Molecular Intervention in Mouse Models of Amyotrophic Lateral Sclerosis and Alzheimer’s Disease – Neuropathology and Behavior

by

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<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
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<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
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<tr>
<td>CAA</td>
<td>Cerebral amyloid angiopathy</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
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<td>APP</td>
<td>Amyloid precursor protein</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>CC</td>
<td>Cingulate cortex</td>
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<td>H</td>
<td>Hippocampus</td>
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<td>EC</td>
<td>Entorhinal cortex</td>
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<td>GFAP</td>
<td>Glial fibrillar acidic protein</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TGF</td>
<td>Tumor growth factor</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<tr>
<td>i.c.v</td>
<td>intracerebroventricular</td>
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<tr>
<td>i.p.</td>
<td>Interperitoneal</td>
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<tr>
<td>MN</td>
<td>Motor neuron</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage colony stimulating factor</td>
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<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>ApoE</td>
<td>ApolipoproteinE</td>
</tr>
<tr>
<td>ACT</td>
<td>α-1 antichymotrypsin</td>
</tr>
<tr>
<td>Serpin</td>
<td>Serine protease inhibitor</td>
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<td>RAWM</td>
<td>Radial arm water maze</td>
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<td>PS1</td>
<td>Presenilin 1</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
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<tr>
<td>PHF</td>
<td>Paired helical filaments</td>
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<tr>
<td>FTD</td>
<td>Frontal temporal dementia</td>
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<tr>
<td>CBD</td>
<td>Cortical basal degeneration</td>
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<tr>
<td>PSP</td>
<td>pProgressive supranuclear palsy</td>
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<tr>
<td>pSer</td>
<td>Phosphorylated serine</td>
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<tr>
<td>pThr</td>
<td>Phosphorylated threonine</td>
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<td>MAPT</td>
<td>Microtubule associated protein tau</td>
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<tr>
<td>DTG</td>
<td>1,3-di-o-tolylguanidine</td>
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<tr>
<td>ERK</td>
<td>Extracellular related kinase</td>
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<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
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MCAO  Middle cerebral arterial occlusion
NO  Nitric oxide
LPS  Lipopolysaccharide
PrPc  “Normal” prion protein
PrPsc  “Scrapie” or misfolded prion protein
TSE  Transmissible spongiform encephalopathy
WT  Wild type
KDI  Lysine-aspartic acid-isoleucine
NMDA  N-methyl-D-aspartic acid
AMPA  $\alpha$-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
LTP  Long term potentiation
EGFP  Enhanced green fluorescent protein
TDP-43  TARP DNA binding protein 43 kDa
DOB  Date of birth
DOD  Date of death
NTG  Nontransgenic
LS  Lifespan
C3  Complement C3 convertase
MOLECULAR INTERVENTION IN MOUSE MODELS OF
AMYOTROPHIC LATERAL SCLEROSIS AND
ALZHEIMER’S DISEASE – NEUROPATHOLOGY AND BEHAVIOR

Steven Prescott Bennett

ABSTRACT

Neurodegeneration describes the progressive loss of structure and function of neurons, leading ultimately to cell and organism death. Although the initiating factors of neurodegenerative diseases such as Alzheimer’s, Parkinson’s, Huntington’s, and Amyotrophic Lateral Sclerosis may be different, they share common pathophysiology. Proteinopathies, as these diseases are now termed, are characterized by atypical deposits of proteins, often due to misfolding. Associated with these deposits are dysfunctional mitochondria, oxidative stress, disrupted axonal transport, inflammation, and apoptotic cell death. If this occurs in motor neurons, as in ALS, ataxia precedes death with little or no change in cognition. On the other hand, if the deposits are found in cortical neurons, as in Alzheimer’s disease, the outcome is dementia and motor function remains largely intact. Each disease is selective for particular types of neurons and brain regions. Although research has elucidated much of the molecular biology involved in these diseases, their initiating causes remain largely unknown.

Most of our current understanding originated with the identification of gene mutations that cause rare familial forms of these diseases. As a result, numerous strains of transgenic animals have been developed to study neurodegenerative disease phenomena and were central to the studies presented in this body of work. Novel routes of drug and gene delivery are described here as well as characterization of the mouse models studied. In particular, this work demonstrates that the blood brain barrier is
disrupted in ALS followed by the formation of autorosettes in ALS mice. In various Alzheimer’s disease mouse models, it was demonstrated that the acute phase reactant alpha-1-antichymotrypsin (ACT) not only interacts with amyloid plaques, but also induces tau phosphorylation in vivo; tying together these disease hallmarks. It was also shown that small fragments of Aβ (1-11) could disrupt the formation of mature amyloid plaques in these mice. Lastly, it was demonstrated that mature plaques could also be decreased by intracranial delivery of granulocyte-macrophage stimulating factor (GM-CSF). My dissertation research goal was to understand and develop these treatment strategies based on protein disaggregation, neuroprotection, and inflammation, meanwhile developing novel methods for targeted delivery of molecules into the CNS of mice.
CHAPTER 1 – INTRODUCTION

1.1. Introduction to neurodegenerative disease

While all diseases, especially those that are fatal, are devastating, there is an additional sense of loss when those diseases affect our minds – often considered the core that makes us human. Although the exact pathophysiology of different neurodegenerative diseases varies, they share certain commonalities, and all involve selective vulnerability of neurons in distinct brain regions. For example, there are properties of motor neurons that make them selectively vulnerable to cell death in amyotrophic lateral sclerosis, and properties of cortical and hippocampal neurons that make these neurons susceptible to Alzheimer’s disease. In either case, the outcome is progressive neuronal death that is terminal to the patient.

Collectively, neurodegenerative diseases are a leading cause of death in the U.S. There are currently over 5 million Alzheimer’s cases, 1.5 million Parkinson’s, 30,000 ALS, and 30,000 Huntington’s patients. In addition 150,000 people are annually diagnosed as “at risk” patients and about 300 cases of prion disease are counted annually (Centers of Disease Control and Prevention). Despite decades of research dedicated to each of these diseases, there are no cures to date. Indeed, those drugs currently prescribed provide modest, if any, alleviation of symptoms and may delay death for just weeks or months. Therefore, novel therapies are urgently needed and strongly sought, some with the intent to be effective for multiple neurodegenerative disorders.

The most common molecular mechanism of neurodegenerative diseases, and a primary focus of my work, is the misfolding of proteins that leads to extracellular and/or intracellular proteinaceous aggregates. Typically, these inclusions/plaques include molecular chaperones and polyubiquitinated proteins, indicating that the cellular
mechanisms for protein folding, quality control and protein degradation are overwhelmed in protein misfolding diseases (PMDs)/proteinopathies (reviewed in Soto and Estrada, 2008). Another component of neurodegenerative diseases is inflammation, which is both a response to aggregates and contributes to their formation. The activation of microglia and astrocytes and the subsequent release of various cytokines and chemokines induce a local, chronic state of inflammation in the affected brain regions (McGeer and McGeer, 1995). The involvement of astrocytes ties inflammation to another aspect of neurodegeneration: glutamate excitotoxicity (Abraham et al., 2001). Astrocytes are known to surround glutaminergic synapses where they express GLT-1 (EAAT2) - glutamate transport molecules which function to rapidly clear this neurotransmitter. Failure to adequately clear glutamate can lead to abnormally elevated glutamate levels at the synapse, in turn causing surges of calcium into post-synaptic neurons (reviewed in Amara and Fontana 2002). Although, \( \text{Ca}^{2+} \) entry partly underlies the process of synaptic plasticity, disruption of calcium homeostasis by excess \( \text{Ca}^{2+} \) has a pronounced deleterious impact on neurons and surrounding glia. Calcium reacts within the cell, altering enzymatic activities, inducing damage to DNA and organelles, and negatively affecting axonal transport and cytoskeletal organization (Gennarelli et al., 1993).

Excess intracellular calcium is also one of several conditions that lead to dysregulation of mitochondria. Mitochondrial dysfunction is problematic because it burdens the cell with reactive oxygen species (ROS) and induces oxidative stress. Moreover, an intrinsic apoptotic pathway can be activated by the release of cytochrome c from the mitochondrial inner membrane, leading to programmed cell death (PCD) or apoptosis (reviewed by Onyango et al., 2006). Indeed, neuronal apoptosis is a common, final event in virtually all neurodegenerative diseases.

While all neurodegenerative diseases have a genetic component, only 5-10% of patients represent cases where the genetic mutation leading to the autosomal dominantly inherited familial forms of both AD and ALS are known. Additional genetic and epigenetic components as well as environmental contributions still have to be determined. It is for this reason that most of the knowledge acquired about ALS and AD comes from the finding and analysis of genetic mutations throughout human populations. Because
the phenotypes of familial and sporadic cases are so similar for each disease, those mutations have been the most useful means to study disease progression and develop possible therapeutic interventions for both familial and sporadic forms of the diseases. Protective as well as risk enhancing environmental or life style factors for each disease have also been described. For example, educational/environmental “enrichment” and developing Alzheimer’s disease seem to be negatively correlated, i.e. these factors have a protective effect, while in ALS a positive correlation, a disease causing/enhancing impact of physical exertion as has been reported for example with soldiers and athletes (Belli and Vanacore, 2005; Haley, 2003).

Once mutations in the disease-related genes were identified and the genes were cloned, \textit{in vitro} and \textit{in vivo} models were developed to test various hypotheses on disease progression and therapy. Cell culture and organotypic tissue culture have proven useful in answering very targeted scientific questions. However, for the study of protein aggregation in CNS tissues, a more systemic approach with animal studies was needed. Accordingly, several different lines of transgenic mice have been developed over the past 15 years to analyze the formation of protein aggregates in vivo. Such mouse models of AD and ALS were the main subjects of my work which investigated causes and treatments for Protein Misfolding Diseases in the brain.

1.2. \textit{Amyotrophic Lateral Sclerosis}

1.2.1. \textit{ALS Pathology}

Amyotrophic Lateral Sclerosis (ALS) is currently the most common motor neuron disease, affecting roughly 30,000 people in the U.S. with a lifetime risk of 1 in 2000. Originally called Charcot’s disease after the French neurobiologist and physician Jean-Martin Charot, who first characterized the disease in 1869, it has more recently been named Lou Gehrig’s disease after the famous Yankees baseball player who was diagnosed with ALS in 1939 and died 2 years later at the age of 37. ALS is characterized by disruption of the cytoskeleton in motor neurons (Williamson et al., 1998), reactive
gliosis (Leigh and Swash, 1991), accumulation of intracellular proteinaceous inclusions (Bruijn et al., 1998), neurofilament abnormalities and formation of axonal spheroids (Carpenter, 1968; Gonatas et al., 1992; Hirano et al., 1984; Leigh et al., 1991), loss of myelinated fibers in the ventral roots of corticospinal tracts (Delisle and Carpenter, 1984), and disruption of the blood brain barrier (Garbuzova-Davis et al., 2007c).

Selective death of motor neurons occurs in the motor cortex, brainstem, and spinal cords of affected individuals (Rowland and Shneider, 2001b).

ALS is a devastating disease, causing spasticity, hyperreflexia, generalized weakness, muscle atrophy and paralysis (Mulder et al., 1986), all leading to death within 2-5 years of onset, most commonly from respiratory failure. ALS primarily targets the large caliber alpha motor neurons in the anterior horn of the spinal cord, 35-100μm in diameter (Ravits et al., 2007). The average age of diagnosis varies, but generally symptoms occur between 40-60 years of age (Hirtz et al., 2007). 90-95% of ALS cases are sporadic, that is, there are no known genetic or environmental causes, despite the high prevalence of the disease in certain populations (Belli and Vanacore, 2005; Haley, 2003; Horner et al., 2003; Mulder and Kurland, 1987; Vanacore et al., 2006). Most knowledge about the disease has so far been gained from mutations in 6 different genes that underlie ALS (Gros-Louis et al., 2006). These genetic predispositions accounts for only 5-10% of all ALS cases, but because the phenotypes of familial and sporadic ALS are so similar, these mutations have been valuable tools in understanding the disease pathogenesis.

1.2.2. Major Genes and Proteins in ALS

The most common gene that harbors mutations leading to familial ALS is superoxide dismutase (SOD1) (Rosen, 1993). SOD1 functions as a 32 kD homodimer and each subunit of 153 amino acids is encoded by 5 exons on chromosome 21q22 (Siddique et al., 1991). The primary function of SOD1 is to convert toxic superoxide radicals, produced by mitochondria during oxidative phosphorylation, to molecular oxygen and hydrogen peroxide. Appropriately, the protein is found in the cytosol, nucleus, mitochondrial inner membrane, and peroxisomes of cells to exert its antioxidant
effects (Bruijn et al., 2004b). The precise role of SOD1 in the progression of ALS is not completely known. However, the prevalence and number of mutations in the human gene encoding SOD1 strongly indicated a role for the protein in the disease progression.

Although the protein is relatively small, there have been over 150 mutations identified in hSOD1 since the first was identified over 15 years ago (Rosen, 1993). Collectively, mutations in SOD1 account for about 3% of all ALS cases (Andersen, 2006). SOD1 is a ubiquitously expressed Cu/Zn metalloenzyme with zinc providing structural stability and copper coordinating the alternating transfer of electrons to both oxidize and reduce superoxide (Beckman et al., 1993). Each SOD1 monomer contains one Cu/Zn active site. However, mutations within this active site do not lead to any different ALS phenotype than mutations in other parts of the molecule (Borchelt et al., 1994; Bowling et al., 1995). Therefore it is likely not the activity of the enzyme itself that promotes the disease, but rather a structural change in the molecule with strong downstream effects. This information, together with the fact that SOD1 knockout mice do not develop disease symptoms, supports the hypothesis that the involvement of SOD1 in the pathogenesis of ALS is a result of a toxic-gain-of-function (Shaw, 2005). This view leads to the conclusion that irrespective of the specific mutation, mutant SOD1 has taken on a new function, independent of its enzymatic activity, and that this function is toxic to the cell. The inheritance pattern of SOD1 mutations, like most neurodegenerative diseases, is autosomal dominant (Siddique et al., 1996)

1.2.3. Genes and Major Mouse Models of ALS

1.2.3.1. SOD1

Currently 12 mouse models of ALS have been developed that harbor some of the indentified mutant forms of human SOD 1 and are used to study ALS and potential therapeutics. The most intensely studied genetic variations are three point mutations leading to the single amino acid substitutions G93A (Gurney, 1994), G37R (Wong et al., 1995), G85R (Bruijn et al., 1997), all of which are used to study disease progression at
different rates. The G93A mouse model, for example, begins showing symptoms as early as 10 weeks and the animals die at 13-16 weeks. The G85R model, on the other hand, begins showing symptoms at 5 months and the mice die by 8-9 months. There are advantages to all different SOD1 mice, because some studies require longer therapeutic windows and others aim for fast responses.

The pathophysiology of SOD1 mutant mice is very similar to that of ALS patients, i.e. the primary pathological event is a progressive loss of both upper and lower motor neurons (Gurney, 1994) and mitochondrial vacuolization (Dalcanto and Gurney, 1995) leading to severe hind limb weakness. These events are accompanied by reactive microgliosis and astrogliosis and localized inflammation (Hall et al., 1998a), the formation of intracellular proteinaceous inclusions (Johnston et al., 2000), neurofilament positive inclusions (Tu et al., 1996), and fragmentation of the Golgi apparatus (Mourelatos et al., 1996). Other events are glutamate excitotoxicity (Rothstein et al., 1990), abnormal axonal transport (Williamson and Cleveland, 1999), and abnormal regulation of growth factors (Lambrechts et al., 2003). Importantly, in all cases the pathological cascade culminates finally in the activation of caspase 3 (Li et al., 2000; Pasinelli et al., 2000; Vukosavic et al., 2000), and the induction of cell death. Although it is generally disputed if aggregate formation is the cause, a byproduct, or even a protective mechanism in neurodegenerative disease, there is no doubt that the presence of inclusions in ALS and the downstream effects of mutant SOD1 on subcellular organelles as well as the activation of programmed cell death (PCD) make SOD1 a very attractive molecule for the investigation of this disease.

1.2.3.2. ALS2

In another ALS related gene, ALS2 (alsin) some disease related mutations have been identified. ALS2 is located on chromosome 2 and was recently shown to cause rare, juvenile onset forms of motor neuron disease which progress slowly, and strongly resemble the symptoms of ALS (Hadano et al., 2001; Yang et al., 2001). The ALS2 encoded protein, alsin, is a 184-kD protein that has been shown to have 3 putative
guanine-nucleotide-exchange factor domains (GEFs). Alsin binds to the periphery of endosomes and is involved with the Ras superfamily of G protein receptors (Yamanaka et al., 2003). Specifically, GEFs catalyze the dissociation of GDP from G-proteins and act, \textit{in vitro}, as an exchange factor for Rab5 (Otomo et al., 2003), a molecule known to be involved in endosomal trafficking. Alsin is enriched in nervous tissue and the mutations that have been identified so far are very unstable (Yamanaka et al., 2003), suggesting a loss of activity in one or more of the GEF domains. Recently, animal models harboring mutations in alsin (ALS2) have been generated for further investigation.

1.2.3.3. \textit{TDP-43}

Another protein that has drawn some attention over the past few years is TDP-43 (TAR DNA Binding protein 43). TDP-43 is a 43 kDa protein encoded by the TARDBP gene located on chromosome 1 and is expressed ubiquitously, including the CNS, where it is found in neuronal and glial nuclei and to a lesser extent in the cytoplasm (Geser et al., 2009). The diverse functions of TDP-43 are not completely understood, but it is believed to stabilize low molecular weight neurofilaments (Strong et al., 2007), modulate cdk6 expression (Ayala et al., 2008a; Ayala et al., 2008b), act as a scaffold for nuclear bodies (Wang et al., 2004), and affect microRNA biogenesis (Gregory et al., 2004), cell cycle, apoptosis (Ayala et al., 2008b), and mRNA transport and regulation of its translation at synapses (Wang et al., 2004). The protein, which can be abnormally phosphorylated and misfolded, was originally discovered in patients with Frontotemporal Dementia (FTD) and has recently been discovered in the inclusions of both familial and sporadic cases of ALS (Arai et al., 2006; Neumann et al., 2006). Research results involving the role(s) of TDP-43 in neurodegenerative disease have been somewhat conflicting, but its presence in the inclusions of ALS patients has made it a very appealing target for therapeutic interventions.
1.2.4. *ALS and excitotoxicity*

The most convincing link between sporadic and familial forms of ALS involves glutamate excitotoxicity (Rothstein et al., 1990). The neurotransmitter glutamate has been implicated in ALS because excess glutamate levels were discovered in the cerebrospinal fluid (CSF) of roughly half of the ALS patients sampled (Rothstein et al., 1991; Rothstein et al., 1990; Shaw et al., 1995). Excess glutamate induces repetitive firing of neurons leading to increases of intracellular calcium levels through increased permeability of calcium-permeable glutamate receptors (Beers et al., 2001). A supporting phenomenon towards glutamate excess as a possible cause for the selective death of motor neurons in ALS is the fact that excess glutamate is cleared much less efficiently from synapses in sporadic and familial ALS cases and accordingly in most ALS cases, glutamate clearance is dysregulated. Typically, finger-like projections from astrocytes rich in the glutamate transporter EAAT2 (GLT-1) surround synapses in order to rapidly clear glutamate (Rothstein et al., 1996; Tanaka et al., 1997). This transport molecule, however, shows pronounced down-regulation in ALS (in response to caspase 3), supporting glutamate excitotoxicity as a potential cause for ALS. Riluzole, a drug that functions to deactivate NMDA receptors and reduce synaptic glutamate levels, is currently the only effective drug for ALS that is FDA approved (Gurney et al., 1998). Further studies revealed that glutamate may also be responsible for other neurodegenerative diseases, namely Alzheimer’s and Parkinson’s disease (Beal, 1998; Hynd et al., 2004).

1.2.5. *ALS and the cytoskeleton*

Most neurodegenerative proteinopathies, including both sporadic and familial ALS (Carpenter, 1968; Chou and Fakadej, 1971; Hirano, 1991; Hirano et al., 1984) show some disturbance of neurofilament content and assembly. Neurofilaments are neuron-specific intermediate filaments and are the most abundant structural proteins found in neurons. They are thought to add rigidity, tensile strength and intracellular guidance for
transport along axons and dendrites. Neurofilaments are cytoskeleton proteins that act together to form and maintain cell shape and facilitate the transport of various molecules and organelles within the cytoplasm of neurons. Abnormal assembly of neurofilaments is of particular consequence in ALS as they are primarily responsible for establishing the proper diameter in axons (Lee and Cleveland, 1996). Lower alpha motor axons are the thickest in the body and proper Neurofilament assembly are critical for adequate neurotransmission through these fibers. Abnormal assembly is prevalent in sporadic ALS cases and in most mouse models. Normally, Neurofilament assembly occurs between NF-L (light), NF-M (medium), and NF-H (heavy) chains (in order of molecular weight-68kDa, 160 kDa, and 200kDa, respectively) (Harris et al., 1991). The first event is dimerization of NF-L filaments or dimerization of NF-L with either NF-M or NF-H filaments, followed by assembly of dimers in a staggered antiparallel fashion allowing NF-H and NF-M filaments to associate (Fuchs and Weber, 1994). Elongation occurs by head to tail assembly of NF-L filaments and phosphorylation occurs on the NF-M and NF-H filaments, the latter of which is one of the most extensively phosphorylated proteins in neurons (Strong et al., 2005b). In ALS, neurofilament assembly can be altered drastically due to reduced steady state levels of NF-L in affected motor neurons. Moreover, mutant SOD1 has recently been shown to destabilize NF-L, providing a potential connection between familial ALS mutations and alteration in NF ratios (Ge et al., 2005).

1.2.6. ALS and intracellular inclusions

The formation of intracellular inclusions has so far been the most prominent feature of ALS, even though there is still no clear understanding of their importance or contribution to the disease. In familial cases of ALS, the inclusions are immunopositive for SOD1. Otherwise, they are comprised mainly of Neurofilaments (Liu et al., 2004), ubiquitin (Bruijn et al., 1998; Jonsson et al., 2004; Mather et al., 1993), peripherin (Robertson et al., 2001), dorfin (Niwa et al., 2002), molecular chaperones (HSP 40 and HSP 90) (Namba et al., 1991; Shinder et al., 2001; Takeuchi et al., 2002) and other
proteins. These inclusions, similar to the amyloid deposits in AD and the α-synuclein positive plaques found in Parkinson’s (Shimura et al., 2001), have been subject of intensive studies to elucidate the pathophysiology of this neurodegenerative disease. While the cause for these plaques/inclusions is still unknown, much emphasis has been placed on investigating those deposits and agents that can exacerbate or ameliorate plaque growth as well as their relative effects on behavior and pathology. Intracellular deposits in ALS could negatively affect the cell in many ways. They could contribute to the loss of protein function, thereby possibly enhancing further deposition and causing other downstream effects within the cell, such as mitochondrial dysfunction (Dal Canto and Gurney, 1994; Kong and Xu, 1998; Wong et al., 1995). Another potential effect of protein aggregation might be the disruption of active sites and thereby enzymatic activity of the involved proteins. In ALS, altering binding regions for copper and zinc in the active site of SOD1 was found to cause protein misfolding and aberrant activity regarding specific substrates (Wang et al., 2003). It has been proposed, for example, that mutant SOD1 interacts with other molecules, such as nitric oxide (Estevez et al., 1999), and forms toxic byproducts, such as peroxynitrate. Peroxynitrate can then react with tyrosine residues to form nitrotyrosine, and thereby block the activity of the nitrated protein (Beckman et al., 1993).

Another consequence of protein aggregation is the binding of various chaperones, thereby leading to the depletion of, for example, of HSP 40 and HSP70. A related observation describes evidence that in proteinopathies and inclusions/deposits the proteasome, the major protein degradation machinery in the cell, is overwhelmed by an overload of ubiquitinated proteins targeted for degradation (Leigh et al., 1991).

1.2.7. ALS and inflammation

The activation of microglia and astrocytes has led researchers to the understanding that ALS is not a cell autonomous disease (Clement et al., 2003). i.e. the disease does not progress solely within neurons, but is exacerbated by supporting glia in the brain. Astrocytes and microglia have also been shown to express mutant SOD1 and
astrocytes even develop inclusions similar to those found in neurons. Activated microglia are known to release proinflammatory cytokines such as TNFα, NO, Cox-2, and IL-1β and IL-6 (Sargsyan et al., 2005), all of which are a part of a localized immune response. TNFα can drive peripherin expression in neurons (found in inclusions) (Robertson et al., 2001), again providing a link between non-neuronal cells and ALS.

Transforming growth factor beta 1 (TGFβ-1) and Macrophage colony-stimulating factor (MCSF) are actually upregulated and released by microglia in presymptomatic mice (Elliott, 2001). Initial studies involving administration of LPS into ALS mice, a known microglial activator, worsened the symptoms and shortened lifespan (Weydt et al., 2004). Studies have also shown that mutant SOD1 in microglia and astrocytes accelerates the release of these molecules and worsens the disease, whereas normal supporting cells surrounding a mutant SOD1 expressing neuron greatly slowed disease progression, but had no change on the onset of the disease. Similarly, introducing mutant SOD1-expressing microglia and astrocytes to normal neurons did not induce motor neuron death suggesting that the supporting cells play a role in the disease, but are not the cause (Boillee et al., 2006a; Boillee et al., 2006b). Some current therapies are being used to target microglial activation. Minocycline, a broad spectrum antibiotic, has been proven to inhibit microglial activation in vivo (Van Den Bosch et al., 2002; Zhu et al., 2002) and its use for treating ALS has been proposed for clinical investigation.

1.2.8. ALS and cellular respiration

The role of mitochondria in the pathology of ALS is not entirely clear, but the presence of vacuolated mitochondria in ALS patients and mouse models (Dalcanto and Gurney, 1995; Wong et al., 1995) and the concomitant interruption of cellular respiration in the presence of mutant SOD1 provides a strong indication that the organelle is involved in the disease process. Specifically, cellular respiration is reduced significantly from both decreased activity of cytochrome oxidase, leading to decreased ATP production, and release of cytochrome c in response to oxidative stress and caspase activation (Jung et al., 2002). Moreover, mutant SOD1 has been shown to bind within
the mitochondrial inner membrane space in spinal cord motor neurons (MNs) (Higgins et al., 2002). Lastly, partial deficiency in the mitochondrial matrix manganese-containing SOD2 enzyme exacerbated ALS-like pathology in SOD1 mutant mice (Zimmerman et al., 2007). Current therapies for ALS targeted to mitochondria include the non-protein amino acid creatine, to enhance mitochondrial energy storage (Adhihetty and Beal, 2008; Klivenyi et al., 1999), and the antibiotic minocycline. Minocycline, a tetracycline derivative previously shown to inhibit microglial activation, has also been proven to inhibit the release of cytochrome c from mitochondria; a final step of programmed cell death (Kriz et al., 2002). Despite promising effects in mice, neither therapy has provided patients with alleviation of symptoms or extended lifespan.

1.2.9. ALS and axonal transport

Another deficiency in ALS, impaired axonal transport, is common to neurodegenerative diseases. Given the asymmetrical nature of motor neurons, an enormous metabolic load is placed on cell bodies to transport molecules as far as 1 meter (fast and slow transport) to and from synapses (Bruijn et al., 2004b). The retrograde, dynein-mediated, transport of components such as multivesicular bodies and trophic factors (NGF, VEGF) back to the cell body is impaired by mutant SOD-1, leading to the conclusion that diminished transport correlates well with the development of motor neuron disease (Williamson et al., 1998). Dynein has many roles, including positioning of the ER and Golgi, and the assembly of the mitotic spindle. Two point mutations have been identified in dynein that cause motor neuron disease (Haiferast et al., 2003). Moreover, the disruption of the dynactin complex, an activator of cytoplasmic dynein, inhibits retrograde axonal transport and leads to a late-onset progressive motor neuron disease (King and Schroer, 2000). A single point mutation in the p150 subunit of dynactin has similarly been linked to a lower motor neuron disorder (Puls et al., 2003).
1.2.10. ALS and the vascular system

Unexpected risk factors contributing to ALS have been revealed in growth and neurotrophic factors. Since astrocytes and microglia are such important sources of these factors, damage to supporting cells and interruption of the trophic support provided by them is a primary underlying cause of motor neurodegeneration. VEGF, a growth factor known to control the growth and permeability of blood vessels and to be protective of motor neurons, has been found to induce motor neuron disease following targeted deletion of the hypoxia-response element (Oosthuysse et al., 2001). Under normal conditions, hypoxia will induce VEGF expression under transcription factors that respond to the oxygen tension in order to maintain or restore vascular perfusion and trigger the growth of blood vessels. If this function is ablated in mice, however, classic ALS symptoms will ensue within 5 months of age (Lambrechts et al., 2003).

Neurotrophic factors, on the other hand, though not shown to cause ALS pathology can ameliorate symptoms in mice. The most prominent of these factors are CNTF (Anand et al., 1995), GDNF (Elliott and Snider, 1996), and IGF-1 (Oppenheim, 1996), all of which have been used for retrograde AAV delivery from muscle to motor neurons, though the only positive effects seen on disease onset and survival were attributed to IGF-1 (Kaspar et al., 2003).

1.2.11. ALS and the blood brain barrier

Recently, it was discovered that the blood brain barrier is compromised in ALS patients (Garbuzova-Davis et al., 2007c). Indeed, impairment of the BBB occurs in most neurodegenerative diseases (Popescu et al., 2009). Capillary endothelial cells are distributed along the length of the vessels, completely encircling the lumen and connected via adherents and tight junctions to form a diffusion barrier into the brain for most blood-borne substances (Ueno, 2007). Increasing evidence points to immune system involvement in ALS pathogenesis. In the spinal cord and brain of both ALS patients and animal models, the presence of T-cell lymphocytes (Alexianu et al., 2001;
Engelhardt et al., 1993a), deposits of IgG (Donnenfeld et al., 1984a; Engelhardt et al., 2005b), complement components C3 and C4 (Engelhardt and Appel, 1990), and monocyte/macrophage and dendritic cells (Troost et al., 1990a) were observed that may have a critical role in motor neuron damage. IgG was detected in the perikarya of motor neurons of the lumbar spinal cord in mice after intraperitoneal injection of IgG derived from sera of ALS patients. Moreover, the uptake of IgG in multivesicular bodies in endothelial cells in the affected areas of the spinal cord was found in both ALS patients and mice injected with human ALS IgG. Additionally, significantly increased levels of albumin, IgG, and C3c have been noted in the cerebrospinal fluid of ALS patients (Annunziata and Volpi, 1985a; Leonardi et al., 1984a). These findings suggest that barrier permeability is affected in ALS. These phenomena were also identified in spinal cord microvessels of G93A mice at early (13 weeks of age) and late (17–18 weeks of age) stage disease, although more leakage, visible with Evans Blue dye, was found in lumbar spinal cords of mice at terminal stage disease. Additionally, basement membrane disruption was noted at both early and late stage disease, as shown by the loss of laminin staining in the G93A mice. Down regulation of Glut-1 and CD146 expressions in spinal cord endothelial cells was also found in G93A mice at early and late stage disease, suggesting an altered endothelial lining leading to vascular leakage (Garbuzova-Davis et al., 2007c)

1.2.12. Environmental components of ALS

Although unexplained, there have been environmental links to acquiring ALS. First described by Mulder and Kurland in 1987, there are “clusters” of the disease in given populations that increase the chance of developing ALS. In the 1950’s for example, there was a 50-fold increase in developing ALS on the island of Guam. More recently there has been a 2 fold increase in ALS patients returning from duty in the Gulf Wars (Haley, 2003; Horner et al., 2003). Additionally, it was reported that ALS was unusually common amongst Italian soccer players (Belli and Vanacore, 2005; Vanacore et al., 2006). Factors such as length of time playing/fighting, intensity of exercise, and
use of drugs or supplements were taken into account, though nothing was conclusively identified as causing ALS.

1.2.13. ALS and neuronal death

Apoptosis or PCD, is a common pathway for neuronal death in neurodegenerative disease and is the final event in ALS. As a presymptomatic event, Caspase 1 was shown to be activated in neurons (Pasinelli et al., 2000; Vukosavic et al., 2000). Activation of caspase 1 is later followed by activation of caspase 3 in astrocytes and motor neurons (Guegan et al., 2001). Caspase 3 subsequently triggers the release of cytochrome C from the mitochondrial respiratory chain, which activates caspase 9, known as “intrinsic” caspase activation. Caspase 9 may act as an effector for subsequent activation of caspase 7 and increased release/activation of caspase 3 (executioner caspases), which are responsible for the degradation of cellular constituents and cell death. Alternatively, apoptosis can be stimulated “extrinsically” by extracellular queues activating caspase 8 followed by caspase 3 and then following the same path as the intrinsic pathway. Further evidence to support apoptosis in ALS was determined by long-term, intrathecal delivery of the pan-caspase inhibitor (N-benzylocarbonyl-Val-Ala-Asp- fluoromethylketone) or zVAD-fmk, which significantly slowed the progression of the disease in G93A mice (Li et al., 2000). Further support for apoptosis in ALS was demonstrated by BCL-2 overexpression in this same model. BCL-2 (B-cell lymphoma 2) is a known anti-apoptotic factor that slowed onset and promoted survival of G93A mice to a similar extent as the pan-caspase inhibitor zVAD-fmk (Kostic et al., 1997).

1.3. Alzheimer’s Disease

1.3.1. Alzheimer’s Pathology

Alzheimer’s disease is currently the most common form of neurodegeneration, affecting over 5 million people nationwide. Approximately 1 person in 8 will be
diagnosed with AD by the age of 65, and 1 in 2 by the age of 85 (National Institutes of Health U.S. Department of Health and Human Services). The disease is characterized, initially, by loss of short term memory and attention span, although affected individuals function with relative normal capacity for up to 3 years following diagnosis. Typically, individuals die within 7-10 years of AD diagnosis, during which time they gradually withdraw from daily life. Slowly and inexorably, the inability to remember names and follow conversations culminates to the inability to carry out familiar tasks, to reason, and to exercise judgment. It is a gradual, but severe cognitive decline, leading ultimately to dementia and death. The cause of death, in most cases, is AD directly, but some AD patients die of cancer, stroke, heart disease, and pneumonia as their general health deteriorates.

Pathologically, AD is characterized by neuronal loss with extracellular deposits of amyloid and intracellular accumulation of neurofibrillary tangles in the hippocampus, amygdala, and cerebral cortex (Selkoe, 1986). Curiously, these classical hallmarks of AD were already described in 1907 by Alois Alzheimer himself following an autopsy of a woman who had died with severe dementia. It was not until 75 years later, however, that the biochemical tools were developed to determine the components of these proteinaceous aggregates (Glennar and Wong 1984)( reviewed by Avila et al., 2004). Moreover, it has since been discovered that other processes common to neurodegeneration including excitotoxicity, oxidative stress, inflammation, and apoptosis all have a role in the pathogenesis of AD.

1.3.2. Major Genes and Proteins in AD

Several proteins have been identified within the amyloid plaques including Aβ, apolipoprotein E (apoE) (Wisniewski and Frangione, 1992) and Alpha 1-antichymotrypsin (ACT) (Abraham et al., 1988), LRP (low density lipoprotein receptor-related protein), activated compliment, and numerous cytokines. Aβ is the primary component of the plaques and is a 38-43 amino acid peptide proteolytically derived from the amyloid precursor protein (APP) (Glennar and Wong, 1984)(Hardy and Selkoe,
APP is a transmembrane protein of unknown function expressed in almost all tissues. In mammals, there are three major isoforms, APP695, APP751, and APP770, which vary by the presence or absence of the Kunitz protease inhibitor (KPI) domain and a chondroitin sulfate glycosaminoglycan (CS GAG) attachment site (Sisodia and Price, 1995). In the brain, primarily APP695 is expressed on the surface of neurons.

APP is processed proteolytically by three enzymes, α, β, and γ-secretases to generate either amyloidogenic or non-amyloidogenic peptides. During amyloidogenic processing, APP is cleaved first by β-secretase, releasing a large N-terminal ectodomain, sAPPβ, into the extracellular space. The remaining C-terminal fragment of 99 amino acids still bound is then cleaved by γ-secretase in the cell membrane to yield Aβ at 39, 40, or 42 amino acids in length. γ-Secretase cleavage between Val637 (in the APP695 isoform) and Ile638 generates Aβ40, and cleavage after Ala639 results in production of Aβ42. The C terminus remaining in the membrane would be expected to be 59 or 57 amino acids in length, respectively, although only a 50 amino acid sequence has been identified. This APP intracellular domain (AICD) presumably plays an important role in the regulation of multiple genes that are involved in the disease development (Slomnicki and Leśniak 2008). Gamma-secretase has been discovered to exert further action by cleaving at the ε-site of APP (Leu646-Val647), followed by cleavage at the ζ-site (Val643 and Ile644). Finally the peptide is cut at the γ-site, releasing both Aβ and AICD. In the non-amyloidogenic pathway, the production of Aβ peptides is precluded because the APP ectodomain is cleaved by α-secretase at the α-site of APP (Lys613-Leu614) which resides within the Aβ sequence. α-secretase cleaves APP into the released ectodomain (sAPPα) and a remaining membrane bound fragment, C83. The remaining fragment is then processed by γ-secretase, similar to the amyloidogenic pathway, to generate AICD.

Some mutations in the APP gene result in an increased production of pathogenic forms of Aβ (Tanzi et al., 1991). To date there are 160 known mutations in APP and related cleavage enzymes, presenilin 1 (PS1) and presenilin 2 (PS2), that lead to familial AD. In fact, most inherited forms of AD result from mutations in PS1. Together with APH-1 (anterior pharyx defective 1 protein) and nicastrin, the presenilins constitute an integral part of the γ-Secretase, an aspartyl protease with low specificity that cleaves
substrates within the sequence of their transmembrane domains. PS1 has nine transmembrane spanning domains and is thought to harbor the active site of \( \gamma \)-secretase. Two highly conserved aspartate residues in PS1 (257 and 385) within the 6\(^{th}\) and 7\(^{th}\) transmembrane domains of PS1 comprise the catalytic core of the enzyme. Nicastrin is necessary for the proper assembly of the \( \gamma \)-secretase complex and has been proposed to modulate the traffic of substrates to the active site, and to serve as a substrate receptor (Wolfe, 2008). The functions of APH1 and PEN2 are not fully elucidated. APH1 has been implicated in stabilizing PS1 and plays a role during assembly of the \( \gamma \)-Secretase complex, while PEN2 stabilizes the final complex and is involved in PS1 endoproteolysis (Spasic and Annaert, 2008). Because processing of APP is effected by secretase activity, mutations in individual components of this protein complex can lead to increases in A\( \beta \) similar to mutations in APP.

ApoE4 is the major genetic risk factor for Alzheimer’s disease. While it serves systemically as a lipid transport protein, in the brain, ApoE is released by astrocytes and microglia as part of an immune response. It also plays a key role in the maintenance and repair of neurons. The \( \varepsilon \)4 allele of ApoE confers the greatest risk of AD at the lowest age of onset. ApoE2, on the other hand, is associated with the lowest risk and highest age of onset, and apoE3 with intermediate risk and age of onset (Strittmatter et al., 1993). The exact mechanism behind the effect of ApoE isoforms as risk factors for AD remains under investigation.

1.3.3. Extracellular Aggregates in AD

A\( \beta \) is prone to aggregation under physiological conditions. The C-terminal 12 amino acid residues from the transmembrane region of the A\( \beta \) peptide are hydrophobic and promote self-aggregation and polymerization into amyloid fibrils. The use of “\( \beta \)” in A\( \beta \) denotes its propensity to form cross \( \beta \)-sheet arrays and polymerize into amyloid fibrils. Specifically, 1-42 has a stronger propensity for aggregation than the more common 1-40 peptide and is the key seed-forming A\( \beta \) species in amyloid fibrils and plaques.
Aβ peptides behave aberrantly in AD, likely leading to a protein misfolding event that serves to nucleate further misfolding. Because of the numerous ways that an amino acid sequence can orient in 3 dimensions, misfolding of proteins is a common event in cells. The first line of defense against the formation of misfolded proteins is their prevention via the utilization of molecular chaperones. Chaperones associate with nascent polypeptides during translation as they emerge from the ribosome and promote proper folding. In the event that a protein is misfolded faster than a chaperone can prevent, a second line of defense, the ubiquitin/proteasome system, is employed. Misfolded proteins are ubiquitinylated and thereby (in most cases) labeled for degradation by the proteasome. Both chaperones and ubiquitin are found in amyloid plaques (as well as intracellular tangles), suggesting that these systems were overwhelmed in affected cells (Pallares-Trujillo et al., 1998).

Alzheimer’s amyloid fibrils are filamentous structures comprised of Aβ about 10 nm wide and 100-1000 nm long. Previous studies using x-ray fiber diffraction analysis originally determined the presence of a β-sheet structure within fibrils. These ribbon-like β sheets form when Aβ peptides align perpendicular to the length of the filament and are held together with hydrogen bonds that run parallel to the length of the fibril. These fibrils then aggregate with themselves and other molecules, such as Apolipoprotein E (ApoE)(Wisniewski and Frangione, 1992) and ACT (Ma et al., 1994), to form insoluble amyloid plaques that average 50μm in diameter (Rambaran and Serpell, 2008).

Amyloid plaques have been the focus of AD research for over 100 years. However, more recent data suggest that it might not be mainly the plaques that are neurotoxic, but the more soluble forms of Aβ. Oligomers, trimers, and dimers of Aβ have been shown to be toxic to cultured neurons since they can readily pass through the cell membrane and cause neuronal damage (Walsh and Selkoe, 2007). Moreover, it is unclear if disaggregation of plaques could possibly lead to an increase in soluble Aβ species. In either view, however, it is clear that the toxic starter molecule is Aβ, whether in aggregates, oligomers or, most likely, both. Therapies aimed at reducing monomeric Aβ would reduce the amount available for oligomers and fibrils alike.
1.3.4. AD and the cytoskeleton (tau)

In addition to extracellular plaques, another central hallmark of Alzheimer’s disease is the accumulation of intracellular aggregates of hyperphosphorylated tau. Tau is a cytosolic protein involved in the assembly and stability of microtubules. In AD and other tauopathies, tau metabolism is altered by overexpression of tau, mis-sense mutations, and posttranslational modifications. Upon phosphorylation, tau undergoes misfolding events, dissociates from microtubules, and aggregates into filaments (reviewed in Chung, 2009). These filaments are the primary component of neurofibrillary tangles which disrupt the cytoskeleton and inhibit axonal transport, leading to cell death as reviewed (Gendron and Petrucelli, 2009).

Tau is encoded by 16 exons located on chromosome 17 and is primarily expressed in neurons. Exons 2, 3, and 10 are alternatively spliced, leading to 6 isoforms of tau. Exon 10 encodes one of the regions involved in the binding of tau to microtubules, and alternative splicing of this exon produces tau isoforms containing either three (tau 3R without exon 10) or four (tau 4R with exon 10) microtubule binding domains (Andreadis, 2005). The contribution of the various tau isoforms to the development of tauopathies is evidenced by several tau mutations that cause frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Kar et al., 2005). In this disease, an increase in the splice variant of exon 10 leads to an elevated ratio of 4R with respect to 3R isoforms. This effect is mainly attributed to intronic mutations that result in the forced expression of exon 10. In contrast, in Pick’s disease tau mutations have been identified that lead to decreased expression of exon 10. Aggregates are predominantly tau filaments composed of the 3R isoform. In AD, no mutations causing changes in tau isoforms have been identified, although increased expression of the 4R tau isoform has been detected in the affected brain region. Imbalance among tau isoforms (3R:4R) and the types of neurons involved are distinct for each tauopathy (Hernandez and Avila, 2007).

Several phosphorylation sites have been identified in tauopathies and respective kinases are implicated in the disease pathogenesis. Primarily, tau is phosphorylated by glycogen synthase kinase 3 (GSK 3), cdk5, CAMKII (Wang et al., 2007) and less
commonly by ERK1, CK1, and PKA (Li et al., 2004; Liu et al., 2006; Pei et al., 2002; Trojanowski et al., 1993). In total, 45 phosphorylation sites have been identified that contribute to AD pathogenesis. They represent more than half of the total 85 phosphorylation sites identified on tau. Regulation of tau also occurs by dephosphorylation via PP2A and, to a lesser extent, PP1 and PP2B. Pin-1, a member of the peptidyl-prolyl cis-trans isomerase family of proteins, is involved in the assembly, folding and transport of certain proteins, including tau. Pin1 binds tau when it is phosphorylated at the AD-related thr 231 residue and facilitates its dephosphorylation by PP2A (Butterfield et al., 2006). It is therefore through phosphorylation and dephosphorylation events that tau can be regulated in the CNS and effect aggregation.

1.3.5. Major Mouse Models of AD

In order to study Alzheimer’s disease, many different strains of transgenic mouse models have been developed over the past several years. The animals have been generated following the identification of numerous genes involved in familial (early onset) AD, where the patient develops the disease before the age of 65. In some studies, Tg2576 mice were used, which harbor the “Swedish” mutations in the Amyloid Precursor Protein (APP) gene (Mullan et al., 1992). These mutations (K670D and M671L) lead to increased processing of the APP gene, resulting in the extracellular accumulation of peptides Aβ1-40 and Aβ1-42, the latter of which is more toxic. One of the main pathological hallmarks of AD is the formation of neuritic plaques, termed amyloid, found primarily in the hippocampus and cortex and comprised predominantly of the Aβ peptides. Tg2576 mice also show cognitive impairment similar to that of AD patients. Typically, plaques are detectable in their brains at approximately 12 months of age and correlate with behavioral deficits (Dewachter et al., 2001).

Mouse models of Alzheimer’s disease have been generated for many of the APP and PS1 mutations identified in the human population. Another common model is based on an APP gene in which valine is encoded instead of a phenylalanine at position 717, known as the “London” mutation (V717F) (Crawford et al., 1991). The PDAPP mouse is
used throughout the experiments presented here. There are also mutations in PS1 and PS2 genes that give rise to altered processivity of APP and increased generation of Aβ peptides and correlating plaque load. PS1 M146L is the model used in our laboratory. Double or triple transgenic mice harbor several of these mutated genes to determine the roles of these proteins in AD and aid in the design of therapeutics.

Additionally, transgenic mice have been generated to model the other hallmark of AD- tauopathy. htau mice (B6.Cg-Maptm1(GFP)Klt Tg(MAPT)8cPdav/J) are homozygous for knock out of the targeted endogenous mtau alleles and hemizygous for the transgene. Although no endogenous mtau is detected, all six isoforms (including both 3R and 4R forms) of human MAPT are expressed. Hyperphosphorylated tau is detected in cell bodies and dendrites by three months of age. Paired helical filaments of aggregated insoluble MAPT can be isolated from brain tissue as early as two months of age (Andorfer et al., 2003).

1.3.6. AD and inflammation

ApoE and ACT are a part of a local inflammatory process in the brain. Aβ, in turn, has domains that bind ACT and ApoE, and these proteins serve to catalyze the conversion of Aβ into filaments in vitro (Ma et al., 1996; Wisniewski et al., 1994) and in vivo to form amyloid deposits in the formation of beta-pleated sheets (Bales et al., 1999; Nilsson et al., 2001).

ACT is a 68 kDa acute phase serum glycoprotein from the family of serine protease inhibitors (serpins). Systemically, it is released by hepatocytes and monocytes in response to inflammation, and is released by neutrophils to function as a suicide inhibitor of cathepsin G (Kalsheker, 1996). In the brain, ACT is known to be produced by and released from astrocytes in response to IL-1β stimulation by microglia, following trauma, infection, or amyloidoses (Abraham, 2001; Das and Potter, 1995). ACT is highly upregulated in AD brain and is involved both in the formation of amyloid plaques (Abraham et al., 1988) and in the phosphorylation of tau (Padmanabhan et al., 2006).
Microglia have been traditionally regarded as resident CNS macrophages. They comprise about 10% of the adult CNS cell population (Pessac et al., 2001). Microglia normally remain in a quiescent state in the healthy CNS, and are characterized morphologically by small cell bodies and elaborate processes. However, upon “activation” in response to infection or CNS injury, microglia experience a change in morphology including shortening of cellular processes and enlargement of their somas. Activated microglia up-regulate a host of cell surface activation antigens (major histocompatibility complex (MHC) class I and II, B7.1, B7.2, and CD40) and produce innate cytokines and chemokines (e.g., tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-12, and IL-18 (Janeway and Medzhitov, 2002) (Medzhitov and Janeway, 2000).

1.3.7. **GM-CSF and Alzheimer’s disease**

Numerous studies have found Rheumatoid arthritis (RA) to be a negative risk factor for Alzheimer’s disease (AD) pathogenesis (Myllykangas-Luosujarvi and Isomaki, 1994), but the mechanisms for RA’s protective effect are unknown. Although two decades of research have intensely focused on anti-inflammatory approaches against AD pathogenesis, NSAID clinical trials have failed to show therapeutic benefit in AD. This suggests that cyclooxygenase 2 activation is not the causative agent for the inverse relationship between RA and AD (McGeer et al., 2006). This outcome suggests that it might be intrinsic factors within RA pathogenesis that protect against AD. Because RA is a peripheral disease that prevents the onset of a neurodegenerative disease, the most logical approach suggests immunological phenomena. Keeping in mind that it seems unlikely for specific secreted factors to be produced in the synovial pannus and maintain sufficient concentrations during travel through the circulatory system and across the blood brain barrier to inhibit AD pathogenesis, we determined that up-regulation of local cellular populations in RA would have the highest potential to enter into the brain and inhibit the development of AD pathology and/or neuronal dysfunction. We therefore
focused our attention primarily on leukocytes involved in both diseases, and secondarily on their secreted inflammatory mediators.

1.3.8. Environmental components of AD

Similar to ALS, sporadic AD cases are thought to have an environmental component in addition to genetic risk factors. It is generally believed that individuals who remain physically and mentally active, maintain strong social ties, and have a history of higher education are less likely to develop AD. Accordingly, groups that are isolated and have limited social interactions have a strong propensity to develop the disease. Studies with transgenic mice (PS/APP) in enriched environments (large cages, larger groups of animals, toys) compared to standard housing (individually caged), indicated that the enriched animals performed better in working memory tasks (RAWM) than controls (Costa et al., 2007). In addition to lifestyle, nutritional components have also been implicated in conferring risks or benefits to the development of AD. For example, Resveratrol, a polyphenol in red wine, epigallocatechin-3-gallate (EGCG) in green tea, caffeine, and moderate ethanol consumption have all been shown to decrease the likelihood of developing AD (Arendash et al., 2006; Marambaud et al., 2005; Rezai-Zadeh et al., 2008). However, their exact mechanisms of interference have still to be determined.

1.3.9. AD and the vascular system

The importance of environmental and medical components for the prevention and cure of Alzheimer’s disease leads to the question of how the blood brain barrier (BBB) is involved in the development and progression of Alzheimer’s disease. It has been known that the BBB in AD, similar to ALS, is compromised. In fact, vascular dysfunction has been implicated in the progression of AD patients for over 20 years (Kalaria, 1992). Cerebral hypoperfusion and impaired LRP or ApoE-mediated transport of Aβ across the BBB likely contribute to the progression of AD as well. In cerebral amyloid angiopathy
(CAA), amyloid deposits are found lining the microvessels within the brain, potentially decreasing cerebral blood flow (CBF). Recent data from clinical imaging experiments (MRI, SPECT) have shown that AD patients have a resting CBF that is significantly reduced when compared to healthy controls (Bell and Zlokovic, 2009). The selective permeability of the BBB to certain molecules is of critical importance for maintaining homeostasis within the CNS. Disruption of this barrier, as seen in ALS and AD, can inhibit normal transport of molecules to and from the brain, or deteriorate and allow indiscriminate interaction between the brain and periphery, exacerbating inflammation.

1.3.10. AD and oxidative stress

Both fibrillar and oligomeric Aβ species stimulate the formation of reactive oxygen species, including free radicals, within neurons (Moreira et al., 2008). Oxidative stress occurs when the antioxidant defense system is overpowered by free radicals. Oxygen and nitrogen based molecules with unpaired electrons are very unstable and highly reactive. If they are not removed properly, they will react with lipids, proteins, and nucleic acids and disrupt membrane fluidity, enzymatic activity, and cause damage to organelles and DNA. Alternatively, dysfunctional mitochondria could alter enzymatic activity in the antioxidant defense system. Either way, there is a clear role of oxidative stress in AD pathogenesis as reviewed (Swerdlow, 2009).

1.3.11. Integration of many pathways

Although it has been known for quite some time that both plaque and tangle pathologies exist in AD patients, it has not always been clear if the events occur independently or in close relationship. It is now known that Aβ can interact with neurons to induce kinase activity and tau phosphorylation (Zheng et al., 2002). Moreover, Aβ has been shown to exert similar effects intracellularly (Laferla et al., 2006). Therefore, according to the amyloid cascade hypothesis of AD (Hardy, 2006), the
overproduction of Aβ leads directly to the formation of amyloid plaques and triggers intracellular aggregation of tau, leading to neuronal death.

Other evidence also supports the fact that amyloid and tau pathologies occur not completely independently. ACT, a protein previously described to be upregulated in the CSF of AD patients and a component of amyloid plaques, has recently been shown to induce tau pathology. Cortical neurons isolated from human embryos and neonatal mice treated with ACT showed increased tau phosphorylation at the AD relevant sites ser 202, thr 231, and thr 396/404 (PHF-1) that was triggered by the cellular kinases ERK1 and GSK3 (Padmanabhan et al., 2006). Moreover, it has been shown that ACT expression is upregulated in other tauopathies (FTDB, PSP, CBD). Collectively, these data support that ACT can induce the phosphorylation of tau, providing another pathological link between the seminal hallmarks of AD, namely plaques and tangles.

1.3.12. AD and neuronal death

Apoptosis is the last pathway activated in AD and other neurodegenerative diseases, other biochemical processes are activated earlier in the disease progression. Like ALS, apoptosis can be activated by the intrinsic and extrinsic pathways and is the final event in the disease progression.

1.4. Research Rationale- Amyotrophic Lateral Sclerosis

As described above, Amyotrophic Lateral Sclerosis and Alzheimer’s disease are neurodegenerative diseases that share certain features and therefore might be prevented or cured with related procedures or medications. It is with this perspective that I focused my research on the promotion and inhibition of protein aggregation in Amyotrophic Lateral Sclerosis and Alzheimer’s disease.
1.4.1. Transmission and induction of ALS

Proteinopathies, i.e. diseases that are based on diverse forms of protein aggregation and misfolding or the secondary modifications that are based on those features, can be caused by environmental and/or genetic components. For example, prion diseases involve the transformation of specific proteins into more aggregate-prone conformations, leading to the nucleation of beta-pleated sheet structures.

Based on the transmissible nature of prion diseases after ingestion of a prion protein and given the aggressive and progressive nature of ALS, we sought to determine if the intracellular inclusions in ALS that contain SOD1, ubiquitin, and neurofilaments, could be isolated from the spinal cords of affected transgenic ALS mouse models and injected into young transgenic mice that do not yet carry the pathogenic form of SOD1. The goal was to assess if the disease could be induced in the host. If these high molecular weight proteins could be isolated from ALS mice and delivered via a single bolus injection into the lateral ventricles of recipient mice, new avenues might be opened to study this devastating disease.

The literature on spinal cord diseases reported the route of protein delivery we had thought out for this experiment as rather problematic. Because the CNS relies on passive diffusion and the animal’s movement to circulate CSF, delivering small amounts of a test substance into the lateral ventricles was not likely to diffuse the substance in sufficient quantity to the lumbar cistern- the initial site of degeneration in ALS. Additionally, it was questionable if exposure to a single injection could trigger a response in the animals. For these reasons, we preferred to develop a technique allowing chronic infusion of homogenates into the lumbar cistern at L3 and L4. Catheters were fashioned to be placed directly into the intrathecal space, sutured into place, and attached to osmotic minipumps for 2 and 4 week delivery. Changes in motor ability (rotarod, latency to/ability to turn over) as well as life-span and enhancement of pathology (increased inclusions and vacuolization of dysfunctional mitochondria) were investigated.
1.4.2. Disruption of the blood brain barrier

The blood-brain barrier (BBB), blood-spinal cord barrier (BSCB), and blood-cerebrospinal fluid barrier (BCSFB) control cerebral/spinal cord homeostasis by selective transport of molecules and cells. In the spinal cord and brain of both ALS patients and animal models, infiltration of T-cell lymphocytes, monocyte-derived macrophages and dendritic cells, and IgG deposits have been observed that may have a critical role in motor neuron damage. Additionally, increased levels of albumin and IgG have been found in the cerebrospinal fluid in ALS patients. These findings suggest altered barrier permeability in ALS. Recently, using electron microscopy, we showed disruption of the BBB and BSCB in areas of motor neuron degeneration in the brain and spinal cord in G93ASOD1 mice modeling ALS at both early and late stages of disease (Garbuzova-Davis et al., 2007c). Examination of the capillary ultrastructure revealed endothelial cell degeneration, which, along with astrocyte alteration, compromised the BBB and BSCB. However, the effect of these alterations upon barrier function in ALS is still unclear. The aim of this study was to determine the functional competence of the BSCB in G93A mice at different stages of disease.

1.4.3. Neuroprotective effects of DTG (1,3-di-o-tolylguanidine)

DTG (1,3-di-o-tolylguanidine) is a high affinity sigma receptor agonist that has often been reported to ameliorate stroke damage in rodents as reviewed (Willing and Pennypacker, 2007). We therefore thought to investigate if DTG could serve as a potential treatment to reduce the robust pathology in ALS mouse models. Because DTG was known to pass the blood brain barrier (BBB), direct delivery into the CNS was not required and subcutaneous release over a period of 28 days was expected to be sufficient. In mouse models of stroke, DTG had been reported to block the inflammatory response evoked by MCAO, as measured by the decreased number of reactive astrocytes and activated microglia (Ajmo et al., 2006). Neuronal survival is specifically enhanced if DTG is administered 24 hours after an ischemic stroke. Because the efficacy of DTG for
stroke treatment was likely the result of combined neuroprotective and anti-inflammatory properties of sigma receptors, and because these receptors are found ubiquitously in the CNS, we hypothesized that enhancing the activity of sigma receptors might provide an effective means for the treatment of ALS as well.

1.4.4. Novel therapeutic laminin derivative, KDI

Laminin is a heterotrimeric extracellular matrix glycoprotein involved in numerous biological processes including cell adhesion, differentiation, migration, and, most importantly, neurite outgrowth. Our collaborator, Paivi Liesi, elucidated that it is, in fact, the soluble tripeptide fragment composed of the amino acids lysine, aspartic acid and isoleucine (KDI) at the carboxy terminus of the laminin γ-chain that promotes neurite outgrowth (Liebkind et al., 2003). They showed that KDI could protect the brain from a kainic acid induced lesion in a dose dependent manner (Wiksten et al., 2004a) and that local administration of KDI to a fully transected rat spinal cord would actually heal the tissue, promote the growth of neurons through the glial scar, and restore lower limb function to the animals (Wiksten et al., 2004a). Because the pharmacokinetics of this tripeptide had not yet been established, direct delivery into the intrathecal space (lumbar cistern) via customized catheters instead of systemic application was desired. Kainate is a glutamate analog and glutamate excitotoxicity (dysregulation) is a common feature of both sporadic and familial ALS. The prospect of using KDI to treat ALS was therefore worthwhile for 2 reasons: Neuroprotection from dysregulated glutamate excitotoxicity and re-growth of damaged motor neurons via laminins and integrins.

1.5. Research Rationale- Alzheimer’s Disease

1.5.1. Development of a bilateral infusion system in mice

Many lines of transgenic mice harboring the gene for human amyloid precursor protein (APP) with different mutations causing familial Alzheimer’s disease have been
developed over the past decade to study plaque deposition and other aspects of AD. However, variations in size, density (fibrillar and dense core), plaque number, and total amyloid load between animals of the same age and genotype have been reported, and significant pathological analyses thus often require very large numbers of mice. Therefore, to study AD therapies for example, it was imperative to develop a technique that would allow each brain hemisphere to receive different infusions. To this end, we developed a catheter technique that facilitates simultaneous bilateral infusion in mouse brains, thereby using the contralateral hemisphere of the same animal as an internal control. The infusions occurred over a period of 28 days with less than 5% mortality.

1.5.2. Intracranial infusion of KDI, a glutamate receptor antagonist and electroporation of a KDI expressing plasmid

A) Memantine was the first in a novel class of Alzheimer's disease medications that acts on the glutamatergic system by blocking NMDA glutamate receptors. Blocking these receptors causes an upregulation in dormant AMPA receptors in post-synaptic neurons. In the dentate gyrus, this upregulation is part of the process of neurogenesis. Trying KDI as potential therapeutic for Alzheimer’s disease was based on the non-competitive, ionotropic glutamate receptor antagonist properties of KDI. KDI was infused intracranially to induce its effect while conferring general neuroprotection to increased glutamate levels.

B) In vivo electroporation has proven to be an effective means of delivering plasmids to virtually any tissue or organ without the need for attenuated viral vectors. The nucleotides encoding the six amino acids representing the longer c-terminus of γ-1 laminin (EIIKDI) were successfully cloned into PCDNA 3.1+. After optimizing the electroporation conditions with an EGFP expression vector, the plasmid encoding the KDI-peptide was introduced for sustained expression into the hippocampus of PS/APP mice via electroporation.
1.5.3. Alzheimer\'s Disease and α-1 antichymotrypsin

1.5.3.1. ACT and tauopathies

Recent work in our lab has shown, in vitro, that tau becomes phosphorylated at several AD-related sites when primary neuronal cultures are incubated with ACT (Padmanabhan et al., 2006). In other, non-Alzheimer\’s human tauopathies, ACT is also upregulated, suggesting that it may have a role in tying together the mayor players in Alzheimer\’s pathology, i.e. amyloid and tau. In this experiment, the same technique was employed as previously (bilateral infusions), but the infusions were performed in different transgenic animals. Here I used mice expressing the human tau protein (htau), and knocked out for the endogenous mouse tau (mtau), to determine if ACT has a similar effect on the phosphorylation of tau in vivo as was shown in cell culture, potentially leading to NFTs.

1.5.3.2. Inhibition of the ACT-Aβ interaction

ACT is a serine protease inhibitor that has been show to act as a pathological chaperone for the development of Alzheimer\’s disease. ACT promotes the fibrillization of Aβ in vitro, is upregulated in the CSF of AD patients and is an integral part of amyloid plaques. It is not known, however, how ACT reacts with existing plaques. While transgenic animals are useful for studying these phenomena, they have two disadvantages: they are constructed to overexpress the transgene throughout their lifetime and show a strong variability in plaque load between animals. To view ACT-induced changes in plaque number, plaque size, and plaque morphology and to allow direct comparison in the same animal, ACT was infused unilaterally into the parenchyma of the (ipsilateral) hippocampus and CSF as control into the (contralateral) hippocampus of the same animal. The purpose of this experiment, ultimately, is to test various compounds for their ability to inhibit the ACT-Aβ interaction. Previous in vitro work showed that fragments of Aβ, when incubated with ACT or ApoE and full length Aβ, can
inhibit the formation of fibrils and increase survival of transfected cells (Ma et al., 1996). In this experiment, fragment 1-11 which is known to be the ACT binding fragment of Aβ, was co-infused with ACT into PS/APP mice to disrupt amyloid formation. Other experiments entailed infusion of the 1-11 peptide alone to see if it might inhibit the endogenous “ACT-equivalent” in the mouse.

1.5.4. Colony stimulating factors and amyloid clearance and cognitive enhancement

Numerous studies have found Rheumatoid arthritis (RA) to be a negative risk factor for Alzheimer’s disease (AD), but the mechanisms for RA’s protective effect are unknown. Much research has proposed that medication with NSAIDs, which is common for RA patients, is protective against AD. However most NSAID clinical trials have proven unsuccessful in AD patients and have therefore led to the hypothesis that intrinsic components of RA pathogenesis may be protective against AD. In these experiments, we proposed that cytokines are upregulated in RA and confer neuroprotection to AD. Multiple colony stimulating factors that induce leukocyte populations and are involved in RA’s pathogenesis, such as GM-CSF, are upregulated in RA patients and were the natural target of our investigation. First we performed bolus intrahippocampal administration of rmGM-CSF into one hemisphere and control aCSF into the other hemisphere to compare plaque load and reactive gliosis. In a second approach we used sub-cutaneous rmGM-CSF injection into aged cognitively-impaired PS/APP mice in an attempt to reverse the cognitive impairment. Both standard radial-arm water maze task and a novel Cognitive Interference task were employed to measure any cognitive enhancement.
CHAPTER 2 – TRANSMISSIBILITY OF MISFOLDED SUPEROXIDE DISMUTASE INCLUSIONS IN A MOUSE MODEL OF ALS

2.1. Introduction

My first investigations related to ALS focused on the proteinaceous inclusions formed within neurons and astrocytes in the CNS of ALS patients. Of particular interest was the possibility that deposits of mutant or misfolded SOD1 might carry the capacity to transmit the disease in a prion like fashion. In prion disease, the native prion protein, PrPc, (PrPc, = cellular prion protein) features a predominantly alpha-helical structure, while PrPSc, as in scrapie, a prion disease in sheep, features a predominantly beta-pleated sheet structure. The only difference between these proteins is a conformational change and according to the “protein only” hypothesis, the agent responsible for misfolding native prion protein to the aggregate prone form is the misfolded protein itself (Prusiner, 1998). PrPSc initiates and sustains the propagation of the pathogenic misfolding of the PrPc, molecule in vivo and causes neurodegeneration in a classic manner: Progressive loss of neurons, local inflammation (microgliosis/astrocytosis), deposition of abnormally folded prion protein (PrPsc), and, notably, transmissibility in most forms of the disease (transmissible spongiform encephalopathy- TSE).

Seeded disease transmissibility/promotion has also been demonstrated in Alzheimer’s mouse models. However, the diseases could only be induced if the mice were genetically predisposed to develop the disease. In young APP transgenic mice, cerebral amyloidosis can be triggered with intracranial infusion of dilute cortical extracts from AD patients and they develop amyloid pathology within a few months. Aβ-rich brain extracts from APP transgenic mice produce seeding similar to that from human extracts, indicating that the inducing agent is not uniquely present in the human brain.
(Walker et al., 2002). Infusion of brain extracts from WT or NTG mice into APP mice, or extracts from APP transgenic mice into Wt or NTG mice induced no phenotypic change, however, there is evidence that injecting brain homogenates from plaque producing mice induces tau pathology in tau transgenic mice (Bolmont et al., 2007). Moreover, direct infusion of Aβ 1-42 into AD transgenic mice, although sufficient to cause cognitive decline and neuronal death, will not form plaques.

Taken together, the data from a number of neurodegenerative diseases indicated that abnormal protein conformation could be acquired and induced in other animals. I therefore sought to determine if a transmissible element could be identified within the motor neuron inclusions of ALS mice. Spinal cord homogenates from symptomatic SOD1 G93A mice were homogenized, determined to contain high molecular weight species of SOD1, and injected into the lateral ventricles of SOD1 and NTG mice in an attempt to induce or speed the development of the ALS phenotype. Additionally, the extracts were infused directly into the lumbar cistern of these animals. Finally, spinal cord (SC) homogenates from symptomatic ALS mice were intrathecally infused into presyptomatic SOD1 transgenic mice with the intention to hasten the disease onset and/or progression.

2.2. Materials and Methods

2.2.1. Intrathecal Catheter Construction

Recent work by our and other groups has shown that compounds that are injected into the cerebrospinal fluid (CSF) do not diffuse efficiently throughout the spinal cord (unpublished observations). It was therefore necessary to infuse the peptide continuously into the CSF of the intrathecal space (lumbar cistern) of the spinal cord of the ALS models. This approach turned out to be a very arduous task for many research groups, and successful long-term intrathecal catheterization in mice, particularly in conjunction with Alzet osmotic minipumps, had not been reported at this time. Due to meticulous attention to surgical details and assistance from the ALS Therapy Development
Foundation, I succeeded in performing long-term intrathecal catheterization in mice. To this end, intrathecal catheters were fashioned by first inserting a 10 cm, 0.005” diameter tungsten wire (Small Parts, Inc. Miramar, Fl) into a 3 cm length of polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD.) with an internal diameter of 0.011” and stretching the material so it was snug with the wire. The un-stretched portion of the PE-10 (outer diameter 0.024”) was then inserted into a 4 cm long polyvinyl tubing (Durect Corp. Cupertino, Ca.) (internal diameter 0.027”) with approximately 2 cm of overlap. The space difference of 0.003” in the step-up to the larger polyvinyl tubing is filled with Locktite 454 adhesive (Plastics One. Roanoke, VA.) and secured with a silk suture (figure 1). After the assemblies were left to dry overnight, sterile water was forced through to assure that the lines were not occluded or damaged.

2.2.2. Spinal Cord Extracts

After an overdose of sodium pentobarbital (~150mg/kg) (i.p.) 6 animals (4 hSOD1 G93A mice and 2 hSOD1WT) were transcardially perfused with 0.9% saline. The spinal cords were microdissected from a group of 5 late disease stage animals and 2 age-matched controls, immediately dounce homogenized in cold 300μM sucrose, aliquoted, and frozen at -80°C until use. Equal amounts of protein from each sample were mixed with 2x sample buffer (Invitrogen, Carlsbad, CA), boiled for 5 min, and subjected to 10-20% Tris Tricine SDS gel electrophoresis (Invitrogen). The electrophoresed proteins were wet transferred onto 0.2 µm nitrocellulose (Whatman, Dassel, Germany) and probed with a rabbit anti-mouse polyclonal antibody to SOD1 (1:1000). The secondary antibody was horse radish peroxidase-conjugated donkey anti-rabbit IgG (Pierce, Rockford, IL). The immuno-labelled proteins were visualized with an enhanced chemiluminescence detection kit (SuperSignal pico, Pierce, Rockford, IL). After different exposures, autoradiographs were developed with a Konica/Minolta SRX101A (Tokyo, Japan) and scanned using a Ricoh Aficio MP3010. Homogenates that showed mostly higher weight SOD1 immunoreactivity were considered to be aggregates and used for subsequent infusions (figure 2).
2.2.3. Osmotic Minipumps

Sterile Alzet osmotic minipumps-model 2004 (Durect Corp. Cupertino, Ca.), with a flow rate of 0.25μL/hr were filled with 200μg of total protein from homogenates of G93A mutant and wt transgenic SOD1 mice, diluted in artificial CSF (Harvard Apparatus, Holliston, MA) and incubated at 37°C in sterile saline for 48 hours. Typically, catheters are attached and primed at this time, but the tungsten wire needed to remain in place for stability during insertion into the spine.

2.2.4. Mice

Another 10 SOD1 G93A mice and 4 controls (C57/B6 background) were purchased from Jackson Labs (Bar Harbor, ME.). These mice express 18 copies of mutant SOD1 and develop ALS pathology at approximately 100 days. Their average lifespan is about 130 days and the phenotype closely resembles a more aggressive familial form of the disease. This model was chosen because at the time it was widely accepted by ALS investigators and the therapeutic/pathological window was short enough to run multiple studies.

2.2.5. Intrathecal Infusions

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (90 days old- 25g) were anesthetized with 1-2% isoflurane and shaved and scrubbed with 10% Betadine solution at the site of incision. A small (3 cm) incision was made, exposing the lumbar region of the vertebral column, and double bladed scissors were used to form a subcutaneous pocket along the back of the animal between the scapula and towards the head. A puncture was then made between the L2 and L3 vertebrae with a 23 gauge needle that allows the small, stretched portion of PE-10 (stabilized with the tungsten wire) to be inserted 0.9 cm into the intrathecal space and being stopped by the silk suture used to adjoin the two different sized tubings. Accurate
placement of the catheter was visualized in every instance by a brief tail flick or muscle
twitch, at which point the tungsten wire was removed and an Alzet pump attached.
Another silk suture was used to “anchor” the catheter into the superficial erector
musculature dorsal the vertebral column. The placement of the second suture into the
musculature immediately caudal to the first “assembly” stitch on the catheter prevented
them from being pulled out during the course of the experiment. The pumps infused for
28 days, around which time the animals were at the terminal stages of paralysis.

2.2.6. Histology and Immunohistochemistry

Mouse spinal cords were carefully removed and fixed in 10% formaldehyde
(formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%)
over another 72 hours. Tissues were frozen to the peltier stage (Physitemp, Clifton, NJ)
of a histoslide (Leica, Heerbrugg, Switzerland) and sectioned coronally at 25 μm. After
incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat
serum, 0.1% Triton X-100, and 0.02% sodium azide) for 60 min, primary antibodies
against SOD1 (1:500) were applied and incubated at 4°C overnight. After thorough
washing, the sections were incubated with secondary antibody, Alexa 488 and 594
fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours.
This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear
stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy
Sciences, Hatfield, PA). Cresyl violet staining was used to analyze the general
morphology of motor neurons and vacuolated mitochondria (figure 3). The slides were
first stained with cresyl violet (Sigma, St. Louis, MO- 1 gram/400mL water at pH 3.6) for
3 minutes, rinsed in water, and placed in 95% ethanol, successively, 3 times, for 4
minutes each. Then they were placed into 100% ethanol twice for 2 minutes each and
xylene twice for 2 minutes each before being coverslipped with Cytoseal (Electron
Microscopy Sciences, Fort Washington, PA) and left to dry at ambient temperature
overnight. The images were analyzed on a Zeiss Imager Z1 microscope with a Zeiss
Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7.
2.2.7. Behavior

Motor deficits and the health of the animals were assessed throughout the experiment. Body weights before and after a 28 day treatment were measured daily as well as the biweekly performance on a rotarod test. The rotarod is designed to assess motor coordination, balance and equilibrium. The test mouse is placed on the rod and the rotarod accelerates gradually to a pre-set maximum speed. The mice’s performance abilities are recorded as latencies in falling from the rod. The rotarod consists of a semi-enclosed chamber which contains a beam made of ribbed plastic and flanked by round plates on either side to prevent any escape. The rod is suspended at a height of 20 cm above the counter and the mouse is placed on top of the beam, facing in the orientation opposite to that of its rotation, so that forward locomotion is necessary to avoid falling (figure 4). The rotarod spins consistently at 1 of 10 pre-set speeds (#5 was chosen) during three 2-minute trials. Latencies for the mice to fall from the rod are recorded automatically once the fallen animal interrupts a laser beam, thereby stopping the timer. Each mouse is given 3 trials with a 20 minute interval between trials. Once it is evident that the mouse is unable to perform, the animal is removed from the study.

2.3. Results and Conclusions

Mating experiments in which mice harboring the wild type human SOD1 gene were mated with mice harboring the wild type human SOD 1 gene G85R resulted in offspring with a more aggressive phenotype than the G85R alone (Wang et al., 2009). Disease onset time and age of death were both significantly reduced in the litter, suggesting that G85R’s interaction with WT SOD1 enhances its pathogenicity. With similar rationale, we sought to determine if there was an element within the inclusions, if not mSOD1 itself, that could, similar to prion diseases induce a misfolding event, thereby accelerating the disease process.

In these experiments, we wished to determine if the induction of misfolding in the spinal cords of SOD1 G93A, another mutant human SOD1, was transmissible, i.e. if a misfolded protein from a host can be introduced into a recipient to nucleate further
misfolding and cause neurodegeneration. Transmissibility, not necessarily infectivity, of misfolded proteins might provide insight into how the process of nucleation occurs in proteinopathies. Recent work with tau tangles, for example, in AD demonstrates that once the tangles are injected into the brain of a presymptomatic mouse, newly formed tangles form immediately, indicating that the first exposure to the tangles triggers a positive feedback loop of tangle formation (Clavaguera et al., 2009).

We sought to determine if exposure to mutant, aggregated SOD would nucleate the formation of characteristic intracellular inclusions in the spinal cord of ALS mice. This was approached first with intracerebroventricular bolus injections of homogenized spinal cords (figure 2) from symptomatic mice into WT and non-symptomatic mice in order to accelerate the phenotype. We found, however, that this was an ineffective means of delivery in this model, since the pathology begins in the anterior spinal column and molecules travel in the CSF diffusely. For this reason, customized catheters in combination with osmotic minipumps needed to be made in order to chronically and effectively deliver the homogenates directly to the target tissue and to infuse the homogenates for a month rather instead of single injections at a distal site.

Table 1 below shows that mutant mice receiving mutant homogenates lived an average of 129.6 days whereas mutant mice receiving WT homogenates lived an average of 124 days. The average lifespan of animals in this colony was 128. No significant change in disease onset (or time of death) were apparent between the groups behaviorally (data not shown- device figure 4), and histological examination of the spinal cords was not warranted. Furthermore, no other group has reported transmissible elements of ALS since these experiments were conducted. In sum, unlike AD and prion disease, SOD1 inclusions cannot seed or nucleate the formation of new aggregates, suggesting a divergence in pathology from other neurodegenerative diseases. At the same time intrathecal delivery of molecules into mice, at this time, a novel technique only practiced by a few researchers was proven to be a valuable tool for drug delivery.
2.4. Figures

Figure 1. Intrathecal catheter assembly

Adapted from Wu et al. 2004
Figure 2. Western Blot of spinal cord homogenates from nontransgenic, SOD1WT, and mSOD1 G93A mice which were subsequently used for chronic intrathecal infusions.
**Figure 3.** 20 μm sections from the lumbar region of spinal cords of nontransgenic (left) and SOD1 G93A mice (right) at 5x magnification. Below is increased magnification (40x) from the ventral horn showing dying motor neurons with mitochondrial vacuoles in the SOD1 G93A mouse (right).
Figure 4. A standard rotarod for individual mice (Med Associates Inc.)
Table 1. Comparing lifespan from SOD1 G93A mice infused with SOD1 G93A spinal cord homogenates (AGG-M) to animals infused with WT infusions (AGG-S).
CHAPTER 3 – EVIDENCE OF COMPROMISED BLOOD-SPINAL CORD BARRIER IN EARLY AND LATE SYMPTOMATIC SOD1 MICE MODELING ALS

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3.1. Abstract

The blood-brain barrier (BBB), blood-spinal cord barrier (BSCB), and blood-cerebrospinal fluid barrier (BCSFB) control cerebral/spinal cord homeostasis by selective transport of molecules and cells from the systemic compartment. In the spinal cord and brain of both ALS patients and animal models, infiltration of T-cell lymphocytes, monocyte-derived macrophages and dendritic cells, and IgG deposits have been observed that may have a critical role in motor neuron damage. Additionally, increased levels of albumin and IgG have been found in the cerebrospinal fluid in ALS patients. These findings suggest altered barrier permeability in ALS. Recently, we showed disruption of the BBB and BSCB in areas of motor neuron degeneration in the brain and spinal cord in G93A SOD1 mice modeling ALS at both early and late stages of disease using electron microscopy. Examination of capillary ultrastructure revealed endothelial cell degeneration, which, along with astrocyte alteration, compromised the BBB and BSCB. However, the effect of these alterations upon barrier function in ALS is still unclear. The aim of this study was to determine the functional competence of the BSCB in G93A mice at different stages of disease. Methodology/Principal Findings: Evans Blue (EB) dye was intravenously injected into ALS mice at early or late stage disease. Vascular leakage and the condition of basement membranes, endothelial cells,
and astrocytes were investigated in cervical and lumbar spinal cords using immunohistochemistry. Results showed EB leakage in spinal cord microvessels from all G93A mice, indicating dysfunction in endothelia and basement membranes and confirming our previous ultrastructural findings on BSCB disruption. Additionally, downregulation of Glut-1 and CD146 expressions in the endothelial cells of the BSCB were found which may relate to vascular leakage. Conclusions/Significance: Results suggest that the BSCB is compromised in areas of motor neuron degeneration in ALS mice at both early and late stages of the disease.

3.2. Introduction

The central nervous system (CNS) is an immunologically privileged zone, which is normally protected from entry of immune cells and serum proteins by the blood-brain barrier (BBB), blood-spinal cord barrier (BSCB), and blood-cerebrospinal fluid barrier (BCSFB). These barriers are specialized structures of the CNS that control cerebral/spinal cord homeostasis by selective transport of molecules and cells from the systemic compartment (reviewed in (Ballabh et al., 2004; Engelhardt, 2006; Pardridge, 1999, 1988; Rebenko-Moll et al., 2006; Sharma, 2005; Vorbrodt and Dobrogowska, 2003). This control is possible due to the unique structural elements of the microvasculature – endothelial cells of brain capillaries and epithelial cells of the choroids plexus, astrocyte end-feet, and pericytes. Brain (spinal cord) capillary endothelial cells are distributed along the length of the vessels, completely encircling the lumen and connected via adherens and tight junctions. Adherens junctions support intercellular adhesion, as tight junctions form a diffusion barrier for most blood-borne substances. The basement membrane (i.e. basal lamina), surrounding the endothelial cells and pericytes, supports the abluminal surface of the endothelium. The basement membrane, which consists of laminin, fibronectin, collagens, and proteoglycans (Dermietzel and Krause, 1991; Rutka et al., 1988), separates adjacent tissues, acting as a barrier to the passage of macromolecules and cell migration. The astrocyte perivascular end-feet ensheathing approximately 95% of the vessel wall appear to have an important role for maintenance
of the BBB (Prat et al., 2001). Thus, functional integrity of all BBB/BSCB elements is critical for protection of the CNS from various harmful blood substances.

Impairment of the BBB occurs in various pathological CNS conditions. Accumulation of collagen in vascular basement membranes and focal necrotic changes in endothelial cells were found in Alzheimer’s patients (Claudio, 1996). Degradation of the extracellular matrix may be concomitant with BBB disruption and tissue softening, setting the stage for the most pronounced forms of brain swelling and leading to the development of severe cerebral edema over subsequent hours and days in stroke patients (reviewed in (Avata and Ropper, 2002)). In multiple sclerosis (MS), which is characterized by inflammatory lesions within the CNS, BBB disruption enables leakage of the serum protein fibrinogen into the brain parenchyma (Kirk et al., 2003; Kwon and Prineas, 1994) and may precede myelin damage aggravating the inflammatory process. Recently, Vos et al. (Vos et al., 2005) demonstrated that BBB dysfunction in MS patients is apparent not only in focal lesions but also in diffuse abnormalities in white matter which were detected by postmortem MRI. BSCB breakdown was found in an experimental model of traumatic spinal cord injury (Noble and Wrathall, 1989; Olsson et al., 1992). It has been shown that spinal nerve lesions also alter BSCB function (Gordh et al., 2006). Results of these studies showed increased microvascular permeability leading to blood protein extravasation and the formation of vasogenic edema that play important roles in the pathophysiology of the diseased or injured spinal cord.

Amyotrophic lateral sclerosis (ALS) is a progressive degenerative disease affecting motor neurons in the spinal cord, motor cortex and brainstem that leads to paralysis and death within five years of disease onset (Miller et al., 2000; Rowland and Shneider, 2001a). Although numerous hypotheses about the etiology and pathogenesis of ALS have been proposed (reviewed in (Bruijn et al., 2004a; Cleveland and Rothstein, 2001; Strong et al., 2005a)), increasing evidence points to immune system involvement in ALS pathogenesis (Alexianu, 1995; Alexianu et al., 2001; Appel et al., 1994; Kawamata et al., 1992). In the spinal cord and brain of both ALS patients and animal models, the presence of T-cell lymphocytes (Alexianu et al., 2001; Engelhardt et al., 1993b), deposits of IgG (Donnenfeld et al., 1984b; Engelhardt and Appel, 1990; Engelhardt et al., 2005a;
Mohamed et al., 2002), complement components C3 and C4 (Donnenfeld et al., 1984b), and monocyte/macrophage and dendritic cells (Engelhardt et al., 1993b; Henkel et al., 2004; Troost et al., 1990b) were observed that may have a critical role in motor neuron damage. Recently, IgG was detected in the perikarya of motor neurons of the lumbar spinal cord in mice after intraperitoneal injection of IgG derived from sera of ALS patients (Engelhardt et al., 2005a). Moreover, the uptake of IgG in multivesicular bodies in endothelial cells in the affected areas of the spinal cord was found in both ALS patients and mice injected with human ALS IgG.

Additionally, significantly increased levels of albumin, IgG, and C3c have been noted in the cerebrospinal fluid of ALS patients (Annunziata and Volpi, 1985b; Apostolski et al., 1991; Leonardi et al., 1984b; Meucci et al., 1993). These findings suggest that barrier permeability may be affected in ALS. Recently, we showed disruption of the BBB and BSCB in areas of motor neuron degeneration in the brain and spinal cord in G93A SOD1 mice modeling ALS at both early and late stages of disease using electron microscopy (Garbuzova-Davis et al., 2007b). Examination of capillary ultrastructure revealed endothelial cell degeneration, which, along with astrocyte alteration, compromised the BBB and BSCB. However, the effect of these alterations upon barrier function in ALS is still unclear. The aim of this study was to determine the functional competence of the BSCB in G93A SOD1 mice modeling ALS at different stages of disease.

3.3. Results

3.3.1. Disease symptom progression in G93A mice

The G93A mice were monitored weekly for initial disease symptoms and disease symptom progression by body weight and extension reflex, beginning at 7 weeks of age. Body weights of G93A mice gradually increased until 11 weeks of age and then stabilized for 2 weeks (12-13 weeks of age). Tremor, an initial sign of disease, was seen in some mice as early as 13 weeks of age. After this time, mice consistently lost body
weight due to muscle atrophy. By 18 weeks of age, G93A mice had lost approximately 14% (p=0.06) of their 13-weeks-of-age body mass, dropping from 25.4±0.38 g to 21.8±0.55 g (Figure 5, A). Eighteen weeks old G93A mice weighed 28% less than C57BL/6J mice of the same age (p=0.007), which continued to maintain their body weights until 20 weeks of age. Deterioration in extension reflex started to appear in G93A mice at 13 weeks of age (Figure 5, B). During the next 5 weeks, mice demonstrated slowly declining hindlimb extension and by 18 weeks of age, showed no extension (p<0.001) and exhibited hindlimb paralysis. Thus, G93A mice at about 13 weeks of age showed initial signs of disease such as tremor, weight loss, and reduced hindlimb extension. Terminal stage of disease in these mice was observed at 17-18 weeks of age, as demonstrated by complete hindlimb paralysis, significant reduction of body weight and absence of hindlimb extension.

3.3.2. Motor neurons in the cervical and lumbar spinal cords of G93A mice

Nissl body staining was performed to identify motor neuron condition in the spinal cords of G93A mice in early and late stages of disease. In control C57BL/6J mice, many healthy motor neurons with large soma and neuritic processes were visible in the cervical and lumbar ventral horn of the spinal cords at 12-13 weeks of age (Figure 6A, Figure 7A) and 19-20 weeks of age (Figure 6B, Figure 7B). In the cervical spinal cord of G93A mice, numerous motor neurons with vacuolization were found at 13 weeks of age, concurrent with initial disease symptoms (Figure 2C). When disease symptoms progressed and mice were paralyzed, usually at 17-18 weeks of age, the number of motor neurons had decreased and motor neurons of various sizes displayed vacuolization (Figure 6D). Only a few healthy motor neurons were identified at this time. More dramatic changes in motor neuron condition were found in the lumbar spinal cords of ALS mice. Most motor neurons in 13 week old mice showed signs of degeneration (Figure 7C). In G93A mice at 17-18 weeks of age, the ventral horn was essentially devoid of motor neurons (Figure 7D).
3.3.3. **Fluorescent detection of Evans Blue in the cervical and lumbar spinal cords of G93A mice**

Evans Blue, the tracer used to assess disruption of the BSCB, was injected into G93A mice at initial (13 weeks of age) and late (17-18 weeks of age) stages of disease symptoms prior to euthanasia. C57BL/6J mice were also administered the EB dye. In the cervical spinal cord, EB was clearly detected within the blood vessels in the control C57BL/6J mice at 12-13 weeks of age (Figure 8A, B, C) or in the cross-sectioned capillaries at 19-20 weeks of age (Figure 8D, E). Vascular leakage of EB was distinguished in G93A mice with early disease symptoms (Figure 8F, G) and at end-stage of disease (Figure 8H, I, J) when more EB extravasation was noted. Interestingly, vessel permeability was perceived in both initial (Figure 8F) and late (Figure 8I) symptomatic G93A mice. In the lumbar spinal cord, EB dye was observed intravascularly in control C57BL/6J mice at 12-13 weeks of age (Figure 9A, B) and 19-20 weeks of age (Figure 9C, D), similar to results in the cervical spinal cord, while EB extravasation abnormalities were found in G93A mice at 13 weeks of age (Figure 9E, F). Significant EB diffusion into the parenchyma of the lumbar spinal cord from many blood vessels was detected in G93A mice at end-stage of disease, 17-18 weeks of age (Figure 9G, H). Considerable vessel permeability was seen in early (Figure 9F) as well as late (Figure 9G) symptomatic G93A mice. Thus, vascular leakage of EB was detected in G93A mice not only at end-stage of disease but also concurrent with early disease symptoms.

3.3.4. **Immunochistochemical characteristics of basement membranes, endothelial cells, and astrocytes in the spinal cords of G93A mice**

Immunofluorescent staining for laminin-1 (major non-collagenous basement membrane glycoprotein) demonstrated well organized microvasculature networks in the cervical and lumbar ventral horn of the spinal cords in C57BL/6J mice at 12-13 weeks of age (Figure 10A, Figure 11A) and 19-20 weeks of age (Figure 10B, Figure 11B). In these control animals, many capillaries of different calibers were visible by laminin detection.
Conversely, a marked reduction of labeled vessels was observed in both cervical and lumbar spinal cords of G93A mice at early (Figure 10C, Figure 11C) and late (Figure 10D, Figure 11D) stages of disease, suggesting a loss of vascularization or disruption of vascular basement membrane integrity. In some cervical spinal cord sections of G93A mice with initial disease symptoms, blurred spots around capillaries were apparent in the ventral horn of the spinal cord (Figure 10C), probably due to vascular leakage.

Immunofluorescent staining for Glut-1 (glucose transporter 1) showed high expression of Glut-1 in microvascular endothelia of the cervical and lumbar ventral horn of the spinal cords in C57BL/6J mice at 12-13 weeks of age (Figure 12A, B, H, I) and at 19-20 weeks of age (Figure 12C, J). The Glut-1 immunoreaction of endothelial cells lining the many capillaries of different calibers was low, or none, in both cervical and lumbar spinal cords of G93A mice at early (Figure 12D, E, K, L) and late (Figure 12F, G, M, N) stages of disease.

Immunohistochemically, endothelial cells (CD146) and astrocytes (GFAP) were of normal appearance in the cervical and lumbar ventral horn of the spinal cords in the control C57BL/6J mice at 12-13 weeks of age (data not shown) and 19-20 weeks of age (Figure 13A, B; Figure 14A, B, C). Delineated astrocytes and astrocytes with perivascular end-feet on the vessel wall were clearly observed. However, endothelia surrounding capillaries were partially revealed in the cervical spinal cord of G93A mice at initial (Figure 13C, D) or late (Figure 13E, F) stages of disease. Although CD146 antigen expression was detected in some endothelial cells of early symptomatic G93A mice, indistinct immunoreaction for CD146 was observed in G93A mice at end-stage of disease. Additionally, swollen endothelial cells (Figure 13F) were found in the cervical spinal cord of late symptomatic G93A mice. A small number of delineated astrocytes were also noted. In the lumbar spinal cord of G93A mice at initial (Figure 14D, E) and late (Figure 14F, G) stages of disease, decreased or unclear CD146 antigen expression was noted in endothelial cells. Notably, increased astrocyte activation in both cervical (Figure 13F) and lumbar (Figure 14F, G) spinal cords was detected in G93A mice at late stage of disease.
3.4. Discussion

In the present study, we investigated the functional competence of the BSCB in G93A SOD1 mice modeling ALS at different stages of disease. We observed microscopic evidence of BSCB impairment in cervical and lumbar spinal cords, areas of motor neuron degeneration, of ALS mice at initial disease symptoms and, more severely, at late stage disease. Our data show EB leakage in cervical/lumbar spinal cord microvessels in G93A mice at early (13 weeks of age) and late (17-18 weeks of age) stage disease. More leakage was found in lumbar spinal cords of mice at terminal stage disease. Additionally, basement membrane disruption was noted at both early and late stage disease, as shown by the loss of laminin staining in the G93A mice. Downregulation of Glut-1 and CD146 expressions in spinal cord endothelial cells was also found in G93A mice at early and late stage disease and may relate to altered endothelial lining leading to vascular leakage. Small numbers of delineated astrocytes were also established. These results confirm our previous ultrastructural findings (Garbuzova-Davis et al., 2007b) on disruption of the BSCB showing functional incompetence of BSCB structural elements in ALS mice.

Significant death of motor neurons in G93A mice occurs at the onset of clinical disease (90 days) and by end-stage disease (136 days), mice show up to a 50% loss of cervical and lumbar motor neurons (Chiu et al., 1995; Gurney et al., 1994; Weydt et al., 2003). In G93A mice, motor deficits have been observed in tests of muscle strength and coordination as early as 8 weeks of age (Barnéoud et al., 1997). These results extend those of our previous studies (Chen, 2003; Garbuzova-Davis et al., 2001; Garbuzova-Davis et al., 2002), showing initial signs of disease, such as tremor, weight loss, and reduced hindlimb extension, in G93A mice at about 13 weeks (90 days) of age. At this age, numerous vacuolized motor neurons were found in the cervical lumbar spinal cord and most motor neurons in the lumbar spinal cord showed signs of degeneration.

The primary BBB/BSCB function is control of the CNS homeostasis by selective transport of molecules and cells from the systemic compartment. Substances with a molecular weight higher than 400 Da generally cannot cross the barriers by free
diffusion. However, certain endogenous large molecules, such as insulin, leptin, transferrin, and insulin-like growth factors, enter the brain from blood via specific endothelial carrier-mediated or receptor-mediated transporters (reviewed in (Ballabh et al., 2004; Pardridge, 2005, 1999, 1988)). Recently, IgG was detected in the perikarya of motor neurons of the lumbar spinal cord in mice 24 hours after intraperitoneal injection of IgG derived from sera of ALS patients (Engelhardt et al., 2005a). The injected IgG was found in the axon terminals of the lumbar ventral horn motor neurons, localizing in the microtubules and rough endoplasmic reticulum. Furthermore, IgG was similarly detected in spinal cord motor neurons of ALS patients. There was also evidence of IgG intake in endothelial cells in affected areas of the spinal cord in both ALS patients and mice injected with human ALS IgG. Engelhardt et al. (Engelhardt et al., 2005a) suggest that “there may be multiple antibodies targeting a variety of epitopes of motor neurons in ALS”. The molecular weight of IgG is 150,000 Da and it is unlikely that these molecules could cross an intact brain capillary endothelium even by receptor-mediated transcytosis. However, Pirttila et al. (Pirttila et al., 2004) showed that insulin-like growth factor (IGF)-1, IGF binding protein-2, or nitric oxide were not elevated in CSF of ALS patients, suggesting that there is not a major disruption in the BCSFB. In another report (Kirkinezos et al., 2004), an ALS mouse model with a permissive BBB was created by crossing G93A mouse with the mdr1a/b knockout mouse and showed that cyclosporine A (CsA), which cannot cross an intact BBB, BSCB or BCSFB, reached the CNS when delivered intraperitoneally into this combined mouse model. Since the authors did not investigate BBB or BSCB condition in the original transgenic G93A mice, it is possible that disruption or dysfunction of these barriers occur in ALS. The 1200 Da molecular weight of CsA is much smaller than that of IgG. Further investigation is needed to resolve this apparent discrepancy in ability to cross the BBB/BCSFB.

Our finding of Evans blue extravasation in early symptomatic G93A mice may suggest that large molecules such as IgG and other blood proteins appear in the spinal cord due to vascular leakage, one possible mechanism accelerating motor neuron damage. However, it is unclear if BBB/BSCB disruption appears prior to motor neuron degeneration or as result of motor neuron dysfunction. Also, differences between the
BBB and BSCB in endothelial protein concentrations may impact observed pathological changes in G93A mice. It has been shown that microvascular endothelial cells, isolated from murine spinal cord, morphologically similar to BBB endothelial cells, express reduced amounts of several prominent BBB proteins such as tight junction-associated proteins ZO-1 and occluding, adherens junction-associated proteins beta-catenin and VE-cadherin, and the efflux transporter P-glycoprotein (Racke et al., 2006).

Reduction in immunofluorescent labeling of basement membrane of affected G93A mice suggests possible membrane disruption. The basement membrane is part of the extracellular matrix and is composed of collagens, proteoglycans, elastin and several glycoproteins, of which laminin is the most abundant (Dermietzel and Krause, 1991; Rutka et al., 1988). Reduced laminin labeling was observed in cervical and lumbar spinal cords of both early and late symptomatic G93A mice, possibly indicating vascularization changes leading to capillary wall permeability. Interestingly, Ono et al. (Ono et al., 1998) showed fragmented and widely separated collagen bundles in capillaries and decreased amounts of collagen in postmortem posterior half of the lateral funiculus and in the anterior horn of cervical enlargements from patients with sporadic ALS. Although the role of these aberrations in the pathogenesis of ALS remains to be determined, the authors suggested that abnormalities of collagen in the perivascular spaces of capillaries “may be secondary to neuronal degeneration as a underlying mechanism in ALS”.

It is well known that glucose transport through the BBB (BSCB) is mediated by glucose transporter isoform 1 (Glut-1) (Gerhart et al., 1989; Pardridge, 1991). Glut-1 is associated mainly with the brain capillary endothelial cells and is asymmetrically distributed between the luminal and abluminal membranes (Farrell and Pardridge, 1991). This asymmetric intracellular pool of glucose transporter may provide for rapid transport of glucose across the abluminal plasmalemma to the brain parenchyma (Stewart et al., 1994). The alteration of Glut-1 may be related to the pathogenesis of microvascular permeability as has been shown, for example, in cerebral edema (reviewed in (Dwyer et al., 2002)). In the present study, we found low and mostly absent expression of Glut-1 in capillaries of both cervical and lumbar spinal cords of G93A mice at early and late stages of disease. This downregulation of Glut-1 expression in the endothelial cells of the BSCB
may be related to altered endothelial lining leading to vascular leakage. Alternatively, decreased Glut-1 expression may result from aggravated alterations of the BSCB in G93A mice. Although additional experiments such as quantitative analysis of Glut-1 distribution and density in the endothelial plasma membranes are needed to elucidate the regulatory mechanisms of Glut-1 expression in the spinal cord, the present study indicates that alteration of Glut-1 could be involved in the pathogenesis of ALS.

Another of our findings was that endothelia surrounding capillaries were partially revealed by CD146 antigen expression in the cervical and lumbar spinal cords of G93A mice at initial and, more markedly, at late stages of disease. Moreover, small numbers of delineated astrocytes were established. These results may indicate that degeneration or, at least, partial dysfunction, of non-neuronal cells in ALS occurs. Evidence of widespread inflammatory reactions in ALS already exists. The presence of monocyte/macrophage cells, activated microglia, and reactive astrocytes was established in the spinal cord tissue of most ALS patients (Engelhardt et al., 1993b; Henkel et al., 2004; Ince et al., 1996; Sasaki et al., 2000; Troost et al., 1990b). In a mouse model of ALS, immune/inflammatory responses (Hall et al., 1998b; Hensley et al., 2006) are present even before any evidence of motor dysfunction (Alexianu et al., 2001; Almer et al., 2001). Strategically, astrocytes are located at the interface between the blood vessels and the brain as well as in the spinal cord parenchyma, influencing both the entry of blood cells into the CNS and the activity of invading cells once they have entered the brain parenchyma (reviewed in (Aschner, 1998; Mucke and Eddleston, 1993)). It has been shown that activated astrocytes and microglia “in response to signals derived from the immune system or generated within the CNS” produce various inflammatory molecules that may increase the permeability of the endothelial cell barrier (Prat et al., 2001). Inhibition of microglia activation, for example, as recently shown in vitro and in vivo using minocycline, may protect the brain after ischemic stroke by improving BBB viability and integrity (Yenari et al., 2006). It is possible that glial cell activation in ALS could lead to vessel leakage. Additionally, decreased numbers of delineated astrocytes and their perivascular end-feet at the blood capillaries could affect vessel permeability.
Thus, our results confirm our previous ultrastructural findings on disruption of the BSCB showing functional incompetence of BSCB structural elements in ALS mice. A breakdown in the BSCB is clearly indicated by EB leakage in cervical/lumbar spinal cord microvessels in G93A mice at early and late stages of disease. Laminin labeling suggests that basement membrane of vessels in the spinal cords of the diseased G93A mice may be affected. Additionally, downregulation of Glut-1 and CD146 expressions in the endothelial cells of the BSCB may be related to altered endothelial lining leading to vascular leakage. Degeneration of astrocytes could influence BSCB integrity.

Importantly, is BSCB breakdown a primary or secondary mechanism to motor neuron degeneration in G93A mice? Demonstrating BSCB disruption prior to the onset of disease symptoms and other pathological processes would indicate that BSCB disruption plays a primary role in ALS pathogenesis.

3.5. Materials and Methods

3.5.1. Animals

All described procedures were approved by the Institutional Animal Care and Use Committee at USF and conducted in compliance with the Guide for the Care and Use of Laboratory Animals. Transgenic male mice B6SJL-TgN (SOD1-G93A) 1GUR (G93A; Jackson Laboratories), over-expressing human SOD1, carrying the Gly93→Ala mutation, were used. Fourteen mice at 7 weeks of age were assessed on sensitive indicators of degenerative state (body weight and extension reflex). Six C57BL/6J male mice of the same age were used as controls. All mice were maintained on a 12:12 h dark:light cycle (light on at 06:00 hours). Room temperature was 23° C. Food and water was available ad libitum.
3.5.2. Characteristics of disease progression

Body weight was measured weekly throughout the study. Extension reflex was also observed weekly, as we previously described (Garbuzova-Davis et al., 2003a; Garbuzova-Davis et al., 2003b). Briefly, extension of the hindlimbs was observed while the mouse was suspended by its tail. A score (0-2) was given to each mouse indicating: normal (2), partial (1) or absent (0) hindlimb extension. Eight G93A mice were euthanatized at initial signs of disease (tremor, weight loss, and reduced hindlimb extension) at approximately 13 weeks of age, while six were allowed to reach the endpoint of hindlimb paralysis at 17-18 weeks of age, when they were also euthanatized. Measures of body weight and extension reflex were recorded for C57BL/6J mice according to the same schedule as G93A mice. C57BL/6J mice were euthanatized at 12-13 weeks (n=3) or 19-20 weeks (n=3) of age.

3.5.3. Evans Blue dye

Evans Blue dye (EB, Aldrich Chemical), 961 Da, was used as a tracer for assessing BBB disruption (Saria and Lundberg, 1983). G93A mice with initial or late stage disease and C57BL/6J mice were intravenously injected with 2% EB in saline solution via the jugular vein 30-40 min prior to euthanasia. This surgical procedure was performed as we previously described (Garbuzova-Davis et al., 2003b). Briefly, mice were anesthetized with Isoflurane delivered using a calibrated vaporizer equipped induction chamber and nose cone and administered at 2-5% in O₂ (2 L/min) to induce anesthesia and then decreased to 2% to maintain the anesthesia. The jugular vein was exposed and isolated using blunt dissection. The vein was ligated and a 31-gauge needle, attached to a 100-µl Hamilton syringe, was placed into the lumen of the vein and sutured in place. Evans Blue was delivered (0.2 ml/100g, 40µl/mouse) during 2 min. The needle was withdrawn, the suture tightened, and the incision closed with Vetbond.
3.5.4. *Euthanasia and tissue preparation*

Euthanasia of all mice was achieved under deep pentobarbital anesthesia and perfusion was not performed to avoid mechanical disruption of blood capillaries. The cervical/lumbar spinal cords were removed, fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.2, and then cryoprotected in 20% sucrose in 0.1 M PB (pH 7.2) overnight. Coronal sections of the cervical (C2-C3) and lumbar (L4-L5) spinal cords were cut at 30 μm in a cryostat.

3.5.5. *Immunofluorescence staining*

For identification of EB leakage, serial tissue sections of the spinal cords were thaw-mounted on slides, washed with deionized water to remove the freezing medium, and then rinsed several times in phosphate-buffered saline (PBS). The slides were coverslipped with Vectashield (Vector) and examined under an epifluorescence microscope.

Some spinal cord tissues were used for immunofluorescent analysis of the basement membrane (laminin), endothelial cells (Glut-1, CD146), and astrocytes (GFAP). Briefly, sections of the cervical/lumbar spinal cords were labeled with rat anti-mouse laminin-1 (α and β chains) monoclonal antibody (1:50, Chemicon), rabbit anti-mouse Glut-1 polyclonal antibody (1:100, Alpha Diagnostic Int.), or double-stained with mouse monoclonal CD146 (1:30, Chemicon) and rabbit polyclonal antibodies against glial fibrillary acidic protein (GFAP, 1:500, Dako). The next day, the slides were incubated for 2 hrs with appropriate secondary antibodies conjugated to either rhodamine (1:1500, Alexa 594, Molecular Probes) or FITC (1:500-700, Alexa 488, Molecular Probes) and, after several rinses in PBS, coverslipped with Vectashield or Vectashield with DAPI (Vector) and examined under epifluorescence using an Olympus BX60 microscope.
3.5.6. Staining of motor neurons in the spinal cord

Coronal sections of the C2-C3 and L4-L5 spinal cords were rinsed in PBS and then stained with 0.1% cresyl violet (30 sec) for routine histological analysis of motor neurons. Sections were then washed several times in deionized water, air-dried, dehydrated, and coverslipped using Permount.

3.5.7. Statistical analysis

Data are presented as means ± SEM. The nonparametric Mann-Whitney unpaired test was used to compare medians.

Acknowledgments:

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3.6. Figures

**A**

*Body weight*

**B**

*Extension reflex*
Figure 5. Characteristics of disease progression in G93A mice. (A) Body weight and (B) extension reflex of G93A and control C57BL/6J mice. G93A mice at about 13 weeks of age showed initial signs of disease such as weight loss and reduced hindlimb extension. Terminal stage of disease was observed at 17-18 weeks of age, as demonstrated by complete hindlimb paralysis, significant reduction of body weight and absence of hindlimb extension. Arrows indicate the age of mice when euthanatasis was performed. The five pointed star in A indicates difference (p=0.06) in body weights between G93A mice at 13 weeks of age and 18 weeks of age; the four pointed star indicates a significant difference (p=0.007) in body weights between G93A and C57BL/6J mice at 18 weeks of age. The four pointed star in B indicates a significant difference in extension reflex (p<0.001) between G93A mice at 13 weeks of age and 18 weeks of age.
Figure 6. Motor neurons in the cervical spinal cord of G93A mice at early and late stage of disease (cresyl violet staining). In the cervical spinal cord, many healthy motor neurons with large soma and neuritic processes were identified in the control C57BL/6J mice at (A) 12-13 weeks of age and (B) 19-20 weeks of age. In G93A mice, numerous motor neurons with vacuolization (asterisks) were found at (C) 13 weeks of age and (D) decreased numbers of motor neurons were noted in 17-18 week old mice. Motor neurons of various sizes displayed vacuolization (asterisks). Scale bar on left side is 200 µm, right side is 50 µm.
Figure 7. Motor neurons in the lumbar spinal cord of G93A mice at early and late stage of disease (cresyl violet staining). In the lumbar spinal cord, C57BL/6J mice at (A) 12-13 weeks of age and (B) 19-20 weeks of age showed numerous motor neurons with strong Nissl body staining. Most degenerated or swollen motor neurons (asterisks) were found in G93A mice at (C) early (13 weeks of age) and (D) late (17-18 weeks of age) stages of disease; most surviving motor neurons were small. Scale bar on left side is 200 μm, right side is 50 μm.
Cervical spinal cord

A  B  C

D  E

F  G

H  I  J
Figure 8. Evans Blue fluorescence in the cervical spinal cord of G93A mice at early and late stages of disease. In the cervical spinal cord, EB was clearly detected within the blood vessels (red, arrowheads) in the control C57BL/6J mice at (A, B, C) 12-13 weeks of age or (D, E) in the lumen of vessels (brilliant green) at 19-20 weeks of age. In G93A mice, vascular leakage of EB (red, arrows) was detected (F, G) at early (13 weeks of age) disease symptoms and (H, I, J) at end-stage of disease (17-18 weeks of age) when more EB extravasation was seen. Arrowheads in F and I indicate vessel permeability. Scale bar in A-J is 25 μm.
Figure 9. Evans Blue fluorescence in the lumbar spinal cord of G93A mice at early and late stages of disease. In the lumbar spinal cord, EB dye (red, arrowheads) was determined intravascularly in the control C57BL/6J at (A, B) 12-13 weeks of age and (C, D) 19-20 weeks of age similar to the cervical spinal cord. EB extravasation abnormalities were found in G93A mice at (E, F) 13 weeks of age (red, arrows). (G, H) Significant EB diffusion (red, arrows) into the parenchyma of the lumbar spinal cord from many blood vessels was detected in G93A mice at end-stage of disease (17-18 weeks of age). Arrowheads in F and G indicate vessel permeability. Scale bar in A-H is 25 μm.
Figure 10. Immunofluorescence staining for laminin in the cervical spinal cord of G93A mice at early and late stages of disease. Many blood vessels of different diameter were immunoreactive for laminin-1 (red) in the control C57BL/6J mice at (A) 12-13 weeks of age and (B) 19-20 weeks of age. In G93A mice at (C) initial or (D) late stages of disease, capillaries appear to be less numerous. In some early symptomatic G93A mice, (C) blurry spots around capillaries were found. The nuclei in A-D are shown with DAPI. Scale bar in A, B, C, D is 200 µm; inserts a, b, c, d is 50 µm.
Lumbar spinal cord

A  a

B  b

C  c

D  d
Figure 11. Immunofluorescence staining for laminin in the lumbar spinal cord of G93A mice at early and late stages of disease. Various laminin-positive vessels (red) were observed in the control C57BL/6J mice at (A) 12-13 weeks of age and (B) 19-20 weeks of age similar to cervical spinal cord results. Fewer blood vessels were labeled in G93A mice at (C) early or (D) end-stage of disease. The nuclei in A-D are shown with DAPI. Scale bar in A, B, C, D is 200 µm; inserts a, b, c, d is 50 µm.
Figure 12. Immunofluorescence staining for Glut-1 in the cervical and lumbar spinal cords of G93A mice at early and late stages of disease. *Cervical spinal cord.* High expression of Glut-1 (red) was determined in endothelial lining of many blood vessels of various diameters in the cervical spinal cord of the control C57BL/6J mice at (A), (B) 12-13 weeks of age and (C) 19-20 weeks of age. In G93A mice at (D), (E) initial or (F), (G) late stages of disease, immunoreaction for Glut-1 in the endothelial cells appear to be low, or nonexistent. The nuclei in A-G are shown with DAPI. Scale bar in A and E is 50 µm; B, C, D, F, G is 25 µm. Outline of white dots indicates configuration of blood vessels. *Lumbar spinal cord.* Similar to the cervical spinal cord, most Glut-1-positive endothelial cells (red) were observed in the control C57BL/6J mice at (H), (I) 12-13 weeks of age and (J) 19-20 weeks of age. Less Glut-1 expression was found in G93A mice at (K), (L) early or (M), (N) end-stage of disease. The nuclei in H-N are shown with DAPI. Scale bar in J, M, N is 50 µm; H, I, K, L is 25 µm.
Figure 13. Immunohistochemical staining for endothelial cells (CD146) and astrocytes (GFAP) in the cervical spinal cord of G93A mice at early and late stages of disease. (A, B) Normal appearance of endothelial cells (green, arrowheads) and delineated astrocytes (red, asterisk) was observed in the control C57BL/6J mice at 19-20 weeks of age. Endothelia (green, arrowheads) surrounding capillaries were partially revealed in G93A mice at (C, D) initial or (E, F) late stages of disease. Note: increased astrocyte activation in the cervical spinal cord (F, asterisks) was detected in G93A mice at late stage of disease. The nuclei in A, C, and E are shown with DAPI. Scale bar in A, C, E is 50 µm; B, D, F is 25 µm.
Lumbar spinal cord
Figure 14. Immunohistochemical staining for endothelial cells (CD146) and astrocytes (GFAP) in the lumbar spinal cord of G93A mice at early and late stages of disease. (A, B, C) Similar to cervical spinal cord, endothelial cells (green, arrowheads) and astrocytes (red, asterisk) in C57BL/6J mice at 19-20 weeks of age appeared normal. In G93A mice at (D, E) early or (F, G) end-stage of disease, decreased CD146 antigen expression by endothelial cells (green, arrowheads) was observed. Note: increased astrocyte activation in the lumbar spinal cord (F, G, asterisks) was detected in G93A mice at late stage of disease. The nuclei in A, C, D, and F are shown with DAPI. Scale bar in A, C, D, F is 50 µm; B, E, G is 25 µm.
3.7. References


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CHAPTER 4 – THE NEUROPROTECTIVE EFFECTS OF THE SIGMA RECEPTOR AGONIST DTG (1,3-DI-O-TOLYLGUANIDINE) ON A MOUSE MODEL OF ALS

4.1. Introduction

Other aspects of ALS have been of interest to our lab including inflammation and excitotoxicity. Treatments that have been beneficial in rodent models of stroke, for example, have potential therapeutic benefits to ALS models. It has been shown by our colleagues (Keith Pennypacker et al.) that in middle cerebral artery occlusion (MCAO) sigma receptor agonists are both neuroprotective and anti-inflammatory (Ajmo et al., 2006). We proposed that sigma receptor activation will protect motor neurons from excitotoxicity and microglia mediated inflammation, the key pathological components shared by stroke and ALS. Specifically, we wished to test whether this class of drugs was effective at reducing motor neuron degeneration and secondary inflammation in the SOD1 G93A mouse model of ALS.

Inflammatory responses are common to all types of neuronal injury and enhance neurodegeneration (DeLegge and Smoke, 2008). This response is mediated by microglia, the endogenous macrophage in the CNS, by astrocytes, and by infiltrating immune cells, which include macrophages and lymphocytes. Resting, nonphagocytic, microglia do not contribute to neurodegeneration. However, upon injury-induced activation, they can contribute to the destruction of neurons with the release of TNF-α (Bezzi et al., 2001; Hide et al., 2000; Noda et al., 2000), leading to the activation of the transcription of pro-inflammatory genes, such as inducible nitric oxide synthase and proinflammatory cytokines (Gebicke-Haerter, 2001). Histological analysis of spinal sections obtained from SOD1 transgenic mice have shown a three fold increase in activated microglia per slice compared to their wild type littermates. Release of tumor necrosis factor alpha
(TNF-α) and nitric oxide (NO) can induce glutamate release from astrocytes (Shibata and Kobayashi, 2008). Further neurodegeneration is elicited through macrophages and other immune cells infiltrating into the ALS-damaged area of the spinal cord.

Sigma receptors, first identified by Martin et al., 1976, are found in numerous tissues and organs. These pleiotropic receptors have since been classified into two types on the basis of pharmacology, sigma-1 and sigma-2, (Bourrie et al., 2004). Both subtypes have high to moderate affinity for antipsychotics, such as haloperidol, and guanidines, such as DTG (1,3-di-o-tolylguanidine). Sigma-1 receptors have two putative transmembrane domains and are different than any other known mammalian protein. While the endogenous ligand for sigma receptors has not been clearly identified, various candidates have been suggested. Progesterone, for example, and other neurosteroids, including pregnenolone and dehydroepiandrosterone, have high affinity for sigma receptors (Maurice et al., 1997). Given the general neuroprotective properties of neurosteroids and their abundance in the cortex, these sigma ligands would be obvious candidates to be investigated as possible endogenous ligands to reduce microgliosis and ensuing inflammation.

The current FDA approved therapy for ALS, Riluzole, is thought to modulate excitotoxicity by acting as a glutamate receptor antagonist, although use of this drug results only in a three month increase in lifespan. Riluzole treatment, however, does nothing for the secondary inflammatory processes, which are critical in the pathology of neurodegenerative disorders such as ALS, AD, and stroke. Taken together, effective treatment regimens for ALS (and possibly other neurodegenerative disorders) should include strategies to minimize both excitotoxicity and inflammation.

Recently, it has been shown that microglia express sigma-1 receptors, making DTG a molecule of therapeutic interest in ALS (Hall et al., 2009). Additionally, sigma receptor activation has been proven to modulate cytokine progression and inhibit the secretion of TNF-α, NO, and interferon-γ by macrophages induced by lipopolysachharide (LPS) injection (Bourrie et al., 1995). Overall, DTG is an exceptional candidate for ALS treatment because it promotes neuronal viability even after neurodegenerative and inflammatory processes have already begun. Furthermore, recent experiments have
shown that stimulation of sigma receptors decreases the excitability of neurons. Thus, the treatment is two-fold in modulating the excitability of neurons and modulating neuroinflammation. In the following experiments, life expectancy, weight retention and motor skills of SOD1 mice were examined following chronic systemic administration of DTG.

4.2. Materials and Methods

4.2.1. Osmotic Minipumps and subcutaneous implantaion

Sterile Alzet osmotic minipumps-model 2004 (Durect Corp. Cupertino, Ca.), with a flow rate of 0.25μL/hr were filled with 100mM DTG (1,3-di-o-tolylguanidine- a sigma 1 receptor agonist) in 3% lactic acid as vehicle and incubated at 37°C in sterile saline for 48 hours. All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (90 days old- 25g) were anesthetized with 1-2% isoflurane and shaved and scrubbed with 10% Betadine solution at the site of incision. A small (3 cm) incision was made, exposing the neck, and double bladed scissors were used to form a subcutaneous pocket along the back of the animal between the scapula and towards the tail for placement of the pump.

4.2.2. Mice

40 SOD1 G93A mice (C57/B6 background) and 10 NTG controls were purchased from Jackson Labs (Bar Harbor, ME.). These mice express 18 copies of mutant SOD1 and develop ALS pathology at approximately 100 days. The average lifespan is approximately 130 days and the phenotype closely resembles a more aggressive familial form of the disease. The model was chosen because it was so widely accepted in the field at the time and the therapeutic/pathological window was short enough to run multiple studies in succession. All surgeries were performed at disease onset (~100 days) and run
for 30 days or until the animal reached endpoint stage. Half of the mice received sham infusions (3% lactic acid only).

4.2.3. Histology and Immunohistochemistry

Mouse spinal cords were carefully removed and fixed in 10% formaldehyde (formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%) over another 72 hours. Tissues were frozen to the peltier stage (Physitemp, Clifton, NJ) of a histoslide (Leica, Heerbruug, Switzerland) and sectioned coronally at 25 μm. After incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat serum, 0.1% Triton X-100, and 0.02% sodium azide) for 60 min, primary antibodies against SOD1 (1:500) were applied and incubated at 4°C overnight. After thorough washing, the sections were incubated with secondary antibody, Alexa 488 and 594 fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours. This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy Sciences, Hatfield, PA). The images were analyzed on a Zeiss Imager Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7. FluoroJade staining was used to identify “dying” motorneurons and Cresyl violet staining was used for general morphology of motor neurons and vacuolated mitochondria (as previously described).

4.2.4. Behavior

Motor deficits and health of the animals were assessed throughout the experiment. Body weights were taken biweekly during the 28 day treatment as well as daily performance on a rototod test. The rotorod is designed to assess motor coordination, balance and equilibrium. The mouse is placed on the rod and the rotorod accelerates gradually to a pre-set speed. Latencies for the mice to fall from the rod are recorded. The rototod consists of a semi-enclosed chamber, which contains a beam made of ribbed
plastic and flanked by round plates on either side to prevent any escape. The rod is fixed at a height of 20 cm above the counter and the mouse is placed on top of the beam facing in the orientation opposite to that of its rotation, so that forward locomotion is necessary to avoid falling. The rotorod spins consistently at 1 of 10 pre-set speeds (#5 was chosen) for three 2-minute trials. Latencies in falling from the rod are recorded automatically once the fallen animal interrupts a laser thereby stopping the timer. Each mouse has 3 trials with a 20 minute interval between trials. Once it is evident that the mice are unable to perform, animals are removed from the study.

4.3. Results and Conclusions

Although preliminary data (n=10) with DTG indicated that prophylactic treatment of SOD1 G93A mice with DTG increased lifespan and retained body weight compared to sham treated littermates, we were unable to confirm either observation in this study (n=40). As table 2 below indicates, the average lifespan for the DTG treated group was ~134 days as compared to the sham treated group which was ~130 days. This modest increase was not statistically significant (figure 15). The behavioral data (not shown) and weight retention data were similar in that no significant detectable differences could be found between the two groups. Immunohistochemical analysis, though not performed to any great extent given the outcome of the study, indicate using Flurojade, that most motor neurons were dead or dying by the end of the experiment, proposed earlier as “scorched earth” (figure 16). That is, the damage done to the spinal cord motor neurons was so great, that a minor therapeutic benefit could not be observed. If these studies were to be repeated, administering DTG prior to disease endpoint or using a different model with a larger therapeutic window may be more effective.
4.4. Figures

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**Table 2** Lifespan and body mass of mice receiving DTG or sham infusions
Figure 15. Graphical representation of lifespan (Y-axis) in table 2 showing modest benefit with DTG, but without significance.
**Figure 16.** The “scorched earth” of degenerating neurons (as well as glia) in the spinal cord of SOD1 G93A mice were detected by Fluoro-Jade staining. A) Fluoro-Jade detects tracts of degenerating neurons. B) At higher magnification, individual neurons are visible. (Borrowed from an ALSA grant application- Pennypacker et al. 2006)
CHAPTER 5 – THE NEUROPROTECTIVE EFFECTS OF THE CARBOXYL TERMINUS OF GAMMA LAMININ, KDI, ON A MOUSE MODEL OF ALS

5.1. Introduction

Because the exact cause of Amyotrophic Lateral Sclerosis (ALS) is unknown, effective treatments are yet to be developed. It is for this reason that multiple approaches for therapies have been investigated. Although ALS is a proteinopathy, glutaminergic dysregulation and motor neuron death also occur and therefore various neuroprotectants have been of interest. In the following experiments, we investigated the use of laminin, namely a fragment of γ-laminin, as a therapy for ALS. Laminin is a heterotrimeric extracellular matrix glycoprotein involved in numerous biological processes. Recently, it has been elucidated that it is, in fact, the soluble tripeptide fragment-lysine, aspartic acid, and isoleucine (KDI) on the carboxyl terminus of the γ-laminin chain that promotes neurite outgrowth (Liesi et al., 2001). To date, there are 15 known isoforms of laminin that are expressed in mammals in a tissue-specific manner. Five different α subunits have been described, 4 β, and 3 γ which arrange as a cruciform heterotrimer (αβγ) of ~900KD in total mass as intact laminin molecules (Miner, 2008). The molecules are the main non-collagenous components of basement membranes and also play diverse roles in the extracellular matrix relating to cell structure, adhesion, differentiation, migration, signaling, metastasis, and neurite outgrowth. It has also been shown that laminin is upregulated during development and repair of most tissues, and portions of the molecule can maintain biological activity following cleavage events. Some laminin cleavage products retain biological activity (Wiksten et al., 2003). Over the past decade, our collaborator, Päivi Liesi, has worked extensively with laminin. In particular, she has elucidated that the carboxyl terminus of γ1-laminin is responsible for neurite outgrowth
both in vitro and in vivo (Liebkind et al., 2003; Wiksten et al., 2004b). Original work focused on the decapeptide of the molecule exerting these effects; however, subsequent studies of the hexapeptide and finally the tripeptide, KDI, still showed activity as a neurite outgrowth factor. KDI has also proven to be a glutamate receptor antagonist via patch clamp studies. In these studies, it was demonstrated that KDI non-competitively inhibited ionotropic glutamate receptors (at 1mg/mL) ~100% of AMPA –alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid and 50% of NMDA- N-Methyl-D-Aspartate receptors (Moykkynen et al., 2005). In vitro work on primary human embryonic spinal cord cultures and organotypic slices of human embryonic spinal cord embedded in Matrigel showed measurable tracts of extending neurites. The most recent experiment performed by Liesi et. al. (2006) showed that rat spinal cords, following transsection, could heal with the continued administration of KDI to the site. In this experiment, glial scarring was minimal and the animals were in most cases able to completely recover from paralysis (Wiksten et al., 2004b). The molecule has thus shown promise as a therapeutic for neurodegeneration, specifically ALS and AD, and we have subsequently investigated its effect in different mouse models of neurodegenerative disease.

Because it is not yet known whether KDI can cross the blood-brain barrier following systemic administration, direct infusion of the molecule into the cerebrospinal fluid (CSF) of these mice was required. Recent work by other groups has proven, however, that compounds in the CSF do not diffuse efficiently. It was therefore necessary to continuously infuse the peptide into the intrathecal space (lumbar cistern) of the spinal cord of the mice. This has proven to be an arduous task for many research groups and was achieved here with assistance from the ALS Therapy Development Foundation. To the authors’ knowledge, successful long-term intrathecal catheterization in mice, particularly in conjunction with Alzet osmotic minipumps, has not been reported elsewhere.

The pathologies of ALS begin in the lower lumbar region of the spinal column and progress rostrally causing hindlimb paralysis and eventually death. A series of transgenic animals have been developed over the years to mimic the disease; the most
frequently used is the SOD1 G93A model. This animal has a glycine to alanine substitution at the 93rd position of the 153 amino acid SOD1 monomer. The mice become paralyzed at approximately 100 days and live another 20-30 days with ALS-like symptoms. Due to the aggressive disease progression in this model, they were the most commonly used model to investigate the disease at the time. The use of KDI in this mouse model of ALS was promising for 2 reasons: First, because KDI was shown to be a neurite outgrowth promoter (Liebkind et al., 2003), enhanced survival, if not MN regrowth was anticipated in these mice dying of motor neuron loss. Second, given KDI’s potent ability, similar to riluzole and memantine, to lower synaptic glutamate levels, it was reasonable to anticipate KDI might reduce excitotoxic insults in ALS.

5.2. Materials and Methods

5.2.1. Intrathecal Catheter Assembly

Intrathecal catheters were fashioned as described previously by first inserting a 10cm, 0.005” diameter tungsten wire (Small Parts, Inc. Miramar, Fl) into a 3 cm length of polyethylene tubing (PE-10, Becton Dickinson, Sparks, MD.) with an internal diameter of 0.011” and stretching the material so it was snug with the wire. The unstretched portion of the PE-10 (outer diameter 0.024”) was next inserted into a 4cm long polyvinyl tubing (Durect Corp. Cupertino, Ca.) (internal diameter 0.027”) with approximately 2 cm of overlap. The difference of 0.003” in the step-up to the larger polyvinyl tubing was filled with Locktite 454 adhesive (Plastics One. Roanoke, VA.) and secured with a silk suture. The assemblies were then left to dry overnight and sterile water was forced through to assure that the lines were not occluded or damaged.

5.2.2. Osmotic Minipumps

Sterile Alzet osmotic minipumps-model 2004 (Durect Corp. Cupertino, Ca.), with a flow rate of 0.25μL/hr were filled with 200ug (1mg/mL) of KDI (NeoMPS, San Diego,
CA) diluted in artificial CSF (Harvard Apparatus, Holliston, MA) and incubated at 37°C in sterile saline for 48 hours. Typically, catheters were attached and primed at this time, but the tungsten wire needed to remain in place for stability during insertion into the spine.

5.2.3. Mice

20 SOD1 G93A mice (C57/B6 background) were purchased from Jackson Labs (Bar Harbor, ME.). These mice express 18 copies of mutant SOD1 and develop ALS pathology at approximately 100 days. The average lifespan is about 130 days, and the phenotype closely resembles a more aggressive familial form of the disease. This ALS mouse model was chosen because at the time it was widely accepted in the field and the therapeutic/pathological window was short enough to run high throughput studies in succession very timely. All surgeries were performed at disease onset (~100 days) and run for 28 days or until the animal reached endpoint stage.

5.2.4. Intrathecal Infusions

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (90 days old- 25g) were anesthetized with 1-2% isoflurane and shaved and scrubbed with 10% Betadine solution at the site of incision. A small (3 cm) incision was made, exposing the lumbar region of the vertebral column, and double bladed scissors were used to form a subcutaneous pocket along the back of the animal between the scapula and towards the head. A puncture was then made between the L2 and L3 vertebrae with a 23 gauge needle that allows the small, stretched portion of PE-10 (stabilized with the tungsten wire) to be inserted 0.9 cm into the intrathecal space and stopped by the silk suture used to adjoin the two different sized tubings. Accurate placement of the catheter was visualized in every instance by a brief tail flick or muscle twitch, at which point the tungsten wire was removed and Alzet pump attached. Another
silk suture was used to “anchor” the catheter into the superficial erector musculature dorsal the vertebral column. The placement of the second suture into the musculature immediately caudal to the first “assembly” stitch on the catheter prevented them from being pulled out during the course of the experiment. The pumps infused for 28 days, around which time the animals were at the terminal stages of paralysis.

5.2.5. Histology and Immunohistochemistry

Mouse spinal cords were carefully removed and fixed in 10% formaldehyde (formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%) over another 72 hours. Tissues were frozen to the peltier stage (Physitemp, Clifton, NJ) of a histoslide (Leica, Heerbruug, Switzerland) and sectioned coronally at 25 μm. After incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat serum, 0.1% Triton X-100, and 0.02% sodium azide) for 60 min, primary antibodies against SOD1 (1:500) were applied and incubated at 4°C overnight. After thorough washing, the sections were incubated with secondary antibody, Alexa 488 and 594 fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours. This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy Sciences, Hatfield, PA). FluoroJade staining was used to identify “dying” motorneurons. The images were analyzed on a Zeiss Imager Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7.

5.2.6. Behavior

Motor deficits and the animals’ health were assessed throughout the experiment. Body weights were taken daily during the 28 day treatment as well as biweekly performance on a rototod test. The rotorod is designed to assess motor coordination, balance and equilibrium. The mouse is placed on the rod and the rotorod accelerates gradually to a pre-set speed. Latencies for the mice to fall from the rod are recorded.
The rotorod consists of a semi-enclosed chamber which contains a beam made of ribbed plastic and flanked by round plates on either side to prevent any escape. The rod is fixed at a height of 20 cm above the counter, and the mouse is placed on top of the beam facing in the orientation opposite to that of its rotation, so that forward locomotion is necessary to avoid falling. The rotorod spins consistently at 1 of 10 pre-set speeds (#5 was chosen) during three 2-minute trials. Latencies for the mice to fall from the rod are recorded automatically once the fallen animal interrupts a laser, thereby stopping the timer. Each mouse was given 3 trials with a 20 minute interval between trials. Once it was evident that the mice were no longer able to perform the task, they were removed from the study.

5.3. Results and Conclusion

KDI was of particular interest to us as a therapeutic for ALS for several reasons. First and foremost, it was demonstrated both in vitro and in vivo that KDI was a potent neurite outgrowth factor. Previous work by Liesi et all demonstrated that neurons would extend long processes when embedded in matrigel containing KDI as compared to standard media (figure 17). More work from her laboratory revealed that KDI, following a full transection of rat spinal cords at L5-L6, could restore the animal’s ability to walk within a couple of weeks with drastic changes in gross morphology of the SC (figure 18). Hematoxylin eosin staining revealed that glial scarring was also minimized and that neurons were actually able to grow through the wound (figure 19). Further investigation from her lab revealed, using patch clamp studies on human neocortical neurons, that KDI was a potent inhibitor of glutamate receptors (both AMPA and NMDA). The only FDA approved drug for ALS, riluzole, functions by lowering synaptic levels of glutamate. Memantine is another glutamate reducing agent that has shown benefit to transgenic ALS mice. Glutamate excitotoxicity unequivocally plays a role in the pathogenesis of ALS and the therapeutic effects of these compounds supports that. We were confident that we had a small molecule of similar biological capability that would benefit the animals in two ways: reduced glutamate concentrations at synapses and promotion of axonal growth.
As indicated in the table below (table 3), KDI had no significant effect (p=0.62) on lifespan in the treated cohort. The animals were monitored daily and given a score 1-10 based on their condition. Their ability to walk, to groom, and to control bodily functions were taken into account and a clinical endpoint was established when the animal was paralyzed completely, unable to attain food and water. There were no differences between the groups in this analysis regarding the progression of the disease in these animals (data not shown). Rotarod performance in these studies were inconclusive. Extensive histological and biochemical analysis of tissues were not warranted, at this time, based on survival and progression data and studies with KDI and ALS were halted. Fluorojade analysis revealed a “scorched earth” effect in the tissues of mice at the end stage in previous studies. That is, the number of dying or dead motor neurons was so high that any changes induced by KDI would have been imperceivable.

Transgenic mouse models, we have found, are limited in most cases to answering the questions that they were designed for. The SOD1 G93A mouse model, for example, has about 18-22 copies of the mutant gene being expressed, which does not correlate in any way with fALS cases, let alone sporadic cases in which the inclusions have little or no WT SOD1 bound (Bruijn et al., 2004b). Moreover, using a model of protein aggregation to study glutamate reducing compounds can be misleading in assessing the effects. There is some justification that glutamate is dysregulated in these mice based upon levels of mutant SOD1, though results have varied in that determination (Tortarolo et al., 2006) (Tovar-y-Romo and Tapia, 2006). In retrospect, the phenotype in this model was so aggressive that it was difficult to test therapeutic compounds with it. The mice would begin to be paralyzed at 90 days of age, which was quite young for the surgeries involved. Then they would quickly decline and need to be euthanized as quickly as 15-20 days later (statistically no different than untreated animals). The value of the SOD1 G93A mouse to the ALS research community has since been questioned. Personal communication with the inventor of the SOD1 G93A transgenic mouse in July 2006 revealed that his lab no longer uses this particular mouse for ALS studies supporting our decision to discontinue working with them.
5.4. Figures

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Table 3. Lifespan of KDI-treated (blue) and sham treated (black) SOD1 G93A mice
Figure 17. Llesi et al. 2003 (previous work and rationale for experiment) TUJ1-immunoreactive (tubulin/axon labeling) neurons from the human embryonic spinal cord on top of the injured monolayers of the human spinal cord glial cells after 48 hr in vitro. In a, a neuron in the lower right corner of the photograph extends a long neurite in the presence of 0.1 μg/ml of the KDI peptide in the culture medium. Note that the moderate TUJ1 immunoreactivity of the majority of the underlying glial cells cannot be seen because of dark exposure of the photograph. Only the intense neuronal and some more intense glial immunoreactivity can be seen. In b, a spinal cord neuron fails to extend long neurites when no KDI peptide is present in the culture medium. Scale bar = 17 μm.
Figure 18. Liesi et al. 2004 (previous work and rationale for experiment) Stereo microscopic images of paraformaldehyde-fixed KDI-treated (a, b) and placebo-treated (c, d) spinal cords 14 weeks after operation. Dorsal images (a, c) show a connective tissue scar that forms at the site of laminectomy (arrow). It is obvious from these stereo images that dorsal (a) and ventral (b) poles of the spinal cord of the KDI-treated animal (motor score 3/4) are both better preserved than are dorsal (c) and ventral (d) poles of the spinal cord of the placebo-treated animal (motor score 1/1). Placebo-treated spinal cord consists of semitranslucent bloody opaque tissue at the transection site whereas KDI-treated spinal cord looks more intact.
Figure 19. Liesi et al. 2004 (previous work and rationale for experiment) Examples of longitudinally cut hematoxylin-eosin-stained paraffin sections (4 μm) through the ventral spinal cord show extent of damage in KDI-treated (a–c) and placebo-treated (d–f) animals. The motor score of each animal after 14 weeks is indicated in the left upper corner of each figure. Results indicate that spinal cords were transected completely and confirm that all KDI treated spinal cords (even the one in c) show less damage and smaller scar areas than do any placebo-treated spinal cords. KDI treatment thus reduces tissue damage and cavity formation and improves the chances for the tissue to heal. Scale bar 500 μm.
6.1. Abstract

A common problem faced by researchers using transgenic models to study disease is the phenotypic variability that exists within a group or colony of animals. Significant pathological analyses thus often require large numbers of mice to perform. Many lines of transgenic mice harboring the gene for human amyloid precursor protein (APP) with different mutations causing familial Alzheimer’s disease have been developed over the past decade to study plaque deposition and other aspects of AD. However, variations in size, density, plaque number, and total amyloid load between animals of the same age and genotype have been identified by our lab and others. Therefore, to study the effects of compounds on amyloid pathology, it was imperative to develop a technique that would allow each brain hemisphere to receive different infusions. We have developed catheters that facilitate simultaneous bilateral infusion in mouse brains, thereby using the contralateral hemisphere of the same animal as an internal control while studying, for example, the effect of compounds on amyloid plaques, a pathological hallmark of the progression of Alzheimer’s disease (AD). Several
molecules have been identified within the plaques including the major component, the Aβ peptide, and two inflammation-related proteins, apolipoprotein E (apoE) and the serine protease inhibitor α-1 antichymotrypsin (ACT). In these experiments, ACT was infused unilaterally over a period of 28 days into the parenchyma and lateral ventricles of PS/APP mice and observed to associate with amyloid plaques, with minimal mortality. Utilizing the ACT/Aβ interaction, an outline of this procedure is discussed here in detail.

6.2. Introduction

Alzheimer’s disease is the most common form of neurodegeneration, affecting over 5 million people nationwide. Approximately 1 person in 8 will be diagnosed with AD by the age of 65, and 1 in 2 by the age of 85 (National Institutes of Health U.S. Department of Health and Human Services). The disease is characterized by severe cognitive decline, leading to dementia and death within an average of 8 years from diagnosis. Pathologically, the disease outcome is massive neuronal loss, extracellular deposits of amyloid, and intracellular accumulation of neurofibrillary tangles (Selkoe, 1986). Several proteins have been identified within the plaques including Aβ, apolipoprotein E (apoE) (Wisniewski and Frangione, 1992) and Alpha 1-antichymotrypsin (ACT) (Abraham et al., 1988). Aβ is the primary component of the plaques and is a 40-43 amino acid peptide proteolytically derived from the amyloid precursor protein (APP) (Hardy and Selkoe, 2002). Mutations in the APP gene that cause AD result in an increased production of pathogenic forms of Aβ (Tanzi et al., 1991). ApoE and ACT are part of a local inflammatory process in the brain. Aβ binds to ACT and ApoE, and these proteins serve to catalyze the conversion of Aβ into filaments \textit{in vitro} (Ma et al., 1996; Wisniewski et al., 1994), and \textit{in vivo} to form beta-pleated structured amyloid deposits (Bales et al., 1999; Nilsson et al., 2001).

ACT is a 68 kDa acute phase serum glycoprotein from the family of serine protease inhibitors (serpins). Systemically, it is released by hepatocytes and monocytes in response to inflammation and functions as a suicide inhibitor of cathepsin G released by neutrophils (Kalsheker, 1996). In the brain, ACT is known to be produced by and
released from astrocytes in response to IL-1β stimulation by microglia following trauma, infection, or amyloidoses (Abraham, 2001; Das and Potter, 1995). ACT is highly upregulated in AD brain and is involved both in the formation of amyloid plaques (Abraham et al., 1988) and in the phosphorylation of tau (Padmanabhan et al., 2006). Using a transgenic mouse carrying a mutated human presenilin gene (PS) and a mutated human APP gene (PS/APP), we observed the co-localization of ACT on existing amyloid plaques in adult PS/APP mice, utilizing a novel brain infusion technique.

Amyloid plaque load can vary significantly from animal to animal of the same APP transgenic genotype as well as from hemisphere to hemisphere of the same animal (Figure 20). We analyzed the brains of 6 PS/APP mice at 10 months of age at 5 sections per brain front to back for variations in plaque load. Although variability does exist from hemisphere to hemisphere in this model per a given section (11%), we have demonstrated that the variability from animal to animal is almost 3 times that from that of total hemisphere to hemisphere comparison (78% from mean compared to 28%, respectively) (Figure 20B). For this reason, we designed a bilateral delivery technique that would allow one of the two hemispheres to serve as an internal control. This technique allows researchers to use fewer mice than an experiment of this nature would typically require and with more accurate results. Thus ACT, for the purposes of our work, could be delivered to one hemisphere and the vehicle, artificial cerebrospinal fluid (aCSF), to the other. Current systems for drug/compound delivery to the mouse brain use pedestal cannulae which are commercially available, but tend to be bulky and irritating to the animal, restrictive to the researcher, and lead to high mortality rates. Furthermore, use of two independent pedestal-based cannulae infusions in the same mouse is not possible due to their large size. Therefore, we designed and constructed novel catheters that are implanted subcutaneously and contoured to the skull. They are minimally invasive and virtually eliminate iatrogenic mortality. Our data show that this method is reliable for studying the chronic delivery of ACT into PS/APP mice to study its effects on amyloid pathology. Moreover, because the catheters are custom made, they can easily be adapted to study the impact of virtually any compound in other mouse models of
neurodegeneration or brain disease as well as on non-transgenic mice in order to study the effects of these compounds on normal physiology.

6.3. Materials and Methods

6.3.1. Catheter Construction

The catheters (patent pending- PCT/US08/73974) used for this experiment were made by first taking a 1.25 cm length of 30 gauge stainless steel tubing (Small Parts, Inc. Miramar, Fl) and carefully inserting it into a 20g needle (tip removed) to the appropriate depth under a dissecting microscope (Leica, Heerbruug, Switzerland), and bending it at 2.5 mm to approximately 90 degrees being very careful not to crimp the tube. The remaining length of the tube was bent again at 5 mm to an angle of 120-160 degrees, approximating the contour of each animal’s skull. These metal cannulae were inserted into a 3 cm length of polyethylene (PE-10) tubing with an internal diameter of 0.28 mm and an outer diameter of 0.61 mm. One centimeter of the PE-10 was then itself inserted into a 4.5 cm length of sterile polyvinyl tubing (PV-50-I.D. 0.69 mm/ O.D. 1.14 mm, Durect Corp., Cupertino, Ca), held in place using a bead of Locktite 454 adhesive (Plastics One. Roanoke, VA.), and cured overnight. The following day, sterile water was forced through each catheter assembly to ensure that the lines were not obstructed. The osmotic pumps were then filled with solution, attached to the catheters and primed and implanted as described below. The proven catheters were later custom manufactured by Braintree Scientific (Braintree, MA) with some minor changes: The adhesive was supplanted by heat sealing PE-10 to PE-50 tubing and, because the diameters of the PV-50 and new PE-50 were not exactly the same, a sheath of silicone (SIL 047) was placed over the PE-50 with an extra 2 mm overhang to attach it to the flow modulator of the pump, forming a tight, leak-proof seal (Figure 21).
6.3.2. **Transgenic Mice**

PS/APP (presenilin 1/amyloid precursor protein) mice and PS/APP/ACT mice were generated by crossing heterozygous PDGF-hAPP (V717F) mice with PDGF-hPS1 (M146L) on both Swiss Webster and C57BL/6 backgrounds. In some cases, PS/APP mice were bred with mice harboring a gene for human ACT (hACT) under a GFAP promoter (Nilsson et al., 2001) were used to be compared to ACT-infused animals. Genotyping was performed using comparative real-time PCR (Bio-Rad iCycler-Hercules, CA). Pathogenically, these Alzheimer's mouse models are characterized by robust accumulation of amyloid plaques and the development of microgliosis between 6-10 months. The mice used in these studies ranged from 9-10 months of age.

6.3.3. **Materials**

Purified human α-1 antichymotrypsin (ACT) was purchased lyophilized from Fitzgerald (Concord, MA) and was reconstituted in artificial CSF (Harvard Apparatus, Holliston, MA) to a concentration of 1mg/mL. ACT was infused directly into the hippocampal parenchyma or lateral ventricles for 28 days using the novel catheters attached to Alzet osmotic minipumps (Alzet model 1004, Durect Corp. Cupertino, CA) with an average flow rate of 0.12μL/hour. The pumps and catheters were submerged in 0.9% sterile saline at 37°C and primed for 48 hours prior to implantation.

6.3.4. **Intracranial Infusions**

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (9 month-old PS/APP- 25-35g) were anesthetized with 1-2% isoflurane, shaved and scrubbed with 10% Betadine solution at the site of incision, and placed into a dual arm stereotaxic frame (Kopf Instruments. Tujunga, Ca.). A small (5 cm) incision was made, exposing the skull and neck, and double bladed scissors (10
cm curved Strabismus, Fine Science Tools, Foster City, CA.) were used to form a subcutaneous pocket along the back of the animal into which 2 osmotic minipumps were inserted with catheters attached. One of the stereotaxic arms held an Ideal Micro-drill (Roboz Surgical Instrument Co., Gaithersburg, MD) holding a 0.32 mm diameter carbide drill bit. Using the drill bit on the stereotaxic arm to find the proper coordinates (from Bregma -2.2-2.5 mm anterior-posterior, +/- 2.2-2.5 mm medial-lateral), two holes were carefully drilled into the skull and a 30 gauge needle from a Hamilton syringe (Hamilton Co., Reno, Nevada) was attached to the second arm of the stereotaxic frame. The needle was inserted to the appropriate depth (2.2-2.5 mm corresponding to the posterior portion of the CA1/CA2 hippocampus) and allowed to sit for 5 minutes for the surrounding tissue to adjust. Alternatively, holes were drilled corresponding to the lateral ventricles at the following coordinates: from Bregma -0.2 mm anterior-posterior, +/- 1.0 mm medial-lateral, and to a depth of 2.2-2.5 mm. After removing the needle, the tips of the cannulae were held directly over the holes with forceps and then gently inserted straight into the preformed holes. Pulling back slightly on the catheters by the base of the osmotic pumps while inserting the cannulae provided stability which prevented them from moving and potentially damaging tissue while they were affixed. The cannulae were firmly affixed to the skull with Locktite 454 adhesive (Plastics One, Roanoke, VA.) and secured down with a piece of nitrile, approximately 1 cm in diameter. After the adhesive cured, the scalp was closed with 6-7 silk sutures. For analgesia, the animal received an immediate subcutaneous dose of ketoprofen (10mg/kg) and up to every 6 hours, as needed, for a maximum of 48 hours post-operatively. After the 28 day period, the animals were given an overdose (~150mg/kg) of sodium pentobarbital (i.p.) and were transcardially perfused with 0.9% saline. The brains were carefully removed and analyzed as outlined below. At this time, the integrity of the catheters was confirmed again by forcing water through them, and the pumps were also determined to be empty.
6.3.5. Preparation of Brain Extracts and Western Blot Analysis

The neocortex and hippocampus were each microdissected on ice, immediately snap frozen in powdered dry ice, and stored at -80°C until use. The tissue samples were then dounce homogenized in cold RIPA buffer (50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) in the presence of a protease inhibitor cocktail (Complete mini protease inhibitor tablet -Roche Diagnostics, Indianapolis, IN). Samples were subsequently spun at 20,000 g for 30 min (Eppendorf 5417R, Westbury, NY) and the supernatants removed, aliquoted, and frozen at -80°C. Equal amounts of protein from each sample were mixed with 2x sample buffer (Invitrogen, Carlsbad, CA), boiled for 5 min, and subjected to 10-20% Tris Tricine SDS gel electrophoresis (Invitrogen). Purified hACT and nontransgenic brain homogenates were used as controls. The electrophoresed proteins were wet transferred onto 0.2 µm nitrocellulose (Whatman, Dassel, Germany) and probed with a rabbit anti-mouse polyclonal antibody to ACT (1:1000- Dako, Glostrop, Denmark). The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Pierce, Rockford, IL). The immuno-labelled proteins were visualized with an enhanced chemiluminesence detection kit (SuperSignal pico, Pierce, Rockford, IL). After different exposures, autoradiographs were developed with a Konica/Minolta SRX101A (Tokyo, Japan), scanned using a Ricoh Aficio MP3010 (Tokyo, Japan) and quantified using Image J.

6.3.6. Histology and Immunohistochemistry

Mouse brain tissues were fixed in 10% formaldehyde (formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%) over another 72 hours. Brains were frozen to the peltier stage (Physitemp, Clifton, NJ) of a histoslide (Leica, Heerbruug, Switzerland) and sectioned coronally at 25 µm. After incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat serum, 0.1% Triton X-100, and 0.02% sodium azide) for 60 min, primary antibodies against ACT (1:500- Dako, Glostrop, Denmark) and Aβ (6E10,1:1000- Covance, Princeton, NJ) were applied and
incubated at 4°C overnight. After thorough washing, the sections were incubated with secondary antibodies, Alexa 488 and 594 fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours. This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy Sciences, Hatfield, PA). The images were analyzed on a Zeiss Imager Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7 software and quantified using Image J. Based on previous plaque analyses performed in our lab and others, for animal to animal, hemisphere to hemisphere plaque comparison, 5 sections from 6 mice (30 total) were stained using Thioflavin S to label the beta-pleated amyloid deposits from anterior to posterior hippocampus in each hemisphere of each mouse. Since the analysis is pairwise, i.e. every data point is compared to every data point, this number was sufficient to show relative variance. Coronal sections of brain (25µm) were mounted to slides and let to adhere overnight. The sections were then rinsed with deionized water and submerged in a 1% Thioflavin S aqueous solution (Sigma-Aldrich, St. Louis, MO) for 5 minutes. The sections were differentiated in 70% ethanol for 5 minutes, rehydrated in 30% ethanol for 5 minutes, washed with deionized water coverslipped, imaged, and quantified as described previously.

6.4. Results

6.4.1. Animal Recovery

Intracranial delivery of molecules, though invasive, is a common procedure to confirm results from in vitro experiments in an in vivo system and to test various compounds in transgenic animals via targeted delivery. As an additional advantage, the novel method described here bypasses concerns related to the blood-brain barrier permeability of the administered compound. ACT, a serine protease inhibitor that is known to contribute to the formation of amyloid pathology in Alzheimer's disease, was infused into the hippocampi or lateral ventricles of 9-month-old PS/APP mice for a
period of 28 days. ACT was infused into one hemisphere and aCSF (vehicle) into the other hemisphere, thus allowing a comparative analysis to be made within the same animal. In all cases, the animals recovered very quickly from the procedure and resumed eating and grooming within minutes of awakening. All animals were monitored closely during the 4 week infusion. In only a few instances did animals scratch at the incisions, but in no case were they able to open the wound and remove or damage any of the implanted pumps and catheters. In most cases, the hair grew back within 2 weeks, and the animals became virtually indistinguishable from non-treated animals, with the exception of bulges on the haunches from the implanted osmotic pumps (Figure 22A). The implants caused minimal discomfort to the animals, and procedural mortality was practically eliminated from these studies. When harvesting tissues following the 28 day infusion, the catheters were checked to ensure they were still connected to the pumps (Figure 22B) and to the cannulae (Figure 22C). The nitrile cap is indicated by the arrow in figure 22D. The entire assembly is shown attached to an Alzet osmotic minipump model 1004 (Durect Corp., Cupertino, CA.) in Figure 22E. Finally, when removing the brains only 2 small holes are visible (Figure 22F) showing that there is no lateral movement of the cannulae during infusion. No serious damage or impairment was caused by the cannula, although tissue is displaced where inserted. Figure 23 shows, on the left hemisphere, exactly where the cannula was placed and that surrounding tissue was intact following infusion. Figure 24 A and B show a region of the hippocampus anterior to the infusion site which also does not show signs of damage. To be noted, the gauge of the cannulae used in this experiment (30g or 0.30 mm O.D.) is the same of those used in commercially available cannulae (Alzet, PlasticsOne), but without the irritation to the animal, inflammation, occasional motor impairment, and high mortality rates associated with them.

6.4.2. Bilateral Brain Infusion

Due to the increased variability in plaque load between animals, even within a largely inbred transgenic mouse line, it is often difficult to compare treated and untreated
mice with a reasonably small number of animals. This phenomenon can be caused by phenotypic variability and/or differential expression of transgenes. Upon analysis of 6 untreated PS/APP mice at 10 months of age, we found that plaque load can vary significantly from animal to animal (78% mean standard deviation in pixel count). This fact makes it difficult to draw conclusions on the effects that different molecules might have on amyloid deposition or other features of the transgenic brain when comparing animals where hemispheres vary much less (28%) when compared. Figure 20A and B demonstrates the variation in plaque load between hemispheres and within animals of this genotype. Therefore, in order to study promoters and inhibitors of AD pathology directly in the brain, it would be preferable (almost twice the efficiency) to compare the individually-treated hemispheres of each animal rather than to compare the brains of littermates or matched cohorts from other litters. Western blots and immunocytochemical techniques demonstrated that our catheters functioned properly and that ACT was delivered successfully to the ventricles and parenchyma, respectively (Figures 23, 24, and 25). If ACT is infused into a PS/APP mouse that has plaques for it to interact with, it binds to the plaques and diffuses very little (Figure 24C & D). However, if ACT is delivered into the hemispheres of nontransgenic mice it diffuses, but still remains in the intended hemisphere (Figure 24A and B).

6.4.3. Interaction of ACT and amyloid

It is well established that ACT is localized, together with the Aβ peptide, in the amyloid plaques of Alzheimer’s disease (Abraham et al, 1988). However, the mechanism and effect of this interaction are still under investigation. Previous in vitro experiments indicated that ACT can bind to Aβ and become integrated into amyloid filaments as they are formed, and the same mechanism was presumed to occur in vivo (Mucke et al., 2000). Using the application method described here, we successfully delivered ACT into individual brain hemispheres of adult PS/APP mice and found that ACT binds to the perimeter of existing plaques in a similar manner as found for ACT association with amyloid plaques in PS/APP/ACT transgenic mice (Nilsson et al., 2001)
While the application of exogeneous ACT did not lead to increased amyloid load during the time frame of these experiments, it should be noted that ACT was infused for only 28 days. A longer exposure could result in an increased plaque load and is currently being investigated. At the same time, while ACT is known to enhance plaque formation, the perimetrical binding of ACT to pre-exisiting Aβ plaques, as shown here, might indicate that ACT involvement in AD pathogenesis not only occurs concurrent with plaque formation but also afterwards. This conclusion is consistent with the fact that ACT is highly upregulated in adult AD brains (Abraham et al, 1988) and also induces the phosphorylation of tau (Padmanabhan et al, 2006).

Although there is evidence that ACT's involvement in AD is not restricted to its participation in plaque formation, the purpose of using ACT in this model was to demonstrate the utility of our infusion system. The catheters and cannulae described here served as an outstanding tool for targeted delivery of ACT into individual brain hemispheres and will facilitate further examination of the mechanistic involvement of ACT and other molecules in the pathology of Alzheimer's disease.

6.5. Discussion

The variability of plaque load (i.e. size, density, and number) between brain hemispheres in PS/APP mice, though a factor to consider in these types of experiments, is substantially less than plaque variability from mouse to mouse (Figure 20A and B.). Based on our analysis of 6 untreated, unrelated animals in our colony, we determined the plaque load data from 5 brain sections through 6 ten-month-old PS/APP mice and compared the standard deviation (plaque variance) between sides of the same slice (11%), between total hemispheres of the same animal (28%), and between individual animals (78%). All of the data from the left hemisphere were compared with the right for each mouse (Hemi-Hemi, 1 data point for each mouse); the left half of a single slice is compared with the right side (Left-Right, 1 data point for each slice); and a permuted comparison of each mouse with every other mouse (Mouse-Mouse, 1 data point for each pair of mice). Where data were aggregated, (Hemi-Hemi and Mouse-Mouse), the mean of
the aggregated counts was used to make the standard deviation comparable to the single slice data (standard deviation of two numbers). The data indicate that evaluation of whole hemispheres in the same mouse is about 4 times less varied than comparing different animals to each other. Because of this variability, it was advantageous to develop a technique for the delivery of drugs and small molecules that would allow the treatment of individual brain hemispheres. By exposing one hemisphere to an experimental compound and the contralateral hemisphere to the vehicle only, our novel technique described here easily allows each animal to serve as its own histological and biochemical control.

Previously, bilateral treatment, either into lateral ventricles or brain parenchyma was difficult to accomplish with mice. Because transgenic mice provide an important experimental system for disease studies, such as Alzheimer’s disease, it was imperative to develop a durable and reliable technique for simultaneous bilateral delivery into the brain that is well-tolerated by mice. Commercially available bilateral delivery devices for mice are pedestal cannulae, which have proven problematic for a variety of reasons: 1) The pedestals are bulky and require large amounts of adhesive to fix into place because the cannula surface that comes into contact with the skull is flat while the skull is curved. This design requires that enough adhesive be applied to fill the gaps and to secure the applicator in place. Frequently, the adhesive does not have a chance to cure completely and reacts with surrounding tissue, causing irritation and the need for animals to be removed from the experiment. 2) The pedestals and tubing remain partially exposed, allowing the animals access to the catheters potentially compromising experiments and also causing concern for their health (inflammation, cachexia, death). 3) The bilateral pedestals have limited flexibility for the spacing of the catheters. If they are designed with fixed distances and fixed depths, their placement in a 25 gram mouse is very different compared to a 35 gram mouse, leading to improper placement of the cannula. 4) Other systems require specialized arms to lower the catheter into place, whereas these are best manipulated individually by hand to be reliably and reproducibly positioned. 5) Moreover, the entire catheters and pumps we used can be completely removed without terminating the life of the animal. This is generally not true of
commercially available catheters, which, when removed cause damage to the skull and do not leave the scalp in healthy enough condition to be sutured. 6) Lastly, since these catheters remain completely subcutaneous, behavioral testing can be safely performed while an animal is being treated, which is not a possibility with pedestals. Overall, the stereotaxic placement of pedestal cannulae, particularly bilateral pedestal cannulae, into a mouse brain is very restrictive and problematic.

Our catheter system described here facilitates targeted compound delivery to individual mouse hemispheres and is remarkably adaptable to reaching any region of the mouse brain and overcomes the challenges listed above. Because the stainless steel catheters can be fashioned to any depth and are implanted individually, variability in the size of the mouse brains can be easily accounted for and accurate catheter placement and proper contouring is ensured. Most importantly, the entire system is implanted subcutaneously with little if any protrusion. The use of the non-reactive nitrile cap to secure the catheters in place requires only a fraction of the adhesive compared to the pedestal method, thereby giving ample time for it to cure properly before the scalp is sutured. Our observations demonstrate that, in most cases, the animals re-grow hair over the scalp in a very short time and never scratch or over-groom the site or damage the catheters. The mortality in experiments using this method is closer to 2-3% as opposed to 30-40% using conventional techniques over the 28-day infusion period.

Here we demonstrated that ACT binds to pre-existing amyloid plaques in APP transgenic mice only in the infused hemisphere. We also showed that ACT could be delivered both to the parenchyma of the mouse hippocampus and into lateral ventricles using our new catheters. Changes in total plaque load, however, in the presence of ACT were not observed over the 28 day infusions. Although ACT is known to be involved in the process of fibrillization of Aβ both in vitro and in vivo, we did show that mature plaques retain binding capacity for ACT. The morphology of the amyloid plaques after ACT infusion strongly resembles the plaque morphology in PS/APP/ACT transgenic mice (Figure 26). Our observation suggests that our delivery system is an effective method to test various compounds in mouse models of neurodegenerative and other
diseases. Ongoing investigations with inhibitors of the Aβ/ACT interaction will help to
determine ACT's role in AD both during and beyond plaque promotion.

In sum, using our novel catheter system, we have studied the infusion of a protein
known to be a component of AD pathology into a mouse model of AD. The potential
application of this system, however, is quite broad. While we have focused on
intrahippocampal and intracerebroventricular (ICV) delivery, our catheters can
conceivably be inserted in any depth and be bent to any angle to deliver compounds to
any region of the brain in other mouse models of neurodegenerative disease. Bilateral
pedestal cannulas do not provide users with such adaptability, since accounting for
variability in animal size poses a problem. Likewise, using 2 independent pedestal
catheters to achieve bilateral delivery in mice is prohibited due to the large size of the
pedestals. The technique described here even allows for the implantation of 2 cannulae
per hemisphere (4 per brain), which would allow delivery of multiple agents to either
hemisphere in the same mouse with minimum mortality.

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References


Figure 20. A) Thioflavin S staining on 25μm coronal sections (montages) of 3 different PS/APP mice at 10 months of age showing plaque variability in the hippocampus and neighboring cortex. B) Scatter-plot of plaque load data from 5 brain sections through 6 ten-month-old PS/APP mice comparing standard deviation (plaque variance) between total hemispheres of the same animal (28%), between sides of the same section (11%) and between animals (78%). All of the data from the left hemisphere are compared with the right for each mouse (Hemi-Hemi, 1 data point for each mouse); the left half of a single slice is compared with the right side (Left-Right, 1 data point for each slice); and a permuted comparison of each mouse with every other mouse (Mouse-Mouse, 1 data point for each pair of mice). Where data were aggregated, (Hemi-Hemi and Mouse-Mouse), the mean of the aggregated counts was used to make the standard deviation comparable to the single slice data (standard deviation of two numbers).
Figure 22. A 10 month-old PS/APP mouse with 2 osmotic pumps following 28 days of infusion while still active (A) and after transcardial perfusion showing the Alzet pumps (B) and cannulae (C) still securely attached. The arrow in figure D shows the nitrile cap used to affix the catheters to the skull and Figure E shows the entire assembly attached to an Alzet 1004 osmotic minipump. When the brain is removed, only small holes are visible where the cannulae were inserted (F).
Figure 23. A) A montage of images taken at 10x from 25μm sections of an animal treated with ACT on the left and aCSF control on the right. The top section is immuno-labelled with the Aβ antibody 6E10 and the bottom with ACT antibodies. Inlays (40x) show Aβ in green, ACT in red, and Hoechst nuclear stain in blue. B) Semi-quantitative analyses of ACT and Aβ immunostaining in the hippocampus and cortex of PS/APP mice (n= 4) infused with ACT into the parenchyma of one hemisphere and aCSF in the contralateral hemisphere.
Figure 24. Montage images of 25μm coronal sections of 10 month old nontransgenic (A & B) and PS/APP mice (C & D) immuno-labeled with antibody against α-1 antichymotrypsin following 28 day infusion demonstrating that ACT stays on the infused hemisphere with or without the presence of plaques.
Figure 25. A) Western Blot of PS/APP of RIPA homogenized mouse hemispheres receiving ACT (red arrows) or aCSF (white arrows) into lateral ventricles (ICV). The green arrow denotes ACT taken from an osmotic pump implanted for 28 days to show minimal degradation of the protein during the experiment.

B) Quantification of ACT in hemispheres infused with ACT or aCSF.
Figure 26. Comparison of ACT associated with amyloid plaques (40x) from a PS/APP/ACT transgenic mouse (A) and a PS/APP ACT-infused mouse (B) both at 9 months of age. Immuno-labeling of Aβ is shown in green (6E10 antibody), ACT in red, and Hoechst nuclear stain in blue.
CHAPTER 7 – THE NEUROPROTECTIVE EFFECTS OF THE CARBOXYL TERMINUS OF GAMMA LAMININ, KDI, ON A MOUSE MODEL OF ALZHEIMER’S DISEASE

7.1. Introduction

Although AD is classified as a proteinopathy, the two currently FDA approved medications used to treat the disease, Aricept and Namenda, act on distinctly different aspects of the disease. Aricept (donezepil HCL) is an acetylcholinesterase inhibitor and is believed to improve cognition in patients by increasing synaptic concentrations of the neurotransmitter acetylcholine. The exact mechanism of this effect is not known, but it has been suggested that Aricept increases the expression of IGF-1 in hippocampal neurons, increases neurogenesis in the dentate gyrus, and increases cerebral blood flow in parenchymal arterioles (Narimatsu et al., 2009). Namenda (memantine) functions differently as its primary function is believed to be inhibition of NMDA receptors in glutamate neurotransmission. Blocking these receptors in a noncompetitive, voltage mediated (ionotropic) manner thereby inhibiting the prolonged influx of Ca2+ ions that induce excitotoxic damage to the neuron.

Over the past decade, our collaborator, Päivi Liesi, has worked extensively with laminins. In particular, she has elucidated that the carboxyl terminus of γ1-laminin is responsible for neurite outgrowth both in vitro and in vivo. To date, there are 15 known isoforms of the laminin expressed in mammals in a tissue-specific manner. Five different α subunits have been described, 4 β, and 3 γ which arrange as a cruciform heterotrimer (αβγ) of ~900KD in total mass as intact laminin molecules. The molecules are the main non-collagenous components of basement membranes and also play diverse roles in the extracellular matrix relating to cell structure, adhesion, differentiation, migration, signaling, metastasis, and neurite outgrowth. It has also been shown that laminin is
upregulated during development and repair of most tissues and portions of the molecule can maintain biological activity following cleavage events.

Original work focused on the gamma laminin decapeptide exerting these neuroregenerative and neuroprotective effects; however, subsequent studies of the hexapeptide and finally the tripeptide, KDI, still showed activity as a neurite outgrowth factor and functioned as a non-competitive ionotropic glutamate receptor antagonist. *In vitro* work, using patch clamp analysis, revealed that KDI could inhibit ~50% of NMDA receptors and 100% of AMPA receptors (Moykkynen et al., 2005). Further work showed in vivo that pretreatment of the rat hippocampus with bolus injections of KDI protected the brain region from subsequent injections of kainic acid, a glutamate analog (Wiksten et al., 2004a). If indeed dysregulation of glutamate is an etiology found in AD and Namenda has proven to have some efficacy, we proposed that KDI would work in a similar manner to ameliorate the symptoms of AD and improve cognition.

In these experiments, KDI was used to treat PS/APP mice. Since it is not yet known whether this molecule can cross the blood-brain barrier following systemic administration, direct infusion of the molecule into the hippocampal parenchyma of these models was required. In order to determine if KDI could improve working memory, similar to memantine, the animals were tested in a RAWM following 28 days of chronic infusion. Preliminary data suggested improvement with treatment of KDI, but without change in plaque load. Therefore, since KDI was reported to bind glutamate receptors, another avenue was investigated- Long term potentiation. Long-term potentiation (LTP) is a molecular mechanism involving synapse strengthening upon recent patterns of activity. It serves as a model for memory and learning in several brain areas, described first in the hippocampus (McKay, 1997). Briefly, it involves the binding of glutamate to ionotropic glutamate receptors, post-synaptic depolarization, influx of calcium, phosphorylation of Thr286 and constitutive activation of CaM Kinase II, resulting in the upregulation of more AMPA receptors and activation of relevant genes (Schlett, 2006).

During the time that the KDI infusions were being performed, EIIKDI was cloned into an expression vector. This was done in anticipation of achieving longer term expression of the molecule in the brain. In vivo electroporation has been used in
numerous tissues for gene expression (Heller and Heller, 2006). Briefly, the technique works by injecting plasmid into tissue and surrounding the site with an electric field to perforate the cells and allow the plasmid to enter cells and be expressed. This method has many advantages over traditional means of transfection that involve developing viral vectors and contending with an immune response. Electroporation is effective as a “proof-of principle” that introduced genes will be a) expressed and b) exert a physiological response within the cell before developing more complicated transfection strategies. Previous work in ours and other labs has demonstrated that this technique is safe in the CNS of mice and was optimized first using an EGFP expression vector (figure 27).

7.2. Materials and Methods

7.2.1. Catheter Construction

The catheters (patent pending- PCT/US08/73974) used for this experiment were made by first taking a 1.25 cm length of 30 gauge stainless steel tubing (Small Parts, Inc. Miramar, Fl) and carefully bending it at 2.5 mm under a dissecting microscope (Leica, Heerbrugg, Switzerland), to approximately 90 degrees, being careful not to crimp the tube. The remaining length of the tube was bent again at 5 mm to an angle of 120-160 degrees, approximating the contour of each animal’s skull. These metal cannulae were inserted into a 3 cm length of polyethylene (PE-10) tubing with an internal diameter of 0.28 mm and an outer diameter of 0.61 mm. One centimeter of the PE-10 was then itself inserted into a 4.5 cm length of sterile polyvinyl tubing (PV-50-I.D. 0.69 mm/ O.D. 1.14 mm, Durect Corp., Cupertino, Ca), held in place using a bead of Locktite 454 adhesive (Plastics One. Roanoke, VA.), and cured overnight. The following day, sterile water was forced through each catheter assembly to ensure that the lines were not obstructed. The osmotic pumps were then filled with solution, attached to the catheters and primed and implanted as described below. The proven catheters were later custom manufactured by Braintree Scientific (Braintree, MA) with some minor changes: The adhesive was
supplanted by heat sealing PE-10 to PE-50 tubing and, because the diameters of the PV-50 and new PE-50 were not exactly the same, a sheath of silicone (SIL 047) was placed over the PE-50 with an extra 2 mm overhang to attach it to the flow modulator of the pump, forming a tight, leak-proof seal.

7.2.2. Osmotic Minipumps

Sterile Alzet osmotic minipumps-model 2004 (Durect Corp. Cupertino, Ca.), with a flow rate of 0.25uL/hr were filled with 200ug (1mg/mL) of KDI (NeoMPS, San Diego, CA) diluted in artificial CSF (Harvard Apparatus, Holliston, MA) and incubated at 37°C in sterile saline for 48 hours. Typically, catheters are attached and primed at this time, but the tungsten wire needed to remain in place for stability during insertion into the spine.

7.2.3. Transgenic Mice

PS/APP (presenilin 1/amyloid precursor protein) mice were generated by crossing heterozygous PDGF-hAPP (V717F) mice with PDGF-hPS1 (M146L) on both Swiss Webster and C57BL/6 backgrounds. Genotyping was performed using comparative real-time PCR (Bio-Rad iCycler- Hercules, CA). Pathogenically, these Alzheimer's mouse models are characterized by robust accumulation of amyloid plaques and the development of microgliosis between 6-8 months of age.

7.2.4. Intracranial Infusions

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (8 month-old PS/APP- 25-35g) were anesthetized with 1-2% isoflurane, shaved and scrubbed with 10% Betadine solution at the site of incision, and placed into a stereotaxic frame (Kopf Instruments. Tujunga, Ca.). A small (3 cm) incision was made, exposing the skull and neck, and double bladed scissors were used to
form a subcutaneous pocket along the back of the animal into which 2 osmotic minipumps were inserted with catheters attached. Two holes were drilled into the skull (from Bregma -2.2-2.5 mm anterior-posterior, +/- 2.2-2.5 mm medial-lateral, and the 30 gauge catheters were inserted to a depth of 2.2-2.5 mm) corresponding to the posterior portion of the CA3 hippocampus. Alternatively, holes were drilled corresponding to the lateral ventricles (from Bregma -0.2 mm anterior-posterior, +/- 1.0 mm medial-lateral, and to a depth of 2.2-2.5 mm). Once they were inserted, the cannulae were affixed to the skull with Locktite 454 adhesive (Plastics One. Roanoke, VA.) and secured down with a piece of nitrile, approximately 1 cm in diameter (Fig 3D). After the adhesive cured, the scalp was closed with 6-7 silk sutures. For analgesia, the animal received an immediate dose of ketoprofen (10mg/kg) and up to every 6 hours, as needed, for a maximum of 48 hours post-operatively. After the 28 day period, the animals were given an overdose (~150mg/kg) of sodium pentobarbital (i.p.) and were transcardially perfused with 0.9% saline. The brains were carefully removed and analyzed as outlined below. At this time, the integrity of the catheters was confirmed again by forcing water through them, and the pumps were also determined to be empty.

7.2.5. Electroporation

The mouse codons for the longer peptide, EIIKDI, were determined and oligomers were synthesized with a small portion of a leader sequence to a length of 98 nucleotides (IDT, San Jose, CA). The sense and antisense strands were annealed and blunt-end cloned into a PCDNA3.1+ expression vector between the XbaI and ecoRV sites and confirmation of insertion, copy, and orientation was confirmed by DNA sequencing. An EGFP clone was used to optimize the electroporation procedure as it was roughly the same size (5 kB) and provided easy visibility of transfection efficiency (figure 26) The procedure is similar to what was mentioned above. Animals (8 month-old PS/APP- 25-35g) were anesthetized with 1-2% isoflurane, shaved and scrubbed with 10% Betadine solution at the site of incision, and placed into a stereotaxic frame (Kopf Instruments. Tujunga, Ca.). A small (3 cm) incision was made, exposing the skull and
3 holes were made, the first at from Bregma -2.2-2.5 mm anterior-posterior, + 2.2-2.5 mm medial-lateral, corresponding to the CA3 region of the hippocampus. The other 2 holes were drilled at 1mm caudal and 1mm rostral to the first hole and were for placement of the electrodes (at a depth of 2.5mm). 5 μg of EGFP DNA (1 μg/μL) was injected into the center hole to a depth of 2.5 mm and the plasmid is pulsed into the surrounding cells. The square pulse generator (BTX, Hollister, Maine) is then set to 225V with 10 pulses, 2Ms each and 250Ms intervals between pulses. The animal was given an overdose (~150mg/kg) of sodium pentobarbital (i.p.) 24 hours later and the brain carefully removed under a fluorescence dissecting scope (Leica, Heerbruug, Switzerland) and photographed with a Leica stereoscopic camera (Leica, Heerbruug, Switzerland). EGFP was electroporated both in the hippocampus and subventricular zone (figure 27).

7.2.6. Behavior

For the RAWM task of spatial working memory (Arendash et al., 2007; Arendash et al., 2001; Ethell et al., 2006), an aluminum insert was placed into a 100cm circular pool to create 6 radially-distributed swim arms emanating from a central circular swim area (Figure 28). An assortment of 2-D and 3-D visual cues surrounded the pool. The number of errors prior to locating which one of the 6 swim arms contained a submerged escape platform (9cm diameter) was determined for 5 trials/day. There was a 30-min time delay between the 4th trial (T4; final acquisition trial) and 5th trial (T5; memory retention trial). The platform location was changed daily to a different arm, with different start arms for each of the 5 trials semi-randomly selected from the remaining 5 swim arms. During each trial (60 s maximum), the mouse was returned to that trial’s start arm upon swimming into an incorrect arm and the number time required to locate the submerged platform was recorded. If the mouse did not find the platform within a 60-s trial, it was guided to the platform for a 30-s stay. The numbers of errors and escape latency during trials 4 and 5 are both considered indices of working memory and are temporally similar.
to standard registration/recall testing of specific items used clinically in evaluating AD patients.

7.2.7. Preparation of Brain Extracts and Western Blot Analysis

The neocortex and hippocampus were each microdissected on ice, immediately snap frozen in powdered dry ice, and stored at -80°C until use. The tissue samples were then dounce homogenized in cold RIPA buffer (50 mM TrisHCl pH7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) in the presence of a protease inhibitor cocktail (Complete mini protease inhibitor tablet -Roche Diagnostics, Indianapolis, IN). Samples were subsequently spun at 20,000 g for 30 min (Eppendorf 5417R, Westbury, NY) and the supernatants removed, aliquoted, and frozen at -80°C. Equal amounts of protein from each sample were mixed with 2x sample buffer (Invitrogen, Carlsbad, CA), boiled for 5 min, and subjected to 10-20% Tris Tricine SDS gel electrophoresis (Invitrogen). Aβ and plaques were immunolabeled using 6E10 and 4G8 antibodies (1:1000) (Covance, Princeton, NJ). The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit IgG (Pierce, Rockford, IL). The immuno-labelled proteins were visualized with an enhanced chemiluminescence detection kit (SuperSignal pico, Pierce, Rockford, IL). After different exposures, autoradiographs were developed with a Konica/Minolta SRX101A (Tokyo, Japan), scanned using a Ricoh Aficio MP3010 (Tokyo, Japan). Quantification was not performed due to a lack of correlation between plaques of treated and untreated animals.

7.2.8. Histology and Immunohistochemistry

Mouse brain tissues were fixed in 10% formaldehyde (formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%) over another 72 hours. Brains were frozen to the peltier stage (Physitemp, Clifton, NJ) of a histoslide (Leica, Heerbruug, Switzerland) and sectioned coronally at 25 μm. After incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat serum, 0.1% Triton X-
100, and 0.02% sodium azide) for 60 min, primary antibodies against ACT (1:500- Dako, Glostrop, Denmark) CAMKII (1:500), pCAMKII (1:1000), GlutR1 (1:1000), pGlutR1 (1:1000), synaptophysin (1:250), synaptopodin (undiluted), synaptobrevin, PSD-95 (1:500), β-tubulin (1:1000) and GAP-43 (1:500) and Aβ (6E10,1:1000- Covance, Princeton, NJ) were applied and incubated at 4 °C overnight. After thorough washing, the sections were incubated with secondary antibodies, Alexa 488 and 594 fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours. This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy Sciences, Hatfield, PA). The images were analyzed on a Zeiss Imager Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7 software and quantified using Image J.

7.3. Results and Conclusions

Memantine (Namenda) functions through inhibition of NMDA receptors in order to reduce the effects of glutamate on post synaptic neurons. Glutamate excitotoxicity is a common pathology for most neurodegenerative diseases and is generally accepted as part of the AD pathogenesis (Schaeffer and Gattaz, 2008). This issue, however, is debated since earlier experiments with transgenic mice were somewhat conflicting and the effects of memantine on AD patients are modest at best (Farlow et al., 2008). In these experiments, we used the tripeptide carboxyl terminus of gamma laminin, KDI, to determine if a) glutamate excitotoxicity is a factor in a mouse models of Alzheimer’s and b) if KDI would reduce the excitotoxic damage and reverse cognitive decline in these mice. Previous work with the peptide fragment revealed that it is a potent non competitive ionotropic glutamate receptor antagonist that inactivates most AMPA receptors and ~50% of NMDA receptors (Moykkynen et al., 2005). In another set of experiments, this same group showed that pretreatment of the rat hippocampus with KDI followed by an injection of kainate, a glutamate analog, prevented excitotoxicity and neuron loss in a dose-dependent manner. This evidence, taken together with the fact that
KDI is a potent neurite outgrowth factor, indicated it had excellent therapeutic potential in AD.

Initially, the transgenic KDI-treated group was showing improvement in cognition from RAWM trials (figures 29 and 30). This was true when comparing individual animals before and after treatment as well as between sham and treated groups of animals. This prompted some excitement and although no change in plaque load was detected, the idea that cognition was improved through the glutamate pathway led us towards thinking about LTP as a possible mechanism for cognitive enhancement. LTP has been identified as a possible molecular mechanism for learning in the DG. Briefly, glutamate binds to AMPA and NMDA receptors and triggers the influx of sodium and calcium, respectively into the postsynaptic cell, which causes a short-lived depolarization called the excitatory postsynaptic potential (EPSP). With repeated stimulation, the next EPSP will occur prior to the decay of the previous. The sum of this is that postsynaptic neurons can be more readily depolarized following a succession of EPSPs and the influx of ions causes a temporary upregulation of the cellular kinases CamKII, PKC, and PKA which function to phosphorylate dormant AMPA receptors, bringing them activated to the synapse and essentially amplifying the signal in the post-synaptic neuron (Kerchner and Nicoll, 2008). To this end, changes in expression of CamKII, pCamKII, GluR1, and pGluR1 were looked into, but not observed. Similarly, there were no detectable changes in synaptophysin, synaptotagmin, synaptobrevin or GAP-43, suggesting that there was not increased synaptogenesis occurring in the treated mice. Furthermore, subsequent behavioral trials failed to repeat what was seen in the first cohort and it was decideded, conclusively, that KDI was of no therapeutic benefit to AD mice.
7.4. Figures

**Figure 27.** Demonstration of successful EGFP vector electroporation in the hippocampus and subventricular zone of mouse brain.
Figure 28. Schematic of radial arm water maze for rodents in testing working (short-term) memory.
Figure 29. Preliminary latency plots in the RAWM of KDI treated and untreated Tg2576 AD mouse models. Results were not repeatable in 2 subsequent cohorts- no change was seen between groups (data not shown).
Figure 30. Preliminary error plots in the RAWM of KDI treated and untreated Tg2576 AD mouse models. Results were not repeatable in 2 subsequent cohorts- no change was seen between groups (data not shown).
CHAPTER 8 – ACT INDUCES THE PHOSPHORYLATION OF TAU IN MOUSE MODELS OF AD

8.1. Introduction

Alpha 1-antichymotrypsin (ACT) is an acute phase serum glycoprotein and serine protease inhibitor that has been shown conclusively to accelerate amyloid fibril and plaque formation in vitro and in vivo (Ma et al., 1994; Nilsson et al., 2004). ACT is upregulated in the injured and AD brain and released by astrocytes surrounding the neurotoxic insult. Whereas ACT has a direct role in the formation of amyloid plaques, recent work from our lab has shown that it can also induce the phosphorylation of tau, which is an important step in the formation of neurofibrillary tangles (NFTs), the other classical protein misfolding hallmark of AD. NFTs are comprised mainly of the microtubule binding protein tau. Tau activity is regulated by phosphorylation and hyperphosphorylation of tau promotes its misfolding and dissociation from microtubules. ptau assembles into paired helical filaments (PHF) that eventually aggregate into insoluble NFTs causing disruption of the cytoskeleton, abnormal axonal transport, and neurotoxicity. The upregulation of soluble ACT in the AD brain, the resulting hyperphoshorylation of tau and the inclusion of ACT in the formation of amyloid plaques, make ACT a link between the amyloidogenic and and tauopathic pathways in Alzheimer’s disease.

Further evidence for ACT’s involvement in the phosphorylation of tau comes from examination of post mortem brain tissue from individuals of other tauopathies. Known to be closely involved with AD pathology, ACT was found to be upregulated and colocalized to tau deposits in frontotemporal dementia (FTD), corticobasal degeneration (CBD), and progressive supernuclear palsy (PSP) patients in neurons and astrocytes.
Moreover, these inclusions are all PHF-1 immunopositive, suggesting that an interaction between ACT and tau might be partly responsible for the pathogenic events in these diseases. ACT is a suicide protease inhibitor, which binds to its target, changes its conformation, and is then, together with the bound protease, endocytosed. Although ACT has been found in astrocytes (Abraham, 2001), it was initially not found in neurons. While early northern blot analyses failed to detect ACT mRNA in neurons, subsequent immunocytochemical analyses demonstrated its presence both in neurons and astrocytes. This observation strongly suggests that ACT, after inhibition of its target protease, enters neurons and then interacts with tau. Alternatively, tau phosphorylation could occur through signal transduction cascades once ACT binds to a receptor, though no evidence for these hypotheses has been yet obtained. Nonetheless, human cortical neurons and mouse embryonic neurons treated with ACT demonstrate increased phosphorylation of tau at pSer202, Pthr 231, and psr 396/404 (PHF-1) as well as intraneuronal ACT staining (Padmanabhan et al., 2006).

In the following experiments, we wished to intracranially infuse ACT into the hippocampal parenchyma of transgenic mice that express human tau, but no mouse tau, to determine if tau phosphorylation and NFT formation would ensue. ACT is well established in the AD field as an amyloid promoter. If it could also be proven to be a part of the ensuing Alzheimer’s tauopathy it would link these two pathologies. Demonstrating that ACT serves as such a link might make it a viable therapeutic target for multiple forms of tauopathies and neurodegeneration.

8.2. Materials and Methods

8.2.1. Catheter Construction

The catheters (patent pending- PCT/US08/73974) used for this experiment were made by first taking a 1.25 cm length of 30 gauge stainless steel tubing (Small Parts, Inc. Miramar, Fl) and carefully bending it at 2.5 mm under a dissecting microscope (Leica, Heerbruug, Switzerland), to approximately 90 degrees, being careful not to crimp the
tube. The remaining length of the tube was bent again at 5 mm to an angle of 120-160 degrees, approximating the contour of each animal’s skull. The metal cannulae were inserted into a 3 cm length of polyethylene (PE-10) tubing with an internal diameter of 0.28 mm and an outer diameter of 0.61 mm. One centimeter of the PE-10 was then itself inserted into a 4.5 cm length of sterile polyvinyl tubing (PV-50-I.D. 0.69 mm/ O.D. 1.14 mm, Durect Corp., Cupertino, Ca), held in place using a bead of Locktite 454 adhesive (Plastics One. Roanoke, VA.), and cured overnight. The following day, sterile water was forced through each catheter assembly to ensure that the lines were not obstructed. The osmotic pumps were then filled with solution, attached to the catheters and primed and implanted as described below. The proven catheters were later custom manufactured by Braintree Scientific (Braintree, MA) with some minor changes: The adhesive was supplanted by heat sealing PE-10 to PE-50 tubing and, because the diameters of the PV-50 and new PE-50 were not exactly the same, a sheath of silicone (SIL 047) was placed over the PE-50 with an extra 2 mm overhang to attach it to the flow modulator of the pump, forming a tight, leak-proof seal.

8.2.2. Transgenic Mice

10 htau mice (B6.Cg-Mapt\textsuperscript{pm1(GFP)Klt} Tg(MAPT)8cPdav/J) were purchased from Jackson Labs. These mice are homozygous for knock out of the the targeted endogenous mtau alleles and hemizygous for the transgene. Although no endogenous mtau is detected, all six isoforms (including both 3R and 4R forms) of human MAPT are expressed. Hyperphosphorylated tau is detected in cell bodies and dendrites by three months. Paired helical filaments of aggregated insoluble MAPT can be isolated from brain tissue as early as two months of age. Overall, these mice develop tau pathology very quickly and are fertile and viable for studies where they are bred with other AD transgenics.
8.2.3. Materials

Purified human α-1 antichymotrypsin (ACT) was purchased lyophilized from Fitzgerald (Concord, MA) and was reconstituted in artificial CSF (Harvard Apparatus, Holliston, MA) to a concentration of 1mg/mL. ACT was infused directly into the hippocampal parenchyma for 28 days using the novel catheters attached to Alzet osmotic minipumps (Alzet model 1004, Durect Corp. Cupertino, CA) with an average flow rate of 0.12μL/hour. The pumps and catheters were submerged in 0.9% sterile saline at 37°C and primed for 48 hours prior to implantation.

8.2.4. Intracranial Infusions

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (3 month-old htau- 20-25g) were anesthetized with 1-2% isoflurane, shaved and scrubbed with 10% Betadine solution at the site of incision, and placed into a stereotaxic frame (Kopf Instruments. Tujunga, Ca.). A small (3 cm) incision was made, exposing the skull and neck, and double bladed scissors were used to form a subcutaneous pocket along the back of the animal into which 2 osmotic minipumps were inserted with catheters attached. Two holes were drilled into the skull (from Bregma -2.2-2.5 mm anterior-posterior, +/- 2.2-2.5 mm medial-lateral, and the 30 gauge catheters were inserted to a depth of 2.2-2.5 mm) corresponding to the posterior portion of the CA3 hippocampus. Once they were inserted, the cannulae were affixed to the skull with Loctite 454 adhesive (Plastics One. Roanoke, VA.) and secured down with a piece of nitrile, approximately 1 cm in diameter (Fig 3D). After the adhesive cured, the scalp was closed with 4-6 silk sutures. For analgesia, the animal received an immediate dose of ketoprofen (10mg/kg) and up to every 6 hours, as needed, for a maximum of 48 hours post-operatively. After the 28 day period, the animals were given an overdose (~150mg/kg) of sodium pentobarbital (i.p.) and were transcardially perfused with 0.9% saline. The brains were carefully removed and analyzed as outlined below. At
this time, the integrity of the catheters was confirmed again by forcing water through, and the pumps were also determined to be empty.

8.2.5. Preparation of Brain Extracts and Western Blot Analysis

The neocortex and hippocampus were each microdissected on ice, immediately snap frozen in powdered dry ice, and stored at -80°C until use. The tissue samples were then dounce homogenized in cold RIPA buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.1% SDS) in the presence of a protease inhibitor cocktail (Complete mini protease inhibitor tablet - Roche Diagnostics, Indianapolis, IN). Samples were subsequently spun at 20,000 g for 30 min (Eppendorf 5417R, Westbury, NY) and the supernatants removed, aliquoted, and frozen at -80°C. Equal amounts of protein from each sample were mixed with 2x sample buffer (Invitrogen, Carlsbad, CA), boiled for 5 min, and subjected to 10-20% Tris Tricine SDS gel electrophoresis (Invitrogen). Purified hACT and nontransgenic brain homogenates were used as controls. The electrophoresed proteins were wet transferred onto 0.2 µm nitrocellulose (Whatman, Dassel, Germany) and probed with a rabbit anti-mouse polyclonal antibody to monoclonal/polyclonal antibodies to PHF-1 (1:1000- Peter Davies, Albert Einstein Inst. NY), pSer202/CP-13 (1:1000-Peter Davies), MC-1 (1:500- Peter Davies), Tg5 total tau (1:1000- Peter Davies), pGSK3α/β (1:1000- Cell Signaling, Beverly, MA), pAKT (1:1000- Cell Signaling), pERK (1:1000- Cell Signaling), pThr231 (1:1000-Invitrogen), pSer422 (1:1000- Invitrogen), pSer262 (1:1000- Invitrogen), AT-100 (1:1000- ThermoScientific, Waltham, MA), ACT (1:1000- Dako, Glostrop, Denmark). The secondary antibodies are horseradish peroxidase-conjugated goat anti-mouse IgG and donkey anti-rabbit IgG (Pierce, Rockford, IL) and the bands were visualized with an enhance SuperSignal pico (Pierce, Rockford, IL). The immuno-labelled proteins were visualized with an enhanced chemiluminescence detection kit (SuperSignal pico, Pierce, Rockford, IL). After different exposures, autoradiographs were developed with a Konica/Minolta SXR101A (Tokyo, Japan), scanned using a Ricoh Aficio MP3010 (Tokyo, Japan) and quantified using
Image J. Alternatively, the secondary antibodies used were infrared (Licor, Lincoln, NE) and visualized at both 680 and 800 nm using the Odyssey (Licor).

8.2.6. Histology and Immunohistochemistry

Mouse brain tissues were fixed in 10% formaldehyde (formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%) over another 72 hours. Brains were frozen to the peltier stage (Physitemp, Clifton, NJ) of a histoslide (Leica, Heerbrugg, Switzerland) and sectioned coronally at 25 μm. After incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat serum, 0.1% Triton X-100, and 0.02% sodium azide) for 60 min, a set of primary monoclonal/polyclonal antibodies was applied. Primary antibodies (mono and polyclonal) included antibodies to PHF-1 (1:250- Peter Davies, Albert Einstein Inst. NY), pSer202/CP-13 (1:250-Peter Davies), MC-1 (1:100- Peter Davies), Tg5 total tau (1:250- Peter Davies), pGSK3α/β (1:250- Cell Signaling, Beverly, MA), pAKT (1:250- Cell Signaling), pERK (1:250- Cell Signaling), pThr231 (1:250-Invitrogen), pSer422 (1:250-Invitrogen), pSer262 (1:250-Invitrogen), AT-100 (1:250-ThermoScientific, Waltham, MA), ACT (1:500-Dako, Glostrop, Denmark). All were applied and incubated at 4 °C overnight. After thorough washing, the sections were exposed to secondary antibodies, Alexa 488 and 594 fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours. This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy Sciences, Hatfield, PA). The images were analyzed on a Zeiss Imager Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7 software.

8.3. Results and Conclusions

It was also discovered recently that ACT is not only upregulated in AD, but other tauopathies including FTDP, CBD, and PSP (Padmanabhan et al., 2006). When
considering that ACT is highly overexpressed in the AD brain and is part of amyloid plaques, taken together with ACT’s role in tauopathies, we hypothesized that ACT could be a trigger for tau phosphorylation in AD. A primary focus in AD research has been finding relationships between the two primary hallmarks of the disease, amyloid and tau. Whereas it was believed for some time that these pathologies existed independently of each other, new evidence suggests that both amyloid aggregates and intracellular AB can stimulate the phosphorylation of tau. In these experiments, we demonstrate that ACT, an acute phase serum glycoprotein, contributes to phosphorylation of tau in vivo.

Previously, it was demonstrated in human and PDAPP mouse cortical neurons that adding ACT to the culture media resulted in AD-specific phosphorylations of tau known to contribute to AD pathogenesis. When ACT is chronically infused into the hippocampus of an htau mouse, these same phosphorylation events occur, although not due to the activity of the same intracellular kinases.

Immunohistochemical analyses revealed that increased levels of phosphor-tau were found in the hippocampus of the ACT infused hemisphere of 5 month old htau mice and in 12 month old PDAPP mice. Specifically, strong staining for pSer202 (CP-13 antibody) was detected in htau mice (figure 32) and pThr231 in PDAPP Mice (figure 36). Western blot analysis confirmed the results in the htau mice. Brain homogenates from ACT-treated and untreated htau brain hemispheres were run side by side. Pser202 was increased in each case where ACT was infused (figure 33). Moreover, it was also determined that there was an increase in pSer396/Thr404 (PHF-1) in the presence of ACT (figure 31), a phosphorylation event closely associated with pre-tangle formation. This site has been shown to be phosphorylated by GSK-3β and activation of GSK-3β (indicated by dephosphorylation at 51KDa) is evident in this study (figure 34). All of these data are consistent with previous in vitro data. One difference between the studies was that ERK was activated (indicated by increased phosphorylation at 42/44KDa) in the presence of ACT in vitro and appears inactivated in vivo following ACT infusions (figure 35). The exact mechanism by which tau becomes hyperphosphorylated in Alzheimer’s disease is not known. In vitro analysis of tau phosphorylation have shown that several kinases can induce phosphorylation of tau at numerous sites, roughly half of which are
observed to be hyperphosphorylated in Alzheimer’s disease brains. Inhibition of GSK-3β, for example, was shown to reduce the phosphorylation of tau and increase cell viability. GSK-3β, amongst other kinases, is elevated in the brains of Alzheimer patients, which suggests that molecules, in this case inflammation-related, can trigger their activation, contributing to tau hyperphosphorylation and tangle formation.

In these experiments, we build upon our previous work in which we propose that the increased Aβ level in Alzheimer’s disease brain activates microglia to express inflammatory proteins like IL-1B. IL-1B, in turn, activates astrocytes to release large amounts of ACT into the surrounding parenchyma (Abraham, 2001). ACT then triggers increased expression or activation of certain kinases, such as GSK-3, which results in tau hyperphosphorylation, tangle formation and neuronal degeneration (Hooper et al., 2008). Therefore, our data support the theory of an inflammatory cascade in AD, brought on by Aβ. More experiments are currently underway to determine which other kinases are activated in response to ACT that result in tau phosphorylation. Inhibition of kinase activity is currently being pursued as a potential therapy for AD (Perez-Martinez, 2009). These results suggest that ACT could be a candidate as an upstream target in both the formation of amyloid plaques and phosphorylation of tau.

Taking together the in vitro and in vivo tau data, these results suggest that inhibiting ACT upstream from tangle formation (and amyloid plaque formation- chapter 9) could be a potential therapy for AD.
8.4. Figures

Figure 31. ACT infusion induces increases in phospho tau (PHF-1) in htau mice. Whole brain homogenates from ACT treated (right) and untreated (left) hemispheres of htau mice are run alongside each other in a western blot and visualized using infrared secondary antibodies (Lycor). The white arrows indicate where ACT (in red) was infused. PHF-1 is shown in green and the signal intensity increases in the presence of ACT. Data is not quantified due to the small number of animals used.
Figure 32. Immunohistochemical analysis of an htau mouse following ACT infusion (left side). The montage was taken at 5x and the inlays taken at 40x. ACT is shown in red, pSer202 is shown in green and is upregulated in the presence of ACT. Data is not quantified due to the small number of animals used.
Figure 33. Whole brain homogenates from ACT treated) and untreated hemispheres of htau mice are run alongside each other in a western blot and visualized using an infrared secondary antibody (Lycor). PSer202 signal intensity (green) increases in the presence of ACT.
Figure 34. ACT infusion leads to a dephosphorylation, thus activation, of GSK3 as indicated by the arrows on a western blot of whole brain homogenates from htau mice.
Figure 35. ACT infusion leads to decreased phosphorylation, thus inactivation, of ERK as indicated by the arrows on a western blot of whole brain homogenates from htau mice.
**Figure 36.** Infusion of ACT into the hippocampus of a PDAPP mouse induces phosphorylation of endogenous mouse tau at Thr231 (left) as compared to aCSF into the contralateral hemisphere (right).
CHAPTER 9 – THE EFFECTS OF CHRONIC INTRACEREBRAL INFUSION OF ACT ON AMYLOID PLAQUES: INHIBITION WITH A-BETA RELATED PEPTIDES

9.1. Introduction

Previous work in our laboratory and others have demonstrated that inflammation and an acute phase response are initiated in the extracellular periphery of amyloid plaques (Abraham et al., 1988; Potter et al., 2001). This inflammation appears to be initiated when increased levels of oligomeric/fibrillar Aβ activate microglia, inducing them to release cytokines such as IL-1β, IL6 and TNFα. IL1 and IL6, in turn trigger the production and release of ACT and apoE from astrocytes (Das and Potter, 1995). These two products of inflammation have been termed pathological chaperones because they accelerate the fibrillization of the Alzheimer Aβ peptide and subsequent amyloid plaque formation. ACT, a serine protease inhibitor (serpin) is serum glycoprotein that is normally made in the liver in response to inflammation and inhibits proteases in order to maintain homeostasis and prevent undue tissue destruction during the acute phase response. ACT is upregulated significantly in the brain of AD patients (Porcellini et al., 2008). ApoE is also upregulated and released from astrocytes and microglia during brain damage and inflammation. The ApoE4 isoform has been shown to be the strongest heritable risk factor for the disease and is also released by astrocytes and microglia during the acute phase response (Abraham, 2001). Evidence that polymorphisms in the ACT gene are also risk factors for developing is accumulating but does not come near the strength of the apoE4 effect (Nilsson et al., 2004).

It has been demonstrated that synthetic Aβ peptides self-polymerize in vitro under various physiological conditions to form filaments that resemble the beta-pleated amyloid
structures found in AD by transmission electron microscopy (TEM) (Castelletto et al., 2008) and attenuated total reflection-FTIR spectroscopy (Cerf et al., 2009). These preparations of filaments have also proven to be neurotoxic to primary neuronal cell cultures. The polymerization of Aβ, it was found, could be accelerated by ACT, and to a greater extent by ApoE4. Moreover, it has been established that ACT and ApoE each bind to different regions on the AB peptide (Ma et al., 1994). ACT binds to the hydrophilic N-terminus (amino acids 2-9) and ApoE to the 12-28 region of the Aβ peptide. These small fragments, when incubated with full length Aβ and their respective pathological chaperones (ACT and ApoE) inhibit the catalyzed polymerization of fibrils in a dose-responsive manner. When these reactions were added to cultures of human cortical neurons, it was found that the two inhibitory peptides prevented apoE and ACT respectively from promoting Aβ polymerization and neurotoxicity, thus enhancing cell survival, as visualized by MTT assays (Ma et al., 1996).

Work with transgenic mice has extended and supported the cell culture findings described above. Breeding PDAPP mice with and without endogenous mouse ApoE and with and without a hACT transgene demonstrated that both ACT and ApoE act independently, but synergistically to facilitate the formation of amyloid plaques. Moreover, it was determined that the regions of the brain containing plaques were somewhat different. In the absence of ApoE, ACT-positive plaques were localized to the hippocampus and in the presence of ApoE, ACT-positive plaques were found in the hippocampus and cortex (Nilsson et al., 2001). These experiments also showed that cognitive deficits in the PDAPP mice, as assayed by the Morris Radial Arm Water Maze and the Radial Arm Water Maze, depended on the presence of ACT and/or apoE-promoted amyloid deposits (Bales et al., 1999; Nilsson et al., 2001).

In December 2006, Wiesniewski et al. created a compound named Aβ12-28P representing a modified 12-28 peptide that reduced amyloid burden when injected systemically into APP-expressing mice. The modifications to the peptide included exchanging the valine in position 18 for proline, thus rendering the peptide nonfibrillogenic and preventing the molecule from binding to plaques. Additionally, AB12-28P was synthesized in a slightly modified version by using D-amino acids and
end-protected by acetylation and amidation of the N and C termini, respectively. These modifications decreased the potential for immunogenicity and extended the serum half-life of the peptide. AB12-28P was, however, still able to cross the BBB and to inhibit apoE/Aβ binding with these modifications. Specifically, the compound was able to decrease amyloid burden by 51.6% and 45.6% in the cortex and hippocampus, respectively, of 7 month old Tg2576 mice and 50.7% and 40.0% in PS/APP (PS-Tg2576) mice (Sadowski et al., 2006). Evidently, the ability of Aβ 12-28 to serve as a decoy peptide and prevent apoE from binding to Aβ in vitro was duplicated in vivo, and suggests that the modified peptide designed by Wisniewski and colleagues is a potential therapy for Alzheimer’s disease.

Because the effect of the interaction of Aβ peptides with ACT on the formation of amyloid was well established, we desired to determine whether this interaction could be exploited to develop a novel Alzheimer therapy. We first studied the effects of ACT on existing plaques by infusing purified human ACT intracranially into PDAPP mice at ~ 8 months of age and assessing the morphology of the plaques. In the next phase of the research, we wished to determine whether the N-terminus of Ab could inhibit the ACT/Aβ interaction in a manner similar to the inhibition of the ApoE/Aβ interaction in vivo by the modified 12-28 peptide. To this end, experiments were designed in which ACT was co-infused with Aβ 1-11 into the hemispheres of PS/APP mice as was the Aβ 1-11 fragment alone.

9.2. Materials and Methods

9.2.1. Catheter Construction

The catheters (patent pending- PCT/US08/73974) used for this experiment were made by first taking a 1.25 cm length of 30 gauge stainless steel tubing (Small Parts, Inc. Miramar, Fl) and carefully bending it at 2.5 mm under a dissecting microscope (Leica, Heerbruug, Switzerland), to approximately 90 degrees, being careful not to crimp the tube. The remaining length of the tube was bent again at 5 mm to an angle of 120-160
degrees, approximating the contour of each animal’s skull. These metal cannulae were inserted into a 3 cm length of polyethylene (PE-10) tubing with an internal diameter of 0.28 mm and an outer diameter of 0.61 mm. One centimeter of the PE-10 was then itself inserted into a 4.5 cm length of sterile polyvinyl tubing (PV-50-I.D. 0.69 mm/ O.D. 1.14 mm, Durect Corp., Cupertino, Ca), held in place using a bead of Locktite 454 adhesive (Plastics One. Roanoke, VA.), and cured overnight. The following day, sterile water was forced through each catheter assembly to ensure that the lines were not obstructed. The osmotic pumps were then filled with solution, attached to the catheters and primed and implanted as described below. The proven catheters were later custom manufactured by Braintree Scientific (Braintree, MA) with some minor changes: The adhesive was supplanted by heat sealing PE-10 to PE-50 tubing and, because the diameters of the PV-50 and new PE-50 were not exactly the same, a sheath of silicone (SIL 047) was placed over the PE-50 with an extra 2 mm overhang to attach it to the flow modulator of the pump, forming a tight, leak-proof seal.

9.2.2. Transgenic Mice

PS/APP (presenilin 1/amyloid precursor protein) mice and PS/APP/ACT mice were generated by crossing heterozygous PDGF-hAPP (V717F) mice with PDGF-hPS1 (M146L) on both Swiss Webster and C57BL/6 backgrounds. Genotyping was performed using comparative real-time PCR (Bio-Rad iCycler- Hercules, CA). Pathologically, these Alzheimer's mouse models are characterized by robust accumulation of amyloid plaques and the development of microgliosis between 6-8 months of age.

9.2.3. Materials

Purified human α-1 antichymotrypsin (ACT) was purchased lyophilized from Fitzgerald (Concord, MA) and was reconstituted in artificial CSF (Harvard Apparatus, Holliston, MA) to a concentration of 1mg/mL. The Aβ 1-11 was suspended at 5 mg/mL (Rpeptide, Bogart, GA) ACT was infused directly into the hippocampal parenchyma for
28 days using the novel catheters attached to Alzet osmotic minipumps (Alzet model 1004, Durect Corp. Cupertino, CA) with an average flow rate of 0.12μL/hour. The pumps and catheters were submerged in 0.9% sterile saline at 37°C and primed for 48 hours prior to implantation.

9.2.4. Intracranial Infusions

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Animals (8 month-old PS/APP- 25-35g) were anesthetized with 1-2% isoflurane, shaved and scrubbed with 10% Betadine solution at the site of incision, and placed into a stereotaxic frame (Kopf Instruments. Tujunga, Ca.). A small (5 cm) incision was made, exposing the skull and neck, and double bladed scissors were used to form a subcutaneous pocket along the back of the animal into which 2 osmotic minipumps were inserted with catheters attached. Two holes were drilled into the skull (from Bregma -2.2-2.5 mm anterior-posterior, +/- 2.2-2.5 mm medial-lateral, and the 30 gauge catheters were inserted to a depth of 2.2-2.5 mm) corresponding to the posterior portion of the CA3 hippocampus. Alternatively, holes were drilled corresponding to the lateral ventricles (from Bregma -0.2 mm anterior-posterior, +/- 1.0 mm medial-lateral, and to a depth of 2.2-2.5 mm). Once they were inserted, the cannulae were affixed to the skull with Locktite 454 adhesive (Plastics One. Roanoke, VA.) and secured down with a piece of nitrile, approximately 1 cm in diameter (Fig 3D). After the adhesive cured, the scalp was closed with 6-7 silk sutures. For analgesia, the animal received an immediate dose of ketoprofen (10mg/kg) and additional doses once every 6 hours, as needed, for a maximum of 48 hours post-operatively. After the 28 day period, the animals were given an overdose (~150mg/kg) of sodium pentobarbital (i.p.) and were transcardially perfused with 0.9% saline. The brains were carefully removed and analyzed as outlined below. At this time, the integrity of the catheters was confirmed again by forcing water through, and the pumps were also determined to be empty.
9.2.5. Histology and Immunohistochemistry

Mouse brain tissues were fixed in 10% formaldehyde (formalin) for 72 hours and then passed through a series of sucrose solutions (10-30%) over another 72 hours. Brains were frozen to the peltier stage (Physitemp, Clifton, NJ) of a histoslide (Leica, Heerbruug, Switzerland) and sectioned coronally at 25 μm. After incubating the sections with blocking buffer (Tris-buffered saline with 10% normal goat serum, 0.1% Triton X-100, and 0.02% sodium azide) for 60 min, primary antibodies against Aβ (6E10 and 4G8, 1:1000- Covance, Princeton, NJ) as well as Iba1 (1:1000) (Wako Chemicals, USA. Inc., Richmond, VA.) were applied and incubated at 4 °C overnight. After thorough washing, the sections were incubated with secondary antibodies, Alexa 488 and 594 fluorophores (1:1000, 1:4000-Invitrogen) in the dark at ambient temperature for 2 hours. This incubation was followed by a Hoechst (1:10,000, Sigma, St. Louis, MO) nuclear stain, and the sections were washed and sealed with Gel-Mount (Electron Microscopy Sciences, Hatfield, PA). The images were analyzed on a Zeiss Imager Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7 software and quantified using Image J.

9.3. Results and Conclusions

Previous work from our and other laboratories has shown that Aβ is the primary component of amyloid plaques found in the AD brain. It has been shown in vitro that Aβ can self polymerize into fibrils and that this process is accelerated by extracellular pathological chaperones (Wisniewski and Frangione, 1992). The term “pathological chaperone” which was coined by Thomas Wiesnieski in 1992 is used to describe molecules that promote a pathological change in a protein of interest, in our case Aβ. Molecular chaperones, on the other hand, are responsible for promoting the folding of proteins into their physiological, rather than a pathological conformation. Previous work has demonstrated that both ACT and ApoE have roles as pathological chaperones in AD. Not only can they accelerate the formation of Aβ fibrils in vitro (Ma et al., 1994), but
transgenic animal studies have also revealed that these molecules bind directly to Aβ fibrils and become part of the ensuing amyloid plaques (Nilsson et al., 2004).

ApoE and ACT are both upregulated in the AD brain and released by residing glia as part of the chronic inflammation induced by Aβ (or Aβ oligomers). The normal physiological roles of these molecules are also known. ApoE is released by hepatocytes and is the major binding protein of LDL during cholesterol transport to tissues. ACT, on the other hand, is released by hepatocytes and neutrophils to inhibit cathepsin G during inflammation to remodel damaged tissues and possibly engulf pathogens (Kalsheker, 1996). ACT is a serine protease inhibitor that is produced in concert with several serine proteases that would otherwise go out of homeostatic balance during growth and repair and cause damage to cells and tissues. The normal roles of ApoE and ACT in the brain, however, are not as clear. One possibility for the presence of ACT was proposed by our lab some time ago: Because the structural homology between cathepsin G, chymotrypsin and Aβ is striking, Aβ might be recognized by ACT as a protease and bound to inhibit its action (Ma et al., 1994).

To interfere with the interaction of ACT and Aβ, thereby the formation of amyloid, novel strategies have been developed to inhibit the binding of these molecules. Previous therapeutic approaches have been focused on inhibiting the secretase enzymes that overproduce Aβ, on immunizing patients against the Aβ peptide, or on manipulating cholinergic and glutaminergic neurotransmission. Inhibiting the interaction of Aβ with ApoE and ACT respectively, was a new approach that has also proven effective in mouse models. Early experiments showed ACT and ApoE could accelerate the formation of Aβ fibrils in vitro, but also that this promotion could be inhibited with small fragments of the Aβ peptide itself. The amino acids that correspond to aminoacids 12-28 in the Aβ 1-42 peptide, attenuated the formation of fibrils significantly when incubated with ApoE (Ma et al., 1996). Similarly, when Aβ 1-42 was incubated with ACT and the amino acids that correspond to positions 2-9 of the Aβ 1-42 fragment, attenuated fibril formation was observed as well. Additionally, when these reactions were cultured with human cortical neurons, MTT cell survival assays determined that the cells were more viable with increasing concentrations of the inhibiting peptides. In sum, these experiments
demonstrated that “decoy” fragments of Aβ could bind ApoE and ACT, thereby preventing these proteins from binding to full length Aβ and accelerating the formation of amyloid.

The next phase of this research, in vivo, was carried out by Wiesnieski et al. in 2006. In this set of experiments, the modified 12-28 peptide, previously described, was delivered systemically in mice harboring the Swedish APP mutation and was found to drastically reduce amyloid load and improve cognition. In my experiments described here, the rationale was very similar in that the an Aβ fragment of 11 amino acids, covering the 2-9 region of Aβ was used to interrupt the Aβ/ACT interaction. However, in these experiments, the peptide was delivered without modification and via direct infusion into the hippocampus of PS1/PDAPP mice. Though preliminary, the delivery of the 1-11 peptide in a small group of animals (n=4) virtually eliminated plaques in the vicinity of the infusions (figures 37 and 38- quantitation figure 39) as well as the presence of microglia (Iba1 staining- figure 40). The use of both 6E10 (figure 37) and 4G8 (figure 38) antibodies to show plaque clearance is notable because they bind the 1-16 and 17-24 residues on Aβ, respectively, which correspond to the binding sites of ACT and ApoE. One might expect that the epitope for ACT be bound to the decoy peptide and not available for the antibody, but staining was strong using both antibodies. Because these mice did not carry the human ACT gene, the interpretation of this finding is not perfectly clear, but it is known that mice have a serpin homologue (Serpin A3N) that is found in plaques that cross-reacts with the ACT antibody. Perhaps the Aβ 1-11 peptide binds to this endogenous protein, and prevents it from promoting amyloid filament formation. Moreover, it appears that the application of the Aβ 1-11 peptide not only interferes with the formation of plaques, but also contributes to the clearance of already existing plaques. The lack of microglial staining on the treated hemisphere would suggest that microglia engulfed amyloid deposits and then departed. Further clarification is currently being pursued with additional experiments. However, it is already evident from this study and the work performed by Wiesniewski et al. that Aβ fragments make effective decoys to bind to the pathological chaperones ApoE and ACT and provide another potential therapy for AD.
9.4. Figures

**Figure 37.** Infusion site of Aβ 1-11 into 8 month old PS/APP mice (n=4) immunolabeled with 6E10 showing marked plaque reduction.
**Figure 38.** Infusion site of Aβ 1-11 into 8 month old PS/APP mice (n=4) immunolabeled with 4G8 showing marked plaque reduction.
Figure 39. Semi-quantification of 4 mice treated with Aβ 1-11 stained with both 6E10 and 4G8 antibodies, with significance of $p=0.024$ and $p=0.058$ respectively.
Figure 40. A) Immunostaining of Aβ 1-11 treated mice with the microglial marker Iba1 showing an absence of microglia on the treated side.
Figure 40. B) Quantification (semi) of above immunohistochemical analysis of Iba1 (n=2)
10.1. Abstract

Objectives: Rheumatoid arthritis (RA) is a negative risk factor for the development of Alzheimer’s disease (AD). While commonly assumed that RA patients’ usage of non-steroidal anti-inflammatory drugs (NSAIDs) helped prevent onset and progression of AD, NSAID clinical trials have proven unsuccessful in AD patients. To determine whether intrinsic factors within RA pathogenesis itself may underlie RA’s protective effect, we investigated the activity of colony-stimulating factors, up-regulated in RA, on the pathology and behavior of transgenic AD mice.

Methods: 5µg bolus injections of macrophage, granulocyte, and granulocyte-macrophage colony- stimulating factors (M-CSF, G-CSF, or GM-CSF) were administered unilaterally into the hippocampus of aged cognitively-impaired AD mice and the resulting amyloid load reductions determined one week later, using the artificial cerebrospinal fluid (aCSF)-injected contralateral sides as controls. 20 daily subcutaneous injections of 5µg of GM-CSF (the most amyloid-reducing CSF in the bolus experiment) were administered to balanced cohorts of AD mice after assessment in a battery of cognitive tests. Reductions in amyloid load and improvements in cognitive function were assessed.
Results: G-CSF and more significantly, GM-CSF reduced amyloidosis throughout the treated brain hemisphere one week following bolus administration to AD mice. Moreover, subcutaneous GM-CSF administration significantly reduced brain amyloidosis and completely reversed the cognitive impairment, while increasing hippocampal synaptic area and microglial density.

Interpretation: These findings, along with two decades of accrued safety data using Leukine, recombinant human GM-CSF, in elderly leukopenic patients, suggest that Leukine should be tested as a treatment to reverse cerebral amyloid pathology and cognitive impairment in AD.

10.2. Introduction

Although numerous studies have reported Rheumatoid arthritis (RA) to reduce the risk of Alzheimer’s disease (AD), the mechanisms for RA’s protective effect are still unknown (McGeer et al., 2006). These reports have commonly assumed that RA patients’ usage of non-steroidal anti-inflammatory drugs (NSAIDs) may help prevent the onset and progression of AD (McGeer et al., 2006), but the largest NSAID clinical trials have not demonstrated efficacy in reducing the incidence of dementia, and recently Naproxen was reported to be detrimental, with increased risk of cardiovascular and cerebrovascular events (Martin et al., 2008). These results suggested to us that intrinsic, probably immunological factors within RA pathogenesis itself, may underlie RA’s protective effect against AD. We surmised that up-regulated local cellular populations in RA would have the highest potential to enter into the brain and inhibit the development of AD pathology and/or neuronal dysfunction.

Alzheimer’s disease is an age-related, progressive neurodegenerative disorder that presents as increasing decline in cognitive and executive function. Alzheimer dementia is associated with cerebrovascular dysfunction (Humpel and Marksteiner, 2005), extracellular accumulation of amyloid β (Aβ) peptides in the brain parenchyma and vasculature walls (Rhodin et al., 2000; Scheuner et al., 1996) (predominantly Aβ1-42 and Aβ1-40), and intraneuronal accumulation of neurofibrillary tangles consisting of
hyperphosphorylated Tau proteins (Padmanabhan et al., 2006). Associated neuroinflammation may contribute to AD pathogenesis (Griffin et al., 1989), as the inflammatory proteins apolipoprotein E (apoE) and α1-Antichymotrypsin (ACT) catalyze the polymerization of Aβ peptides into amyloid filaments in vivo and in vitro (Ma et al., 1996; Nilsson et al., 2004; Padmanabhan et al., 2006; Potter et al., 2001; Wisniewski et al., 1994). Conversely, it has also been shown that amyloid plaques form rapidly and then become decorated by microglia (Koenigsknecht-Talboo et al., 2008; Meyer-Luehmann et al., 2008), both resident and bone marrow-derived, suggesting an ability and intention to remove amyloid (Malm et al., 2005; Simard and Rivest, 2004; Simard et al., 2006). Thus it is unclear whether neuroinflammation is deleterious or beneficial in the AD brain, and indeed the role of microglia in AD is complex and may involve different states of activation with different activities.

Rheumatoid arthritis is an autoimmune disease in which inflamed synovial tissue and highly vascularized pannus form, irreparably damaging the cartilage and bone. In this inflammatory pannus, leukocyte populations are greatly expanded, and many proinflammatory factors are produced that work together in feed-forward mechanisms, further increasing leukocytosis, cytokine/chemokine release, osteoclastogenesis, angiogenesis, and autoantibody production (rheumatoid factors and anti-citrullinated protein antibodies) (Schellekens et al., 2000; Szekanecz and Koch, 2007). Additionally, the adaptive immune system presents a Th17 phenotype within CD4+ lymphocytes, with ultimate production of interleukin 17 (IL-17) which is then responsible for inducing much of the pro-inflammatory effects (Cox et al., 2008; Parsonage et al., 2008). Further enhancements of leukocyte populations come from increased expression of structurally-unrelated colony-stimulating factors: M-CSF (macrophage), G-CSF (granulocyte), and GM-CSF (granulocyte-macrophage) (Kawaj et al., 1995; Nakamura et al., 2000; Olszewski et al., 2001; Xu et al., 1989).

Although up-regulated leukocytes in RA could potentially enter into the brain and inhibit development of AD pathology and/or neuronal dysfunction, lymphocytic infiltrates into AD patient brains have not been reported. The lack of infiltration suggests that proliferation and activation of the innate immune system might be responsible for
preventing AD pathology in RA patients. Evidence supporting the innate immune system’s role in AD pathogenesis show that complement proteins are up-regulated in AD brain, and that inhibition of C3 convertase significantly increases amyloid pathology in AD mice (Wyss-Coray et al., 2002). Bone marrow-derived microglia also play a critical role in restricting amyloid deposition, but this association and many associated receptors and enzymes, such as CD36, scavenger receptor A, and receptor for advanced glycation end products, or neprilysin, insulysin, and matrix metalloproteinases, decline with age, while AD pathology increases (El Khoury et al., 2007; Hickman et al., 2008; Simard et al., 2006).

To investigate the interplay of the innate immune system and AD, we studied the effects on AD pathology of the three colony-stimulating factors (M-CSF, G-CSF, and GM-CSF), which are up-regulated during RA pathogenesis (Kawaji et al., 1995; Nakamura et al., 2000; Olszewski et al., 2001; Xu et al., 1989). These CSFs enhance the survival and function of their respective leukocytes and drive their proliferation and differentiation from myeloid lineage precursors. GM-CSF induces dendritic cells, macrophages, and granulocytes (neutrophils, basophils, and eosinophils), while M-CSF and G-CSF respectively induce the macrophage and granulocyte subsets of the innate immune system. These innate cells have the ability to diapedese from the circulatory system and to differentiate further into various specialized immune cells within organs (microglia, Langerhan’s cells, etc.). GM-CSF and G-CSF are also known to be involved in erythropoiesis, and GM-CSF and erythropoietin act synergistically in the maturation and proliferation of the burst-forming and colony-forming erythroid units to the normoblast stage of erythropoiesis (Fisher, 2003). Circulating Aβ binds to complement opsonin C3b in an antibody-independent fashion, and C3-opsonized particles bind to the complement receptor CR1 on erythrocytes and to CR1g on liver-resident kupffer macrophages (Helmy et al., 2006; Rogers et al., 2006). Thus GM-CSF could function in both the peripheral clearance of Aβ and in bone marrow-derived microglial activity, since it is involved in the proliferation, differentiation, and maintenance of most innate leukocytes.
Here, we report on experiments that investigated the effect of CSF administration on amyloid plaque deposition, microglial activation, synaptic function, and associated cognitive decline in a mouse model of AD. Our results, particularly with GM-CSF, provide a compelling explanation for RA’s inverse relationship with AD. Moreover, the reduction of amyloidosis and enhancement of cognition by GM-CSF warrant clinical investigation of Leukine for the treatment of Mild Cognitive Impaired (MCI) and AD patients, especially with Leukine’s long-standing safety history in leukopenic patients.

10.3. Materials and Methods

All procedures involving experimentation on animals were performed in accordance with the guidelines set forth by the University of South Florida Animal Care and Use Committee. Transgene detections were performed using QPCR (Bio-Rad iCycler, Hercules, CA).

10.3.1. Transgenic Mouse Studies Involving Intracerebral Administration of CSFs

PS/APP mice in this study, which begin accumulating robust amyloid plaques at 6-8 months, were generated by crossing heterozygous PDGF-hAPP (V717F) mice with PDGF-hPS1 (M146L) on both Swiss Webster and C57BL/6 backgrounds. M-CSF was bilaterally infused directly into the lateral ventricles (5 µg/day) for 14 days using a novel intracranial catheter infusion system (Bennett, 2009). This completely subcutaneously-contained system allows bilateral intracerebral infusion of test substances ipsilaterally and vehicle contralaterally, and overcomes the problem of amyloidosis variance between animals, effectively making each animal its own control (Supplementary Figure 1). All three CSFs were stereotaxically-injected (5 µg/injection) into the (ipsilateral) hippocampus, with aCSF vehicle injected contralaterally into four PS/APP mice each (all 10-12 months old, 25-35 g, both genders). Two holes were drilled into the skull (from bregma -2.5 mm anterior-posterior, +/- 2.5 mm medial-lateral, and the 30 gauge needle inserted to a depth of 2.5 mm). Mice were perfused with 0.9% cold
saline 7 days later and their brains placed in 10% neutral buffered formalin. Recombinant mouse GM-CSF (rmGM-CSF), recombinant murine G-CSF (rmG-CSF), and recombinant mouse M-CSF (rmM-CSF) (R&D Systems, Minneapolis, MN) will be referred to as GM-CSF, G-CSF, and M-CSF throughout this publication.

10.3.2. Immunohistochemistry and Image Analysis of Intrahippocampal-injected Mice

Formalin-fixed brains were either coronally cryosectioned at 14-µm, or paraffin-embedded and sectioned at 5-µm, with standard deparaffination and antigen retrieval steps (boiled in 10mM Sodium Citrate buffer for 20 minutes) performed before immunohistochemical staining of 5 sections at 150 µm intervals. To significantly reduce cost of reagents and antibodies with paraffin-embedded slides, a novel magnetic immunohistochemical staining device was developed (patent pending, Tech ID# 09A015). Standard fluorescent immunohistochemical techniques used primary anti-Aβ antibodies 6E10 (Covance, Emeryville, CA, 1:1000), and MabTech’s 3740-5 (MabTech, Cincinnati, OH, 1:5000) to immunolabel amyloid deposition coupled with Alexa fluorophore-labelled secondary antibodies (Molecular Probes, Eugene, OR, 1:1000, 1:4000), and Hoechst (Sigma) nuclear staining. Immunofluorescence was detected on a Zeiss Imager.Z1 microscope (Oberkochen, Germany) using Axiovision 4.7 software and digital images quantified using ImageJ (Supplementary Figure 5-6 Appendix B).

10.3.3. Behavioral Transgenic Mouse Study Involving GM-CSF Treatment

Mice in this study were derived from the Florida Alzheimer’s Disease Research Center mouse colony, wherein heterozygous mice carrying the mutant APPK670N, M671L gene (APPsw) are routinely crossed with heterozygous PS1 (Tg line 6.2) mice to obtain APPsw/PS1, APPsw, PS1, and non-transgenic (NT) genotype offspring with a mixed C57/B6/SW/SJL background. Eleven APPsw, 4 APPsw/PS1, and 17 NT mice, all 12-months old, were selected and evaluated for 8 days in the RAWM task of working memory as previously described (Arendash et al., 2001) and Supplementary Figure 7).
Numerous experiments have revealed that various genotypes of AD mice perform equally once they reach cognitive impairment. Thus, the 15 Tg mice were divided into two groups, balanced in RAWM performance, with 2 APPsw/PS1 mice included in each group. The 17 NT mice were also divided into two groups, balanced in RAWM performance. Two weeks following pre-treatment testing, one group of Tg mice \((n = 7)\) and one group of NT mice \((n = 9)\) were started on a 10-day treatment protocol with GM-CSF \((5 \mu g/day \text{ given subcutaneously})\), while animals in the control Tg and NT groups \((n = 8 \text{ per group})\) concurrently received daily vehicle (saline) treatment subcutaneously. On the 11\(^{th}\) day of injections, all mice began four days of RAWM evaluation, were given 2 days of rest, then evaluated in 4 days of Cognitive Interference task testing as previously described (Echeverria et al., 2009; Loewenstein et al., 2004). Daily GM-CSF and saline injections were continued throughout the behavioral testing period. After completion of behavioral testing at 20 days into treatment, all mice were euthanatized, brains fixed as described above, and paraffin-embedded. Careful visual examination of all tissues upon necropsy revealed no morphological abnormalities, and the mice tolerated daily subcutaneous injections well. Each analysis was done by a single examiner blinded to sample identities, and statistical analyses were performed by a single examiner blinded to treatment group identities. The code was not broken until analyses were completed.

10.3.4. Immunohistochemistry and Image Analysis of Subcutaneous GM-CSF-treated Mice

Five 5-\(\mu m\) sections \((150-\mu m \text{ apart})\) were made of formalin-fixed, paraffin-embedded sections throughout the hippocampus of each mouse and immunoreactivity was developed using the Vectastain ABC \textit{Elite} kit (Vector Laboratories, Burlingame, CA) coupled with the diaminobenzidine reaction, according to the manufacturer’s protocol. Immunostaining used biotinylated anti-A\(\beta\) clone 4G8 (Covance, Emeryville, CA, 1:200), synaptophysin (DAKO, Carpinteria, CA, undiluted), and Iba1 (Wako, Richmond, VA, 1:1000) as primary antibodies. Images were acquired using an Olympus
BX60 microscope and digital images were quantified using SimplePCI software (Compix Inc., Imaging Systems, Cranberry Township, PA), according to previous methods (Sanchez-Ramos et al., 2009). Each analysis was done by a single investigator blinded to sample identities and genotype.

10.4. Results

10.4.1. Intracerebral administration of CSFs

Following bilateral intracerebroventricular infusion of M-CSF for two weeks into PS/APP mice, immunohistochemical analysis of both experimental and control mice showed considerable variances of amyloid deposition between mice of similar age (Supplementary Figure 1-Appendix B), significantly compromising our ability to determine M-CSF’s effect in a limited mouse cohort. While improving our drug delivery system by developing novel bilateral brain infusion catheters (Bennett, 2009), we found that parenchymally-infused recombinant peptides remained localized to the infused hemisphere. These findings led us to administer the CSFs as a unilateral intrahippocampal bolus with a contralateral injection of vehicle as control, thus obviating the need for large numbers of transgenic mice and age-matched littermate controls to obtain statistical significance. Each CSF was stereotaxically injected into the hippocampus of 4 mice, with artificial cerebrospinal fluid vehicle (aCSF) injected contralaterally. The mice were sacrificed 7 days post-injection.

M-CSF injections into mice resulted in swelling of the entire hemisphere as compared to the control side, and in one mouse, an apparent hyperplasia had formed at the injection site (Supplementary Figure 2-Appendix B). Quantification of amyloid plaque loads from anterior to posterior of each mouse showed similar deposition in the M-CSF-injected hemispheres as compared to the control sides (data not shown). In contrast to M-CSF, G-CSF injections did not induce swelling and showed some modest reductions of amyloid deposition (Supplementary Figure 3- Appendix B), which was
subsequently corroborated by independent observations from fellow investigators (Sanchez-Ramos et al., 2009).

GM-CSF-injections, however, demonstrated pronounced decreases in amyloid deposition, as compared to control hemispheres, in visual observations of coronal tissue sections (Figure 41a; Supplementary Figure 4- Appendix B). Quantification of amyloid plaques anterior to posterior revealed significant reductions within individual mice and overall significant reductions for all plaque parameters measured (Figure 41b and Supplementary Figure 5- Appendix B).

10.4.2. Daily subcutaneous injection of GM-CSF

Based upon the positive results from intrahippocampal injections, we investigated the effect of subcutaneous GM-CSF injection on AD pathology and cognitive function. Prior to GM-CSF treatment, APPsw+PS1(Tg) mice were first confirmed by RAWM testing to be cognitively-impaired for working memory. Both the non-transgenic control mice (NT) and the Tg mice were then sub-divided into two cognitively-balanced groups, for either GM-CSF or saline treatment. RAWM testing post-injection re-confirmed that Tg control mice were substantially impaired compared to NT control mice. This impairment was evident in individual blocks of testing, but also over all 4 days of testing (Figure 42a). In sharp contrast, GM-CSF-treated Tg mice performed equally well or better than NT control mice during individual blocks and overall. GM-CSF-treated NT mice performed as well as or slightly better than NT controls (Figure 42a).

Before evaluation in the Cognitive Interference Task, the mice rested two days. This task mimic human interference testing, which discriminates between normal aged, MCI, and AD patients (Loewenstein et al., 2004). In all four cognitive interference measures assessed over 4 days of testing (Figure 42b), Tg control mice were clearly impaired compared to NT mice, and Tg mice treated with GM-CSF exhibited significantly better three-trial recall and delayed recall compared to Tg controls. Indeed, for all four cognitive measures, GM-CSF-treated transgenic AD mice performed similarly to NT mice. A particularly strong effect of GM-CSF treatment in Tg mice was
evident for the proactive interference measure during the first half of testing (Figure 42c),
wherein GM-CSF-treated Tg mice performed substantially better than Tg controls and identically to both groups of NT mice. Proactive interference susceptibility has been reported to be a more sensitive marker for differentiating MCI and AD patients from aged normals than traditional measures of delayed recall and rate of forgetting (Loewenstein et al., 2004). Parenthetically, even the GM-CSF-treated NT mice showed a trend towards improved cognition in behavioral studies, albeit not statistically significant. Subsequent analysis of brains from Tg mice of this study revealed that GM-CSF treatment induced large reductions in amyloid burdens within entorhinal cortex (↓55%) and hippocampal (↓57%) compared to control Tg mice (Figure 43).

The improved cognitive function and reduced cortical amyloidosis of GM-CSF-treated Tg mice were paralleled by increased microglial density as compared to saline-treated Tg mice (Figure 44), implying an augmented ability to bind and remove amyloid deposition (El Khoury et al., 2007; Hickman et al., 2008). The GM-CSF-treated Tg mice similarly demonstrated increased synaptophysin immunoreactivity in both CA1 and CA3 regions (Figure 45), indicating increased synaptic area in these hippocampal areas. Prior work has shown that adult neural stem cells in hippocampal dentate gyrus (DG) express GM-CSF receptors, and GM-CSF increases neuronal differentiation of these cells in a dose-dependent fashion (Kruger et al., 2007). Thus, one mechanism for the observed GM-CSF-induced cognitive improvement is enhanced removal of deposited Aβ in hippocampus, with ensuing neuronal growth/synaptic differentiation of DG mossy fiber innervation to CA3, resulting in increased innervation/synaptogenesis of Schaffer collaterals into CA1. Removal of deposited Aβ from entorhinal cortex may also increase perforant pathway viability to hippocampal projection fields in DG and CA1.

Thus GM-CSF-induced reduction of amyloidosis and enhancement of hippocampal/entorhinal cortex circuitry, critical for working (short-term) memory, may underlie GM-CSF’s reversal of working memory impairment in Alzheimer’s Tg mice.
10.5. Discussion

Since peripheral leukocyte populations are increased in RA and possess the ability to infiltrate into the brain, we initially investigated M-CSF, G-CSF, and GM-CSF to determine which CSF might affect amyloidosis. In the vasculature, all three CSFs work to drive the proliferation, differentiation, and survival of their respective innate leukocytes from monocytic precursors.

In our study, we found different functional effects for each intrahippocampal-injected CSF. In the M-CSF injected mice, there was no effect on amyloidosis but pathological changes were noticed, such as swelling and hyperplasia (Supplementary Figure 2- Appendix B). Parenchymal overexpression of M-CSF in any organ is probably not advisable as overexpression of M-CSF and/or its receptor within mammary glands has similarly resulted in tumor formation and hyperplasia (Kirma et al., 2004). In a study by Boissonneault et al. (2009), the authors published that chronic intraperitoneal injection of M-CSF prevents and reverses amyloid deposition and cognitive impairment and induces brain accumulation of bone marrow-derived microglia (Boissonneault et al., 2009). Although the authors did not relate their findings to RA’s inverse relationship with AD, their data provides evidence that up-regulated M-CSF in RA pathogenesis and systemic administration of M-CSF in AD patients may impart protection against AD onset or progression. Peripheral administration of G-CSF has also been recently found to ameliorate amyloid pathology and reduce cognitive defects in AD models (Sanchez-Ramos et al., 2009), which corroborates our observations of modest amyloid reduction by intrahippocampal injection.

Although the M-CSF and G-CSF findings are encouraging, our GM-CSF intrahippocampal injections into an AD mouse model demonstrated a much more pronounced reduction of amyloidosis. Furthermore, subcutaneous administration of GM-CSF resulted in reversal of cognitive impairment resulting in function similar to that of wild-type mice, and an average of about 50% reduction of amyloidosis. Explanations for these very robust effects are multiple, including the aforementioned augmentation of peripheral erythropoietic amyloid-clearance mechanisms (Fisher, 2003; Helmy et al.,
as well as increased neurogenesis (Kruger et al., 2007), increased cerebral angiogenesis (Schneider et al., 2007), neuroprotection from apoptosis (Schabitz et al., 2008), reduction in amyloidosis (Figures 41, 43, and Supplementary Figure 5- Appendix B) and increased neuronal plasticity (Figure 45). These effects mirror those reported for G-CSF (Sanchez-Ramos et al., 2009), and supports the current practice of interchangeable prescription of either recombinant human GM-CSF (Leukine) or G-CSF (Granocyte, Neupogen, and Neulasta) into patients with depressed bone marrow function. G-CSF primarily treats neutropenia while GM-CSF treats all leukopenia, and both have long records of safety data from two decades of FDA-approved usage. Rare adverse events are usually mild febrile incidents that quickly subside upon cessation of administration. G-CSF is currently in clinical trial for stroke and was recently approved for an AD Phase II clinical trial. However, GM-CSF/Leukine is more effective in the AD mouse model and while neutrophils are short-lived leukocytes, and the fact that GM-CSF induces up-regulation of all innate cells means that it could potentially impart prolonged protective effects against AD.

The beneficial effects of CSFs, especially GM-CSF in mouse models of AD, point to a potential new approach to AD therapy and support our hypothesis that up-regulated leukocytosis, induced in RA, may impart the protective effect of RA against AD. Indeed, they also indicate, with the failure of NSAID clinical trials in AD which encouraged us to develop and test our hypothesis that intrinsic pathogenic properties of RA are protective against AD, that age-linked depressed hematopoiesis may be etiological for AD pathogenesis.

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10.6. References


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10.7. Figures
Figure 41. Intrahippocampal injection of GM-CSF (left) and aCSF (right). (a) Representative coronal tissue cryo-sectioned at 14 µm and stained with MabTech α-Aβ/Alexa 546. Image is a montage of about 145 pictures taken at 10X. White spots indicate amyloid plaque immunolabelling (see Supplementary Figure 4 Appendix B for representative montaged sections of all 4 mice). (b) Overall plaque reductions seen in all 4 plaque parameters measured from 5 quantified sections per mouse (n = 4 mice). Each analyzed picture per coronal section was thresholded equally to the same standard deviation from the histogram mean, and analyzed for area, perimeter, feret diameter, and integrated density parameters of each plaque. Area and Perimeter data were calculated from the total number of plaque values in each hemisphere per section, and Feret Diameter and Integrated Density were calculated from the average values of the plaques measured in each hemisphere per section. Each section quantified contained analysis of 15-25 individual 10X pictures per hemisphere, with less pictures quantified in the anterior brain and more in posterior brain (see Supplementary Figure 6 Appendix B). Error bars are ± Standard Error of the Mean. Statistical significance from ipsilateral GM-CSF administration versus contralateral aCSF-injection hemispheres was obtained by paired Students t-test with p values (Area: p < 1.11E-07; Perimeter: p < 1.41E-06; Feret Diameter: p < 2.36E-09; Integrated Density: p < 1.11E-07).
Figure 42. Behavioral analysis following daily subcutaneous GM-CSF injections. (a) Standard Radial Arm Water Maze errors. Tg control mice (n = 8) show substantial impairment on working memory trials T4 and T5 compared to NT control mice (n = 8) in individual blocks of testing (upper), and over all 4 days of testing (lower). GM-CSF-treated Tg mice (n = 7) performed as well as or better than NT control mice on working memory trials T4 and T5 during individual blocks and over all. GM-CSF-treated NT mice (n = 9) performed similarly to or slightly better than NT controls (Note significantly better performance of NT+GMCSF group versus NT group for T4 of Block 1), although this effect was not significant overall. Statistical significance determined by one-way ANOVA for the 8-days of pre-treatment testing (four 2-day blocks) and the 4-days of post-treatment testing (two 2-day blocks), with evaluation for individual blocks, as well as over all blocks. Thereafter, post hoc pair-by-pair differences between groups were resolved with the Fisher LSD (least significant difference) test. (**p < 0.05 or higher significance versus all other groups; †p < 0.05 or higher significance versus Tg+GM-CSF and NT+GM-CSF). (b) Cognitive Interference Task. Overall (4 Days) Tg control mice are impaired compared to NT mice on all four cognitive measures assessed. GM-CSF-treated Tg mice exhibited significantly better 3-trial recall (A1-A3) and delayed recall (A5) compared to Tg controls and performed similarly to NT mice in all four cognitive measures. GM-CSF treatment of NT mice did not result in significantly better performance compared to NT controls, although trends for a beneficial GM-CSF effect in NT mice were evident overall. For statistical analysis, both 2-day blocks were analyzed separately, as were all four days collectively. One way ANOVA’s were employed for each of the four behavioral measures analyzed, followed by post hoc Fisher’s LSD test to determine significant group differences at p < 0.05. (*Tg significantly different from NT+GM-CSF, **Tg significantly different from all other groups). (c) Cognitive Interference Task. Proactive Interference testing (First 2 days). GM-CSF-treated Tg mice performed significantly better than Tg controls and equally to NT and GM-CSF-treated NT mice. Statistical significance determined by one-way ANOVA (**p < 0.05 or higher significance versus all other groups).
Figure 43. Amyloid deposition in subcutaneous GM-CSF-injected mice. (a-d)
Photomicrographs of coronal 5-μm paraffin-embedded sections immunolabelled with anti-Aβ antibody (clone 4G8 in Entorhinal cortex (E) and hippocampus (H)). Since the 4G8 antibody was obtained with biotin label, the secondary step of the ABC protocol was omitted. However, treatment with 70% formic acid prior to the pre-blocking step was necessary. Phosphate-buffered saline (0.1 mM, pH 7.4) was used instead of primary antibody or ABC reagent as a negative control. Pictures are representative of amyloid load closest to the mean of the GM-CSF- or saline-treated Tg groups. Scale bar = 50 μm.
(e) Percent of amyloid burden from the average of five 5-μm sections (150-μm apart) through both anatomic regions of interest (hippocampus and entorhinal cortex) per mouse of GM-CSF-treated (n = 5) versus saline-treated (n = 6). A threshold optical density was obtained that discriminated staining from background, and each region of interest was manually edited to eliminate artifacts. Data are reported as percentage of immunolabeled area captured (positive pixels) relative to the full area captured (total pixels). Statistical significance was determined by two-tailed homoscedastic Student’s t-test with a p value of < 0.05 considered significant: Entorhinal cortex (*p < 0.026), and Hippocampus (p = 0.12).
**Figure 44.** Microglial immunostaining in subcutaneous GM-CSF-injected mice.

(a-d) Photomicrographs of coronal 5µm paraffin-embedded sections immunolabelled with Iba-1 antibody in Entorrhinal cortex (E) and hippocampus (H). Normal rabbit serum was used instead of primary antibody or ABC reagent as a negative control. Pictures are representative of Iba-1 immunolabelling closest to the mean of the GM-CSF- or saline control- treated groups. Scale bar = 50µm. (e) Percent of Iba1 burden from the average of five 5-µm sections (150 µm apart) through both anatomic regions of interest (H and EC) per mouse of GM-CSF-treated (n = 5) versus saline-treated (n = 6). A threshold optical density was obtained that discriminated staining from background, and each region of interest was manually edited to eliminate artifacts. Data are reported as percentage of immunolabeled area captured (positive pixels) relative to the full area captured (total pixels). Differences between the two groups were statistically significant by two-tailed homoscedastic Student’s t-test with a p value of <0.05 considered significant: H(p < 0.02), EC(p < 0.05).
**Figure 45.** Synaptophysin immunostaining in subcutaneous GM-CSF-injected mice. (a-d) Photomicrographs of coronal 5-µm paraffin-embedded sections immunolabelled with anti-synaptophysin antibody. Normal rabbit serum was used instead of primary antibody or ABC reagent as a negative control. Pictures are representative of synaptophysin immunolabelling closest to the mean of the GM-CSF- or saline control-treated groups. Scale bar = 50 µm. (e) Percent of synaptophysin immunoreactivity from the average of 5 sections per mouse of GM-CSF-treated (n = 5) versus saline control-treated (n = 6). To evaluate synaptophysin immunoreactivity, after the mode of all images was converted to gray scale, the average intensity of positive signals from each image was quantified in the CA1 and CA3 regions of hippocampus as a relative number from zero (white) to 255 (black). Albeit numerically small, differences between the two groups were statistically significant by two-tailed homoscedastic Student’s t-test with a p value of < 0.05 considered significant: CA1(p < 0.0013), CA3(p < 0.0023).
CHAPTER 11 – DISCUSSION

Neurodegeneration describes the selective and progressive loss of structure and function in neurons. Although the initiating factors, symptoms, and pathologies of neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s, Huntington’s, and Amyotrophic Lateral Sclerosis may be different, they share common pathophysiologies. Primary hallmarks of these pathologies are atypical accumulations of protein deposits, often due to misfolding. Often associated with these deposits are dysfunctional mitochondria, oxidative stress, disrupted axonal transport, inflammation, and apoptotic cell death (DeLegge and Smoke, 2008; Gebicke-Haerter, 2001; Gendron and Petrucelli, 2009; Swerdlow, 2009). If these disruptions occur in motor neurons, as in ALS, motor function is impaired and if the deposits are found in cortical neurons, as in Alzheimer’s disease, the outcome is dementia (Soto, 2003; Weisman et al., 2006). Why these neurons are selectively vulnerable in each disease is unknown and, unfortunately for many people, preventions and therapies remain elusive. In the work described here, mouse models of neurodegenerative disease were used to investigate promoters and inhibitor of neurodegeneration in the proteinopathies ALS and AD.

Mouse models are a common and useful research tool for investigating disease pathology and developing potential therapies prior to clinical trials in humans. In comparison to other animals, their short lifespan averaging 2.5 years, high reproductive rate, and relative ease of modification for the incorporation and expression of transgenes make mice ideal for studying human diseases. Additionally, controlled and standardized environments keep behavioral testing rather objective and every mouse is completely naïve to each experiment. Therefore, responses to novel environments are controlled and readily quantifiable. Another benefit of mice, though a potentially confounding variable, is their hardy immune system. They are very capable of resisting iatrogenic infection
during intracranial and intrathecal penetration as well as during subcutaneous implantation of osmotic pumps in conditions where complete sterility is simply not possible.

There are also some clear disadvantages and challenges to using transgenic mouse models. In both AD and ALS, only 5-10% of the disease is caused directly by gene mutations (Gros-Louis et al., 2006; Hardy, 2006). The efficacy of genetic approaches to the study of neurodegenerative diseases is also limited by the fact that many such disorders have a wide display of phenotypes and varied etiology. In addition, the interpretation of results obtained in animal models has to integrate the fact that often non-physiological levels of selected proteins such as Aβ or SOD1 are used to recapitulate a disease in a mouse within a reasonable experimental time frame. Moreover, because it is not yet known if the proteinaceous aggregates in these diseases are causes, byproducts, or neuroprotective factors, one must be mindful of the exact query to the system and of the interpretation of the results. In addition, while it is reasonable to try possible interventions on already established mouse models instead of engaging in the time consuming and tedious process of producing alternative mouse models, approaches with suboptimal mouse models can sometimes fail to produce conclusive answers. It may be in this context that several of the experiments conducted here did not result in favorable outcomes. For example, many animals in my experiments were designed to make amyloid plaques, tau tangles, and SOD1 inclusions. Naturally studies that focused on exacerbating or ameliorating these phenotypes were more successful than testing compounds involved in a different, though important, facet of the disease.

The focus of my work presented here has been on mouse models of neurodegenerative diseases, taking the good with the bad. I successfully studied the permeability of the blood brain barrier and the spontaneous formation of autorosettes in the SOD1 G93A mouse. Other positive results were obtained from practical, engineering approaches to working with the mice. Intrathecal delivery of compounds in mice is arduous, and historically experiments that required delivery of compounds into the CSF would enter through the lateral ventricles. Over some time, I have developed a tool and technique to effectively and reproducibly deliver compounds directly to the site of early
ALS pathology in the lumbar region of the spinal cord. After years of struggling with commercially available intracranial catheters and experiencing high mortality rates, infections, physical damage to hardware or the animal, and pain/discomfort, enough incentive to devise a better means presented itself. Not only can the patented intracranial catheter assembly obviate concern for these pitfalls, but it solved another major issue with the AD mice: plaque variability within the colony. If one hemisphere could serve as the other’s control, differences in amyloid load between genetically identical animals could be compensated. In another novel application, though not a novel concept, I used *in vivo* electroporation, into the CNS, a procedure that had not been published for such use at the time I started out. Although it may appear rather crude, electroporation is an excellent tool for “proof of principle” gene delivery prior to designing attenuated viral vectors (Heller and Heller, 2006).

Ultimately, scientific success was generally achieved by directly focusing queries on what the mouse models were designed for (inclusions, plaques, tangles) or characterization of these pathologies in mice. Human tau mice were used to investigate phosphorylation and tangle formation, and PS/APP mice were used to study the effects of ACT and plaque build up. Most noteworthy are the experiments that reduce plaque load in the animals, which was achieved with 2 completely different approaches (Aβ 1-11 and GM-CSF). It is still unknown whether plaques and tangles are the cause of AD or a bystander effect, but they are nonetheless the focal point of most AD research.

The first set of experiments focused on a well-studied and relatively common aspect of neurodegenerative disease: transmissibility. In 1994, Stanley Pruisner received the Nobel Prize for ground breaking work with prion protein. Bovine spongiform encephalopathy (BSE), scrapie of sheep, and Creutzfeldt-Jakob disease (CJD) of humans are the most commonly known prion diseases (Prusiner, 1998). Prions are proteins that can acquire a misfolded conformation that nucleates the misfolding of other normally folded prion proteins and form beta sheet amyloid structures (Wickner et al., 2008). It has since been discovered that amyloid plaques from post-mortem human or mouse brain, once suspended and injected into the brain of a young PDAPP transgenic mouse, can trigger the early seeding of plaques (Walker et al., 2002). More recently, it has been
shown that a misfolded tau mutant could seed tangle formation when injected into an htau mouse (Clavaguera et al., 2009). We hypothesized that such an outcome could also be triggered by SOD1 aggregates to initiate ALS. After homogenizing spinal cords from end-stage SOD1 G93A animals, the homogenates were infused into the lumbar cistern of younger transgenics to see if ALS pathology could be seeded or accelerated. Prior to our experiments, there were conflicting reports from breeding experiments with mutant and wild type SOD1 mice stating that the mutant SOD1 may exert a conformational change in the WT and accelerate the disease (Wang et al., 2009), whereas other groups found no difference (Bruijn et al., 2004b). When SOD1 aggregates were infused in our experiments, we could not detect any changes in the behavior or lifespan in the animals. Our negative result could be explained as follows: 1) Aggregates were infused into the CSF and not directly into the tissue out of concern about causing damage to the animal. 2) There was already a ceiling effect with ~18 copies of mutant SOD1 being expressed, and the infusions could not enhance this effect any further. Moreover, the human SOD1 gene is flanked by Alu repeats (retrotransposons) (Maguire et al., 2006) and colonies would eventually begin to lose copy number as a result. As mentioned in Chapter 2, Mark Gurney, the investigator who discovered the G93A mutation, disclosed in a personal communication that his own lab has not use this SOD1 transgenic mouse model for quite some time. The SOD1 G93A mouse was designed to form inclusions and develop an aggressive paralytic phenotype after only 100 days of age and persists for 30 days until the animals had to be euthanized. Although this therapeutic window seems appealing to researchers for successive high throughput drug studies, the most effective therapies have been siRNA (Rizvanov et al., 2009) and antisense oligonucleotides (Smith et al., 2006). In addition, these approaches would only be useful in reducing the amount of mutant protein that is being heavily overproduced. Accordingly, these therapies might only translate to individuals with that particular SOD1 mutation, while there are in fact over 150 ALS-causing SOD1 mutations known (Turner and Talbot, 2008). While inflammation and excitotoxicity are very clearly implicated in ALS pathogenesis, we came to conclude that SOD1 aggregation has the most dominant effect. Therefore DTG and KDI, though potentially useful compounds, had only marginal if any effect on this
mouse model. However, given the potential of both of these compounds, further research in other models (G85R, G37R), even prophylactically, may be warranted but was not pursued by our lab.

As previously stated, some of our observations in the SOD1 G93A mouse were noteworthy. Inflammation is a hallmark of all neurodegenerative disorders and is evident in this model from activation of microglia and astrocytes (DeLegge and Smoke, 2008; Gebicke-Haerter, 2001). Recently it was also suggested that the adaptive immune system plays a role in ALS (Banerjee et al., 2008). In chapter 3, we show that the blood brain barrier is compromised in the G93A mouse. Previous work showed that T cells (Donnenfeld et al., 1984b; Engelhardt and Appel, 1990; Engelhardt et al., 2005a; Mohamed et al., 2002) and IgG (Alexianu et al., 2001; Engelhardt et al., 1993b) were actually present in high amounts in the CSF of ALS patients and also in these mice (Garbuzova-Davis et al., 2007a). The increased permeability of the BBB in later stages of the disease would provide a logical explanation for this phenomenon. Furthermore, we have identified the formation of autorosettes in this model (appendix A), which has been shown to be a T cell marker. Taken together, our observations show that ALS may involve activation of both the innate and adaptive immune systems. Unfortunately, the model was of little use for the study of potential therapeutics.

It was at this time that I shifted my efforts from studies on ALS to investigations related to Alzheimer’s disease. Initially I transferred the idea of testing KDI, the tripeptide of γ-laminin, as a potential therapeutic for neuodegenerative diseases to the treatment of cognitive impairment. We investigated the effects of KDI on plaque load in treated PS/APP and Tg2576 mice, no changes were observed. Nonetheless, the reported potent in vitro effects of KDI as a neurite outgrowth factor and NMDA/AMPA receptor antagonist (Moykkynen et al., 2005) (similar to Namenda) left us optimistic that it could provide some benefit to the mice. Because the first cohort of KDI treated animals, as shown in chapter 7, showed marked restoration in cognitive abilities as tested with the RAWM, the effects of KDI on LTP and synaptogenesis were investigated. While the brains from the first cohort were analyzed, the second cohort of animals was starting on their 28 day infusions. Using immunohistochemical analyses, synaptic markers including
synaptophysin, synaptotagmin, synaptopodin, synaptobrevin, GAP-43, and PSD-95 antibodies revealed no changes between treated and untreated mice; the possibility of delivering bilaterally was not yet available at this time. In another hypothesis we speculated that we were interfering with the glutamate neurotransmission in such a manner as to stimulate the phosphorylation, thus activation, of dormant AMPA receptors leading to the possibility that KDI was inducing LTP. GluRI, pGluRI, CamKII, and pCamKII levels were compared between KDI treated and untreated mice, but no differences were observed. Moreover, additional behavior tests that were performed twice with new animal cohorts, but did not show significant differences between KDI-treated and untreated animals either. We interpreted this outcome as follows: 1) If the data published on KDI and its ability to function as a potent non-competitive ionotropic glutamate receptor antagonist is accurate, then our results just show little relevance of glutamate dysregulation in AD, i.e KDI does not yield a therapeutic effect in AD, which, at the same time, helps to explain Namenda’s modest benefit. 2) The chosen mouse model was possibly not the best system to investigate the relevance of excitotoxicity in AD. 3) KDI’s effects had, for the most part, been limited to in vitro studies and cannot be translated to the mice.

When studying the htau mice, we focused directly on phosphorylation events that lead to tangle formation. Tau has ~45 AD-related phosphorylation sites, and numerous kinases and phosphotases are responsible for regulating the phosphorylation status and thus the conformation of tau. Previous in vitro work from our laboratory showed that in most tauopathies, including CBD, PSP, FTDP-17, and AD, antichymotrypsin is upregulated and subsequently found in aggregates in post mortem brain tissues (Padmanabhan et al., 2006). This observation was of particular interest to us, because ACT is well-established to have a role in Aβ fibrillization in vitro (Ma et al., 1996) and amyloid plaque formation in transgenic mice (Nilsson et al., 2001). ACT, like ApoE, has been termed a “pathological chaperone” (Wisniewski and Frangione, 1992) in the development of plaques in AD. In chapter 8, I demonstrated how chronic intracranial infusion of ACT into an htau mouse increased levels of pSer 202, PHF-1, and pThr231 in tau and that the intracellular kinase, GSK3, was activated. GSK3 has been implicated in
the phosphorylation of several sites on tau, including pThr231 and pSer394/404 (PHF-1) (Hooper et al., 2008), and my findings are consistent with these observations. One notable difference between my study and previous in vitro work was that pERK was found in much lower amounts (deactivated) in after infusion of ACT. Thus in cultured mouse cortical neurons, adding ACT to the media increased levels of pERK in a dose dependent fashion (Padmanabhan et al., 2006) whereas in vivo, the opposite effect seemed to occur. These results are from a pilot study of 5 mice, only 4 of which clearly received ACT. This work will be followed up as a full study with more animals in anticipation of revealing other phosphorylation sites as well as putative kinases. Additionally, it is not yet known if it is the physical presence or protease inhibiting activity of ACT that induces these events and whether they occur from ACT interacting with elements on the cell surface of neurons or after endocytosis. These possibilities would need to be addressed in order to determine how to best inhibit ACT-mediated tau phosphorylation and tangle formation.

ACT is of particular interest because it provides a link between the hallmark pathologies of AD. It is directly involved in the fibrillization of Aβ and the formation of plaques and has been shown to induce tau phosphorylation by GSK-3 at sites known to be involved in Alzheimer’s disease (Padmanabhan et al., 2006). Therefore, inhibition or downregulation of ACT, which is highly upregulated during, AD may be of therapeutic interest. Early observations from our lab suggest that Aβ has strong homology in the N-terminus with both cathepsin G and chymotrypsin, both of which are serine proteases and known serine protease targets of ACT (Abraham and Potter, 1989). It is therefore conceivable that ACT is upregulated to bind Aβ in order to sequester it from forming plaques or oligomers, but that ACT becomes outcompeted by the amount of substrates and overwhelmed in the process.

Historically, ridding the brain of plaques and tangles has been the major therapeutic goal, either through stimulation or inhibition of the innate and adaptive immune systems (Maccioni et al., 2009) or more direct mechanisms. The involvement of ACT and ApoE in the formation of amyloid plaques and concomitant activation of microglia and astrocytes and release of cytokines and chemokines support the theory that,
as in all neurodegenerative diseases, inflammation is a major aspect of AD pathogenesis. Whether this is a primary or secondary event to the formation of oligomers and plaques is uncertain, as well as whether or not it is beneficial or detrimental to have triggered an immunological response. Microglia, for example, are known to release proinflammatory cytokines (Hanisch, 2002) and also to engulf amyloid (Herber et al., 2007).

In chapter 9, I describe the use of a “decoy” Aβ peptide (1-11) to reduce the amount of amyloid formed. The rationale for this approach came from previous in vitro work from our laboratory in which the amount of Aβ fibrils was enhanced in the presence of ApoE and ACT, but that such catalyzed fibrilization could be attenuated by adding Aβ fragments 12-28 and 2-9, respectively. These peptides include the binding regions for ApoE and ACT on full length Aβ and they, might block these proteins from binding Aβ and rendering the reaction mixture less toxic to cultured neurons, especially with increasing amounts of the decoy peptides (Ma et al., 1996). In 2006, Wisniewski et al. published a study in which they had taken the 12-28 peptide and, with some modification, injected the molecule systemically into Tg2576 mice (with and without PS1 M146L). This treatment resulted in a significant reduction in plaque load and improved cognition in behavioral tasks (Sadowski et al., 2006). The same rationale was applied in our attempt to interfere with the interaction of ACT and Aβ when we introduced the peptide fragment 1-11 (containing the 2-9 binding site of ACT on Aβ) in PS/APP mice as they were developing plaques. The peptide was infused intrahippocampally into a small pilot of PS/APP mice, unilaterally, with aCSF to the contralateral hemisphere for direct comparison of plaque load. As shown in the figures of chapter 10, the peptide not only significantly reduced new plaque formation, but appears to have cleared existing plaques as shown with both the 6E10 antibody (binds Aβ 1-16) and 4G8 antibody (binds Aβ 17-24). Most likely, the peptide binds to the “ACT equivalent” in the mouse or serpin A3N, which is identified in the plaques of these mice with anti-ACT antibodies. The mechanism for the clearance is unknown, but Iba1 microglial staining is virtually absent from the treated hemisphere, indicating that these cells are not present or that the respective epitope is not accessible. Further investigation is needed to determine the putative mechanisms of clearance as well as to infuse the peptide bilaterally for
behavioral analyses. Ultimately, modification of the peptide to reduce immunogenicity and increase its half life, could allow systemic dosing and yield a potential AD therapy.

In another part of my dissertation research, I investigated the negative correlation between patients with Rheumatoid arthritis and AD. We hypothesized that there were factors upregulated in the autoimmune disease that conferred neuroprotection and reduced if not eliminated the risk of developing AD. Although up-regulated leukocytes in RA could potentially enter into the brain and inhibit development of AD pathology and/or neuronal dysfunction, lymphocytic infiltrates into AD patient brains have not been reported. The lack of infiltration suggests that activation of the innate immune system might be responsible for preventing AD pathology in RA patients. Originally, 3 cytokines were identified as upregulated in RA and of potential interest: G-CSF, M-CSF, and GM-CSF. These CSFs enhance the survival of their respective leukocytes and drive their proliferation and differentiation from monocytic precursors. M-CSF and G-CSF induce specific subsets of the innate immune system, while GM-CSF induces the full range of innate cells. Bolus injections of 5 μg per hemisphere showed significant plaque reductions after only 1 week (see chapter 11). This success led us to begin a subcutaneous dosing regime and performed behavioral analyses. The improved cognitive function and reduced cortical amyloidosis of GM-CSF-treated Tg mice was paralleled by increased synaptophysin immunoreactivity in both CA1 and CA3, indicating increased synaptic density in these hippocampal areas. Further investigation into GM-CSF to elucidate its mechanism in AD is in progress as well as the initiation of clinical trials with Leukine an FDA approved drug of recombinant GM-CSF currently in use for recovery from chemotherapy.

It is a remarkable and unfortunate fact that despite the enormous knowledge that has been acquired about ALS and AD, effective cures or even treatments for both diseases have been elusive. Much debate exists on whether protein aggregation is an initiating factor in neurodegeneration or a side effect. Similar debate questions whether activation of the immune system is beneficial or detrimental to CNS function in neurodegenerative diseases. Our research suggests the former. In AD, some reports state that cognition is closely linked to plaque load in the mice (Gordon et al., 2001),
suggesting that, even without neuron loss, the plaques cause behavioral deficits. Conversely, other studies show no change in plaque load with improved cognition (Costa et al., 2007). Nonetheless, the studies discussed here focused primarily on the use of transgenic mouse models of protein aggregation and on procedures that could exacerbate or inhibit the effect. The specific focus of my work was on the development of novel means of drug and gene delivery into transgenic mice. The fact that certain compounds did not affect protein aggregation still leaves the possibility that they might have therapeutic potential if tested in other systems. However, unquestionably, the delivery systems developed along the way, have implications that reach beyond AD and ALS as they can each be readily adapted for investigations with virtually all mouse models of neurodegenerative disease.
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APPENDICES
Appendix A- Lymphopenia and Spontaneous Autorosette Formation in an SOD1 Model of ALS

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by motoneuron degeneration. Increasing evidence suggests immune system involvement in ALS pathogenesis but information about peripheral blood characteristics has been lacking. We evaluated hematological and morphological parameters in peripheral blood of G93A SOD1 mice. A significant decrease in white blood cells was found at the end stage of disease. The lymphocyte reduction may suggest immunodeficiency in ALS. Spontaneously forming rosettes with autologous erythrocytes were noted in approximately 28% of lymphocytes in SOD1 mice. To our knowledge, this is the first study characterizing hematology and revealing autorosettes in the SOD1 mouse model of ALS at the terminal phase of disease.

Introduction

Currently, amyotrophic lateral sclerosis (ALS) is the most frequently occurring motor neuron disease lacking effective therapy (Mulder et al., 1986). About 10% of cases are dominantly inherited and about 20% of those arise from mutations in the gene for Cu/Zn superoxide dismutase (SOD1). The pathogenic mechanism of ALS is unknown and there
Appendix A: (Continued)

is no identified biological marker of disease progression that can be monitored in ALS patients. In ALS spinal cord tissue, the presence of T cells (Alexianu et al., 2001), IgG deposits (Mohamed et al., 2002; Engelhardt and Appel, 1990), monocytes and activated macrophages (Henkel et al., 2004), suggests that these immune effectors are involved in the inflammatory process. Inflammatory response occurs in the CNS through mechanisms that differ from systemic inflammation (Consilvio et al., 2004), as described for ALS patients (McGeer and McGeer, 2002). Several transgenic mice modeling ALS expressing mutated human SOD1 have been created. The first and most widely used is the G93A mouse that overexpresses human SOD1, carrying the Gly93 Ala mutation and that develops an ALS-like motor neuron disease. G93A SOD1 mice show motor neuron pathology similar to ALS patients (Gurney et al., 1994). Still, we lack information regarding the hematological characteristics of both ALS patients and animals. Characteristics of complete blood count (CBC) and white blood cell (WBC) differential may therefore help elucidate the type of pathogenic processes present during disease, very important for developing a treatment strategy. Previously, an increasing number of circulating neutrophils found in ALS patients had been described (Desport et al., 2001). However, statistically significant differences between the number of neutrophils in ALS patients and neutrophils’ normal range were not shown. The aim of the present work was to evaluate the complex hematological and morphological parameters in peripheral blood from the G93A SOD1 mouse model of ALS2.

Materials and methods

Mice

Transgenic male mice B6SJL-TgN 1GUR (G93A) obtained from Jackson Laboratories, Bar Harbor, MA, overexpressing human SOD1, carrying the Gly93 to Ala mutation (Gurney et al., 1994), were used (n =12). Transgenic mice (BL6/SJL) carrying the normal
Appendix A: (Continued)

allele for human SOD1 gene (n =5, hTg) and C57BL/6 animals (n =7), matched by age and gender to experimental animals were used as controls. All animal experiment procedures were approved by the Institutional Animal Care and Use Committee at USF and conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

Blood samples

The blood samples were obtained from G93A mice at the terminal stage of disease when mice developed hind limb paralysis (18–19 weeks). Samples were also obtained from hTg and C57BL/6 mice of the same age, 18–19 weeks. Mice were sacrificed under deep chloral hydrate (10%) anesthesia and about 300 Al of blood was taken transcardially and collected into blood collection tubes with EDTA(K3) (Sherwood Medical, MO). Analyses for CBC and WBC differential were performed by Antech Diagnostics (NY, USA). Additionally, blood smears (3 from each animal) were taken from the tail vein for morphological analysis. The smears were dried for 30 min, fixed in methanol for 7 min, then stained by Giemsa (Sigma- Aldrich, GS80, St. Louis, MO) as previously described (Brown and Febiger, 1993).

Morphological evaluation of blood smears

After staining, blood smears were rinsed several times in distilled water and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). The morphology of the peripheral blood cells was examined under an Olympus BX-60 microscope. The images were analyzed by Image-Pro Plus version 4.1 for Windows software (Silver Spring, MD). Rosette counts were performed using the full area of the slide.
Statistical analysis

All data on CBC count and WBC differential were analyzed by ANOVA (analysis of variance). The results are presented as means TSEM. Significance between the results for different groups was estimated using the two-tailed Student’s t-test.

Results

Hematological analysis

The blood samples were obtained from G93A mice at the terminal stage of disease (about 18–19 weeks of age). All G93A mice had severe disease progression (complete hind limb paralysis) at the time of blood collection. The main characteristics of the red and white blood cells from G93A and control (hTg and C57BL/6) groups of mice are presented in Table 1. The red blood cell counts did not differ between G93A mice and the two control groups. However, the platelet numbers were increased in G93A mice without statistical significance. In G93A mice, WBC numbers were drastically reduced (0.7_10^3/AL, p <0.001) compared to control hTg mice (2.6_10^3/AL) and C57BL/6 (3.8_10^3/AL).

Lymphocyte density in G93A mice (0.26_10^3/AL) was 8.4 times lower than in hTg mice (2.22_10^3/AL) and 10 times lower than in C57BL/6 mice (2.66_10^3/AL). The absolute number of circulating monocytes and eosinophils also decreased ( p <0.05) in G93A mice. The basophil number was significantly increased in G93A mice compared to hTg mice ( p <0.05), but not to C57BL/6 mice. This dramatic reduction in the number of lymphocytes in peripheral blood may suggest severe immunodeficiency in ALS.

Leukocytes are typically recruited from circulation into local sites of inflammation, where the cells perform their functions to reduce tissue alterations. However, neutrophil levels, which can reflect acute inflammation in peripheral blood of G93A mice, did not significantly differ from control animals.
Appendix A: (Continued)

Morphological examination of the peripheral blood in SOD1 mice

Routine blood smears can still be useful and informative if current views on disease pathogenesis are incorporated during their perusal. Morphological examination of peripheral blood from G93A mice showed a number of lymphocytes spontaneously forming rosettes with autologous erythrocytes or autorosette-forming lymphocytes (aRFL) (Fig. 1A,B). The mean percentage of aRFL was 27.7 ± 3.3 in the peripheral blood from G93A mice. The average number of erythrocytes which formed rosettes around one lymphocyte in G93A mice was 8.6 ± 3.2. A squeezed shape of erythrocytes in rosettes was noted. The aRFL were found in two thirds of G93A mice but not in the peripheral blood of C57BL/6 (Fig. 1C) or hTg (Fig. 1D,E) control animals.

Discussion

It has been shown that the immune system is involved in ALS in human and G93A mice (Alexianu et al., 2001; Mohamed et al., 2002; Engelhardt and Appel, 1990). The clinical significance and degree of involvement of the immune system in ALS is still controversial. Nyland and Naess (1978) reported that T lymphocytes in the peripheral blood of ALS patients were not significantly reduced. However, other clinical studies found decreased totals of blood lymphocytes and increased percentages of B-cells and IgM (Khondkarian et al., 1985) and reductions of T mu cells, which bear Fc receptor for IgM (Westall et al., 1983). Moreover, Provinciali et al. (1988) showed significant marked lymphopenia, reduction of CD2, CD8 and Leu 7 positive cells and an increase of the CD4/CD8 ratio in ALS patients at early disease stage. From these results, it is clear that immunopathological reactions in ALS are occurring and may depend upon duration, severity, and cause of disease. However, biological markers of this disease are still uncertain. Utilizing CBC and WBC differential, we were the first to find a significant
Appendix A: (Continued)

decrease of WBC numbers in G93A SOD1 mice at the end stage of disease. Decreased WBC in G93A mice was mainly due to declines in lymphocyte density, 8.4 times lower than in hTg mice and 10 times lower than in C57BL/6 control mice. Significantly decreased numbers of peripheral monocytes and eosinophils in G93A mice versus control animals were also noted. These findings may suggest severe immunodeficiency in these animals at the terminal stage of disease. Since the transgenic G93A mice have a high number (≥20) of copies of mutant SOD1, it is possible to suggest that peripheral blood results are be linked to ALS gene mutations. This SOD1-mediated toxicity may involve a cascade of events including depletion of peripheral lymphocytes. Also, we cannot exclude the possibility that “consumption” of these cells is extremely high, causing their disappearance from peripheral blood. Monocytopenia and eosinopenia can be considered as prognostic features in ALS. An eosinophil is a type of phagocyte that produces the pro-inflammatory protein histamine, as well as nerve growth factor (NGF) and neurotrophin-3 (NT-3) (Kobayashi et al., 2002). Although most eosinophilic leukocytes are localized in mucosal tissues, perhaps these cells can migrate from blood into neural tissue following chemo-attraction signals, as demonstrated for T-cells (CD4, CD8) (Alexianu et al., 2001) and monocyte/macrophages (CD14, CD68) (Henkel et al., 2004). Also, mucosal immune responses could be involved in ALS pathogenesis and that the redistribution of white cells from the blood may be mediated by mucosal chemo-kines (Belyakov and Berzofsky, 2004). However, the significance of mucosal immunity in the pathogenesis of ALS is likely linked to the possibility of viral infection. The role of basophils is not completely understood. Basophils circulate for a few hours in blood and migrate into tissues where they may reside for several weeks as mast cells. Basophilia commonly indicates the presence of myeloproliferative disease as opposed to leukemoid reaction but can be a consequence of allergy, hypersensitivity reaction, metabolic or endocrine disorders (Bochner and Schleimer, 2001), chronic basophilic leukemia, or Hodgkin’s disease (Pardanani et al., 2003). Basophils complete their differentiation in bone marrow and appear in the peripheral blood as mature basophils. Recently, these
Appendix A: (Continued)

blood cells have been shown to be important regulators of humoral immune responses by their influence on B cell function (Mack et al., 2005) and they can be recruited to sites of inflammation (Wedemeyer et al., 2000). Observed basophilia in G93A mice may reflect the inflammatory process in the spinal cord, involving mast cells, macrophages and T cells, as recently described in ALS patients (Graves et al., 2004). The link between basophilia and lymphopenia observed in G93A mice at end-stage disease needs further investigation, and we will initially focus upon possible alterations in bone marrow function. Interestingly, red blood cell parameters in ALS mice did not statistically differ from both control groups of animals. Only one hematological parameter has been previously noted in ALS patients, an increased number of circulating neutrophils correlating with hypermetabolism (Desport et al., 2001). The reason for the association between elevated neutrophil levels and hypermetabolism is still unknown. However, our data on neutrophils contained in peripheral blood of G93A mice did not support this neutrophil observation in patients. Additionally, the platelet numbers were increased in G93A mice without statistical significance. However, these results have coincident with recently published data (Kiktenko et al., 2005), that platelet activation was found in ALS patients. Platelet activation was more pronounced in ALS than in multiple sclerosis (Kiktenko et al., 2005). It is clear that the presence of white blood cells in the peripheral blood has been significantly reduced in G93A mice at end stage disease, but the mechanism underlying this decline is unclear. Since white blood cells ultimately originate from bone marrow, it is possible to suggest that production of these cells has been impaired. However, we cannot exclude potential involvement of secondary lymphoid organs such as the spleen or lymph nodes in this pathology. Presently, we are performing phenotypic characterization of lymphocytes in peripheral blood, primary and secondary lymphoid organs and degenerating areas of the brain and spinal cord in G93A mice modeling ALS. Correlation of lymphocyte densities among these locations is currently under investigation. It will also be important to determine if reductions in leukocytes, especially lymphocytes, in peripheral blood correlate with disease progression. Our other
finding was spontaneous aRFL in G93A mice at the terminal stage of disease. Rosette-formation has been previously noted in some pathological conditions. The ability of lymphocytes to bind with autologous erythrocytes has been observed in patients with autoimmune thyroiditis and cancer (Endo et al., 1984; Tamura et al., 1984; Ichikawa et al., 1983). Subpopulations of T-lymphocytes (CD4) forming aRFL in peripheral blood from patients with thyroid diseases (Endo et al., 1984), lupus erythematosus (SLE) (Ichikawa et al., 1983) and non-Hodgkin’s lymphomas (Tamura et al., 1984) has been reported. Finally, aRFL evaluation has been proposed as a criterion of autoimmune pathology. However, the usual protocol for aRFL evaluation includes the mitogen-induced activation of lymphocytes in vitro by pokeweed and phytogemagglutinin mitogens for rosette formation (Ichikawa et al., 1983). Our results, showing aRFL in ALS mice without lymphocyte stimulation by mitogens or other activators, may indicate involvement of an autoimmune mechanism in the etiopathology of ALS. As is well known, autologous rosette incidence is also abnormally high in nude (congenitally athymic) mice. The high numbers of aRFL found after adult thymectomy can be normalized by injection of thymic hormones, suggesting that aRFL might be associated with immature T-cell precursors (Charreire and Bach, 1975). The phenomenon of spontaneous aRFL noted in G93A mice suggests that early precursors from thymus or bone marrow may be involved in forming aRFL. However, phenotypical analysis of lymphocyte maturation should be performed to confirm this involvement and a future study will include this analysis. We hypothesize that the lymphopoietic system is involved in ALS and that impairment of this system critically influences immune system function. This is the first evidence that peripheral blood of transgenic G93A mice contains autologous rosette-forming lymphocytes. Further investigation of autoimmunity and pathologically significant antigens in ALS is warranted.
Appendix A: (Continued)

Acknowledgements:

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Appendix A: (Continued)


Gurney, M.E., Pu, H., Chiu, A.Y., Dal Canto, M.C., Polchow, C.Y.,


Appendix A: (Continued)


Appendix A: (Continued)

Table 4. Complete blood cell count and white blood cell differential in SOD1 G93A mice as compared to control mice

<table>
<thead>
<tr>
<th>Hematological parameters</th>
<th>Units</th>
<th>G93A SOD1 (n = 12)</th>
<th>BL6/SJL (hTg) (n = 5)</th>
<th>C57BL/6 mice (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cell count, RBC</td>
<td>10^6/µL</td>
<td>6.9 ± 0.9</td>
<td>7.2 ± 0.4</td>
<td>7.5 ± 1.1</td>
</tr>
<tr>
<td>Hemoglobin, Hb</td>
<td>g/dL</td>
<td>10.3 ± 1.2</td>
<td>12.3 ± 0.6</td>
<td>11.7 ± 1.2</td>
</tr>
<tr>
<td>Mean cell hemoglobin, MCHC</td>
<td>pg</td>
<td>15.2 ± 0.5</td>
<td>16.4 ± 0.3</td>
<td>15.7 ± 0.8</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>%</td>
<td>31.4 ± 6.7</td>
<td>33.4 ± 3.3</td>
<td>33.5 ± 2.2</td>
</tr>
<tr>
<td>Platelet count</td>
<td>10^4/µL</td>
<td>907 ± 282</td>
<td>878 ± 96</td>
<td>714 ± 233</td>
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<tr>
<td>Mean cell volume, MCV</td>
<td>fl</td>
<td>43.9 ± 4.3</td>
<td>47.2 ± 2.7</td>
<td>44.2 ± 2.6</td>
</tr>
<tr>
<td>White blood cell count, WBC</td>
<td>10^3/µL</td>
<td>0.7 ± 0.2**</td>
<td>2.6 ± 1.3</td>
<td>3.8 ± 1.3</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>10^3/µL</td>
<td>0.21 ± 0.04</td>
<td>0.27 ± 0.103</td>
<td>0.26 ± 0.023</td>
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<td>Lymphocytes</td>
<td>10^3/µL</td>
<td>0.26 ± 0.062**</td>
<td>2.22 ± 0.554</td>
<td>2.66 ± 0.425</td>
</tr>
<tr>
<td>Monocytes</td>
<td>10^3/µL</td>
<td>0.018 ± 0.003*</td>
<td>0.026 ± 0.011</td>
<td>0.051 ± 0.07</td>
</tr>
<tr>
<td>Basophils</td>
<td>10^3/µL</td>
<td>0.0065 ± 0.009*</td>
<td>0.001 ± 0.001</td>
<td>0.0038 ± 0.001</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>10^3/µL</td>
<td>0.006 ± 0.0018*</td>
<td>0.014 ± 0.0063</td>
<td>0.0134 ± 0.006</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.001 compared to control C57BL/6 animals and BL6/SJL (hTg) control mice.
Figure 46. Morphological analysis of peripheral blood cells (Giemsa staining).

Spontaneous autorosette-formations were found in G93A mice (A, B, arrows) at end stage of disease. A squeezed shape of erythrocytes in rosettes was noted. Normal morphological characteristics of peripheral blood cells were noted in C57BL/6 (C) and BL6/SJL (hTg) control animals (D, E). 1— autorosette forming by lymphocytes; 2— lymphocyte, 3— platelet, 4— reticulocyte; 5— monocyte; 6— segmented neutrophil; 7— basophil; 8— erythrocyte. Scale bar in A–E is 25 Am.
Appendix B- Arthritis-Induced Hematopoiesis Repairs Pathology and Memory in Alzheimer’s Mice- Supplementary Figures
Appendix B: (Continued)

Supplementary Figure 1. Significant variation of amyloid plaque load between mice. Animals (PS/APP, all 8.8 - 9.6 months, numbered sequentially according to date of birth, 25-35 g, both genders) were anesthetized with 1-2% isoflurane, shaved and scrubbed with 10% Betadine solution at the site of incision, and placed into a stereotaxic frame (Kopf Instruments, Tujunga, Ca.). A small (3 cm) incision was made, exposing the skull, and curved Strabysmus surgical scissors were used to form a subcutaneous pocket along the animal’s back into which 2 osmotic minipumps (Alzet model 1004, average flow rate of 0.12 µL/hour, Durect Corp., Cupertino, CA) were inserted. Two holes were drilled into the skull (from Bregma -0.1 mm anterior-posterior, +/- 0.9 mm medial-lateral), and 30 gauge catheters were inserted at a depth of 3.0 mm, corresponding to the lateral ventricles. Leading from the Alzet pump was a proprietary catheter system (patent pending - PCT/US08/73974) with the delivery tips fashioned to the contours of the skull rather than the commercially-available pedestal cannula. The cannulae are affixed to the skull using Locktite 454 adhesive (Plastics One, Roanoke, VA) and secured with 1 cm diameter nitrile, followed by silk sutures to close the scalp. After 2 weeks of bilateral intracerebroventricular infusions of M-CSF, mice were perfused, brain tissues were fixed in 10% neutral buffered formalin, and cryosectioned at 14 µm. Standard fluorescent immunohistochemistry used 6E10/Alexa 488 and Hoechst nuclear stain (blue). Bright green spots indicate amyloid plaques. Pictures taken at 5X. Mouse numbers 148, 160, 171, and 211 received M-CSF and mice 164, 170, 176, and 177 received aCSF.
Appendix B: (Continued)
Appendix B: (Continued)

Supplementary Figure 2. Intrahippocampal injection of M-CSF (left) and aCSF (right). (A) The image is a montage of ~35 5X pictures and is representative of the effects seen from anterior hippocampus to posterior in all 4 M-CSF-injected mice. (B) This photo shows enlargement of the M-CSF-injected left hemisphere, as seen following saline perfusion. Note the small bump at the site of injection (arrow). (C) Image shows cyst or tumor-like growth formed in the needle track at the site of M-CSF injection. Cryosectioned at 14 μm and stained with 6E10/Alexa 488 and Hoechst. Picture taken at 20X.
Appendix B: (Continued)
Appendix B: (Continued)

Supplementary Figure 3. Intrahippocampal injection of G-CSF (left) and aCSF (right). Amyloid plaques indicated as white spots. Visual observation of amyloid plaques show a modest reduction of plaque in the left G-CSF-injected hemisphere. Cryosectioned at 14 µm and stained with 6E10/Alexa546 and Hoechst nuclear stain. Images are montages of ~35 5X pictures each. Sections numbered 1 through 6 and correspond with anterior to posterior.
Appendix B: (Continued)
Appendix B: (Continued)
Appendix B: (Continued)

Supplementary Figure 4. Intrahippocampal injection of GM-CSF(left) and aCSF(right). Representative sections of each mouse proximal to injection site. Tissue sections stained with MabTech α-Aβ/Alexa 488. White spots indicate amyloid plaque immunolabelling. Images are montages of about 145 pictures taken at 10X. Figures 4A-C are from 14 µm frozen sections, and 4D is from a 5 µm paraffin-embedded section.
Appendix B: (Continued)

![Graph and Table]

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>GM-CSF</th>
<th>aCSF</th>
<th>Individual p-Value (1,1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M224</td>
<td>219,640.41</td>
<td>413,046.79</td>
<td>0.0183</td>
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<tr>
<td>M553</td>
<td>433,471.79</td>
<td>779,562.91</td>
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<td>M649</td>
<td>131,155.13</td>
<td>281,493.85</td>
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<tr>
<td>M708</td>
<td>628,152.56</td>
<td>798,960.00</td>
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Overall p-Value (1,1) = 1.83e-06
Appendix B: (Continued)

![Bar chart showing average plaque area in μm² for different mouse numbers.

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Average Area (μm²)</th>
<th>GM-CSF</th>
<th>aCSF</th>
<th>Individual p-Value (1,1)</th>
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<tbody>
<tr>
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<td>233.28</td>
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<td>M553</td>
<td>229.44</td>
<td>314.32</td>
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<td>M649</td>
<td>188.54</td>
<td>257.85</td>
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<td>M708</td>
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<td>0.0137</td>
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<td>1.10E-07</td>
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</table>
Appendix B: (Continued)

![Graph showing the sum of plaque perimeters (in μm) for different mice.

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>Sum of Plaque Perimeters (μm)</th>
<th>GM-CSF</th>
<th>aCSF</th>
<th>Individual pValue (1,1)</th>
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</thead>
<tbody>
<tr>
<td>M224</td>
<td></td>
<td>58.19547</td>
<td>96.48737</td>
<td>0.0068</td>
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<td>M553</td>
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<td>124.35704</td>
<td>178.03692</td>
<td>0.0019</td>
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<tr>
<td>M649</td>
<td></td>
<td>38.40625</td>
<td>75.97573</td>
<td>0.0035</td>
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<tr>
<td>M708</td>
<td></td>
<td>135.65128</td>
<td>165.03243</td>
<td>0.0384</td>
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</table>

Overall pValue (1,1) 3.57E-08
Appendix B: (Continued)
Appendix B: (Continued)

![Graph showing Feret Diameter (in μm) for different mouse IDs and treatments.]

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Feret Diameter (in μm)</th>
<th>GM-CSF</th>
<th>aCSF</th>
<th>Individual pValue (1,1)</th>
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</table>
Appendix B: (Continued)

![Graph showing integrated density in pixels² for different conditions]

<table>
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<th>Mouse #</th>
<th>GM-CSF</th>
<th>aCSF</th>
<th>Individual pValue (1,1)</th>
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<tr>
<td>M224</td>
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<td>M533</td>
<td>91,270.5</td>
<td>125,037.5</td>
<td>0.0144</td>
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<tr>
<td>M649</td>
<td>75,320.8</td>
<td>102,585.7</td>
<td>0.0035</td>
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<td>M708</td>
<td>142,088.6</td>
<td>171,162.1</td>
<td>0.0090</td>
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<td>Overall pValue (1,1)</td>
<td>1.108E-07</td>
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Appendix B:  (Continued)

Supplementary Figure 5. Quantification of reduced amyloid deposition in GM-CSF-injected left hemispheres versus aCSF-injected contralateral right hemispheres. There were 5 sections per mouse quantified. Each montaged section contained over 140 10X pictures and of these, 15 - 25 pictures per hemisphere were selected to quantify as described in Supplementary Figure 6 online. All pictures per section were taken at the same exposure on a Zeiss Imager.Z1 fluorescence microscope with a Zeiss Axiocam Mrm camera (Oberkochen, Germany) using Axiovision 4.7 software. Each figure shows total or average values from the 5 sections/mouse with significance per individual mouse and overall. Error bars are ±SEM: (a - b) plaque areas (c - d) perimeter values (e) average feret diameters (f) average integrated densities
Appendix B: (Continued)
Appendix B: (Continued)

Supplementary Figure 7. Behavioral Tasks

Radial Arm Water Maze. For the RAWM task of spatial working memory, an aluminum insert was placed into a 100cm circular pool to create 6 radially-distributed swim arms emanating from a central circular swim area. An assortment of 2-D and 3-D visual cues surrounded the pool. The number of errors prior to locating which one of the 6 swim arms contained a submerged escape platform (9cm diameter) was determined for 5 trials/day. There was a 30-min time delay between the 4th trial (T4; final acquisition trial) and 5th trial (T5; memory retention trial). The platform location was changed daily to a different arm, with different start arms for each of the 5 trials semi-randomly selected from the remaining 5 swim arms. During each trial (60 s maximum), the mouse was returned to that trial’s start arm upon swimming into an incorrect arm and the latency time required to locate the submerged platform was recorded. If the mouse did not find the platform within a 60-s trial, it was guided to the platform for a 30-s stay. The numbers of errors and escape latency during trials 4 and 5 are both considered indices of working memory and are temporally similar to standard registration/recall testing of specific items used clinically in evaluating AD patients.

Cognitive Interference Task. This task was designed to mimic, measure-for-measure, a cognitive interference task recently utilized clinically to discriminate between normal aged, MCI, and AD patients. The task involves two RAWM set-ups in two different rooms, with two sets of visual cues different from those utilized in standard RAWM testing. The task requires animals to remember a set of visual cues (in RAWM-A), so that following interference with a different set of cues (in RAWM-B), the initial set of cues can be recalled to successfully solve the RAWM task. Five behavioral measures were examined: A1-A3 (Composite three-trial recall score from first 3 trials performed in RAWM-A), B (proactive interference measure attained from a single trial in RAWM-B), A4 (retroactive interference measure attained during a single trial in RAWM-A), and A5 (delayed-recall measure attained from a single trial in RAWM-A following a 20-min
Appendix B: (Continued)

delay between A4 and A5). As with the standard RAWM task, this interference task involves the platform location being changed daily to a different arm for both RAWM set-ups. For A1 and B trials, the animal is initially allowed one minute to find the platform on their own before being guided to the platform. Then the actual trial is performed in each case. As with the standard RAWM task, animals were given 60s to find the escape platform per trial, with the number of errors and escape latency recorded per trial.
ABOUT THE AUTHOR

Steven Prescott Bennett received his Bachelor’s degree in Biology with a strong minor in Chemistry from Eckerd College in 1997. After 2 years working in a DNA sequencing laboratory, he began his research career at the Roskamp research institute. In the fall of 2001 he joined the Medical Science Ph.D. program at the University of South Florida in the laboratory of Dr. Huntington Potter where he specialized in drug and gene delivery to mouse models of amyotrophic lateral sclerosis (ALS) and Alzheimer’s disease (AD). His formal appointment was in the department of Molecular Medicine within college of Medicine. He successfully defended his doctoral dissertation on October 14, 2009 at the University of South Florida.