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Developing a novel co-culture model and testing the effects of placental derived stem cells on inflammation in Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is an abhorrent disease that robs people of their memories and personalities. Researchers have yet to develop an effective disease-modifying treatment for this multifaceted disease. Inflammation is one of the main hallmarks of AD and is caused by the over-activation of the main immune cells of the brain: neuroglial cells. High concentrations of nitric oxide (NO) associated with this heightened immune response have been known to cause neuronal damage and cellular loss. The aim of this study was to develop a viable co-culture system in which placental derived stem cells (PDSCs) and neuroglial cells are cultured together in systems involving transwells and cover slips for further analysis. A transwell co-culture system resulted in no effect of PDSCs on the NO concentration mediated by LPS stimulation or the modulation of NGF in glia cultures. This suggests that NGF may not necessarily be the neurotrophin causing the decrease of NO in activated glia. A second co-culture system, a cell-to-cell contact cover slip system in which glia were grown in a welled plate and PDSCs grown on cover slips, revealed a decrease in NO concentration in LPS stimulated glia when compared to controls. There were several problems with this model however, including cell disruption stemming from execution flaws. Using Immunocytochemistry, this study also qualified and quantified the morphological differences in activated glial cells that had been treated with stem cells visualized through an induced nitric oxide synthase antibody (iNOS). LPS stimulated glia showed a shrunken, shriveled appearance typical of apoptosis with a high intensity of iNOS stain and a high area percentage covered by stain. Control glia, however, showed healthy spread and flattened glial formations with a minor but widespread intensity of iNOS. These studies, though preliminary, offer a platform on which to conduct further experiments in glia and PDSC co-culture as well as clues about how iNOS gets activated in glial inflammation in a model of AD.

Common Abbreviations: AD- Alzheimer's disease; Aβ- Amyloid Beta; NFT- Neurofibrillary Tangles; NO- Nitric Oxide; FBS- Fetal Bovine Serum; LPS- Lipopolysaccharide; IFN-γ- Interferon-γ; PDSC- Placental Derived Stem Cells; SCCM- Stem Cell Conditioned Media; BDNF- Brain Derived Neurotrophic Factor; NGF- Nerve Growth Factor.

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1. Introduction

1.1 Early History

In 1906, Alois Alzheimer began work with a 51 year old female who was suffering from a strange psychological manifestation (Strassnig and Ganguli, 2005; Möller and Graeber, 1998). Auguste's symptoms began with an unwarranted jealousy towards her husband which expanded to include paranoia about her surroundings and hallucinations. Her memory declined rapidly and she became violent because of her complete "disorientation to time and place." Though she was largely able to recognize objects presented to her, she appeared to have lost the ability to use them correctly. Eventually, she lost all mental function and died after a four and a half year decline in health and intellect. A histological examination of her brain revealed "macroscopic foci" and "a bundle of fibrils… which had formerly been occupied by a ganglion cell" as well as brain atrophy and arteriosclerosis. In 1911, Alzheimer reported another patient named Johann who presented with similar symptoms (Strassnig and Ganguli, 2005). Just like Auguste, Johann experienced a similar rapid decline in memory with intact object recognition. He had a pleasant demeanor but, throughout the course of the disease he became increasingly uncooperative. Johann died after three years of hospitalization and decline in mental status. Alzheimer's examination of Johann's brain came up with similar features that were present in Auguste's with some significant differences that required further investigation and distinction.

Alzheimer observed the same plaque-like formations in Johann and Auguste, but found that there was an absence of neurofibrillary tangles (NFT) in Johann's brain

(Strassnig and Ganguli, 2005). Alzheimer investigated the similarities and differences between his two patients as well as clinically and histologically similar patients who had been studied by other scientists. Senile dementia was ruled out as a possible diagnosis for each patient based on their symptoms, however, molecular differences such as the presence or absence of NFT also distinguished the cases from each other. It was difficult to distinguish these cases as presenile dementia or just an atypically severe case of senile dementia. Alzheimer himself struggled with differentiating the symptoms and pathologies in each of his subjects despite Emil Kraepelin's classification of the findings as "Alzheimer's Disease" (Berrios, 1990). There remained a controversy about the true differences among senile dementia, presenile dementia and the newly dubbed Alzheimer's disease (AD) (Berrios, 1990; Holstein, 1997). In the years following Kraepelin's naming and Alzheimer's case report, there was confusion as to how a case of dementia should be classified. Many seemed to distinguish senile dementia and Alzheimer's disease by the severity and the age of onset. Others distinguished it by the types of plaques found in the post-mortem brain. Without many of the modern research techniques that exist today, there were more questions than answers when it came to Alzheimer's disease. What causes it? How is it different from other dementias? Is it different from other dementias at all?

1.2 Modern Nosology

Today, it is well documented that amyloid-beta plaques, neurofibrillary tau tangles, loss of synaptic connections, inflammation, and cell death are all hallmarks of

Alzheimer's disease (Alzheimer's Association, 2013). These are normally accompanied by profound changes in personality, memory loss and difficulties in behavioral and bodily functions. Many people who go on to develop Alzheimer's disease start off showing mild disturbances in mental ability. This is also known as mild cognitive impairment (MCI). About 10 to 20% of adults have MCI, and 15% of those who report their symptoms to a doctor go on to develop dementia. Several magnetic resonance imaging (MRI) studies have shown that although the symptoms of the disease often appear at around the age of 65, changes manifest in the brain many years prior to diagnosis (NIH, 2012; Drzezga et al., 2003). The changes then become concentrated in the hippocampus, one of the most important brain structures for memory, which leads to symptoms such as confusion, forgetfulness, and some personality changes (NIH, 2012; Wang et al., 2003). Late in the disease, there are widespread atrophy, numerous plaques, and tangles throughout (Thompson et al., 1998; NIH, 2012). At this stage in the disease, those afflicted are left with the inability to communicate and are completely dependent on others. Because the only confirmation of an Alzheimer's disease diagnosis can come from a postmortem analysis of the brain, doctors must rely solely on memory assessments and tests that rule out other diseases that present similarly to AD (Alzheimer's Association, 2013).

Though dementia is often an underlying cause, Alzheimer's disease is currently the 6th leading cause of death in the United States with the immediate cause of death stemming from pneumonia, cerebrovascular, or cardiac disease (Alzheimer's Association, 2013; Thomas et al., 1997). There are currently 5 million people over the

age of 65 living with the disease. This number will increase to an estimated 13.8 million people by 2050. With age being the highest risk factor of the disease and a population that is getting older, there is going to be a higher demand for care if trends persist (Social Security, 2013). People who are 85 and over are 5.4 times more likely to die from AD than those that are between the ages of 75 and 84 (Tejada-Vera, 2013). Care for the people currently living with the disease costs the United States $203 billion which, if the increase in prevalence continues, will turn into a $1.2 trillion cost of care by 2050 (Alzheimer's Association, 2013).

Less than 3% of the AD population is afflicted with early onset Alzheimer's disease which normally presents around the age of 55 or even earlier (Bird, 1999). There are several gene mutations associated with this type of Alzheimer's disease. These include a mutation in the gene for presenilin 1 on chromosome 14, a mutation in the gene for presenilin 2 on chromosome 1, and a mutation in the gene for amyloid precursor protein (APP) on chromosome 21 (Borchelt et al 1996). Presenilin 1 is a protein that is involved in the cleavage of APP into smaller sizes of soluble complexes, including soluble amyloid precursor protein and amyloid beta complex (U.S. National Library of Medicine). A mutation in the PSEN1 gene leads to an overproduction of the amyloid beta peptide, which leads to a toxic accumulation of plaques in the brain. This mutation accounts for about 70% of the early onset Alzheimer's disease cases. Presenilin 2 works in a very similar way to presenilin 1, but only account for about 5% of early onset cases. APP can cause early-onset Alzheimer's disease through 50 different mutations the most common of which changes valine to isoleucine at position 717. This results in a longer,

stickier form of amyloid beta and an overall increase in the peptide. A mutation associated with any of these genes results in a highly probable development of early-onset Alzheimer's disease.

Over 95% of Alzheimer's cases are considered to be of the sporadic type or late-onset (NIH, 2012). There is no known cause for this type of Alzheimer's disease, though scientists have found a gene that correlates to a person's risk of developing the disease later in life. The apolipoprotein E (APOE) gene has several different alleles with APO ε2, APO ε4, and APO ε3 being the most common (Isbir et al., 2001). Each allele has a different correlation with one's risk of developing the disease. The rarest form, APO ε2, can actually have a preventative and/or protective effect on the person's susceptibility to developing Alzheimer's disease. The most common form, APO ε3, is thought to have no effect or a neutral effect on the risk of developing the disease. APO ε4, however, has been associated with 40% of late onset cases (NIH, 2012). The APOE gene is normally involved in creating proteins that carry and transport cholesterol and fat through the bloodstream and is thought to be correlated with an increase in vascular and cerebral Aβ plaques (Schmechel et al, 1993). It is possible that the degree of pathology that is associated with different forms of the APOE proteins could have to do with the degree to which amyloid beta adheres to the proteins during transport (Saunders et al, 1993). Even though having the APO ε4 allele can mean an increased risk of developing the disease, it is not a definitive indicator of imminent development of the disease(Schmechel et al, 1993).

Amyloid beta (Aβ) plaques are one of the most prominent structural hallmarks of Alzheimer's disease (Kowall and Budson, 2011). A popular hypothesis that has dominated AD research for over twenty years is the amyloid cascade hypothesis. It is predicated on the idea that amyloid beta is the main culprit in the development of Alzheimer's disease. It is thought that in familial Alzheimer's disease, the accumulation of Aβ is due to a direct mutation of APP, PSEN 1 or PSEN 2, while in sporadic Alzheimer's disease, the amyloid plaques appear as a result of a malfunction in the normal clearance mechanisms or a defect in the vascular transport proteins. According to the hypothesis, APP is first cleaved by either α-secretase or β-secretase (Galimberti and Scarpini, 2011). Cleavage by these proteolytic secretases causes shedding of the ectodomain of APP and results in α and β C-terminal fragments. The α-secretase cleaved C-terminal end is an 83-residue membrane-associated or a C86 while the β-secretase cleaved fragment is a 99-residue membrane associated or a C99 (Wolfe, 2001). These C-terminal fragments are then cleaved by γ-secretase which releases two proteins into the extracellular space, p3 from C89 and Aβ from C99. PSEN 1 and/or PSEN 2 are thought to be catalytic subunits of γ-secretase and cause cleavage of Aβ resulting in the variants Aβ40 and Aβ42. Aβ42 is oligomerized and deposited in diffuse plaques throughout cortical areas (Kowall and Budson, 2011). These oligomers cause a disruption in synaptic communication and function which leads to an altered ionic homeostatic state which causes kinases and proteases associated with the cell's microtubules to become impaired, aiding in the formation of tau tangles. Toxic inflammation from activated microglia also begins to cause damage as a result of the Aβ deposits. Over time, damage becomes

widespread and atrophy results from a loss of neurons and the symptoms of dementia begin to appear.

There are several problems with this model of AD pathology. One would expect that amyloid plaques would have a higher prevalence in the limbic system since that would correlate with the early symptoms; however, amyloid plaques are found in the frontal cortex in the early stages of the disease while the neurofibrillary tangles are more prevalent in the limbic system (Pimplikar, 2006). Also, the amount of amyloid beta seen in postmortem brains does not often correlate to the severity of the symptoms seen clinically. Even patients who were cognitively normal can have a high number of Aβ plaques in their brains whereas patients who were diagnosed with severe Alzheimer's disease may have no plaques at all. This suggests that there is more to the disease than just amyloid beta. One of the most important aspects of memory is the synaptic connectivity that allows our neurons to communicate with each other and form the connections responsible for memory (Selkoe, 2002). There are a multitude of avenues being explored by scientists in order to come up with effective treatments, though it is likely that the disease involves multiple factors and is not caused by one single mechanism.

Despite the dominating prevalence of the amyloid cascade hypothesis in research, there has been a significant amount of research done that explores the other hallmarks of the disease and how they may contribute to the cause and perpetuation of symptoms. As previously mentioned, one of the other main hallmarks of AD are neurofibrillary tangles (NFTs). NFTs are made up of a protein called tau (Galimberti and Scarpini, 2011). In

normal cells, tau is part of the microtubule network that not only allows for transport of nutrients, mitochondria and other cellular components, but also allows for the stabilization and destabilization of neurites. This growth and retraction of neurites comes as a result of the phosphorylation and dephosphorylation of the tau protein by kinases which allow tau to attach and detach from the tubulin of the microtubules in a cell's axons and dendrites (Mandlekow and Mandelkow, 1995). In AD, the tau protein becomes hyperphosphorylated and aggregates into paired helical filaments, causing an extended state of destabilization and accumulation of the protein in the neurites (Ballatore et al. 2007). Not only does the destabilization of the microtubules cause a loss of communication between neurons, but the paired helical tau filaments disrupt communication and transportation even further, eventually resulting in the cell's death. Research has shown that tau becomes phosphorylated at serine-proline or threonine-proline sites which suggest that the hyperphosphorylated tau could be a result of proline directed kinases, though these results are inconclusive (Steinhilb et al., 2007). With so many conflicting results on the forefront of AD research, finding a cure or even an effective treatment has been made rather difficult.

One of the biggest obstacles in the way of treatment of AD is the fact that there is no accurate way to predict if and when someone will develop the disease (Galimberti and Scarpini, 2011; Lawrence and Sahakian, 1998). Treatment, therefore, can only be given once the disease has already caused a significant amount of damage. Consequently, most of the treatments available today are aimed at treating the symptoms of AD and stalling the progression, but not targeting the underlying pathology causing cell death (Mayo

Clinic, 2013). One of the first attempts at a pharmacological treatment of AD was through a drug called Physostigmine, an acetylcholinesterase inhibitor (Lawrence and Sahakian, 1998). The development of this intravenous and orally administrated drug was predicated on the correlation of acetylcholine (Ach) depletion in the brains of AD patients. Not only did studies show that correlation between Ach depletion and severity of AD symptoms was strong, but they also found that cognitively normal patients that were given an intravenous Ach receptor antagonist showed many of the same memory, language, and other deficits seen in AD patients (Blokland, 1996). However, despite these parallels, clinical trials presented mixed results due to only a slight increase in cognition with a high number of side effects as well as variability in the bioavailability of the drug.

The first drug approved by the FDA for clinical treatment of AD was Tacrine in 1993 (Davis and Powchik, 1995). This acetylcholinesterase inhibitor showed promising cognitive improvements in 14 of 17 AD patients in a study done by Summers et al in 1986 which led to its eventual FDA approval. This drug works by binding to the active enzymatic site of acetylcholinesterase which prevents the breakdown of Ach thereby prolonging the activity of Ach. Tacrine causes a large increase of serum alanine aminotransferase (ALT) in about half of all users, with some patient's levels reaching 20 times the normal limit. This high increase in ALT can result in hepatotoxicity which makes it a less commonly used drug in today's market. Donepezil, also an acetylcholinesterase inhibitor (AChEI), was the second drug approved by the FDA for treatment of AD and is now approved for severe and mild cases of AD (Galimberti and

Scarpini, 2011). Galantamine and rivastigmine have also joined the AChEI class of AD drugs all with similar efficacies.

N-methyl-D-aspartate (NMDA) antagonists, specifically the drug memantine, have been FDA approved and instituted as a treatment for moderate to severe AD (Galimberti and Scarpini, 2011). NMDA receptors are plentiful the hippocampus; over activation of these receptors can cause an overabundance of calcium in the cell which causes it to release free radicals and die (Martinez-Coria et al., 2010). Research shows that Aβ can latch on to the NMDA receptors and cause this chain of events which is why some researchers have turned to NMDA antagonists to decrease the over activation of receptors caused by Aβ accumulation. Increased NMDA receptor activation has been shown to also cause hyperphosphorylation of tau, further justifying the use of the NMDA receptor antagonist as a treatment. However, as with the AChEIs, NMDA receptor antagonists only help to improve cognition to a point and do not treat the underlying cause of the disease; there are no cures or disease-modifying drugs that actually target the cause of the disease.

Because the amyloid cascade hypothesis has been dominating research for many years, there have been several attempts to target Aβ in order to develop a disease-modifying drug. Vaccines have been looked at as one way to attack Aβ, by strengthening a patient's immune system so that it may clear out the over accumulation of Aβ or prevent the plaques from forming at all (Mayo Clinic, 2013). In phase II trials of a vaccine using Aβ42 plus adjuvant, 60% of the 300 patients showed antibody activity; however, the trial was stopped after only 2 or 3 injections because a significant number of

patients developed aseptic meningo-encephalitis (Galimberti and Scarpini, 2011; Mayo Clinic, 2013). Also, despite the antibody activity found in the trial subjects, there was no significant difference in cognition (Galimberti and Scarpini, 2011). After these initial trials, the vaccine was modified to act as a passive immunization rather than active. This vaccine, monoclonal anti-Aβ antibody bapineuzumab, entered phase II clinical trials followed by phase III trials, which started before the phase II trials ended. This vaccine had mixed results, with those who were non-carriers of the APOε4 allele showing the most significant cognitive improvement. Imaging analysis of the AD patients who participated in this clinical trial showed a reduced retention for plaques. There are several other vaccines that are currently in clinical trials in the US, but none have passed phase III clinical trials. Scientists have looked at blood donors for possible treatments. The natural anti-amyloid antibodies found in human intravenous immunoglobin have shown to slow or stop cognitive decline in 7 patients in phase I clinical trials, 6 of whom not only showed a halt in decline, but actually showed improvement.

Selective Aβ42 lowering agents have also been explored as potential treatments for AD (Galimberti and Scarpini, 2011). The first of these types of drugs was Tarenflurbil which altered γ-secretase activity in order to reduce the amount of Aβ42 plaque formation (Galimberti and Scarpini, 2011; Imbimbo, 2009). Tarenflurbil could alter γ-secretase activity at the active site that causes production of Aβ42 without interfering with other γ-secretase substrates. This drug reached phase III clinical trials, but led to little to no cognitive improvement in non-placebo control groups and in some cases, cognitive decline was greater for those given the drug as compared to those on placebo.

Tau has also been targeted in the battle against AD. Inhibitors of kinases such as GSK3 and ERK2 have been explored in order to reduce the number of tangles intercellularly (Mazanetz and Fischer, 2007). Though many of the drugs and treatments that have been tested have been promising, there are currently no FDA-approved disease-modifying drugs for AD. Scientists are constantly looking at potential pathways that may lead to a drug that can target the disease in a way that will not just treat symptoms, but may actually lead to a cure.

1.3 Neuroglia

Neuroglia are the most abundant cells in the brain making up 90% of the cells within the brain (Stork et al. 2012). There are three main types of glia, including astrocytes, oligodendrocytes and microglia each of which has a particular and important role (Heneka et al. 2010). Many functions of neuroglia are yet to be understood, but several have been outlined by extensive scientific research (Stork et al. 2012). Their functions range from structural support, maintenance, development, as well as roles in immunity. Oligodendrocytes are primarily the insulators of the brain (Nagelhus et al. 2013). They serve as the myelin sheath or covering around the axons of neurons and allow for quick transmission of action potentials. Astrocytes provide support for the brain along with other functions, and microglia have a significant role in the brain's immune system. The roles of neuroglial cells have been explored for several neurodegenerative diseases, including amyotrophic lateral sclerosis (Lou Gehrig's disease), Parkinson's disease, and Alzheimer's disease (Heneka et al. 2010).

Astrocytes are the greatest in number not only of neuroglia but of all cells in the brain (Nagelhus et al. 2013; Heneka et al. 2010). Astrocytes hold the brain together by filing in the spaces between neurons and blood vessels. They also help regulate homeostasis in the brain, including water homeostasis, ion concentration, and pH balance. Astrocytes are known for their role in synaptic communication between neurons. Neurons and astrocytes create a cross-talk in which neurotransmitters signal different glial processes and gliotransmitters to regulate neuronal processes. Degradation, clearance of material, and damage repair are also important roles of astrocytes. Damage repair is often coupled with the signaling and recruiting of astrocytes resulting in neuronal inflammation and scarring. Chronic inflammation has been known to cause neuronal loss and other damage including astrogliosis or scarring.

Microglia are considered to be the main immune cells in the brain and therefore contribute the most to the inflammation seen in an AD brain (Heneka et al. 2010). Microglia are constantly surveying the brain for pathogens, bacteria and neuronal damage in order to activate and release inflammatory mediators that protect neurons from further damage (Van Eldik, 2001). Microglia can be found in two states: one of rest or, more accurately, surveillance, or one of response (Hanisch and Kettenmann, 2007). The response state is caused by a shift from the resting state through either a change in the naturally occurring chemical environment of the brain or the introduction of a factor that is abnormal to the brain's environment. Receptors on the surface of the cells cause an inhibition in activity in the normal cellular environment. When a change in the chemical

environment occurs, the receptors on the surface of the microglial cells respond to specific factors causing them to shift to an activated state resulting in inflammation.

1.4 Nitric Oxide

Nitric oxide (NO), a molecule for which one of the major sources is glial cells, has been identified as an important substance for brain health (Law et al., 2003). NO is made when nitric oxide synthase (NOS) enzymes convert L-arginine to L-citrulline. There are three different types of NOS species. Type I is found in neurons and is Ca^{2+} dependent. Type II is found in glial cells while Type III is found in the endothelium and is membrane bound. The NOS species found in glial cells, iNOS, is induced by inflammatory cytokines, causing the conversion of L-arginine to intermediates that lead to L-citrulline, which then leads to the production of NO.

Though NO has many benefits and physiologically normal functions, it has also been well documented as one of the main toxins found in an AD brain (Law et al. 2003; Heneka and O'Banion, 2007). Prolonged activation from glial cells can produce NO concentrations in the brain that end up causing damage rather than fixing it. Damage from NO comes from the combination of superoxide anions and the NO molecule, which creates a reactive oxygen species (ROS). ROS can cause DNA damage and change the function of some proteins, wreaking havoc on the cell and eventually leading to cell death (Law et al., 2003). There have also been several associations found between active glia and Aβ. Accumulations of active glia have been found around groups of tau tangles that mark the area of an apoptotically disintegrated neuron (McGreer and McGreer, 1995).

Active glia have also been found to accumulate around Aβ plaques. Aβ can activate CD4+ T cells, which have been known to produce certain inflammatory cytokines, including interleukin-1β, interleukin-6, tumor necrosis factor α, and interferon-γ. A high microglial activation has even been associated with the APO ε3 and APO ε4 genotypes, further presenting evidence for the connection between the damage of inflammation and AD.

Several routes have been explored in an attempt to quell the over activation of glial cells in the AD brain. Protease inhibitors have been used in order to try and prevent the detrimental changes made in the proteins from ROS (McGreer and McGreer, 2001). Prostaglandins, (fatty acid chains that contribute to inflammation) can be reduced by non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs were found to not only reduce cognitive impairment and delay the severity of disease progression, but they were also found to reduce the accumulation of Aβ by up to 50% using the NSAID ibuprofen (Heneka and O'Banion, 2007; Lim et al, 2000). Ibuprofen was also found to reduce the amount of interleukin-1β as well as glial fibrillary acidic protein (Lim et al., 2000). However, NSAIDs carry many gastrointestinal side effects and do not eradicate the disease, which have made them a less popular choice for treatment (McGreer and McGreer, 2001).

1.5 Laboratory Models

Developing a viable model with which to research Alzheimer's disease has been an ongoing process for many years. Human models are difficult to work with for a number of reasons including a high number of confounding variables and ethical issues that animal models do not possess. Mice and rats are often used in research in order to manipulate desired variables. Transgenic mouse models have been used in a vast majority of Alzheimer's research (Spires and Hyman, 2005). The earliest transgenic model came from a manipulation of the APP peptide, which is altered in both the familial and sporadic type of AD. A 1991 study conducted by Kawabata et al. reported a significant increase in Aβ plaques through the manipulation of the C-terminus of APP through the Thy-1 gene (Kawabata et al., 1991). Importantly, this study did not report concurrent behavioral studies which are essential in creating a new model because the appearance of plaques does not necessarily indicate that the subject will show memory deficits, as previously stated. Hsaio et al. (1996) conducted an experiment using a human APP peptide that was double mutated and inserted it into a hamster prion protein cosmic vector for use in mice. This group conducted behavioral tests on transgenic mice and non-transgenic mice aged 3, 6, and 9 months. Though the 9 month transgenic mice showed longer escape latencies in the Morris water maze at the start, they were still able to perform just as well as non-transgenic mice after several trials. Though these models were able to mimic some of the deficits of AD, there were ultimately missing some aspects of the disease that could only be found with further research.

Because Aβ is only one of the hallmarks of AD, a model that induced the formation of tau tangles was the next logical step in the development of a transgenic animal model. Gotz and colleagues took the first steps in creating a transgenic model that expressed the tau pathology found in AD. They used three different promoters, the best of which, Thy-1 promoter, yielded the highest human tau expression in mice. After analysis of distribution of the human tau protein using *in situ* hybridization, tau was found throughout the cortex, including areas known to accumulate tau tangles in an AD brain. However, the expression of the human mRNA was not distributed as uniformly throughout the mouse brain with some areas expressing higher mRNA than other areas; the severity of plaque accumulation also seemed to favor female mice. Though their model was not perfect, it provided a stepping stone to the next generation of transgenic models which crossed Aβ expressing mice and tau expressing mice (Lewis et al., 2001).

Oddo et al. (2003) introduced a triple-transgenic (3x-Tg) mouse model in which mutations for Aβ, tau, and PSEN 1 lead to a model that presented with both plaques and tangles. The 3x-Tg model not only equally favored both male and female, but the appearance of the plaques and tangles were much quicker than previous models. The 3x-Tg also showed an increased severity of plaques and tangles compared to previous models, with plaques having an earlier prominence and tangles appearing later, similar to the pathology in humans. Transgenic models are invaluable to AD research as they allow for the investigation of new treatments and allow us to better understand the pathology of AD.

Cell culture is an efficient and popular model to use in AD research as well. They allow for the manipulation of variables at the molecular level and can provide insight into cellular mechanisms of which animal models are incapable. Glial cells and neurons cultured from mice or rats serve as a template for manipulation in order to model the disease state seen in AD. In order to mimic the neuroinflammatory response of neuroglial cells, scientists often use lipopolysaccharide (LPS). Bacterial LPS works by activating inflammatory mediators such as interleukin-1β which then in turn causes upregulation of inflammatory cytokines and an increase nitric oxide (Raetz and Whitfeild, 2002). This is often used in conjunction with interferon-γ in order to potentiate the immune response initiated by LPS activation (Jeohn et al., 2000). Together these factors produce the deleterious cellular environment found in AD.

1.6 Stem Cells

Stem cells have been used as a treatment for several different diseases including neurological diseases. They are particularly useful because they have the ability to proliferate and can differentiate into different types of cells in the body (NIH, 2002). There are two main types of stem cells that are used for treatments and are currently being looked into as treatments for diseases such as AD: embryonic stem cells and adult or body stem cells. Both embryonic and adult stem cells have their advantages and disadvantages.

Adult stem cells are cells that are isolated from organs in the body that already contain differentiated cells (NIH, 2002). Adult stem cells can be isolated from bone

marrow, skin, or even areas in the brain. These cells can be used to differentiate into different kinds of tissue, but only into tissue that is related to their origin organ. Adult stem cells also secrete factors that help promote cell health and growth factors such as brain derived neurotrophic factor (BDNF) or nerve growth factor (NGF) which encourages neuronal growth. A study by Burlton-Jones et al. (2011) found that the transplantation of neural stem cells caused an improvement in cognitive function in a triple transgenic mouse model through elevation of BDNF. Although there are few ethical issues associated with adult stem cells, they are found in small quantities in their origin organs and can be difficult to grow in culture, unlike embryonic stem cells which are grown with relative ease in culture (Strauer and Koronowski, 2003). Recently, scientists have found that adult stem cells can be manipulated in order to make them function more like embryonic stem cells by changing their gene expression (NIH, 2002). These are called induced pluripotent stem cells (iPSC). iPSCs can be induced to form any type of cell in the body, though their practical use in medicine is still under review. Both iPSC and tissue derived adult stem cells require a blood match to prevent rejection from the recipient, creating another hurdle in their practical clinical use (Bradely et al., 2002).

Embryonic stem cells have been used as a treatment for neurological diseases because of their proliferation and differentiation power. They can also be easily expanded in culture although they are difficult to isolate (Strauer and Koronowski, 2003). Once isolated, however, embryonic stem cells can grow indefinitely, an invaluable quality when working in the lab. Embryonic stem cells are controversial because they require the termination of a pregnancy prior to isolation and can often times become tumerogenic

(NIH, 2002; Strauer and Koronowski, 2003). Also, if embryonic stem cells are implanted into a non-related patient, they have a high risk of being rejected by that patient (Bradley et al., 2002).

Though embryonic stem cells and adult stem cells have been explored through extensive research, placental derived stem cells have not received as much attention. Placental derived stem cells (PDSCs) have many benefits, including the ease in which they can be isolated and the beneficial neurotrophins they secrete (Fauza, 2004; Parolini et al., 2008). PDSCs can be taken from the post-partum afterbirth, which means they do not require termination of the pregnancy to be isolated (Evangelista et al., 2008). It has been shown however that cells isolated earlier in the gestation process tend to proliferate at an accelerated rate as compared to those that are isolated later in the gestational period (Parolini et al., 2008). Placental or amniotic stem cells can be easily isolated through mechanisms similar to normal prenatal testing in humans (Fauza, 2004). PDSCs have been used in various ways in research for potential treatments of neurological diseases including AD. Transplanted PDSCs in mouse or rat models have shown to have regenerative properties that lead to improved cognition, decreased Aβ deposits, and prevention of disease progression (Nikolic et al., 2008; Reimann et al., 2009).

Stem cells have been found to mediate immunosuppressive effects through the secretion of neurotrophic factors. Neurotrophins in culture with immune cells can allow for a decrease in the production of inflammatory cytokines (Miki, 2011). The mechanism by which this works is still unknown, but the evidence for the immunomodulatory benefits of stem cells is mounting. A study by Yun et al. (2013) revealed that in mice

infused with Aβ-42, several inflammatory cytokines such as TNF-α, IL-10, and IL-17 were reduced when treated with PDSCs. Reduction of inflammatory cytokines was paired with a reduction in iNOS and COX-2 expression as well. Another study by Yan et al (2013) showed a similar reduction in NO and inflammatory cytokines using bone marrow derived stem cell conditioned media. Immunomodulation of NO through stem cell neurotrophins can be largely beneficial in the search for an AD treatment. Although the fundamental mechanisms surrounding the cause of AD are still unknown, an effective disease-modifying treatment may provide insight into the molecular workings of an AD brain.

1.7 Present Study

The development of an effective disease-modifying treatment for AD has eluded researchers for decades. When dealing with a multifaceted disease such as AD, finding a treatment and viable model to test it on is one of the main goals of scientists on the forefront of research. As previously discussed, neuroglial cells contribute to neuronal death and brain atrophy through chronic inflammatory processes. If this inflammation can be reduced, it is possible that we can quell the disease state by slowing neuronal loss, protecting the brain from damage, or even halting the disease process altogether. The medical and research world have been finding more and more benefits of stem cell therapy, from cell replacement to neurotrophic therapy. Although *in vivo* studies using transgenic rat or mouse models can be a very effective resource in research, cell culture can offer certain benefits that *in vivo* studies are incapable of producing. *In vitro* cell

cultures allow for a closer look at morphological differences between cells and more controlled parameters in which the experiments are conducted. Cells in culture can be monitored for changes and can be manipulated with molecular specificity.

Recent studies conducted in the Drew University neuroscience lab showed that media conditioned by placental stem cells can effectively lower the NO content of neuroglia *in vitro* (Frese, 2012). LPS and IFN-γ were used in order to stimulate the glial cells to release toxic amounts of NO typical of AD. The Griess reaction was used to quantify NO by the detection of nitrite, a stable byproduct of NO breakdown, and causing a colorimetric change in solution (Promega, 2009). The detection of nitrite in glial samples represents the relative amount of NO in the samples. Therefore form this point forward, even though the Griess reaction detects the amount of nitrite in samples, we will refer to the results of the Griess reaction as NO concentration or NO content. The aim of this past study was to determine if the neurotrophic factors produced a change in the immunological response of activated glial cells. This study aims to refine the methods used in the previous study and develop an effective co-culture system for glial cells and stem cells in order to asses any interactions that may occur between them. Current effective stem cell treatments have been applied through a transplantation injection into the patient's brain (Lindvall and Kokaia, 2006). Developing a viable co-culture system will more closely resemble the cellular environment in a hypothetical patient who was to receive stem cell treatment for AD. It was hypothesized that neurotrophic factors from rat placental derived stem cells would cause a decrease in NO content produced by

neuroglial cells in co-culture models such as transwells and a direct-contact cover slip model.

Another aim of this study is to use immunocytochemistry for analysis of glial cell structure and iNOS content to better understand the mechanism by which inflammation occurs. In order to visualize iNOS distribution in photos of glial samples, an iNOS monoclonal antibody was used. It was hypothesized that the percent area and average intensity of iNOS stained glial cells would be significantly higher in those stimulated with LPS and IFN-γ. Also, it was predicted that treatment with SCCM will reduce the percent area and average intensity of the stain in LPS stimulated glia. From this we should be able to learn about the morphology of LPS and IFN-γ stimulated neuroglial cells in terms of distribution of iNOS within a cell and throughout the culture as well. Doing so can help direct future studies and may provide insight into mechanisms of iNOS and NO in activated glia.

2. Materials and Methods

2.1 Isolation of Placental Derived Stem Cells

Placentas of E17 rats were isolated and rinsed in ice cold, sterile phosphate buffered solution (PBS) for removal of excess blood. Maternal deciduas, umbilical cord and amniotic sac of each placenta were removed. The placentas were minced using forceps in stem cell growth media (SCGM) containing 10% fetal bovine serum (FBS). The placentas were further dissociated with a glass pipet and plated in 25cm^2 pre-coated tissue culture flasks. Flasks were maintained in a humidified incubator at 37°C, 5% CO_2

for 48 hours or until 70-80% confluency was reached. Once optimal confluency was reached, the media was removed and the flask was rinsed with sterile PBS to remove residual FBS, which has been known to prevent trypsin activity. The flasks were then incubated for five minutes in 37°C, 5% CO_2 with trypsin to release the cells from the bottom of the flask. A small amount of SCGM equal to half of the amount of trypsin contained in the flask was added to neutralize trypsin. All of the media in the flask was then removed and transferred into a centrifuge tube. Media containing stem cells was then centrifuged at 1000RPM for five minutes until a pellet of cells formed at the bottom of the tube. The supernatant was removed and the cell pellet was resuspended in new SCGM. The resuspended cells were then plated in either new tissue culture flasks or welled plates for further experimentation. Those that were replated in tissue culture flasks had their media removed and replaced with new SCGM every 4-5 days.

2.2 Isolation of Neuroglial Cells

Brains of 11 day old rats were isolated in dishes containing Hank's Buffered Saline Solution (HBSS). The meninges of the brains were removed as blood vessels in culture can affect glial health. Using forceps, the brains were minced. Minced brain tissue was then trypsinized for five minutes in a 37°C water bath. The cells were then rinsed in a series of two HBSS washes for two to three minutes each to remove residual trypsin. Cells were subsequently added to plating media (PM) containing Neurobasal media, 10% FBS, and 1% pen-strep antibiotic. The cells were then dissociated and plated in 75 cm^2 cell culture flasks, then incubated for 48 hours at 37°C and 5% CO_2 in a humidified

environment. After an optimal confluency of about 80% was reached, the media was removed from the flasks and the cells were rinsed with sterile PBS to remove residual FBS to prevent trypsin deactivation. Trypsin was added to the flasks and subsequently incubated for five minutes. Media equal to about half of the amount of trypsin contained in the flask was then added to neutralize the trypsin. All of the media was removed and transferred to a centrifuge flask for centrifugation at 1000RPM for five minutes until a pellet of cells was formed. The supernatant above the pellet was aspirated off without disturbance of the pellet. The pellet was then re-suspended in new PM and plated in new cell culture flasks or welled plates for further experimentation. Those that were re-plated in cell culture flasks were fed with new PM after removal of old media every 4-5 days.

2.3 Treatment of Neuroglia with Stem Cell Conditioned Media

Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS was added to stem cell cultures and incubated for 24 hours in a humidified environment at 37°C and 5% CO_2. The supernatant deemed stem cell conditioned media (SCCM) was collected for use on glial cultures. SCCM was added to half of a welled plate containing glia with the remaining wells receiving plain DMEM for control conditions. After 24 hours of pre-treatment, media was removed from each well. Half of the SCCM treated wells and half of the DMEM treated wells received 1ng/mL lipopolysaccharide (LPS) and 500μg/μL interferon-γ (IFN-γ)**.** Going forward when referring to LPS/IFN-γ stimulated conditions, it will instead be referred to as simply LPS. The remaining wells in each condition

received plain DMEM. After 48 hours of stimulation, the supernatant of each well was collected for analysis.

2.4 Co-culture of Placental Stem Cells and Glial Cells in a Transwell System

Cultured glial cells were plated in a welled plate and allowed to attach and settle for 24 hours. In a separate welled plate, stem cell cultures were added to small, porous transwells (0.4μm polyester membrane) and allowed to attach and settle for 24 hours with SCGM in the large well underneath to prevent drying. The transwell stem cell cultures were then added to half of the wells of the plate containing glia and then incubated for 24 hours for pre-treatment. Half of the pre-treated wells and half of the non-pre-treated wells were then stimulated with 1ng/ml LPS in PBS and 500 μg/μL IFN-γ to cause activation of glial immune responses. The remaining wells were stimulated with plain DMEM as controls. Cells were incubated in these conditions in humidified 37°C and 5% CO_2 for 48 hours. The supernatant of the glial cells were then collected for analysis.

2.5 Quantifying NGF in Glial Supernatant: ELISA Assay

Samples from the transwell co-culture system were collected and stored in a -80°C freezer. Once samples and reagents reached room temperature, a rat NGF standard was serially diluted 1:2 to provide a standard curve. The NGF standard and the samples were loaded into a microplate pre-coated with NGF antigen and shaken at room temperature for 45 minutes. Solutions were discarded and wells were rinsed with a 1X wash buffer. Microplate was shaken for 30 minutes with a NGF detection antibody and

subsequently washed with 1X wash buffer. Streptavidin-HRP conjugate was added to each well and shaken for another 30 minutes at room temperature. After another series of washed with 1X wash buffer, 3,3',5,5'-Tetramethylbenzidine (TMB) was added to each well and incubated for 15 minutes in the dark. A stop solution was used to prevent the TMB reaction from progressing and the plate was read at 450nm in a microplate reader.

2.6 Co-culture of Placental Stem Cells and Glial Cells in Cover Slip System

Cover slips were bathed in 0.5M HCl for 24 hours and were subsequently removed. They were then rinsed with distilled H_2O followed by 70% ethanol and allowed to dry. Cover slips were then autoclaved on a dry setting. Parafin wax beads were then added on one face of each cover slip providing space to handle cover slips with forceps in culture. Cultured glial cells were plated in a welled plate and allowed to attach and settle for 24 hours. Cultured stem cells were also plated in a welled plate and allowed to attach and settle for 24 hours. Simultaneously in separate welled plates, glial cultures and stem cell cultures were allowed to settle for 24 hours in wells containing prepared cover slips. After 24 hours, cover slips containing stem cells were flipped over into glial culture wells such that the face of the cover slip containing cells was in direct contact with the glial culture surface. The same procedure was performed for the cover slips containing glia and wells containing stem cells. These cover slip co-culture systems were incubated for 24 hours for pre-treatment. Half of the wells in each glia-stem cell culture were stimulated with 1ng/mL LPS in PBS and 500μg/μL IFN-γ to cause activation of glial immune responses and the remaining wells were treated with plain DMEM as a control.

The cultures were incubated for 48 hours followed by collection of the supernatant of each of the wells for analysis.

2.7 Analysis of Nitric Oxide Content Using the Griess Reaction

A sample of the supernatant from experimental conditions was placed in a 96 well plate. A serial dilution from 100% down to 0% was made from plain DMEM and 10mM sodium nitrite as a comparative control for the samples. A solution containing 50% of 0.2% naphthylethylenediamine dihydrochloride and 50% sulphanilamide in phosphoric acid were mixed in order to create the Griess reagent which was added to each of the wells causing a color change to purple. Nitrite content was assessed using a microplate reader set to read at 540 nm. Data was collected and exported to Microsoft Excel and SPSS for analysis.

2.8 Fixing and Staining Glial Cells

The media in each of the wells on a plate was carefully aspirated off. 4% paraformaldehyde was then added to each well and allowed to sit for about 20 minutes to preserve the cells in their current state. A series of PBS washes was used to remove excess paraformaldehyde from the cells. Cells were then exposed to Triton-100X for 10 minutes in order to permeate the cell membranes. **S**ubsequent washes with PBS were performed three times. A primary antibody, inducible nitric oxide synthase (iNOS) (produced in mouse), was then applied to each well and allowed to shake for one hour at room temperature. The primary antibody was removed and the cells were washed three

times again with PBS. A secondary antibody, Anti-mouse IgG $F(ab')_2$ fragment-Cy3 antibody produced in sheep, was applied to each well and allowed to shake at room temperature for one hour. The secondary antibody was removed and wells were washed with PBS with the final wash left on the cells to keep them moist for later analysis.

2.9 Immunocytochemistry

Wells to be analyzed were selected visually based on number of visible cells in both auto exposure and fluorescent settings. Photos were taken as a representative sample for each condition. All photos were taken at the same exposure of 15 seconds. Percentage of photo area stained and average stain intensity were analyzed for each photo. Intensity parameters were set by choosing a representative picture from a control condition and then adjusting the threshold at which the fluorescence would be read. As the threshold was shifted, the photo area changed the amount of area and range of intensity that appeared around each visible cell. Once the area and range of visible fluorescence matched the shape of the pictured cells, in this case an intensity of <581, this same threshold was used to quantify percent area and average intensity of stain for every image thereafter. Mean for image intensity was calculated by subtracting the background signal or minimum intensity.

3. Results

3.1 Developing and testing novel methods

Developing a viable model in which to test stem cell and glial interactions is vital to the advancement of research in this area. Once parameters such as pre-treatment and stimulation timeline are mapped out, other manipulations can occur including new treatment therapies. One of the ways in which interactions can be analyzed is with a co-culture system. A co-culture system allows for cell-to- cell interactions so that any and all secreted factors can exact their effect on the surrounding cells which could have effects on morphology or in this case immunity. This also more closely models the type of treatments being used as current therapies for neurological diseases. Using previous studies and culture models from the Drew University neuroscience lab, I refined the original model which utilized stem cell conditioned media and also expanded the model to include co-culture designs.

3.1a. Manipulating Stem Cell Conditioned Media

Glial cells were cultured and pre-treated with SCCM for 24 hours followed by stimulation with LPS for 48 hours. Nitric oxide (NO) content in culture supernatant was inferred through the quantification of nitrite through the application of the Griess reagent. NO concentration of samples was normalized based on a 0-100% nitrite curve. There was a significant increase of LPS in both stimulated groups (Two-way ANOVA: $F(1,68)= 6.937$, $p=0.010$) but there was no significant effect of SCCM ($p>0.05$) (Figure 1). There was, however, a significant interaction between SCCM and LPS

($F(1,68)=73.81$, $p<0.001$). In conditions that were stimulated with LPS and treated with SCCM, there was a significant decrease in activation ($p<0.001$). There was also, however, a significant increase in activation in non-LPS stimulated conditions that were treated with SCCM ($p<0.001$). Despite this increase in activation, there was still a significant difference between (+)SCCM/(-)LPS and (+)SCCM/(+)LPS conditions ($p<0.001$).

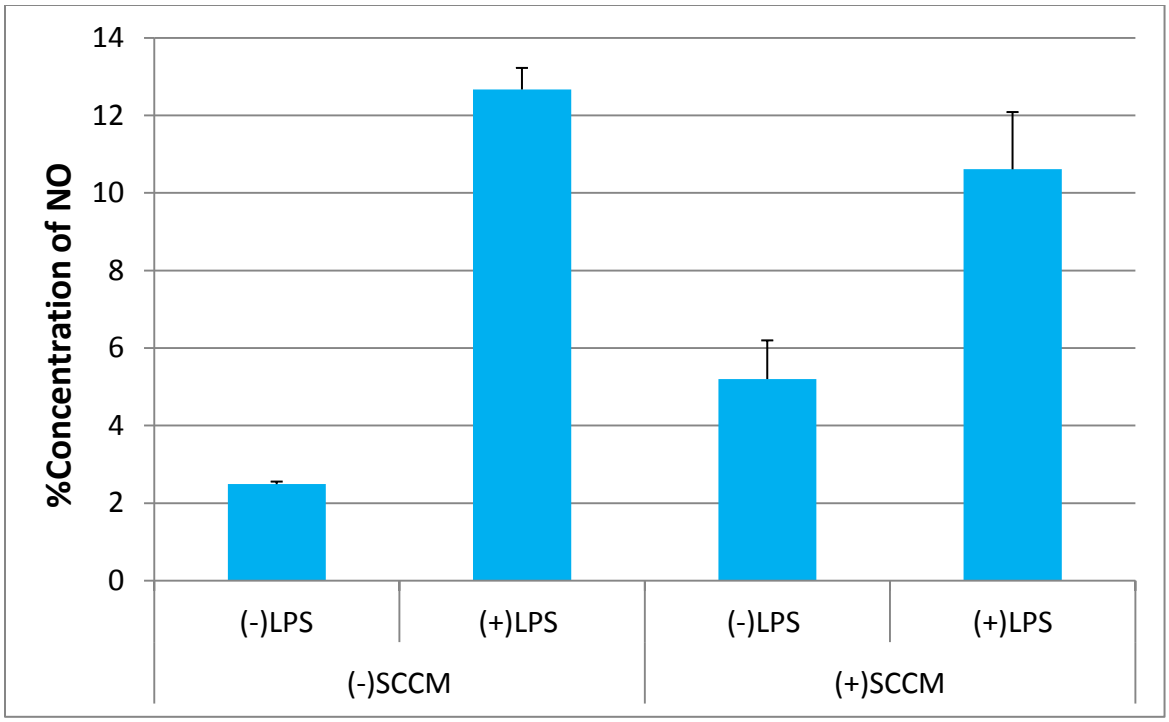


Figure 1: Nitric oxide concentration for neuroglia treated with SCCM- Neuroglial cells were grown for 1 day and then pre-treated with SCCM [(+)SCCM]or without SCCM [(-) SCCM] as a control. Cells within each of the (-)SCCM and (+)SCCM conditions were stimulated with LPS [(+)LPS] or without LPS [(-)LPS]. N=18/condition. Data are mean ±SEM.

Glial activation in the brain of an Alzheimer's patient would hypothetically continue even during stem cell treatment. In order to model this, glia were pre-treated with SCCM both before and during LPS activation (Ptx/Stm) then compared to glia only pre-treated with SCCM before stimulation (Ptx) (Figure 2). There was a significant main effect of SCCM (Three-Way ANOVA: p=0.001) and LPS (p<0.001) in both Ptx/Stm and Ptx conditions. There was also a significant main effect of adding SCCM to the stimulation phase (Ptx/Stm) (p<0.001). A significant interaction between (+)LPS and SCCM also occurred (p<0.001). There was a significant increase in NO concentration for LPS stimulated conditions for both (+)SCCM and (-)SCCM conditions (p<0.001, p<0.001). There was also a significant difference in LPS for both Ptx and Ptx/Stm conditions (p<0.001, p<0.001). There was no difference in activation for non-LPS stimulated conditions for either Ptx or Ptx/Stm, but there was difference in activation for LPS stimulated cells between Ptx and Ptx/Stm (p<0.001).

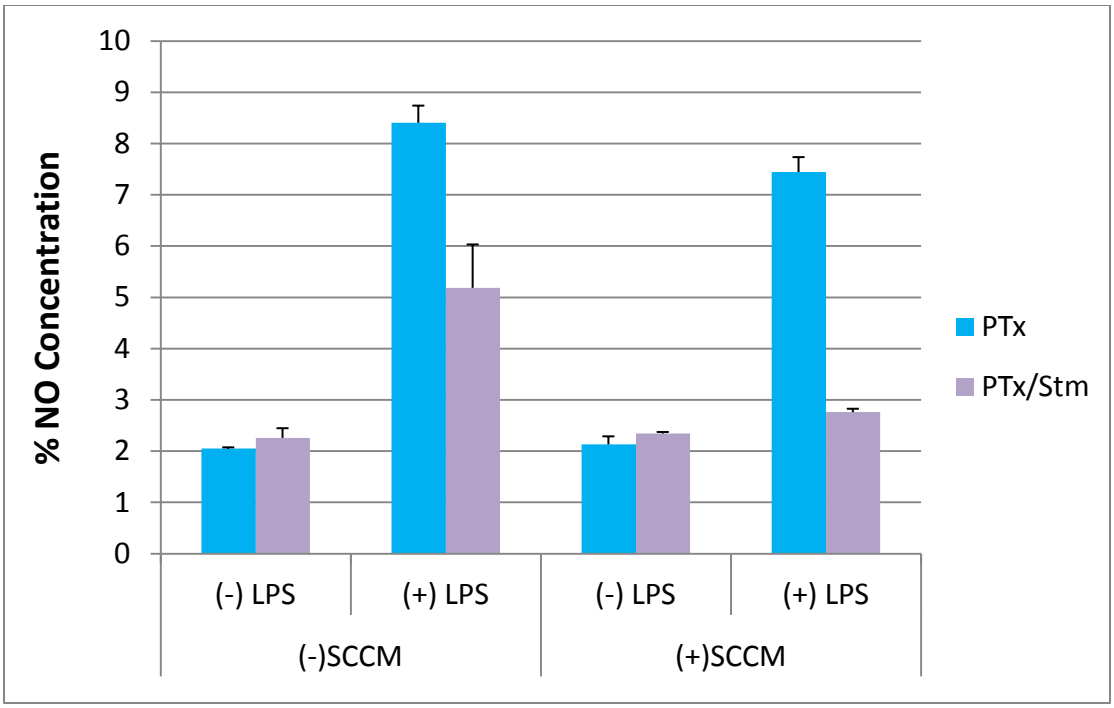


Figure 2: Nitric oxide concentration for neuroglia treated before and during stimulation- Neuroglial cells were grown for 1 day and then pre-treated with SCCM [(+)SCCM] or without SCCM [(-)SCCM] as a control. Cells within each of the (-)SCCM and (+)SCCM conditions were stimulated with LPS [(+)LPS]containing SCCM [PTx/Stm] or with LPS containing DMEM [Ptx]. The remaining cells in each condition were stimulated without LPS [(-)LPS]. N=6/condition. Data are mean ±SEM.

3.1b. Transwell Co-Culture System

Glia cells were grown in a co-culture transwell system in which stem cells were cultured in smaller membranous wells atop glia cultures. Control wells were not stimulated with LPS and either received no pre-treatment, or pre-treatment with stem cells. A positive control consisted of glia that were stimulated with LPS but no stem cell pre-treatment. There was a significant increase in NO concentration for LPS stimulated glia [(+)LPS] in either stem cell condition [(+)SC and (-)SC] (Two-way ANOVA: $p<0.001$). There was no significant effect of stem cell treatment [(+)SC] ($p>0.05$) and there was also no interaction between LPS stimulation and stem cell treatment ($p>0.05$).

Glial supernatants were collected and analyzed for presence of neuronal growth factor (NGF). Optical density was measured in a microplate reader after execution of an ELISA assay on glial supernatants. There was a significant increase of NGF in LPS stimulated conditions (Two-way ANOVA: $p<0.001$). There was not a main effect of stem cell treatment ($p>0.05$) and no interaction between stem cell treatment and LPS stimulation ($p>0.05$).

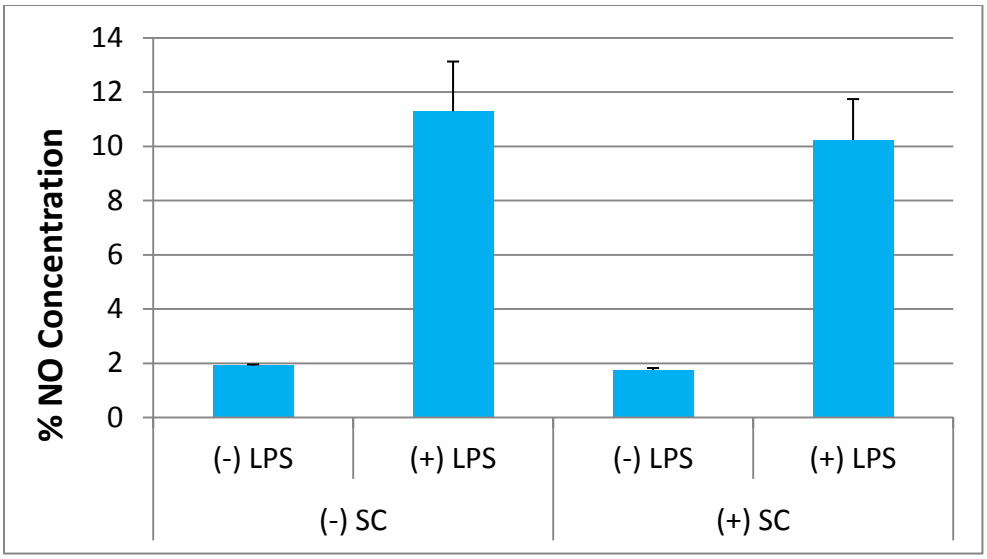


Figure 3: Nitric oxide concentration for neuroglial cells in a transwell co-culture system- Glia were pre-treated with stem cell [(+)SC] cultures or with plain DMEM [(-)SC] in a transwell system for 24 hours followed by stimulation with LPS [(+)LPS] for 48 hours. Controls were not stimulated with LPS [(-)LPS] but with plain DMEM. NO concentration normalized to 100% control. N=24/condition. Data are mean ±SEM.

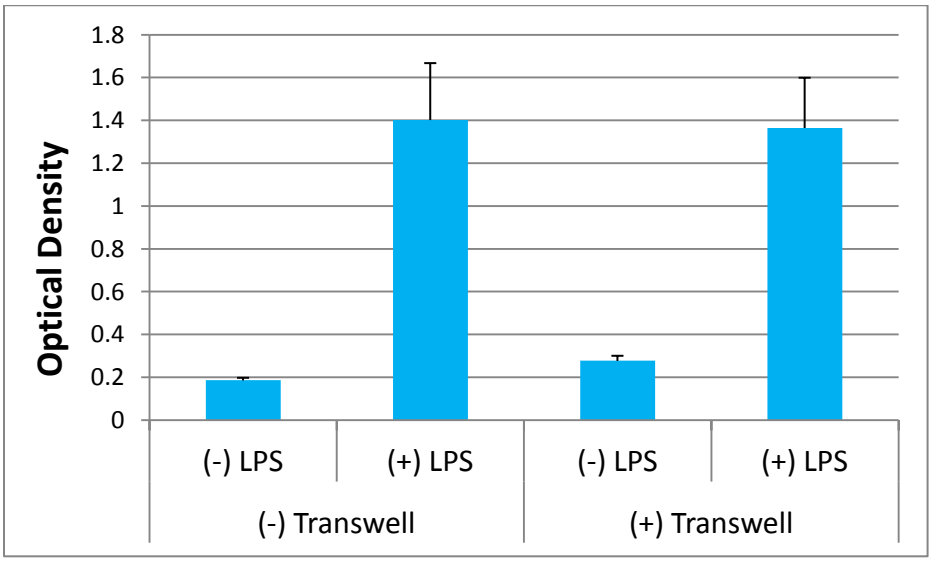


Figure 4: NGF content in glia supernatant of transwell co-culture- Glia stimulated with LPS [(+)LPS] and without LPS [(-)LPS] were each treated with transwells containing PDSCs [(+)Transwell] or without transwells containing PDSCs [(-)Transwell]. An ELISA assay was performed in order to assess the relative NGF content in each sample. N=8/condition. Data are mean ±SEM.

3.1c. Cover Slip Co-Culture System

A cell-to-cell contact co-culture system was developed for stem cells and neuroglia. Cells were grown for either one day or two days before pre-treatment. Model A consisted of glia grown on cover slips which were subsequently turned over onto stem cells grown in a welled plate for treatment (Figure 5A). According to the results of a 2 x 2 x 2 Three-Way ANOVA, there was no significant difference between cells grown for 1 day or 2 days. There was a significant main effect of stem cell treatment [(+)SC] ($F(1,16)= 12.122$, p=0.003) and LPS [(+)LPS] ($F(1,16)= 52.565$, $p<0.001$). There was a statistically significant interaction between stem cell treatment and LPS stimulation as well as one between stem cell treatment and number of days grown. A third interaction was also observed between LPS stimulated conditions and number of days grown. These interactions qualify the main effects of stem cell treatment and LPS stimulation.

In model A, there was no significant increase in NO concentration from LPS stimulation for (-)SC conditions. There was, however, an increase in NO concentration from LPS stimulated conditions for (+)SC conditions ($p<0.001$). In (-)LPS conditions, there was no significant difference between conditions treated with [(+)SC] and without [(-)SC] stem cells. A significant increase was observed, however, for (+)LPS conditions that were treated with and without stem cells ($p<0.001$). In terms of number of days cells were grown, there was a significant difference between days grown for (+)SC conditions ($p=0.011$). There was also a significant difference between (+)SC and (-) SC conditions for cells grown for 1 day ($p<0.001$), but not for 2 days. In conditions stimulated with LPS [(+)LPS] and without LPS [(-)LPS], there was a significant effect of number of days

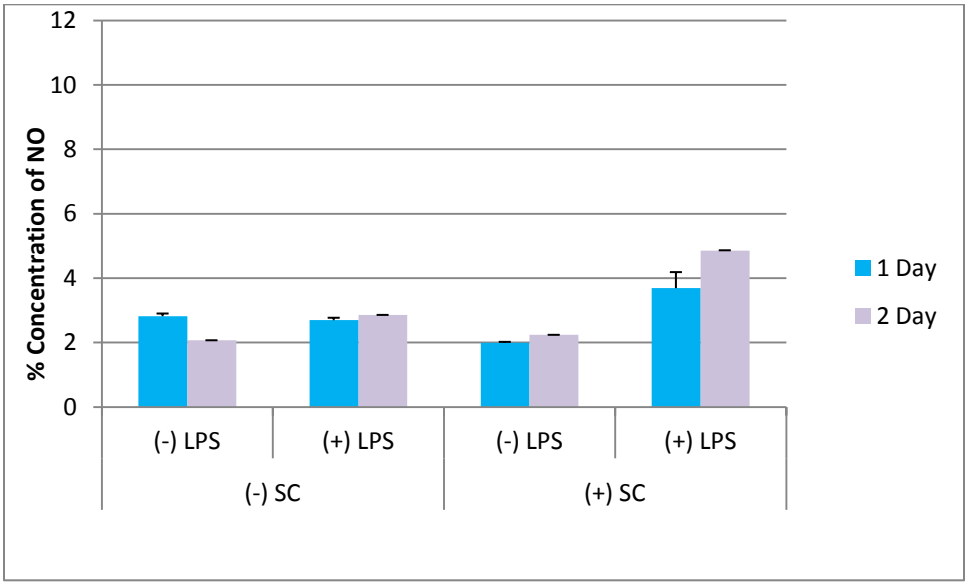
grown for both 1 day ($p<0.001$) and 2 days ($p=0.006$). Also, for LPS stimulated conditions [(+)LPS], there was a significant difference between number of days grown ($p=0.014$).

A second model, model B, included glia grown in a welled plate which were subsequently treated with stem cells grown on cover slips (Figure 5B). There was a significant main effect of LPS stimulation (Three Way ANOVA: $F(1,16)= 3357.642$, $p<0.001$). There was also a significant main effect of number of days grown ($F(1,16)= 248.503$, $p<0.001$). In model B, there was an interaction between stem cell treatment, LPS stimulation, and number of days grown. This three-way interaction qualifies the main effects LPS stimulation and number of days grown.

In model B, there was no significant difference in NO content by the number of days grown for control conditions [(-)SC/(-)LPS]. There was a significant difference by the number of days grown for (-)SC conditions stimulated with LPS [(+)LPS] ($p<0.001$). There was also a significant difference in number of days grown for (+)SC treated conditions both stimulated with LPS [(+)LPS]($p<0.001$) and non-LPS stimulated conditions [(-)LPS]($p<0.001$). For conditions without LPS stimulation [(-)LPS] grown for 1 day, there was a significant difference between (+)SC treatment and (-)SC treatment ($p<0.001$). There was not, however, a significant difference between (+)SC and (-)SC treatment for (-)LPS conditions grown for 2 days. There was a significant difference between (+)SC and (-)SC treatment conditions for conditions with LPS stimulation [(+) LPS] grown for both 1 day and 2 days ($p<0.001$, $p=0.013$). In (-)SC conditions grown for both 1 day and 2 days, there was a significant difference in LPS stimulation

($p<0.001$,$p<0.001$). The same significance was found for (+)SC treatment conditions as well: difference in LPS stimulation for both 1 and 2 days grown ($p<0.001$,$p<0.001$).

A.



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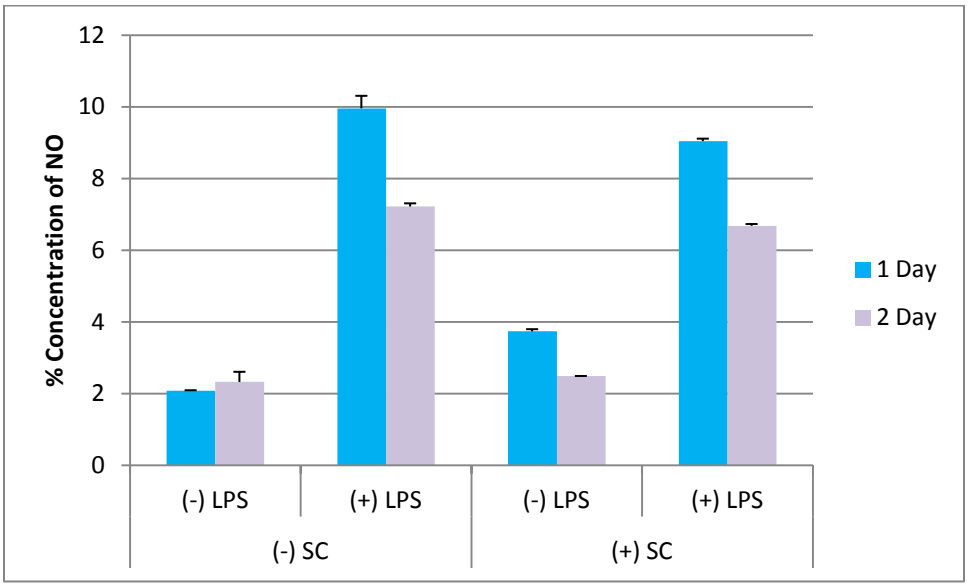

Figure 5: Nitric oxide concentration for neuroglia in a cover slip co-culture system- (A) Glia were grown on a cover slip followed by exposure to stem cells or plain DMEM (control). (B) Stem cells were grown on a cover slip then used as a treatment for glial cells. "1 Day" and "2 Day" refer to the number of days cells were grown prior to pre-treatment. Following pre-treatment cells were stimulated with LPS or DMEM (control). N= 3/condition. Data are mean ±SEM.

3.2 Analysis of stem cell and glial interactions

Along with the several culture models developed in this study, morphological differences between SCCM treated and LPS stimulated glial cells were observed through immunocytochemical fluorescent staining. In the current study, an iNOS antibody fluorescent stain allowed for visualization of iNOS in activated glia. Analyzing the distribution of iNOS in each condition through stain intensity and quantification of percent area stained can reveal answers to how NO can cause damage in AD conditions.

3.2a Qualification of iNOS stain in neuroglia

Glial cells were stained with an iNOS antibody in order to visualize iNOS distribution and cell morphology. Control glia that were not treated with SCCM or stimulated with LPS [(-)SCCM/(-)LPS] showed normal flattened and spread glial formation and presented with a diminished but widespread distribution of iNOS in over 50% of the cells (Figure 6A). Glia that were stimulated with LPS and treated with SCCM in both stimulation and pre-treatment [(+)SCCM/(+)LPS/(Ptx/Stm)] showed normal glial morphology and little to no iNOS distribution in about 75% of the cells (Figure 6B). Glia were stimulated with LPS and did not receive SCCM treatment [(-)SCCM/(+)LPS/] showed a high concentration of iNOS with a shriveled, shrunken appearance in about 35% of the cells (Figure 6C). Glia were stimulated with LPS and contained SCCM only in pre-treatment and not stimulation [(+)SCCM/(+)LPS/(Ptx)] showed a low

concentration and distribution of iNOS, but some morphological shriveling and shrinking in about 25% of the cells (Figure 6D).

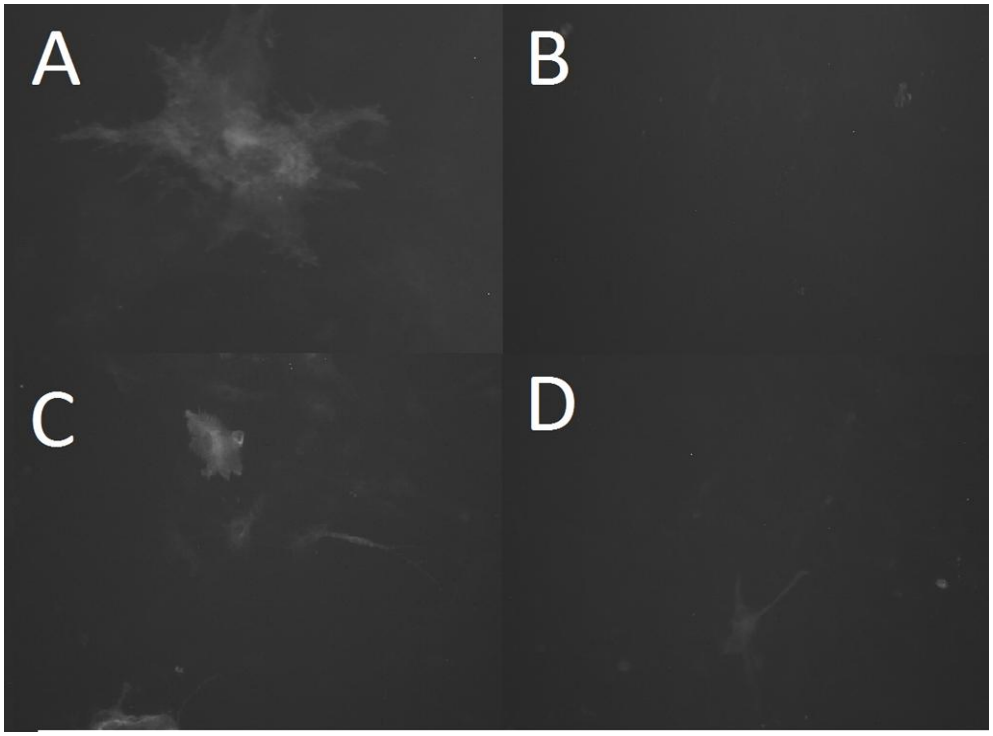

Figure 6: iNOS staining of neuroglia- Glia were fixed and stained with iNOS monoclonal anti-body to visualize iNOS in the cell bodies. All glia pictured were imaged with same exposure of 15s. (A) Control glia without SCCM treatment or LPS stimulation [(-)SCCM/(-)LPS]. (B) Glia that were stimulated with LPS and treated with SCCM in both stimulation and pre-treatment [(+)SCCM/(+)LPS/(Ptx/Stm)]. (C) Glia that were stimulated with LPS but not treated with SCCM at all [(-)SCCM/(+)LPS]. (D) Glia that were stimulated with LPS and treated with SCCM only in pre-treatment [(+)SCCM/(+)LPS/(Ptx)].

3.2b. Quantification of iNOS stain in neuroglia

Glia were pre-treated with SCCM [(+)SCCM] or plain DMEM as a control [(-) SCCM] for 24 hours. They were then stimulated with LPS [(+)LPS] or without LPS [(-) LPS]. Half of the cells were stimulated with LPS containing SCCM (Ptx/Stm) and the other half were stimulated with only LPS (Ptx). After another 48 hours of stimulation, glia were stained with iNOS antibody in order to visualize activation within the cells. Images were collected for each condition and the percent area stained as well as the average intensity were recorded for each image.

In quantifying percent area of image stained, there was no significant main effect of SCCM using a Two-Way ANOVA. There was, however, an interaction between LPS and SCCM ($F(1,19)= 23.941$, $p<0.001$) (Figure 7). For average stain intensity there was a significant effect of SCCM ($p<0.05$) but not of LPS ($p>0.05$)(Figure 8). There was, however, an interaction between LPS and SCCM ($p<0.001$). For conditions without LPS stimulation [(-)LPS] there was a significant increase in percent area stained with the addition of SCCM [(+)SCCM] ($p=0.005$). For conditions stimulated with LPS [(+)LPS], there was a significant decrease in percent area stained with iNOS with the addition of SCCM treatment [(+)SCCM] ($p<0.001$). There was also a significant increase in percent area stained for (+)LPS/(-)SCCM conditions compared to (-)LPS/(-)SCCM conditions ($p<0.001$). Percent area stained decreased significantly in (+)LPS/(+) SCCM conditions compared to (-)LPS/(+)SCCM conditions ($p=0.023$).

In quantifying the average intensity of staining, there was an interaction between SCCM and LPS ($p<0.001$). For both (+)SCCM and (-)SCCM conditions, the effect of

LPS was significant ($p<0.001$, $p<0.001$). There was a slight but significant increase in intensity between (-) LPS conditions with and without SCCM treatment ($p=0.007$). There was a significant decrease in average stain intensity for (+)LPS/(+)SCCM conditions compared to (+)LPS/(-)SCCM conditions.

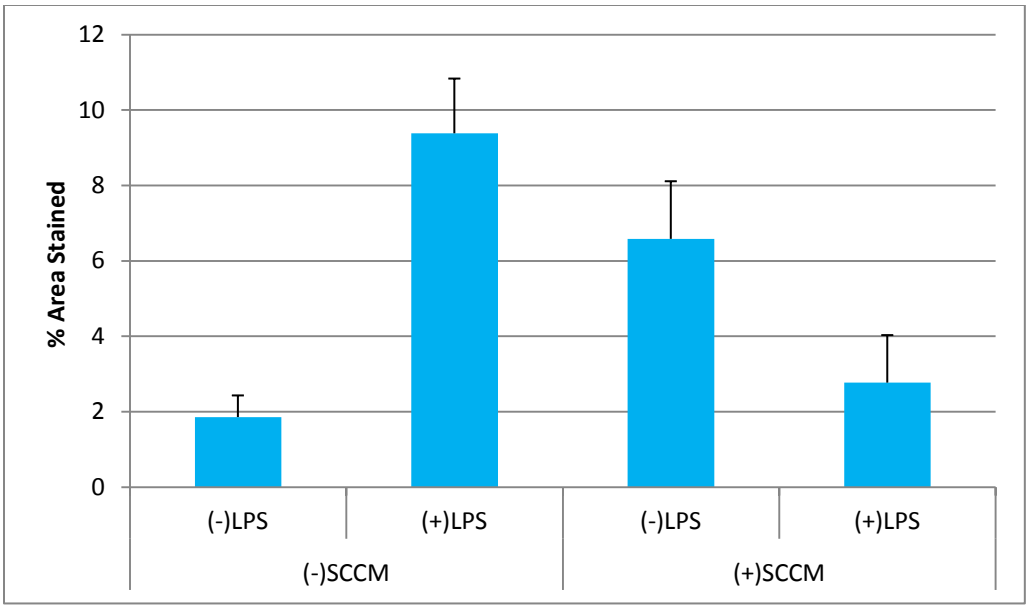

Figure 7: Percent area quantification of iNOS staining of glial cells- Neuroglial cells were grown for 1 day and then pre-treated with SCCM [(+)SCCM] or without SCCM [(-) SCCM] as a control. Cells within each of the (-)SCCM and (+)SCCM conditions were stimulated with LPS [(+)LPS]containing SCCM [PTx/Stm]. The remaining cells in each condition were stimulated without LPS [(-)LPS]. Immunofluorescent images of glia stained with iNOS antibody were collected. The percentage of the image area with stain was recorded for each condition. N=8-25. Data are mean ±SEM.

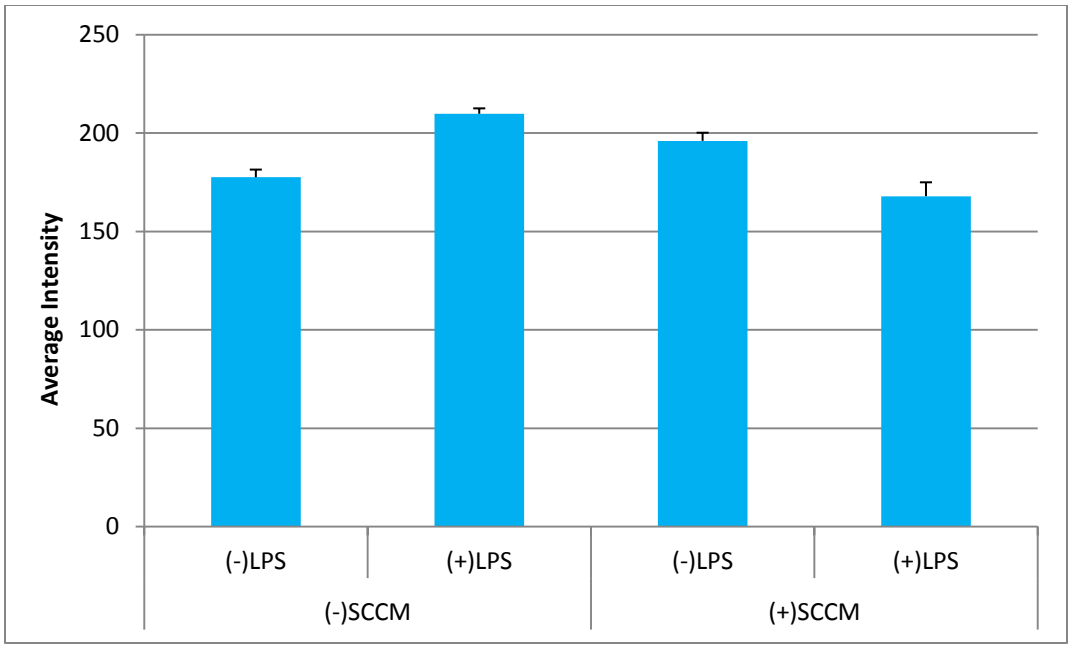

Figure 8: Average intensity quantification of iNOS staining of glial cells- Neuroglial cells were grown for 1 day and then pre-treated with SCCM [(+)SCCM] or without SCCM [(-)SCCM] as a control. Cells within each of the (-)SCCM and (+)SCCM conditions were stimulated with LPS [(+)LPS]containing SCCM [PTx/Stm]. The remaining cells in each condition were stimulated without LPS [(-)LPS]. Immunofluorescent images of glia stained with iNOS antibody were collected. Average intensity of the stain in each image was recorded. N=8-25. Data are mean ±SEM.

4. Discussion

Molecular mechanisms underlying AD phenotypes are not well understood despite continuous research efforts. One such mechanism that has been explored for answers to the AD puzzle is the heightened immune responses and inflammation that are observed in a typical AD brain. Though an active immune system is typically beneficial, in AD, the inflammation that occurs is chronic and severe which leads to more damage than protection. There are many pathways that could give rise to this inflammation and therefore many possibilities for treatment. Stem cells have been cited as a potential treatment for various neurological disorders such as Parkinson's disease, stroke, and Alzheimer's disease (Momin et al., 2010). Stem cell treatments for these various neurological diseases and disorders are typically done through cell replacement therapies in which stem cells are differentiated into neural cells in order to replace cells lost to damages. Several different types of stem cells have been used in research for these therapies (NIH, 2012). Adult stem cells, such as bone marrow derived stem cells, have been used for their innate ability to secrete neurotrophic factors while embryonic stem cells proliferate indefinitely and are easy to grow in culture. Neurotrophic factors have been found to cause a decrease in the immune response of neuroglial cells typical in an AD brain (Burlton-Jones et al., 2011). Each of these types of cells has their drawbacks with both adult stem cells and embryonic stem cells requiring a blood match to use in a patient among other disadvantages. Placental derived stem cells (PDSCs) can be isolated from a post-partum placenta, have the same proliferation power as embryonic stem cells

and secrete the same factors as adult stem cells making them highly suitable for research (Fauza, 2004; Parolini et al., 2008; Evangelista et al., 2008).

Based on past methods used in the Drew University neuroscience lab, stem cell conditioned media (SCCM) was found to decrease NO content, a product of neuroglial cells that were stimulated with LPS (Frese, 2012). An interest of this study was to refine the previous SCCM model and find a system in which neuroglial cells and stem cells could be cultured together in order to provide a platform on which to investigate morphological and response differences. Culturing cells together will serve as a more realistic model because current stem cell therapies use a direct injection of stem cells rather than a treatment with conditioned media. Another aim of this study was to analyze the morphological differences of neuroglia when stimulated with LPS to induce NO secretion in order to evaluate how over-activation of the immune response affects cell morphology and iNOS. The morphology and iNOS distribution within glial cells were also observed for stimulated cells that were then treated with SCCM to explore the effects PDSC treatment.

4.1 Evaluation of novel culture models

AD is a disease that can last decades which is why it is important to find a treatment that can attenuate the effects of the disease on a long-term basis. In order to emulate this, the time at which SCCM was added to culture was manipulated to include the SCCM in both pre-treatment and stimulation. Other novel culture methods were also tested including several co-culture systems. It was hypothesized that a co-culture system

can cause a greater decrease in NO acting as a long term treatment and eventually allow for other manipulation and observations. Co-culture systems such as a transwell system and a cell-to-cell contact cover slip system were developed in order to test this. It was hypothesized that stem cell neurotrophins, such as NGF, would be increased in cultures that received stem cell treatment.

4.1a. Stem Cell Conditioned Media

Studies previously performed at Drew University used media conditioned from PDCSs as a pre-treatment for LPS stimulated glial cells (Frese, 2012). The conditions and parameters set by these previous experiments were re-created in this study to use as a baseline from which a new co-culture system could be compared. A replication of the SCCM experiment yielded similar results to previous experiments with a significant increase in NO concentration in glia stimulated with LPS (Figure 1). Though there was no significant main effect of SCCM, there was a significant interaction between LPS and SCCM with a significant decrease in NO concentration for LPS stimulated cells treated with SCCM. This supported the original hypothesis that SCCM causes a decrease in NO content in LPS stimulated glia. Unexpectedly, however, there was also an increase in NO concentration for glia treated with SCCM without LPS stimulation. This could be due to a trace amount of contaminants that could have caused an increased immune response in the glial cells or it could be pointing to a mechanistic effect of the SCCM on the cellular responses of the glia.

In order to more closely model *in vivo* treatment with SCs, SCCM was used for both pre-treatment phase and during stimulation with LPS (Ptx/Stm) and compared to the previous model in which SCCM was only applied as a pre-treatment (Ptx)(Figure 2). As expected, there was a significant increase in LPS induced NO production in glia for both Ptx and Ptx/Stm conditions as well as a significant main effect of SCCM for these conditions. For control glia not stimulated by LPS, there was a no difference in activation between Ptx/Stm and Ptx conditions which was the expected result. Glia stimulated with LPS in the Ptx/Stm condition had a significantly lower NO concentration as compared to glia stimulated with LPS in the Ptx condition for both (+) SCCM and (-) SCCM treatment. As hypothesized, prolonged exposure to SCCM through both the pre-treatment and stimulation phases caused a more dramatic decrease in NO. This significant decrease of NO indicates that treating with SCCM both before and during stimulation with LPS suggests that this would be a reasonable modification to the protocol. This also suggests that treating with SCCM during only the stimulation phase may be enough to attenuate a decrease in NO content in activated glia. Future experiments should include a timeline study in which glia are treated before, during, and after stimulation.

4.1b. Transwell Co-Culture System

A transwell system in which stem cells were cultured in porous transwells on top of wells with glia was assessed as a viable model for a stem cell-glia co-culture. The bottoms of the transwells were porous in order to allow for the exchange of factors released by the glia and stem cells. Results of this experiment showed the expected

elevation in NO concentration due to LPS stimulation (Figure 3). This elevation in NO was observed for both (+)SC and (-)SC conditions which was not the desired or expected because SC treatment was expected to decrease the effect of LPS stimulation. A possible reason for this unexpected effect may include the small pore size of the transwells which could have prevented exchange of factors in the co-culture. To assess this possibility, an ELISA assay was performed in order to determine if there were sufficient amounts of neurotrophins to exert a suppressive effect on LPS stimulated glia.

The supernatants of glia cells were collected and analyzed for presence of NGF (Figure 7). It was hypothesized that NGF would be increased in conditions that received stem cell treatment, however, this was not the case. Samples that were not stimulated with LPS showed an insignificant amount of NGF in both transwell and non-transwell treated conditions [(+) SC and (-) SC]. On the other hand, NGF levels were comparably high in both LPS stimulated conditions (both [(-) SC and (+) SC]). NGF is naturally produced by both glial cells and stem cells for neuronal growth and protection (Lu et al., 1991; NIH, 2002). It is reasonable to surmise that because NGF content was high in both of the stimulated conditions but not the stem cell treated control, the glia responded to the LPS activation by releasing NGF as normal inflammatory response. Inflammation in controlled, normal levels is actually beneficial to cell health. In these conditions, however, the high levels of NO counter balance the cells attempt at neuroprotection through NGF. If we could find a way to decrease the NO content released by the glial cells, but keep the increase in NGF they produce, we may be able to prevent damage more efficiently with the help of high NGF concentrations. On the other hand, because

NGF was high in both LPS stimulated conditions, but NO was not decreased by any significant amount with stem cell treatment, this could suggest that NGF may not be the main stem cell neurotrophin attenuating the inflammatory response of glial cells. It was originally hypothesized that an insignificant decrease of NO in LPS stimulated glia treated with stem cells may suggest a pore size too small for exchange of factors. Because the molecular size of NGF is 16 x 25 x 18Å and the pore size of the transwells is 0.4µm, the possibility of a pore size too small can be ruled out (McDonald et al., 1991).

4.1c. Cell-to Cell Contact Cover Slip System

Sterilized cover slips treated with HCl were used as culturing surfaces for stem cells or glial cells. Welled-plates were prepared, one plate for stem cells (model A) and one plate for glial cell cultures (model B). Glia in wells were exposed to either blank cover slips, or cover slips with stem cells. Glia grown on cover slips were exposed to wells either with or without stem cells. Sets of glia and stem cells were grown for either 1 day or 2 days. Each of these systems was tested in order to ensure an optimal set up for a cover slip co-culture system.

Overall, the activation of glial cells that were grown on cover slips was much lower than glia grown in wells (Figure 8). Within model A, there was no significant difference between cells that were grown for 1 day versus those grown for 2 days though there was a significant main effect of both SCCM and LPS (Figure 8A). The main effect of LPS was due to an increase in NO concentration in LPS stimulated conditions which would normally be the hypothesized result, however, in this case LPS activation was

higher in (+)SC/(+)LPS conditions which is not the expected result. There was also an interaction between SC treatment and LPS stimulation which is qualified by the main effects which, as just mentioned, were not the main effects that were expected. Because this was a novel co-culture system, several problems arose in the culturing process of these cells. First, tweezers were used in order to remove cover slips from the wells they were grown in to the experimental wells. During this transfer process, many cover slips broke which may have caused cell disruption and cell loss contributing to the low amount of activation seen in Figure 8A. Further cell disruption may have been caused by the insufficient stabilization of the wax beads used to create a buffer space between the stem cell surface and glial cell surface. These wax beads would often come off during the incubation period which left them floating on the surface of the supernatant. The higher activation seen in (+)SC/(+)LPS conditions as compared to (-)SC/(+)LPS conditions could be due to a larger number of surviving glia in those conditions just by chance of fewer broken cover slips and/or more stable wax beading.

Glial cells and stem cells were grown for either 1 day or 2 days before pre-treatment in order to determine if a shorter or longer growth period would affect activation or response to treatment. In model A, there was a significant difference in activation for (+)SC treated conditions between 1 day or 2 days with 2 day growth showing a higher NO concentration than 1 day growth for LPS stimulated conditions. This could also be due to a higher number of cells surviving in the 2 day growth condition because they had more time to proliferate and therefore more cells were available for LPS activation. There was also a significant difference between (+)SC and

(-)SC conditions for cells grown for 1 day, but not for 2 days. In (+)LPS and (-)LPS conditions, there was a significant effect on activation for both 1 day and 2 days, but once again, activation for (+)LPS conditions treated with stem cells was higher than (+)LPS conditions without stem cell treatment, the opposite of the hypothesis and once again most likely due to random variations in cell survival.

In model B, there was a significant effect of LPS and number of days grown which was qualified by a three way interaction between LPS, SC, and days grown (Figure 8B). Control conditions [(-)SC/(+)LPS] showed no significant difference in activation which was expected. LPS stimulated glia without stem cell exposure [(-)SC/(+)LPS] showed a significant increase in NO concentration for 1 day compared to 2 day growth. Glia grown in a welled plate may become slightly insensitive to LPS activation after more than one day of growth. Evidence for this possibility can also be seen in the (+)SC conditions. In the (+)SC/(+)LPS condition, there was a significant difference between 1 and 2 day growth with 1 day growth having a higher activation than 2 day. There was also a difference in NO concentration for (+)SC treated conditions without LPS stimulation.

The question of interest, does PDSC treatment cause a decrease in the NO concentration of LPS stimulated glia, was found to be supported for both 1 day and 2 day growth. There was a significant decrease in NO concentration for glia pre-treated with stem cells and stimulated with LPS [(+)SC/(+)LPS] compared to glia only stimulated with LPS [(-)SC/(+)LPS]. This was true for both 1 day and 2 day growth, though 2 day growth had an overall lower activation than 1 day.

Glia were able to grow on a larger surface undisturbed by breaking cover slips or loose wax beads, which could account for the overall increase in activation in Figure 8B compared to Figure 8A. Cells grown for 1 day had an overall higher NO concentration than cells grown for 2 days in the model B. Future studies should include an MTS assay in order to assess the number of viable glia in 1 and 2 day cultures. There was a significant difference in LPS stimulated glia treated with stem cells compared to those that were not treated in both models, though the difference was much greater in model B compared to model A. Without a sufficient number of surviving cells, a heightened amount of NO for LPS stimulated glia similar to the environment in AD cannot be achieved.

4.2 Morphological and Immunocytochemical Analysis

Glial cells were stained with iNOS antibody in order to visualize iNOS distribution and cell morphology. Control glia [(-)SCCM/(-)LPS] showed normal flattened and spread glial formations and presented with a minor widespread distribution of iNOS (Figure 6A). The trace amounts of fluorescence in these glia are most likely indicative of physiologically normal iNOS activation. In the same conditions for the assessment of NO concentrations measured through the Griess reaction, there were baseline levels of NO which correlates to their appearance (Figure 2). Glia stimulated with LPS and were treated with SCCM in both stimulation and pre-treatment [(+)SCCM/(+)LPS/(Ptx/Stm)] showed normal glial morphology and little to no iNOS distribution (Figure 6B). This could suggest that SCCM reduces the number of cells

expressing iNOS rather than the amount of iNOS expression within each cell after prolonged exposure to SCCM. There were still cells expressing iNOS in the Ptx condition, but there were far fewer in the Ptx/Stm condition. Evidence for cells expressing iNOS can be seen in Figure 2, where the NO concentration was heightened, and in Figure 7 where there was percent area stained comparable to the baseline conditions. Glia that did not receive SCCM in any phase [(-)SCCM/(+)LPS] showed a high concentration of NO with a shriveled, shrunken appearance (Figure 6C). This shrinking and shriveling could be indicative of apoptosis which is characterized by the shriveling and condensing of the cell (Johnson et al., 2002). A study by Hu and Van Eldik (1996) showed an increase in apoptosis with the increase of iNOS expression in glial cells. In this study, a similar effect was observed. Also, the morphological shrinkage is also supported by the significantly high percent area covered by activated glia found in Figure 7. Glia that contained SCCM only in pre-treatment [(+)SCCM/(+)LPS/(Ptx)] showed a low concentration of NO and low distribution of iNOS, but some morphological shriveling and shrinking was still present (Figure 6D). This is consistent with the percentage of area seen covered by activated glia also represented in Figure 7. There was a significant difference in average intensity of stain as an effect of SCCM but not an effect of LPS on stain intensity, thought, there was an interaction between the two (Figure 8). This could be indicating that there is a decrease in number of cells and intensity of stain when activated by LPS in the presence of SCCM, but an increase in number and intensity when SCCM is applied alone. This is once again evidence for SCCM causing an increase in immunity when applied to glia, but can also cause a

decrease in activation when the immune response is highly induced causing a neuroprotective effect.

There were several problems with the execution of the cover slip model including the transfer of the cover slips to wells for treatment, and choosing the number of day for which to grow the cells. Going forward, perhaps larger cover slips and larger wells should be used to decrease the chance of breaking. Also, another medium other than paraffin wax should be used to create a separation between the cover slip surface and the surface of the bottom of the well. This execution of the novel cell-to-cell contact cover slip co-culture system did indicate, however, that glial cells should be grown in wells and stem cells grown on cover slips and not the other way around. As previously stated, an MTS assay should also be included in future experiments of this type in order to verify the number of living glia in culture. Also, inclusion of a stain for a cellular marker such as actin will allow for visualization of all cells including those with activated iNOS and provide a comparative measurement of iNOS. Since many of the cells in the (-) SCCM/(+)LPS condition showed signs of apoptosis, staining for an apoptotic marker within the cells would provide measurable evidence for apoptosis.

4.3 Future Studies

Going forward, similar studies to the present one should include some modifications. Many of these modifications including the addition of an MTS assay and testing a different neurotrophin other than NGF have been outlined in previous sections. Further modifications must be considered as well. In treating an AD patient, the disease is

on its course well before treatment is administered or a diagnosis is even made. So, in order to better represent the environment of an AD brain, future studies should switch the order of pre-treatment and stimulation putting stimulation before treatment with stem cells. A time course experiment in which stem cell treatment is applied 24 hours before, simultaneously, and 24 hours after glial stimulation would provide a valuable comparative experiment. This will more closely model disease conditions and indicate whether stem cell therapy is a feasible treatment for AD.

A co-culture system in which glia are grown directly with stem cells before stimulation may be a practical model to look into in the future. This will allow for cell-to-cell contact without the possible drawbacks of a transwell or cover slip system. Stem cells will then be present not only in the pre-treatment period but also through the stimulation period which could hopefully further decrease NO content through the effect of stem cell neurotrophins and once again more closely model actual treatment conditions. An unfortunate drawback to this system would be the inability to isolate glia once the experiment is complete. This can make it difficult to do immunocytochemical analysis of the glia and act as an obstacle in viewing the morphological differences in stimulated versus non-stimulated glia. Therefore, staining for different types of glia in these conditions will not only allow for the differentiation between glia and stem cells, but also show what types of glia are becoming activated by LPS.

Further *in vitro* models should include the introduction of neurons in culture. Obviously, a human brain consists of more than just glial cells and it is therefore important to explore the interaction between glial cells and neurons together in culture

when treated with stem cells. A possible experiment could include a direct co-culture of glia and neurons and pre-treatment followed by stimulation with LPS. Though there are many benefits to *in vitro* models, another way to observe this interaction would be through *in vivo* studies. These would be an important compliment to *in vitro* studies since *in vivo* studies allow for experimental conditions that more closely model the environment observed in AD and can provide behavioral reinforcement as well as cellular evidence for stem cells as a viable treatment.

Bibliography

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. 2002. Molecular Biology of the Cell. 4th edition. New York: Garland Science. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21054/.

Alzheimer's Association. 2013. "2013 Alzheimer's Disease Facts and Figures." 1-71.

Ballatore C, Virginia MYL, Trojanowski JQ. 2007. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. Neuroscience 8:663-667.

Berrios GE. November 1990. Alzheimer's disease: a conceptual history. The International Journal of Geriatric Psychiatry 5: 355-365.

Bird TD. September 1999 [Updated 2012 Oct 18]. Early-Onset Familial Alzheimer Disease. In: Pagon RA, Adam MP, Bird TD, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2013. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1236/

Bradley JA, Bolton EM, Pedersen RA. November 2002. Stem cell medicine encounters the immune system. Nature Reviews Immunology 2(11): 859-71.

Burlton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Müller FJ, Loring FJ, Yamasaki TR, Poon WW, Green KN, LaFerla FM. August 2011. Neural stem cells inprove cognition via BDNF in a transgenic model of Alzheimer disease. Processdings of the National Academy of Science 100(32): 13594-13599.

Davis KL, Powchik P. March 1995. Tacrine. The Lancet 345: 625-630.

Drzezga A, Lautenschlager N, Siebner H, Riemenschneider M, Willoch F, Minoshima S, Schwaiger M, Kurz A. May 2003. Cerebral metabolic changes accompanying conversion of mild cognitive impairment into Alzheimer's disease: a PET follow-up study. European Journal of Nuclear Medicine and Molecular Imaging 30(8): 1104-1113.

Evangelista M, Soncini M, Parolini O. Spetember 2008. Placenta-derived stem cells: new hope for cell therapy? Cytotechnology 58(1): 33-42.

Fauza D. December 2004. Amniotic fluid and placental stem cells. Best Practice and Research Clinical Obstetrics and Gyneacology 18(6): 877-891.

Frese LJ. 2012. Placental derived stem cells regulate activated glia in an in-vitro inflammatory model of Alzheimer's disease. Thesis Collection Drew University Library 69p.

Galimberti D, Scarpini E. June 2011. Progress in Alzheimer's disease. Journal of Neurology 259: 201-211.

Gotz J, Probst A, Spillantini MG, Schafer T, Jakes R, Burki K, Goedert M. 1995. Somatodendritic localization and hyperphosphorylation of tau protein in transgenic mice expressing the longest human brain tau isoform. The EMBO Journal 14(7): 1304-1313.

Guan Z, Baier LD, Morrison AR. March 1997. P38 mitogen-activated protein kinase down-regulates Nitric Oxide and up-regulates prostaglandin E2 biosynthesis stimulated by interleukin-1β. The Journal of Biological Chemistry 272:8083-8093.

Hanisch UK, Kettenmann H. November 2007. Microglia: active sensory and versatile effector cells in the normal and pathological brain. Nature Neuroscience 10(11):1387-1394.

Heneka MT, O'Banion MK. March 2007. Inflammatory process in Alzheimer's disease. Journal of Neuroimmunology 184(1-2):69-91.

Heneka MT, Rodriguez JJ, Verkhratsky A. May 2010. Neuroglia in neurodegeneration. Brain Research Reviews 63:189-211.

Holstein M. 1997. Alzheimer's disease and senile dementia, 1885-1920: an interpretive history of disease negotiation. Journal of Aging Studies 11(1): 1-13.

Hsiao K, Chapman P, Nilsen S, Eckman C, Harigaya Y, Younkin S, Yang F, Cole G. October 1996. Correlative memory deficits, AP elevation, and amyloid plaques in transgenic mice. Science 274: 99-102.

Hu J, Van Eldik LJ. 1996. S100β induces apoptotic cell death in cultured atrosytes via a nitric oxide-dependent pathway. Biochemica et Biophysica Acta 1313: 239-245.

Imbimbo BP. 2009. Why did Tarenflurbil fail in Alzheimer's disease? Journal of Alzheimer's Disease 17:757-760.

Isbir T, Agaçhan B, Yilmaz H, Aydin M, Kara I, Eker E, Eker D. 2001. Apolopoprotein-E gene polymorphism and lipid profiles in Alzheimer's disease. American Journal of Alzheimer's Disease & Other Diementias 16(2):77.

Jeohn GH, Kim WG, Hong JS. October 2000. Time dependency of the action of nitric oxide in lipopolysaccharide–interferon-γ-induced neuronal cell death in murine primary neuron–glia co-cultures. Brain Research 880(1-2):173-177.

Kawabata S, Higgins GA, Gordon JW. December 1991. Amyloid plaques, neurofibrillary tangles and neuronal loss in brains of transgenic mice overexpressing a C-terminal fragment of human amyloid precursor protein. Nature 354: 476-478.

Kowall AE, Budson NW. 2009 [Updated 2011]. The handbook of Alzheimer's disease and other dementias. Hoboken: Wiley-Blackwell. 659p.

Law A, Gauthier S, Quirion R. March 2001. Say NO to Alzhiemer's disease: the putative links between nitric oxide and dementia of the Alzheimer's type. Brain Research Reviews 35(1):73-96.

Lawrence AD, Sahakian BJ. May 1998. The cognitive psychopharmacology of Alzheimer's disease: focus on cholinergic systems. Neurochemical Research 23(5):787-794.

Lewis J, Dickson DW, Lin W, Chisholm L, Corral A, Jones G, Yen S, Sahara N, Skipper L, Yager D, Eckman C, Hardy J, Hutton M, McGowan E. August 2001. Enhanced neurofibrillary degeneration in transgenic mice expressing mutant tau and APP . Science 293: 1487-1491.

Lim GP, Yang F, Chu T, Chen P, Beech W, Teter B, Tran T, Ubeda O, Hsiao Ashe K, Frautschy SA, Cole GM. August 2001. Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's Disease. The Journal of Neuroscience 20(115):5709-5714.

Lu B, Yokoyama M, Dreyfus CF, Black IB. 1991. NGF gene expression in actively growing brain glia. The Journal of Neuroscience 11(2):318-326.

Mandelkow E, Mandelkow E. 1995. Microtubules and microtubule-associated proteins. Current Opinion in Cell Biology 7:72-81.

Martinez-Coria H, Green KN, Billings LM, Kitazawa M, Albrecht M, Rammes G, Parsons CG, Gupta S, Banerjee P, LaFerla FM. February 2010. Memantine Improves cognition and reduces Alzheimer's-like neuropathology in transgenic mice. The American Journal of Pathology 176(2):870-880.

Mayo Clinic. 2013. http://www.mayoclinic.org/diseases-conditions/alzheimers-disease/in-depth/alzheimers-treatments/ART-20047780?pg=1

Mazanetz MP, Fischer PM. 2007. Untangling tau hyperphosphorylation in drug design for neurodegenerative diseases. Nature 6:464-479.

McDonald NQ, RLapatto R, Murray-Rust J, Gunning J, Wlodawer A, Glundell TL. 1991. New protein fold revealed by a 2.3Å resolution crystal structure of nerve growth factor. Nature 354: 411-414.

McGreer PL, McGreer EG. 1995. The inflammartory response system of brain: implications for therapy of Alzheimer's and other neurodegenerative diseases. Brain Research Reviews 21:195-218.

McGreer PL, McGreer EG. 2001. Inflammation, autotoxicity and Alzheimer disese. Neurobiology of Aging 22:799-809.

Miki T. 2011. Amnion-derived stem cells: in quest of clinical applications. Stem Cell Research and Therapy 2:1-11.

Möller HJ, Graeber MB. March 1998. The case described by Alois Alzheimer in 1911 historical and conceptual perspectives based on the clinical record and neurohistological sections. European Archives of Psychology and Clinical Neruoscience 248: 111–122.

Momin EN, Mohyeldin A, Zaidi HA, Vela G, Quinones-Hinojosa A. 2010. Mesenchymal stem cells : new approaches for the treatment of neurological diseases. Current Stem Cell Research and Therapy 5: 326-344.

Nagelhus EA, Amiry-Moghaddam M, Bergersen LH, Bjaalie JG, Eriksson J, Gundersen V, Leergaard TB, Morth JP, Storm-Mathisen J, Torp R, Walhovd K, Tønjum T. October 2013. The glia doctrine: Addressing the role of glial cells in healthy brain ageing. Mechanisms of Ageing and Development 134(10): 449-459.

Nikolic WV, Hou H, Tan J. June 2008. Peripherally Administered Human Umbilical Cord Blood Cells Reduce Parenchymal and Vascular β-Amyloid Deposits in Alzheimer Mice. Stem Cells and Development 17(3): 423-439.

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, et al. 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. Neuron 39: 409–421

Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM. July 2003. Triple-transgenic model of Alzheimer's disase with plaques and tangles: Intracellular Aβ ans synaptic dysfunction. Neuron 39(3):409-421.

Parolini O, Alviano F, Bagnara GP, Bilic G, Bühring HJ, Marco Evangelista[1], Hennerbichler S, Liu B, Magatti M, Mao N, Miki T, Marongiu F, Nakajima H, Nikaido T, Portmann-Lanz CB, Sankar V, Soncini M, Stadler G, Surbek D, Takahashi TA,Redi H, Sakuragawa S, Wolbank S, Zeisberger S, Zisch A, Strom SC. February 2008. Concise review: isolation and characterization of cells from human term placenta: outcome of the first international workshop on placenta derived stem cells. Stem Cells 26(2): 300-311.

Pimplikar SW. June 2009. Reassesing the amyloid cascade hypothesis of Alzheimer's disease. The international Journal of Biochemistry and Cell Biology 41(6): 1261-1268.

Promega. 2009. Griess Reagent System. Technical Bulletin. Promega Corporation. Madison, WI. 1-9.

Reimann V, Creutzig U, Kogler G. 2009. Stem cells derived from cord blood in transplantation and regenerative medicine. Deutsches Ärzteblatt International 106(50): 831-836.

Saunders AM, Strittmatter WJ, Schmechel D, Pericak-Vance MA, Joo Sh, Rosi BL, GUsella JF, Crapper-MacLachlan DR, Alberts MJ, Hulette C, Crain B, Goldgaber D, Roses AD. 1993. Association of apolipoprotein E allele ε4 with late-onset familial and sporadic Alzheimer's disease. Neurology 43: 1467-1472.

Schmechel DE, Saunders Am, Strittmatter WJ, Crain BJ, Hulette CM, Joo SH, Pericak-Vance MA, Goldgaber D, Roses AD. 1993. Increased amyloid β-peptide deposition in cerebral cortex as a consequence of apolipoprotein E genotype in late-onset Alzheimer disease. Neurobiology 90: 9649-9653.

Selkoe DJ. October 2002. Alzheimer's disease is a synaptic failure. Science 298: 789-791.

Sheng JG, Jones RA, Zhou XQ, McGinness JM, Van Eldik LJ, Mrak RE, Griffin WST. 2001. Interleukin-1 promotion of MAPK-p38 overexpression in experimental animals and in Alzheimer's disease: potential significance for tau protein phosphorylation. Neurochemistry International 39(5-6):341-348.

Social Security. http://www.ssa.gov/planners/lifeexpectancy.htm

Spires TL, Hyman BT. July 2005. Transgenic models of Alzheimer's disease: learning from animals. NeuroRx 2(3):423-437.

Steinhilb ML, Dias-Santagata D, Fulga TA, Felch TA, Feany MB. 2007. Tau phosphorylation sites work in concert to promote neurotoxicity in vivo. Molecular Biology of the Cell 18(12): 5060-5068.

Stem Cell Basics: In *Stem Cell Information* [World Wide Web site]. Bethesda, MD: National Institutes of Health, U.S. Department of Health and Human Services, 2002 [cited January, 2014] Available at http://stemcells.nih.gov/info/basics/pages/basics1.aspx

Stork T, Bernados R, Freeman MR. (ed. Zhang et al.) 2012. Analysis of glial cell development and function in Drasophila. Cold Spring Harbor Protocols 1-17.

Strassnig M and Ganguli M. September 2005. About a peculiar disease of the cerebral cortex. Psychiatry (Edgmont) 2(9): 30–33.

Strauer BE, Koronowski R. 2003. Stem Cell Therapy in Perspective: In *Circulation* Dallas, TX: American Heart Association. [cited February, 2014]. Available at http://circ.ahajournals.org.ezproxy.drew.edu/content/107/7/929.full.pdf+html.

Tejada-Vera B. March 2013. Mortality from Alzhiemer's disease in the United States: Data for 2000 and 2010. Centers for Disease Control and Prevention (CDC). <http://www.cdc.gov/nchs/data/databriefs/db116.htm>.

Thomas BM, Starr JM, Whalley LJ. 1997. Death certification in treated cases of presenile Alzheimer's disease and vascular dementia in Scotland. Age and Ageing 26: 401-406.

Thompson PM, Moussai J, Zohoori S, Goldkorn A, Khan AA, Mega MS, Small GW, Cummings JL, Toga AW. September 1998. Cortical variability and asymmetry in normal aging and Alzheimer's Disease. Cerebral Cortex 8:1047-3211.

Van Eldik LJ. December 2001. Glia and Alzheimer's disease. Neurochemistry International 39:329-331.

Wang L, Swank JS, Glick IE, Gado MH, Miller MI, Morris JC, Csernansky JG. June 2003. Changes in hippocampal volume and shape across time distinguish dementia of the Alzheimer type from healthy aging. NeuroImage 20: 667-682.

Wolfe, MS. 2001. Presenilin and g-secretase: structure meets function. Journal of Neurochemistry 76: 1612-1614.

Xu J, Kochanek KD, Murphy SL, Tejada-Vera B. May 2010. Deaths: final data for 2007. National Vital Statistics Report 58: 1-136.

Yan K, Zhang R, Sun C, Chen L, Li P, Liu Y, Peng L, Sun H, Qin K, Chen F, Huang W, Chen Y, Lv B, Du M, Zou Y, Cai Y, Qin L, Tang Y, Jiang X. December 2013. Bone-marrow derived stem cells maintain the resting phenotype of microglia and inhibit microglial activation. Public Library of Science 8(12): e84116.

Yun HM, Kim HS, Park KR, Shin JM, Kang AR, Lee K, Song S, Kim YB, Han SB, Chung HM, Hong JT. December 2013. Placenta-derived mesenchymal stem cells improve memory dysfunction in an Aβ-42-infused mouse model of Alzheimer's disease. Cell Death and Disease 4: e958.