PS1 mouse model. The stimulation of neurogenesis and synaptogenesis could be attributed to 1) the direct action of G-CSF on hippocampal neural progenitors, which was consistent with the \textit{in vitro} data in the human neuronal cell line, or 2) the direct action of G-CSF on endogenous microglia which reduces amyloid burden and provides an improved neurogenic environment. Therefore future studies comparing the effects of G-CSF on neural progenitor cells and microglia will be important to elucidate the contribution of each of the cell types to the beneficial effects seen with G-CSF treatment in an AD mouse model.
In summary, these studies were designed to explore the mechanism(s) of action of G-CSF responsible for a) enhancement of cognitive performance in a Tg AD mouse model, b) reduction of amyloid burden, c) increased microgliosis and d) stimulation of hippocampal neurogenesis. Working with cellular models of monocytic and neuronal cell lines, we found that a) G-CSF interacts with its cognate receptor with different binding kinetics and with greater affinity for the monocyte G-CSFR, b) the number of G-CSF receptors in neurons is greater than in monocytes, and c) the anti-apoptotic response in neurons occurs at lower concentrations of G-CSF than in monocytes. From these experiments we infer that neuronal G-CSF receptors are activated only when levels of G-CSF ligand in brain are equal to or higher than G-CSF levels in blood (as in severe inflammation, injury to blood brain barrier or after administration of high doses of G-CSF as a therapeutic agent). Moreover, G-CSF appeared not to enhance migratory capacity of monocytes so it is unlikely that the increased microgliosis observed in the earlier studies in Tg AD mice is solely attributable to increased infiltration of monocytes into brain. From these observations, we infer that the direct effects of G-CSF on microglia is most likely to be responsible for microgliosis and decreased amyloid burden.

From a translational perspective, these findings provide an impetus to develop G-CSF as a pro-cognitive agent in diseases associated with decline in hippocampal neurogenesis, such as AD and other dementing illnesses.
References

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About the Author

Amanda Renee Rowe was born in San Antonio, Texas and moved with her family to Bradenton, Florida a few years later. She graduated from Manatee High School in 2003 as valedictorian. She continued her education at the University of Florida (UF) studying Human Nutrition. After graduating with her B.S. from UF in 2006, she continued to work in the department of Food Science and Human Nutrition as a lab manager/technician of Dr. Lynn Bailey, whose laboratory research focused on nutrient-gene interactions affecting folate and vitamin B12-related genes. In 2007, she started the MD/PhD program at the University of South Florida College of Medicine and began working in the laboratory of Dr. Juan Sanchez-Ramos. She was president of the Association of Medical Science Graduate Students 2008-2009 (AMSGS) during her second year of medical school. In 2009, she completed her first two years of the MD program and continued to work full-time on her PhD degree in the department of Molecular Pharmacology and Physiology with a concentration in Neuroscience. In 2011, she was married in Bradenton, Florida to Aaron Pennington and therefore her name was changed to Amanda Renee Pennington. She expects to complete her MD degree in the spring of 2014.