Recombinant AAV Gene Therapy and Delivery for Alzheimer’s Disease

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LIST OF ABBREVIATIONS

AAV: Adeno-associated virus
BACE: Beta site APP cleaving enzyme (beta secretase)
Aβ: Amyloid Beta
AD: Alzheimer’s disease
APP: Amyloid precursor protein
CA: Cornu ammonis
CBA: Chicken β-actin
CED: Convection enhanced delivery
DG: Dentate gyrus
GFAP: Glial fibrillary acidic protein
GFP: Green Fluorescent Protein
ECE: Endothelin converting enzyme
FAD: Familial Alzheimer’s disease
HA: Heamagluttinin
IDE: Insulin degrading enzyme
ITR: Inverted terminal repeat
MMP: Matrix metalloprotease
NEP: Neprilysin
NeuN: Neuronal nuclei
NGF: Nerve Growth Factor
PS: Presenilin
rAAV: Recombinant adeno-associated virus
Alzheimer’s disease (AD), first characterized in the early 20\textsuperscript{th} century, is a common form of dementia which can occur as a result of genetic mutations in the genes encoding presenilin 1, presenilin 2, or amyloid precursor protein (APP). These genetic alterations can accelerate the pathological characteristics of AD, including the formation of extracellular neuritic plaques composed of amyloid beta peptides and the formation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein. Ultimately, AD results in gross neuron loss in the brain which is evidenced clinically as a progressive decline in mental capacity. A strong body of scientific evidence has previously demonstrated that the driving factor in the pathogenesis of AD is potentially the accumulation of Aβ peptides in the brain. Thus, reduction of Aβ deposition is a major therapeutic strategy in the treatment of AD. Recently it has been suggested that Aβ accumulation in the brain is modulated, not only by Aβ production, but also by its degradation. Several important studies have demonstrated that Aβ degradation is modulated by several endogenous zinc metalloproteases shown to have
amyloid degrading capabilities. These endogenous proteases include neprilysin (NEP), endothelin converting enzyme (ECE), insulin degrading enzyme (IDE) and matrix metalloprotease 9 (MMP9). In this investigation we study the effects of upregulating expression of several of these proteases through administration of recombinant adeno-associated viral vector (rAAV) containing both endogenous and synthetic genes for ECE and NEP on amyloid deposition in amyloid precursor protein (APP) plus presenilin-1 (PS1) transgenic mice. rAAV administration directly into the brain resulted in increased expression of ECE and NEP and a substantial decrease in amyloid pathology. We were able to significantly increase the area of viral distribution by using novel delivery methods resulting in increased gene expression and distribution.

These data support great potential of gene therapy as a method of treatment for neurological diseases. Optimization of gene transfer methods aimed at a particular cell type and brain region in the CNS can be accomplished using AAV serotype specificity and novel delivery techniques leading to successful gene transduction thus providing a promising therapeutic avenue through which to treat AD.
INTRODUCTION

ALZHEIMER’S DISEASE

Alzheimer’s disease (AD) is a debilitating and devastating neurodegenerative disorder that leads to a progressive decline in memory, the ability to learn and reason, and loss of overall brain function ultimately resulting in death. AD is the most common form of senile dementia (a group of conditions that gradually destroy brain cells leading to progressive decline in mental capacity) affecting approximately 4.5 million individuals in the United States consequently causing a great financial burden on the U.S. economy. The estimated annual costs of caring for individuals with Alzheimer’s disease are at least $100 billion, according to the Alzheimer’s Association and the National Institute on Aging. The molecular mechanisms underlying Alzheimer’s disease (AD) have been extensively investigated. AD can occur as a result of genetic autosomal-dominant mutations in the genes encoding presenilin 1, presenilin 2, or amyloid precursor protein (APP) (Levy et al., 1990), (Goate et al., 1991), (Hardy et al., 1992), (Scheuner et al., 1996). Interestingly, these autosomal dominant genetic mutations only account for 1-2% of AD cases which are inherited and commonly referred to as early onset Alzheimer’s disease or familial AD (FAD) (Fidani and Goate, 1992). The cause of AD accounting for the majority of the cases remains unknown, although Apolipoprotein E4 is a known risk factor.
These genetic alterations have been found to accelerate the pathological characteristics of AD which include the formation of extracellular amyloid plaques and the formation of intracellular neurofibrillary tangles consisting of hyperphosphorylated tau. The accumulation of these amyloid plaques are not only a crucial factor in the pathology of AD (Selkoe, 2001), (Bard et al., 2003), but have been argued to contribute to the distinctive clinical symptoms of AD such as progressive cognitive decline, loss of memory and decreased mental capacity (Cummings et al., 1996a), (Nicoll et al., 2003), (Masliah et al., 2005). The genetic mutations that result in AD pathology all cause alterations in the normal processing of the amyloid precursor protein (APP) by altering the proteolytic cleavage sites which cause the production and release of Aβ peptides in the brain. Accumulation of these peptides leads to the formation of extracellular deposits commonly referred to as neuritic plaques (Cummings et al., 1996b), (Selkoe, 2001). Another hallmark pathological feature of AD are intraneuronal neurofibrillary tangles which can occur independent of the neuritic plaque. The tau aggregates which compose these tangles are nonmembrane bound fibers arranged as pairs of helical filaments. Although the mechanism by which neuritic plaques and neurofibrillary tangles can eventually lead to neuron loss is debated among researchers, it has been repeatedly demonstrated that reducing amyloid deposits in the brain can significantly improve cognitive deficits (Janus et al., 2000), (Morgan et al., 2000), (Dodart et al., 2002). Consequently, elucidating novel methods of decreasing or preventing amyloid accumulation has been a primary focus in the treatment of Alzheimer’s disease.
AMYLOID PRECURSOR PROTEIN (APP) AND PRESENILIN 1 AND 2

The genetic etiology of AD provides important evidence supporting the “Amyloid Cascade Hypothesis” first put forth by John Hardy in 1991 (Hardy and Allsop, 1991), (Hardy and Selkoe, 2002). Specifically, all genetic mutations that cause early onset or familial AD alter the production of Aβ in the brain (Kimberly et al., 2000). The amyloid precursor protein (APP) gene was the first AD susceptibility gene to be identified (George-Hyslop, 2000). The βAPP gene encodes for a transmembrane glycoprotein that contains 770 amino acids, but has several isoforms which vary in length (George-Hyslop, 2000). The extracellular domain of the protein is the critical portion that normally undergoes processing through a series of proteolytic cleavages. These cleavage pathways involve an α-secretase, β-secretase, and γ-secretase that give rise to several different peptides. The normal processing of the APP molecule only involves the α-secretase cleavage which readily cleaves the βAPP695 isoform of APP between the Lys687 and Leu688 residues in the extracellular domain. This cleavage is subsequently followed by a γ-secretase cleavage which yields a soluble APP molecule and a smaller P3 fragment. The abnormal processing of APP involves a β-secretase cleavage followed by a γ-secretase cleavage yielding Aβ peptide species Aβ1-40 and Aβ1-42 (Selkoe, 1998), (Sinha and Lieberburg, 1999), (George-Hyslop, 2000). Cells that contain the APP mutation readily produce larger amounts of the Aβ 1-42 peptide, the pathogenic species which is slightly more hydrophobic causing it to aggregate, as opposed to the Aβ1-40 species (Wang et al., 1999). This results in a change in the ratio of the
particular species which are normally produced by the cell, causing the peptides to abnormally accumulate forming plaques which are primarily made up of the Aβ 1-42 peptide and deposited within the brain parenchyma (Cummings et al., 1992), (Suzuki et al., 1994), (Cummings et al., 1996b), (Skovronsky et al., 1998). The aberrant accumulation of Aβ peptides in the brain has been recognized as an essential factor in the pathology of Alzheimer’s disease (AD). Due to the fact that γ-secretase catalyzes the final step in the production of Aβ peptides and because it determines the final length of the Aβ peptide variants, understanding this protease has become an important target not only in the molecular genetics of AD but has emerged as an important target in the development of potential AD therapy.

The continued study of the molecular genetics of AD eventually revealed the genes known as presenilin 1 (PS1) and presenilin 2 (PS2) (57 and 55 kDa polypeptides, respectively) which were initially discovered in 1995. The presenilins play an integral role in the molecular mechanisms leading to the development of Alzheimer’s disease (Anwar et al., 1996), (Hutton et al., 1996), (Hutton and Hardy, 1997), (Dewji and Singer, 1998), (Siman et al., 2000). Several different functions have been credited to presenilin including a role in promoting or reducing a neuron’s susceptibility to apoptosis, regulating intracellular calcium signaling, trafficking of membrane proteins, processing of the APP molecule, and involvement in the cleavage of other substrates (Ray et al., 1999), (Siman et al., 2000), (Brunkan and Goate, 2005), (Dewji, 2005). The presenilin 1 mutations account for the earliest and most aggressive forms of AD,
while presenilin 2 mutations have a later and more variable age of onset (George-Hyslop, 2000), (Ishii et al., 2001). Over one hundred different mutations have been reported in the PS1 gene, most of which are missense mutations, and approximately 6 mutations found in PS2. The presenilins have been found to be in part expressed at the cell surface with the majority of the protein being expressed in the intracellular compartment. Reports have also indicated that PS1 and PS2 are expressed in the endoplasmic reticulum and the golgi apparatus as well as in nuclear membranes (Dewji, 2005). Ultimately, the presenilin mutations lead to an increased production of cerebral Aβ1-42 peptides and a 1.5 to 3 fold increase in Aβ42 containing plaques in human brain tissue of FAD human tissue as opposed to sporadic cases of AD (Lamere et al., 1996). The finding that presenilin mutations cause specific increases in Aβ1-42 production lead to the idea that mutant PS must play a role in the modulation of γ-secretase activity and are essential for the γ-secretase cleavage of βAPP (Ray et al., 1999). Results from transgenic mouse models with PS mutations and APP mutations clearly indicate that PS mutations not only increase production and deposition of Aβ species in the brain but also imply that presenilin may act as the γ-secretase enzyme or an essential cofactor for the γ-secretase cleavage of βAPP (Duff et al., 1996), (Ishii et al., 2001), (Brunkan and Goate, 2005). Despite the breakthrough discovery of genes responsible for early onset AD this accounts for only 1-2% of all AD cases and therefore investigation to identify AD susceptibility genes have become an area of intense research.
TRANSGENIC MOUSE MODELS OF AMYLOID DEPOSITION

Almost ten years after the discovery of the FAD mutations in the APP gene and the introduction of transgenic technology, researchers have used knowledge of the numerous mutations on APP, PS1, and PS2 to create murine models that have lead to an increased understanding of AD pathogenesis. The first generation of an AD transgenic mouse model was based on the over expression of the wild type human (hAPP) cDNAs and in most cases these efforts failed to produce mice with the hallmark pathogenicity found in human AD cases with the exception of the NSEAPP (APP751) mouse produced by Quon et al. in 1991. Investigators eventually discovered that the overexpression of the hAPP in addition to either the Swedish mutation (APP_{K670N,M671L}) or the London mutation (APP_{V717F}) was sufficient to produce enough Aβ peptides to cause significant amount of amyloid deposition (Quon et al., 1991), (Higgins and Jacobsen, 2003). This gave rise to the PDAPP mouse first presented by Games et al. in 1995 that contains the London mutation (APP_{V717F}) under control of the platelet derived growth factor promoter (PDGF). The Tg2576 mouse first described by Hsiao et al. in 1996 contains the APP695 isoform with the Swedish mutation (APP_{K670N,M671L}) under the prion protein promoter (PrP) which ensures high CNS expression (Hsiao et al., 1996, Higgins and Jacobsen, 2003). These mice exhibit a similar positive correlation between increased age and amyloid deposition as well as selective distribution of amyloid within specific brain regions such as cortex and hippocampus.
Another single transgenic mouse model of amyloid deposition (APP23 mouse) was developed by Novartis and described by Sturchler-Pierrat et. al., in 1997 and contains the hAPP751 isoform with the Swedish mutation under the control of the Thy-1 promotor to enhance neuronal expression. These mice also emulate the PDAPP and Tg2576 mice in their region specific development of amyloid deposits but unlike the two previous transgenic models the APP23 mouse has been shown to demonstrate neurodegeneration in the CA1 region of the hippocampus which seems to correlate with increased plaque load (between 14-18 months of age) (Calhoun et al., 1998), (Higgins and Jacobsen, 2003). In more recent years double transgenic mouse models of AD which express both mutant APP and human presenilin with FAD mutations have been successfully created (Higgins and Jacobsen, 2003). These mice have significantly enhanced production of the Aβ1-42 peptide and also begin to develop amyloid deposits at a much earlier age compared to their single transgenic counterparts (Borchelt et al., 1997), (Holcomb et al., 1998). The PSAPP mouse overexpresses the PS1 gene with the M146L mutation (Duff et al., 1996) and the Swedish mutation (Hsiao et al., 1996) and show significantly increased plaque pathology, beginning around 3 months of age, which is associated with gliosis (Holcomb et al., 1998). Interestingly, transgenic mice that express only FAD presenilin mutations alone show no signs of amyloid deposition (Duff et al., 1996). The CRND8 transgenic model expresses a double mutated form of the APP695 (APP_{V717F} and APP_{K670N,M671L}) under the PrP promoter. This model also results in accelerated plaque deposition which is clearly evident at 3 months of age. PDAPP mice have also
been shown to exhibit some behavioral deficits as early as three months of age even before the development of amyloid plaque deposits could be detected (Higgins and Jacobsen, 2003). The Tg2576 transgenic model differ from the PDAPP animals in that they begin to develop cognitive deficits between 9-10 months of age and this correlates more closely with the development of amyloid deposits in the brain (Hsiao et al., 1996). In early 2002 Westerman and colleagues proposed that cognitive deficits more likely are the result of increases in soluble forms of Aβ (or small protofibrils of Aβ) rather than plaque deposition (Westerman et al., 2002). More recent reports argue that large Aβ aggregates may in fact play a neuroprotective role providing a mechanism in which smaller soluble Aβ moieties are sequestered into larger insoluble aggregates preventing them from causing destruction. Specifically, the soluble oligomeric Aβ assemblies have been implicated as the driving factor in AD pathogenesis as they exhibit potent toxicity in their capacity to significantly decrease LTP, contribute to learning and memory deficits in vivo and induce neuronal cell death in vitro (Lambert et al., 1998), (El-Agnaf et al., 2000), (Klein, 2002; Glabe, 2005), (Malaplate-Armand et al., 2006), (Townsend et al., 2006), (Cerpa et al., 2008), (Varvel et al., 2008)

ZINC METALLOPROTEASES/ENDOGENOUS Aβ DEGRADING ENZYMES

In recent years a growing number of endogenous proteases have been found to have Aβ degrading capabilities in the brain and other tissues both in vivo
and *in vitro*. Several of these enzymes are members of zinc metalloprotease family and include neprilysin (NEP), insulin degrading enzyme (IDE; insulysin), endothelin converting enzymes (ECE-1 and ECE-2), and angiotensin converting enzyme (ACE). Recently, a number of other proteases have been shown to degrade Aβ in *in vitro*, including matrix metalloproteases 2, 3, and 9, and the serine protease, plasmin but the degree to which they may be involved in the normal catabolism of Aβ remains uncertain (Yan et al., 2006), (Turner and Nalivaeva, 2007). Down regulation of these enzymes within the brain could potentially contribute to Aβ accumulation and lead to development of AD.

NEP, a 92-kDa glycosylated ectoenzyme, was originally found as a kidney enzyme and is capable of degrading multiple peptide hormones (Iwata et al., 2000a). Also known as enkephalinase, it is capable of cleaving enkephalins and termination of peptidergic neurotransmission. It is membrane bound and its active site is oriented outside the cell. NEP has a very large range of regulatory activity as it is capable of degrading numerous bioactive peptides including opioid peptides, tachykinins, substance P, atrial natriuretic peptides, chemotactic peptides and adrenocorticotropic hormone (ACTH) (Matsas et al., 1984a), (Turner et al., 1996). NEP is expressed in the CNS and in the periphery particularly in epithelial cells of the intestines, kidney and lungs (Fulcher et al., 1982), (Matsas et al., 1984b), (Kenny et al., 1985). In the CNS NEP is expressed in neurons, mainly pyramidal neurons, and glial cells types (Turner et al., 2003). Therefore, NEP is involved in regulation of several different physiological processes, such as cardiovascular activity, cell migration and
proliferation, inflammation, and neuropeptide regulation (Turner, 2003). NEP, is a highly conserved protein and the NEP gene is located on chromosome 3 spanning more than 80 kb (Roques et al., 1993) in humans. Several homologues of NEP have also been identified most of which remain uncharacterized (Turner and Nalivaeva, 2007), (Nalivaeva et al., 2008). Although, one homologue named NEP2 (also known as Sep and NL1) has been shown to also degrade Aβ, unlike NEP it is expressed only in specific neuronal populations in the brain and in the spinal cord. Furthermore, NEP2 is expressed in a membrane bound form in the endoplasmic reticulum and a soluble secreted form but very little is known regarding its function or whether it plays a role in Aβ metabolism. 

Iwata et. al., in 2000 first demonstrated the ability of NEP to degrade Aβ peptides in the brain parenchyma and also illustrated that suppressing NEP would lead to an increase in Aβ deposition (Iwata et al., 2000). In 2002 Iwata et. al. also demonstrated that NEP activity decreased with age in regions where Aβ accumulated such as the cortex and hippocampus, however in areas such as the striatum where Aβ did not accumulate NEP was increased (Yasojima et al., 2001b). Evidence from transgenic animal models demonstrate that NEP deficient mice show a significant increase in amyloid deposition as opposed to their wild type counterparts clearly indicating that NEP activity plays a significant role in endogenous Aβ degradation (Iwata et al., 2002), (Iwata et al., 2004), (Marr et al., 2004).

Another important Aβ degrading enzyme is known as endothelin converting enzyme (ECE). ECE, like NEP is a membrane bound
metalloprotease approximately 87 kd in size and exists in several isoforms including ECE-1a, ECE-1b, ECE-1c, and ECE-2. ECE-1 has been found localized in the Golgi and vesicles. Its primary function is to catalyze the conversion of big endothelin (big ET) into vasoactive endothelins (Eckman et al., 2003). ECE-1 has also been reported to hydrolyse other biologically active peptides including bradykinin, neurotensin and substance P. ECE is a homologue protein of NEP, yet an important structural difference between the two is that ECE-1 exists as a disulfide linked dimer. ECE-1 is expressed in endothelial cells as well as several other cell types including neurons and glia within the brain that are particularly relevant to AD disease pathology. Although several other isoforms of ECE exist, ECE-1c is the predominantly expressed mRNA in humans. Each isoform has distinct subcellular localizations; specifically ECE-1a, ECE-1c, and ECE1d are primarily localized at the cell surface, while ECE-1b is found in the intracellular compartment. To date there are no significant differences in the catalytic properties of the different isoforms (Turner et al., 2004). The Aβ degrading capabilities of ECE were discovered when Eckman et al. observed that the metalloprotease inhibitor, phosphoramidon, caused a rapid accumulation of Aβ in a cell line which expressed ECE and did not cause this increase in cells not expressing ECE (Eckman and Eckman, 2005). Subsequent studies revealed that when ECE was overexpressed in the cell lines the Aβ accumulation was reduced by approximately 90%. In vivo data from ECE (+/-) transgenic mice which show about 27% reductions in ECE-1
activity also show significant increases in both Aβ40 and Aβ42 levels in the brain (Eckman and Eckman, 2005).

In addition to NEP and ECE, IDE (insulysin) has been suggested as another Aβ degrading enzyme that may play an important role in the dynamic process of Aβ degradation in the brain. Unlike NEP and ECE-1, IDE is primarily localized in the cytosol but it can also be located, at low levels, in peroxisomes as well as in the plasma membrane and in a secreted form in the extracellular compartment (Turner et al., 2004). IDE is expressed in the human CNS in cortical and subcortical neurons as well as in endothelial cells, pericytes and smooth muscle cells (Morelli et al., 2004). In vitro, IDE is capable of degrading a variety of substrates including insulin and its role in Aβ degradation in the brain was first proposed by Kurochkin et. al., (Kurochkin and Goto, 1994), (Kurochkin, 2001), (Turner et al., 2004), (Farris et al., 2005). Several studies support the importance of IDE activity in Aβ catabolism, including a correlation study presented by Perez et. al. in 2000 revealed that IDE activity which correlated with a decrease in soluble Aβ peptide degradation in the AD brain compared to normal aged matched control brains (Perez et al., 1999). Furthermore, in transgenic mice with IDE (-/-) deficiencies results in almost a 50% decrease in Aβ degradation in not only the brain but in primary neuron cultures as well; while the overexpression of IDE significantly reduces brain Aβ levels in APP mice (Farris et al., 2003), (Leissring et al., 2003), (Farris et al., 2005).

The in vivo data from multiple studies involving NEP, ECE, and IDE transgenic mice provides clear evidence that the activity of these specific
proteases are essential for the regulation of Aβ accumulation in the brain. Therefore, the regulation of these proteases through novel methods such as gene therapy provides a unique avenue through which novel therapeutic treatments can be explored.

GENE THERAPY AND VIRAL VECTORS

Gene therapy has a fairly short and controversial history and was first envisioned as a potentially viable scientific tool for the treatment of hereditary single-gene defects. Beginning in the late 1970’s and 80’s gene therapy made its unofficial clinical debut with two unapproved clinical trials. In the first trials, two young boys with arginase deficiency syndrome were treated using in vivo gene therapy with a wild-type Shope papilloma virus in an effort to replace the missing enzyme with the viral arginase. Another trial involved the use of ex vivo gene therapy in which a bone marrow transplant was performed using bone marrow treated with a β-globin-containing plasmid in order to treat two patients with β-thalassemia. The results were neither efficacious nor deleterious (Scollay, 2001). During the 1990’s the use of gene therapy in approved gene therapy trials steadily increased and by the mid 2000 nearly 4000 patients have been treated with gene therapy in more than 500 trials (Scollay, 2001). The majority of the trials in the 1990’s were unsuccessful and in 1999 the first published death resulting from gene therapy using an adenovirus vector was highly publicized lending to a bad public perception of gene therapy and it’s potential as a viable therapeutic avenue in the treatment of disease and revealing the need to further
understand the viral vector interactions with the human immune system. Fortunately, by 2000 the first successful procedures were conducted in three individuals suffering from severe combined immunodeficiency (SCID), (Scollay, 2001). This progress was the result of improvements in understanding different types of viral vectors and in our understanding of genetics and the biology of transducing cells.

There are several different mechanisms by which gene therapy can be achieved and an important factor in the success of the method involves the type of vehicle used to delivery the therapeutic gene to the target tissue (Thomas et al., 2003). Viral vectors utilized in the first gene therapy attempts were extremely inefficient and did not persist in the host cells and the transgene expression was in most cases for a short time. A thorough understanding of the molecular basis of how viruses and viral vectors interact with the host has been a major focal point in the area of gene therapy. As a result over the past few years, several vectors have been developed aimed at improved efficiency, specificity, and safety which have also resulted in successful treatments (Thomas et al., 2003). The diversity of diseases that gene therapy research has aimed at targeting is expanding from the initial hereditary single gene defect treatment to include acquired diseases such as cancer, cardiovascular disease, infectious disease and neurodegenerative disease (Mandel and Burger, 2004). This means that no one vector method will be the most efficacious for all disease categories. Thus understanding and utilizing unique characteristics of different viral vector types is essential for the success of gene transfer and persistence of the transgene.
There are five major categories of viral vectors which can be categorized into two main groups (Thomas et al., 2003). The groups include viruses whose genome integrates into the host cellular genome known as oncoretroviruses or those which persist apart from the cellular host genome as an episome, or extrachromosomal unit, within the cell nucleus. Retroviruses, such as human T cell leukemia virus, sarcoma virus and HIV, are a class of viruses that can create double-stranded DNA copies of their RNA genomes. These copies of its genome can be integrated into the chromosomes of host cells. Lentivirus is characterized by a long incubation period. Human immunodeficiency virus (HIV) is an example of a lentivirus. Adenoviruses are a class of viruses with double-stranded DNA genomes that cause respiratory, intestinal, and eye infections in humans. The virus that causes the common cold is an adenovirus. Adeno-associated viruses are a class of small non-pathogenic human parvovirus, single-stranded DNA viruses that can insert their genetic material which can persist as an episomal plasmid. Finally, herpes simplex viruses are a class of double-stranded DNA viruses that infect a particular cell type, specifically neurons. Herpes simplex virus type 1 is a common human pathogen that causes cold sores (Thomas et al., 2003). There are advantages and disadvantages to each type of vector which determines it’s suitability for specific treatments. Particularly, vectors such as adeno-associated virus (AAV), adenovirus and herpes virus, which have non-integrating vectors, usually have a persistent transgene expression only in non proliferating cells whereas retroviruses with integrating genomes have persistent transgene expression in dividing cells. The size of the viral vehicle can also be a
determining factor mediating the type of gene that can be expressed. The table below, published by Thomas et. al.. in 2003 summarized the advantages and disadvantages of the five different viral vectors (Thomas et al., 2003).

Adeno-associated viruses are members of the \textit{parvoviridae} family and are subsequently a member of the helper virus genus which indicates that they need a helper virus, in most cases adenovirus or herpes simplex virus, in order to support successful infection and replication. When the helper virus is absent AAVs will generate a latent infection within the cell and in 90% of cases this means the AAV genome will persist as an episomal unit (Thomas et al., 2003), (Tenenbaum et al., 2004a), (Wu et al., 2006).

ADENO-ASSOCIATED VIRAL VECTORS AND SEROLOGY

AAVs are one of the smallest viruses, approximately 25 nm in diameter, and their DNA genome is less than 5 kb which contains two large open reading frames with inverted terminals repeats located at either end (Samulski et al., 1982), (Tenenbaum et al., 1994), (Tenenbaum et al., 2004b), (Wu et al., 2006). AAVs must accomplish several major steps to achieve successful gene expression which include attachment to the cell surface receptors, endocytosis, trafficking to the nucleus, uncoating of virus which releases viral genome, and finally conversion of the genome to double stranded DNA which is transcribed in the nucleus. The efficiencies of rAAV transduction intricately depend on the successful implementation of each step. AAV vectors arrive in a variety of flavors otherwise known as serotypes. The AAV serotype refers to the efficiency
by which the specific AAV can infect a particular cell type through attachment to specific cell surface receptors. A new AAV serotype is defined by the inability of an antibody that is reactive to the viral capsid protein of one serotype in neutralizing those of another serotype (Choi et al., 2005). Through the modifications to the viral genome and the capsids rAAV can be designed to be more efficient at transducing specific cells or tissue types to optimize gene therapy (Choi et al., 2005).

AAV2 serotype was the first to be cloned into bacterial plasmids by Samulski et. al. in 1982 and to date it is the most characterized of all the serotypes of which a total of 10 have been discovered, and has been shown to effectively transducer neurons in the CNS in a number of animal studies (Samulski et al., 1982), (Fu et al., 2003), (Burger et al., 2004), (Tenenbaum et al., 2004b). AAV5 was originally discovered in a human clinical sample in 1984 as a contaminant in adenovirus stock and it contains ITRs (inverted terminal repeats) that are not unlike the structures of AAV2 ITRs and is the most divergent of all of the serotypes (Choi et al., 2005), (Wu et al., 2006). AAV1 and AAV3 were also initially found as contaminants of simian adenovirus 15, while AAV6 was identified as a contaminant of adenovirus type 5 by Rutledge et. al. in 1998. AAV7 and AAV8 were originally isolated from rhesus monkey tissue in 2002 by Gao et. al. AAV9, like AAV5, was originally discovered in human tissue, while AAV10 and AAV11 are the most recently identified serotypes and initially were isolated from cynomolgus monkey tissue in 2004 by Mori et. al. but they are not well characterized (Mori et al., 2004), (Wu et al., 2006).
The fairly recent identification of different AAV serotypes had advanced the study of rAAV vectors which has quickly become a major dominant focus in the field of gene therapy. There are a number of studies aimed at characterizing the different AAV serotypes with respect to transduction efficiency, tissue tropism, cell surface receptors, intracellular processing, and capsid structure. AAV2 vectors are the most extensively characterized and due to their well established safety profile and range of infectivity, approximately 20 clinical trails have been conducted using the AAV2 vector serotypes in numerous patients (Wu et al., 2007). In order to effectively study transduction efficiencies of different serotypes of AAV vectors in different tissue types, scientists have utilized the “cross-packaging” strategy which essentially enables an unbiased direct comparison of the transduction rates without influence of ITRs on transgene expression (Grieger and Samulski, 2005), (Wu et al., 2007). To accomplish this the Cap genes of different serotypes are placed downstream of the AAV2 Rep genes which ultimately allows the generation of serotypes specific capsids while the packaged genomes within the capsids are identical, these are also referred to as hybrid viruses (Rabinowitz et al., 2002), (Rabinowitz et al., 2004), (Wu et al., 2006). In addition to transcapsidation first described by Rabinowitz et. al. in 2002 there are several other techniques to accomplish the formation of a hybrid virus armed with specific modifications to enhance efficiency of gene uptake, transfer, and expression in a specified therapeutic avenue. These include absorption modifications where the capsid surface is modified to carry a foreign antibody that will bind to the cell surface receptor of
interest to increase absorption efficiency. Mosaic capsids are another method to increase transduction efficiency. This technique involves creating a mixture of viral capsid proteins from different serotypes at various ratios in order to combine tissue tropisms of interest (Xiao et al., 1999), (Rabinowitz and Samulski, 2000). Finally, chimeric capsids can also provide a means of increasing transduction of a particular specificity and this technique involves the packaging of capsid proteins with foreign peptide sequences, such as a hemagglutinin (HA) tag fused at either the N or C terminus of the capsid coding sequence to alter tissue tropism (Yang et al., 1999), (Bowles et al., 2003), (Wu et al., 2006).

Although the data regarding AAV serotype specific tissue tropism is subject to different interpretation due to variations in vector titer, promoters, transgenes between studies, in general the transduction efficiencies for most of the AAV serotypes have been determined. In the CNS serotype characterization studies have revealed that AAV1, and 5 have higher transduction efficiencies than AAV2 throughout the all regions and cell types of the CNS (Alisky et al., 2000), (Burger et al., 2004), (Burger et al., 2005) while AAV4 will efficiently transduce specific cell types such as astrocytes within the subventricular zone (Davidson et al., 2000), (Weber et al., 2003), (Wu et al., 2006). Studies by Wolfe et. al. also reveal that AAV7, 8, 9 and Rh10 expressing cDNA for lysosomal enzyme are also capable of transducing neurons within specific regions in the mouse brain. AAV9 and AAVRh10 appeared to have the highest transduction efficiencies and were found to undergo vector genome transport through axonal transport pathways (Cearley and Wolfe, 2006).
Despite the rapidly growing body of knowledge in the field of AAV vector serology there is still much to be done. In addition to the 10 AAV serotypes that have been found over 100 AAV variants have also been discovered and with the technological advances in engineering hybrid vectors, including mosaic AAVs, chimeric AAVs, absorption modifications and transcapsidation AAV vectors appear to have a promising future in the field of gene therapy. (AAV serotypes 6-11 are not currently commercially available).

ENHANCING DISTRIBUTION OF GENE PRODUCT

AAV vectors are a novel mean by which transgenes can be delivered to potentially treat several types of degenerative brain diseases. Unfortunately, one of the major disadvantages of a single intracranial injection into the brain parenchyma using simple diffusion does not allow for efficient uptake of the transgene or significant distribution of the AAV macromolecules to significantly large areas of the affected regions within the brain. Convection enhanced delivery (CED), first described by Bobo et al in 1994, is a method of delivering clinically relevant volumes of therapeutic agents to significantly larger areas of the brain in a direct intracranial injection procedure in comparison to simple diffusion methods (Bobo et al., 1994). The CED technique is designed to utilize the phenomenon of bulk flow and positive pressure to distribute macromolecules to a large area within solid tissue. The CED technique was originally proposed by scientists in the early 1990s as a method of delivering drugs directly to the parenchyma that would not normally cross the blood brain barrier and that
consisted of large macromolecules too large to diffuse throughout the tissue (Raghavan et al., 2006). Due to the lack of approved drugs that can be directly intracranially administered to the brain and the difficulty in predicting methods that ensure delivery of the therapeutic agent to its target site in spite of its use in clinical trials CED remains an experimental procedure and research of CED delivery devices is under current investigation by several researchers (Bankiewicz et al., 2000), (Raghavan et al., 2006).

This CED method has been investigated in gene therapy studies as a way to increase the distribution of AAV vectors in the brain. Studies conducted by Bankiewicz et. al. in 2000 revealed that CED can significantly increase in gene transfer and distribution of AAV expressing AADC in the striatum of MPTP-treated monkeys. The AAV vector was found to be safely distributed throughout the entire region of the striatum compared to the simple injection method where the distribution was severely limited (Bankiewicz et al., 2000). Similar results were replicated in the rat brain by Cunningham et. al. in 2000 with AAV2 expressing thymidine kinase (TK) where the CED method showed robust gene transfer and increased distribution area within the putamen. CED injections in the striatum were found distributed the AAV-TK throughout the striatum after a single injection into this region and TK immunoreactive cells were also found outside the striatum, in the globus pallidus, subthalamic nucleus, thalamus, and substantia nigra (Hadaczek et al., 2006).

One of the mechanistic limitations of the CED method as well as the simple injection method is the reflux of the injected material from the injection
hole upon the removal of the cannula. In 2005 Krauze et. al. developed a reflux free step cannula design which effectively eliminates reflux by placing silicone coated tubing within the cannula creating a step that prevents the backflow of fluid (Krauze et al., 2005b), (Krauze et al., 2005a). The optimization of more efficient cannula designs coupled with the encouraging results from studies showing enhanced gene transfer and distribution emphasizes the therapeutic potential of the CED method in helping overcome some of the mechanical disadvantages of gene delivery in regards to gene therapy (Krauze et al., 2005a).

The use of osmotic agents such as mannitol is an addition method that can be used to increase the area of distribution of macromolecules throughout the CNS. Mannitol is a blood brain barrier interruptive reagent and is also known to temporarily increase vascular pressure subsequently reducing intracranial pressure. High concentrations of mannitol intravenously infused are currently used in patients with traumatic brain disease to reduce intracranial pressure. This osmotic agent pulls fluid from the CNS by increasing vascular osmotic pressure. Several studies have also shown that with intra-arterial infusion of mannitol the blood brain barrier can be opened to enhance the distribution of chemotherapeutics throughout the CNS in both rats and humans (Nilaver et al., 1995), (Rapoport, 2001), (Fu et al., 2003).
PAPER 1:
ADENO-ASSOCIATED VIRAL (AAV) SEROTYPE 5
VECTOR MEDIATED GENE DELIVERY OF ENDOTHELIN
CONVERTING ENZYME REDUCES Aβ DEPOSITS IN
APP+PS-1 TRANSGENIC MICE.

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ABSTRACT

Reduction of Aβ deposition is a major therapeutic strategy in Alzheimer's disease (AD). The concentration of Aβ in the brain is modulated, not only by Aβ production, but also by its degradation. One protease involved in the degradation of Aβ peptides is endothelin converting enzyme (ECE). In the current study, we investigated the effects of an intracranial administration of a recombinant adeno-associated viral vector (rAAV) containing the ECE-1 synthetic gene on amyloid deposition in amyloid precursor protein (APP) plus presenilin-1 (PS1) transgenic mice. The recombinant AAV vector was injected unilaterally into the right anterior cortex and hippocampus of six-month-old mice while control mice received an AAV vector expressing GFP. Immunohistochemistry for the haemagglutinin tag appended to ECE revealed strong expression in areas surrounding the injection sites but minimal expression in the contralateral regions. Immunohistochemistry for Aβ decreased in the anterior cortex and hippocampus of mice receiving ECE synthetic gene. Further, decreases in Congo red positive deposits were also observed in both regions. These results indicate that increasing the expression of β-amyloid degrading
enzymes through gene therapy is a promising therapeutic avenue through which to treat AD.

Key Words: Alzheimer’s disease; Beta Amyloid, Gene Therapy, Viral Vector, Amyloid Degrading Enzyme, Zinc Metalloprotease.

INTRODUCTION

AD is the most common form of senile dementia (a group of conditions that gradually destroy brain cells leading to progressive decline in mental capacity) affecting approximately 4.5 million individuals in the United States. The molecular mechanisms underlying AD have been extensively investigated (Hardy and Selkoe, 2002). Although the mechanism by which neuritic plaques and neurofibrillary tangles can eventually lead to neuron loss is debated, it has been repeatedly demonstrated that reducing amyloid deposits in the brain can significantly improve cognitive deficits in amyloid depositing transgenic mice (Janus et al., 2000),(Morgan et al., 2000),(Westerman et al., 2002),(Wilcock et al., 2006). Consequently, elucidating novel methods of decreasing or preventing amyloid accumulation has been a primary focus in the treatment of AD.

In recent years several endogenous proteases have been found which degrade Aβ in the brain and other tissues both in vivo and in vitro. These zinc metalloproteases, include neprilysin (NEP), insulin degrading enzyme (IDE; insulysin), and endothelin converting enzymes (ECE-1 and ECE-2). Other
proteases that appear to play a role in Aβ metabolism include matrix metalloproteinase-9 (Yan et al., 2006), cathepsin B (Mueller-Steiner et al., 2006) and plasmin (Turner et al., 2004). The overall accumulation of Aβ in the brain is attributed to an imbalance between its production and degradation/clearance. Down-regulation of these degrading enzymes within the brain during aging could potentially contribute to Aβ accumulation eventually leading to development of AD (Caccamo et al., 2005) (Yasojima et al., 2001).

Several current studies have implicated ECE as an important enzyme in the degradation of Aβ and preventing its accumulation (Eckman and Eckman, 2005). ECE is a membrane-bound metallopeptase with an N-terminal cytosolic domain and an extracellular catalytic domain. ECE-1 is expressed in endothelial cells in all organs as well as other cell types including neurons, glia and neuroendocrine cells. Two different genes encoding for ECE-1 and ECE-2 have been identified, however ECE-1 is predominately expressed. ECE was discovered for its ability to catalyze the conversion of big endothelin into vasoactive endothelins (Shimada et al., 1996), (Eckman et al., 2003), (Leissring et al., 2002), (Muller et al., 2003), (Hunter and Turner, 2006). ECE-1 has now been reported to hydrolyse other biologically active peptides including bradykinin, neurotensin and substance P. In humans, ECE-1 exists as four different isoforms including ECE-1a, ECE-1b, ECE-1c, and ECE-1d (Muller et al., 2003), (Hunter and Turner, 2006), (Turner et al., 2004), (Jackson et al., 2006). Expression patterns have shown that these isoforms have distinct subcellular localizations with slight variations depending on the species and cell type (Muller
et al., 2003), (Hunter and Turner, 2006). To date, there are no significant differences reported in the catalytic properties of the different isoforms (Turner et al., 2004). The Aβ degrading capability of ECE was discovered when Eckman (Eckman and Eckman, 2005) observed that the ECE inhibitor, phosphoramidon, caused a rapid accumulation of Aβ in a cell line expressing ECE but not in cells lacking ECE expression. Subsequent studies revealed that overexpression of ECE in cell lines reduced Aβ accumulation by approximately 90%. In vivo data from ECE (+/-) transgenic mice, which show 25% reductions in ECE-1 activity, also show significant increases in both Aβ40 and Aβ42 levels in the brain (Eckman and Eckman, 2005). Moreover, intraventricular injections of phosphoramidon, increase Aβ in wild type and APP transgenic mice (Eckman et al., 2006). These studies indicate a clear correlation between ECE-1 activity and Aβ load in the brain.

In the present study we investigated the effects of upregulating the ECE-1 enzyme by using a rAAV vector, serotype 5, on Aβ load in the brain. rAAV vectors are desirable candidates for gene therapy in the central nervous system because AAV is a nonpathogenic virus; it has low immunogenicity and it is deficient for replication due to the removal of all the viral encoded genes. Further, rAAV vectors have been shown to be very efficient in infecting neuronal cells and maintaining long term expression (Burger et al., 2005), (Mandel et al., 2006). We observed a significant reduction in the levels of Aβ in the mice injected with the ECE virus which shows that this method may offer a promising therapeutic avenue through which to treat AD.
RESULTS

The ECE-1 synthetic gene within the rAAV was under the control of the hybrid chicken β-actin cytomegalovirus (CBA) promoter and was tagged with haemagglutinin (HA) peptide sequence for detection within the brain and discrimination from endogenous ECE. Prior to virus production the rAAV vector was tested in HEK 293 cells to evaluate the expression cassette. Cell lysate and conditioned media from transfected and untransfected cells were examined by Western blot analysis to determine ECE expression. Untransfected cell lysate and media were negative for ECE-HA protein expression. ECE-HA protein expression was only detected in the cell lyate fraction and not the media (Fig 1b).

The rAAV ECE-HA vector was tested for enzyme activity in vivo. Nontransgenic mice (n=12) aged 9 months were injected bilaterally into the right and left hippocampus. Group one received the rAAV-ECE-HA treatment and the second group served as the control group and received no treatment (n=6 per group). All injections were performed as described in Methods with each site of injection receiving a total volume of 2 μl of the vector at a concentration of 1 x 10^{12} vg/ml. Six weeks post injection the animals were sacrificed and frozen hippocampal tissue from the animals was homogenized and separated into membrane and soluble fractions by centrifugation. The ECE specific activity was determined as the nmole of MOCA/ min/µg protein. Within the homogenate and in the membrane fractions of the homogenized tissue, animals receiving the ECE treatment had significantly higher ECE-like specific activity than the control animals (Fig 2). Error bars indicate ± standard error of the mean value from
triplicates for each sample (specific activity/min/μg protein) for each treatment
group (n=6 per group).

rAAV was initially injected unilaterally into the right hippocampus and right
anterior cortical regions of six month old APP + PS1 mice. Each injection site
received 2μl volume and a concentration of 1 x 10^{12} vg/ml of material at a flow
rate of 0.5 μl/min. The expression of ECE was evaluated six weeks following
treatment. Immunostaining of the tissue with an anti-HA antibody revealed ECE-
HA expression in most animals. The expression patterns for the ECE protein
were confined to the areas surrounding the injection site. ECE-HA was detected
in CA4 neurons in the hilus and CA3 neurons of the hippocampus pyramidal cell
layer. In the dentate molecular layer, a number of round cells, possibly,
oligodendrocytes or astrocytes also expressed the label (Fig. 3b). In animals
receiving the rAAV-GFP control vector there was no positive HA staining in the
hippocampus, but the GFP expression pattern was comparable to that of the
ECE vector (Fig 4). In animals receiving the rAAV expressing ECE-HA there
were low levels of positive staining in the contra-lateral dentate gyrus (Fig. 3a).

When the cortical regions were analyzed for expression, there was a large
amount of ECE-HA expression which was also detected over a larger area of the
cortex. ECE positive expression was concentrated in the anterior cortex (Fig 3c),
but was observed also in the striatum, corpus callosum and septum along the
midline (Fig 3d) in the ipsilateral and contralateral hemispheres. In animals
receiving rAAV-GFP, GFP was detected in a similar expression patterns as ECE-
HA. GFP positive cells were limited to the areas immediately surrounding the
injection site in both the cortex and the hippocampus and no GFP positive cells were seen in the contralateral hemisphere (Fig. 4b, 4d and 4f respectively). This data suggests that the rAAV vector serotype 5 is effective at transducing neuronal cell types in vivo and expressing significant amounts of recombinant ECE-1.

Our next goal was to evaluate the effects of a single intracranial administration of the rAAV-ECE-HA vector in APP + PS1 transgenic mice, to determine the effect of over expression of the ECE protein on amyloid deposition. rAAV was injected unilaterally into the right anterior cortex and hippocampus of six month old mice while the left untreated hemisphere. The control group was treated with rAAV containing GFP. Total Aβ load was ascertained six weeks after intracranial injections by immunohistochemical methods using a polyclonal anti-Aβ antiserum which primarily recognizes the N-terminal domain of Aβ, and thus labels both Aβ1-40 and Aβ1-42 (gift of Gottschall, PE, Univ of Arkansas). The regional Aβ distribution and density in APP + PS1 transgenic mice were similar to those reported by Gordon et al. (Gordon et al., 2002). Immunohistochemistry revealed darkly stained compact plaques and more lightly stained diffuse plaque deposits in the APP + PS1 animal tissue. Plaque deposition was distributed throughout the cortical regions as well as in the hippocampus (although most concentrated in the molecular layers of the dentate gyrus and the CA1 region, surrounding the hippocampal fissure). Animals injected with the control rAAV-GFP showed Aβ immunohistochemical staining patterns throughout the cortex and hippocampus comparable to those of untreated APP + PS1 transgenic mice.
of the same age (Fig 5a and 5b). A notable decrease in the amount of hippocampal Aβ staining was observed in animals injected with the rAAV expressing ECE-HA six weeks after the time of injection when compared to animals injected with the control rAAV-GFP vector (Fig. 5c, 5d). The reductions in Aβ deposition were limited to the areas surrounding the cortical and hippocampal injection sites. ANOVA analysis of total Aβ was significantly decreased by 50% in mice receiving rAAV-ECE-HA in both the hippocampus and the anterior cortex (Fig. 5e).

Congophilic plaque load was also analyzed following intracranial injections of rAAV-ECE-HA vector. The area of congophilic labeling was substantially less than Aβ immunochemistry, staining only compacted fibrillar Aβ deposits, as expected (Gordon et al., 2002). Figures 6c and 6d indicate that the positive congophilic staining for the mice receiving the ECE vector was visibly less, especially in the hippocampus, compared to the control animals. The reductions in congophilic deposition were limited to the site of injection in cortex and hippocampus. Quantification of the Congo red staining by ANOVA analysis revealed that animals receiving the rAAV expressing ECE-HA showed significant reductions in the hippocampus (62%) as well as significant reductions in the anterior cortex (46%; Fig 6e).

DISCUSSION

There have been several gene therapy approaches examined for the treatment of Alzheimer's disease. Initial experiments were based on the
observation that application of neuronal growth factors mediated neuroprotection and reduced the loss of cholinergic neurons in experimental lesion models (Hefti and Weiner, 1986);(Williams et al., 1986). Therefore, NGF (nerve growth factor) genes have been delivered to the brain using recombinant viral vectors, such as AAV and lentivirus, or delivered via an ex vivo approach, with transformed fibroblasts or neuronal stem cells (Tuszynski, 2007),(Cenciarelli et al., 2006). These studies have shown rescue of cholinergic neurons and are being further examined as a potential therapy. However, it is unlikely that NGF would cure AD because the widespread neuronal loss that occurs in the later stages of the disease would not be compensated by the early rescue of cholinergic neurons. NGF rescue of neurons is currently being examined in clinical trials because it offers a substantial decline in the rate of neuronal loss and could slow the progression of symptoms of Alzheimer's. Other growth factors that are also of potential interest in AD include brain-derived neurotrophic factor and neurotrophin-4/5 to address the neuronal loss in the cortex and hippocampus.

A second approach to treat Alzheimer's pathology involves the reduction of Aβ through immunization. It was observed that immunization against Aβ would result in a significant reduction in Aβ deposits and improve learning and memory deficits (Morgan et al., 2000),(Janus et al., 2000). Peripheral passive immunization against Aβ with antibody injections has been successful in mouse models and more recently viral vectors have been used as a gene therapy approach to deliver Aβ antibodies (Fukuchi et al., 2006), (Levites et al., 2006). This approach has demonstrated some promising reduction (25-50%) in the
levels of Aβ deposition. AAV vectors have also been used as an Aβ vaccine, with the over expression of Abeta42. This method has shown reduction in Aβ deposition and some reduction in cognitive impairment in Alzheimer Tg mice (Mouri et al., 2007), (Hara et al., 2004), (Zhang et al., 2003).

It has been demonstrated that the apolipoprotein E (apoE) gene is a major risk factor for late-onset AD with the ApoE2 allele decreasing and the ApoE4 allele increasing the morbid risk for developing AD. Therefore to develop a gene therapy approach to Alzheimer’s, Dodart et al. (2005) over expressed ApoE2 using a lentiviral vector. The authors observed a dramatically reduced hippocampal Aβ burden in Tg mice. It is as yet unclear how ApoE2 is reducing the Aβ but it may be increasing the Aβ clearance (Dodart et al., 2005).

A final gene therapy approach for the treatment of Alzheimer’s has been the over expression of Aβ degrading enzymes such as neprilysin. Either using a direct transduction of neurons using a viral vector or an ex vivo approach with transformed fibroblasts, the increase in NEP expression has been shown to significantly reduce the overall Aβ load on Alzheimer Tg mice (Marr et al., 2003), (Iwata et al., 2004),(Hong et al., 2006), (Hemming et al., 2007). In this report we are examining another Aβ degrading enzyme, ECE, for its ability to reduce the Aβ load on Alzheimer APP + PS1 mice using rAAV for increased neuronal expression of ECE.

Recombinant AAV has become widely used for the transduction of neuronal cells. AAV is nonpathogenic, has low immunogenicity, lacks all viral genes and is capable of long term expression in neurons. This makes rAAV a
good candidate for the use as a gene therapy vector for neurological disorders (Mandel et al., 2006), (Burger et al., 2005). Further, rAAV is currently being examined in a number of neurological clinical trials (Mandel and Burger, 2004). Here we are using rAAV for the over expression of the ECE protease which had been previously shown to cleave Aβ peptide (Eckman and Eckman, 2005). We believed that the over expression of ECE using rAAV would enable the clearance of Aβ deposition within the mouse brain and ameliorate the pathogenesis of Alzheimer’s.

rAAV serotype-5 ECE-HA vector was injected unilaterally into the mouse hippocampus and anterior cortex. Examination of the expression profile of the expressed ECE has shown that the rAAV of ECE can transduce several different neuronal cell types within the mouse brain, and possible other cell types in the dentate molecular layer. This is consistent with the previously published serological specificity of this vector (Alisky et al., 2000), (Burger et al., 2004), (Choi et al., 2005). The expression profile of ECE was similar to that observed with our rAAV5 vector expressing GFP.

No noticeable toxic effects were seen in mice receiving the ECE-HA vector compared to control animals. No neuron loss or gross morphometric changes were observed in fixed brain tissue nor were there any signs of general toxicity as evidence by abnormal behavior in the mice. Only one animal died throughout the course of the study but this did not appear to be a result of toxicity from the over expression of the ECE protein. All animals were weighed before treatment and immediately before sacrifice and no significant changes in weight
were noted indicating that up regulation of ECE does not appear to have adverse effects or cause general toxicity in the mouse model, yet further investigation must be done to confirm its safety profile. ECE has many endogenous peptides including opioid peptides, tachykinin, atrial naturetic peptides, and other small regulatory vasoactive peptides. It is uncertain whether interactions between other potential substrates in the brain may limit the ability of ECE to degrade Aβ or cause potential problems by reducing the levels of these other peptides. Previous studies have revealed that in the AD brain as well as in the animal model of AD, ECE (in addition to other Aβ degrading enzymes) are down regulated specifically in areas that are prone to plaque formation (Iwata et al., 2002), (Yono et al., 2004). Therefore, up-regulation of ECE to restore normal levels of this endogenous protease should have minimal adverse effects. In addition, ECE has been shown to have higher affinity for larger peptides such as bradykinin, substance P, and neurotensin, hydrolyzing them at amino acid hydrophobic residues (Johnson et al., 1999), (Lo et al., 1999). Monitoring changes in these peptides between control and treatment groups may help identify other potential toxic effects resulting from increases in ECE that could occur in this model.

Our results from the over expression of ECE suggest that the up-regulation of ECE through rAAV vectors can provide a viable method to decrease total amyloid deposition in the brain. The activity level determined using an ECE specific assay demonstrated that we achieved a several fold fold increase in ECE activity in homogenates, most of which was membrane associated. This increase
in ECE activity was able to significantly reduce total Aβ deposition in the anterior cortex and hippocampal sites of injection (Fig. 5). Similarly, when Congo red stained compacted deposits were measured; the ECE-HA vector significantly reduced staining (Fig. 6). We were able to achieve a 50% reduction in the total Aβ present in the cortex and hippocampal regions. Similarly, we reduced the congophilic compact plaque load to ~50% of that of the controls. We have yet to determine if this reduction in the Aβ levels in the mice with the ECE will lead to significant improvement in behavioral tests such as the Morris water maze. We are currently testing more injections of ECE to see if we can create a significant reduction of whole brain Aβ levels and ameliorate the memory deficits that are observed in the APP + PS1 mice. Our data are consistent with reports that ECE can degrade Aβ in vitro, and that partial knockdown of the ECE gene leads to more rapid accumulation of Aβ (Eckman et al., 2001), (Eckman et al., 2003). The present work adds to the evidence that ECE plays an important role in Aβ deposition by demonstrating that local over expression of the enzyme activity can dramatically reduce the deposition of amyloid in the brains of APP + PS1 transgenic mice. Thus, regulation of ECE may be used as therapeutic target for the treatment of Alzheimer's disease.

MATERIALS AND METHODS

Generation of ECE Constructs and rAAV Production

The ECE-1 coding sequence (GI:4503442) was cloned using polymerase chain reaction (PCR) from a GenePool cDNA library obtained from Invitrogen. The
primers used for the full length ECE were
GAGGAATTACCGGTCCACCATGCGGGGCGTGTTGGCCGCCCCCGGTGTGCT
and GAGATCGATTACCAGACTTCACACTTGTGAGGCGG. The PCR product
was cloned into pBluescript and sequenced to confirm sequence identity. The
ECE was then cloned into the vector called pTR5-MCS at the Eco RI and Cla I
cloning sites. This vector contains the AAV terminal repeats for AAV virus
production and the CBA promoter for ECE mRNA transcription. A Hemagglutinin
(HA) tag was added to the C-terminus of ECE synthetic gene using the following
oligonucleotide
GTGTGAAGTCTGGATGGCTTCTAGCTATCTTATGACGTGCCTGACTATGCCAT
TGTAA and its complement. The recombinant viruses were generated and
purified using the method of Zolotukhin et al. (2002). Infectious rAAV particles
are expressed as vector genomes (v/g)/mL. Vector genomes were quantitated
using the dot plot protocol, with a probe for the CBA promoter, as described by
(Zolotukhin et al., 2002).

*Western Blot Analysis*

Protein samples were boiled in Lamælæi sample buffer prior to loading on a 10%
polyacrylamide gel. The proteins on the gel were transferred to Millipore
Nitrocellulose membrane and probed with anti-HA antibody (Santa Cruz) and
antimouse-HRP conjugate (Amersham). Millipore Immobilon detection reagent
was used to visualize bands.

*Transgenic Mice*
APP + PS1 mice (Holcomb et al., 1998) were acquired from the breeding colonies at the University of South Florida. Multiple mice were housed together whenever possible until the time of the experiment. Mice were then singly-housed 1 week before surgical procedures until the time of sacrifice. Study animals were given water and food *ad libitum* and maintained on a twelve hour light/dark cycle and standard vivarium conditions. Two cohorts of mice were used, the first cohort consisted of APP + PS1 mice aged 6 months ($n=16$) and the second cohort consisted of nontransgenic mice aged 9 months ($n=12$). Animals in each cohort were assigned to two groups. Group one received a control vector expressing GFP (first cohort $n=8$; second cohort $n=6$). Group two received AAV vector expressing the membrane bound endothelin converting enzyme sequence (first cohort $n=8$; second cohort $n=6$). All groups were sacrificed after six weeks post intracranial injection.

*Surgical Procedure*

Immediately before surgery mice were weighed then anesthetized using isoflurane. Surgery was performed using a stereotaxic apparatus. The cranium was exposed using an incision through the skin along the median sagittal plane, and two holes were drilled through the cranium over the right cortex injection site and the right hippocampal injection site. Previously determined coordinates for burr holes, taken from bregma were as follows; frontal cortex, anteroposterior, -1.5 mm; lateral, -2.0 mm, vertical, 3.0 mm, hippocampus, anteroposterior, -2.7 mm; lateral -2.5 mm, vertical, 3.0 mm. Burr holes were drilled using a dental drill bit (SSW HP-3, SSWhite Burs Inc., Lakewood, NJ). Injections of 2 μl of total
volume of each of the viral vectors in sterile PBS at a concentration of $1 \times 10^{12}$

vg/ml were dispensed into hippocampus and cortex over a period of 4 min using

a 26 gauge needle attached to a 10 μl syringe (Hamilton Co., Reno, NV). The

incision was then cleaned and closed with surgical staples. Animals were

recovered within 10 minutes and housed singly until time of sacrifice.

**Immunohistochemistry**

Six weeks post surgery, mice were weighed, overdosed with pentobarbital

(200 mg/kg;) and perfused with 25 ml of 0.9% normal saline solution then 50 ml

of freshly prepared 4% paraformaldehyde. Brains were collected from the

animals immediately following perfusion and immersion fixed in 4%

paraformaldehyde for 24 h. Mouse brains were cryoprotected in successive

incubations in 10%, 20%, 30% solutions of sucrose; 24 h in each solution.

Subsequently, brains were frozen on a cold stage and sectioned in the horizontal

plane (25 μm thickness) on a sliding microtome and stored in Dulbecco’s

phosphate buffered saline (DPBS) with 0.2% sodium azide solution at 4 °C.

Six sections 100 μm apart spanning the site of injection were chosen and

free-floating immunochemical and histological analysis was performed to
determine ECE expression using anti-HA biotinylated rabbit polyclonal antibody

at a concentration of 1:1000 (Roche, Indianapolis, IN), total Aβ using a rabbit

primary anti-Aβ serum at a concentration 1:10,000 and a secondary anti-rabbit

antibody (Serotec, Raleigh, NC). Another series of sections were mounted on

slides and stained with Congo red to assess compact congophilic positive plaque

load. Immunohistochemical procedural methods are analogous to those
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Congo red histology was performed using sections that were premounted on slides then air dried for a minimum of 24 hours. The sections were rehydrated for 30 seconds before beginning staining protocol. For Congo red, hydrated sections were incubated in an freshly prepared alkaline alcoholic saturated sodium chloride solution (2.5mM NaOH in 80% alcohol) for 20 min, then incubated in 0.2% congo red in alkaline alcoholic saturated sodium chloride solution for 30 minutes. Slides with sections were rinsed through three changes of 100% ethanol, and cleared through three changes of xylene and finally coverslipped with DPX. Histological sections from control animals treated with
rAAV expressing GFP were mounted on slides and dehydrated through a series of increasing concentrations of ethanol. The mounted sections were then cleared in three changes of histoclear and coverslipped with DPX.

Stained sections were imaged using an Evolution MP digital camera mounted on an Olympus BX51 microscope at 100X final magnification (10 X objective). Six horizontal brain sections (100µm apart; every 4th section) were taken from each animal and four nonoverlapping images near the site of injection from each of these sections were captured (24 measurements per mouse). All images were taken from the same location in all animals. Quantification of positive staining product surrounding and including the injection sites in the right anterior cortex and the right hippocampus and the corresponding regions in the left hemisphere were determined using Image-Pro® Plus (Media Cybernetics®, Silver Springs, MD). Quantifications of the right regions, ispilateral to the injection site, were calculated and ANOVA statistical analysis was performed using StatView® version 5.0.1 (SAS Institute, Raleigh, NC).

Enzyme Activity Assay

ECE activity was characterized and adapted from a previous fluorometric assay method (Johnson and Ahn, 2000) for a 96 well plate format with slight modifications. Six weeks post injection brain tissue was removed then dissected immediately following sacrifice and frozen at -80 ºC. The tissue from each animal was rapidly thawed and homogenized with the Ultra-Turrax T8 motor-driven homogenizer (IKA-Labortechnik, Germany) in solubilizing buffer containing 20 mM Tris-HCl, pH 7.4, 250 mM sucrose immediately prior to
assaying (Iwata et al., 2002). Samples were then centrifuged at 100,000 x g, 4 °C for 45 min using an Avanti J-30I Centrifuge (Beckman Instruments, Inc., Palo Alto, CA) to obtain a soluble fraction and a membrane fraction. The membrane fraction was resuspended in buffer containing 20 mM Tris-HCl, 250 mM sucrose, pH 7.4. Protein concentration was determined from the homogenate, membrane fraction and soluble fractions from each sample using a general BCA assay (Pierce). Aliquots of the cell lysate (5 μg) were incubated with 10 μM (final) of the fluorogenic peptide (MOCA- Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH), (R&D Systems) in MES buffer (sodium phosphate pH 6.8 containing 0.1M NaCl). MOCA- Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH is efficiently quenched by resonance energy transfer to the dinitrophenyl group and the continuous fluorescent intensity is increased upon internal cleavage of the peptide (ECE-1 cleavage between the Ala-Phe bond). The increased fluorescence produced from cleavage of the substrate was measured using a Molecular Devices fMax spectrofluorometer plate reader (MDS Analytical Technologies, Sunnyvale, CA) with a 60 min time point to normalize independent experiments. A standard curve of (7-methoxycoumarin-4-yl) acetyl (MOCA) was analyzed along with each assay and used to convert the relative fluorescence units (RFU) to the moles of MOCA produced by the respective cell lysates. Parallel assay reactions of all samples (in triplicate) were carried out in the presence of an ECE specific inhibitor (SM19712, Sigma Aldrich) at a concentration of 20 μM (Umekawa et al., 2000), (Matsumura et al., 2000). ECE specific activity was considered to be the difference in RFU between samples
including SM19712 from the total activity samples (no SM19712). Values were calculated and expressed as nanomoles MCA/min/μg protein.

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**FIGURE 1:** (A) DIAGRAM OF ECE CONSTRUCT AND (B) WESTERN ANALYSIS OF ECE EXPRESSION IN HEK 293 CELLS. (A) Diagrammatic representation of the rAAV construct expressing the ECE synthetic gene under the control of the chicken β-actin (CBA) promoter. (B) Western analysis of ECE expressed in 293 cells using an anti-HA antibody. Lane 1, 293 cell lysate; lane 2, ECE transfected cell lysate, lane 3, conditioned media from untransfected cells, lane 4 media from cells transfected with ECE.
**FIGURE 2: ECE ENZYMATIC ACTIVITY OBTAINED FROM MOUSE HIPPOCAMPAL REGIONS AFTER INJECTION OF rAAV VIRUS EXPRESSING ECE PROTEIN.** ECE-like specific activity is higher in the membrane fraction and homogenate of the hippocampal tissue in the ECE treatment group compared to the control group. The star (*) indicates significance with a p-value < 0.05. Error bars indicate ± standard error of the mean value from triplicates for each sample (specific activity/min/μg protein) for each treatment group (n=6 per group).
FIGURE 3: EXAMINATION OF ECE EXPRESSION USING ANTI-HA IMMUNOREACTIVITY IN THE HIPPOCAMPUS AND ANTERIOR CORTEX.

Panel A shows slight ECE expression in granule cells of the dentate gyrus of the left contra lateral hippocampus following intracranial administration of ECE into the right hippocampus. Panel B shows strong ECE expression detected at the site of vector injection. Panel C shows strong ECE expression in the anterior cortex. Panel D shows slight ECE expression along the midline and some expression in the lateral septum of left (contra lateral) side following intracranial administration of ECE into the right anterior cortex. fi = fimbria; LS = lateral septum; CC = corpus callosum; dg = dentate gyrus; CA1 = Cajal Area 1; CA3 = Cajal area 3; Magnification = 40X and Scale bar = 120 μm
**FIGURE 4: ECE AND GFP EXPRESSION PROFILES IN MICE BRAINS FOLLOWING rAAV ADMINISTRATION.** Panels A shows strong ECE expression in the right anterior cortex following intracranial administration of ECE vector. Panel B shows GFP expression in the anterior cortex following intracranial administration of control GFP vector. Panel C shows ECE expression in pyramidal cells in CA2 region of the right hippocampus. Panel D shows GFP expression in the CA3 region of the hippocampus. Panel E shows slight ECE expression in CA4 neurons of the dentate gyrus of the left contra lateral hippocampus. Panel F shows no positive expression in the left uninjected hippocampus following intracranial injection of GFP vector into the right hippocampus. Scale bar = 50μm (panels A, C, E). Scale bar = 25 μm (panels B, D, F).
**FIGURE 5**: TOTAL AMYLOID LOAD IS REDUCED FOLLOWING INTRACRANIAL ADMINISTRATION OF rAAV-ECE-HA VECTOR. Panels A and B show Aβ immunostaining in the right cortex and hippocampus respectively of animals receiving intracranial injection of control vector, GFP. Panels C and D show Aβ immunostaining in right cortex and hippocampal regions respectively of mice receiving intracranial injection of ECE. Scale bar = 120μm. Panel G shows the percent area of positive staining, normalized to the control, for both cortex (left) and hippocampus (right) after AAV-ECE injection. The asterisk (*) indicates significant reduction compared with control values with p-values < 0.05.
**FIGURE 6: CONGOPHILIC COMPACT PLAQUE LOAD IS REDUCED FOLLOWING INTRACRANIAL ADMINISTRATION OF ECE-HA rAAV VECTOR.** Panels A and B show total congophilic staining in the right cortex and hippocampus respectively of animals receiving intracranial injection of control vector, GFP. Panels C and D show congophilic positive staining in right cortex and hippocampal regions respectively of mice receiving intracranial injection of ECE. Magnification = 40X, scale bar = 120μm. Panel E shows the percent area of positive staining, normalized to the control, for the cortical (right) and hippocampal (left) injected regions. The star (*) indicates significance with a p-value < 0.05.
REFERENCE LIST


in the brain by neprilysin and endothelin-converting enzyme but not angiotensin-


Gordon MN, Holcomb LA, Jantzen PT, DiCarlo G, Wilcock D, Boyett KW, Connor 
K, Melachrino J, O'Callaghan JP, Morgan D (2002) Time course of the 
development of Alzheimer-like pathology in the doubly transgenic PS1+APP 

Hara H, Monsonego A, Yuasa K, Adachi K, Xiao X, Takeda S, Takahashi K, 
recombinant adeno-associated virus vector for Alzheimer's disease. 

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: 


Hemming ML, Patterson M, Reske-Nielsen C, Lin L, Isacson O, Selkoe DJ 
(2007) Reducing amyloid plaque burden via ex vivo gene delivery of an Abeta- 
degrading protease: a novel therapeutic approach to Alzheimer disease. 

Holcomb L, Gordon MN, McGowan E, Yu X, Benkovic S, Jantzen P, Wright K, 
Saad I, Mueller R, Morgan D, Sanders S, Zehr C, O'Campo K, Hardy J, Prada 
phenotype in transgenic mice carrying both mutant amyloid precursor protein and 

virus RNAi and neprilysin gene transfer vectors reduce accumulation of 
Alzheimer's disease-related amyloid-beta peptide in vivo. Gene Ther 13:1068- 
1079.

Hunter AR, Turner AJ (2006) Expression and localization of endothelin- 
converting enzyme-1 isoforms in human endothelial cells. Exp Biol Med (Maywood) 
231:718-722.

reduction of A beta-degrading endopeptidase, neprilysin, in mouse hippocampus 

51


minimal vascular consequences in aged amyloid precursor protein transgenic mice. JNeurosci 26:5340-5346.


PAPER 2:

RECOMBINANT ADENO-ASSOCIATED VIRAL VECTOR MEDIATED GENE DELIVERY OF SECRETED NEPRILYSIN REDUCES β-AMYLOID DEPOSITION IN APP + PS1 TRANSGENIC MICE.

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ABSTRACT

The accumulation of β-amyloid (Aβ) peptides in the brain has been recognized as an instigating factor in Alzheimer’s disease (AD) pathology. Recently, it has been argued that clearance of Aβ is partially dependent upon several endogenous zinc metalloproteases. Gene therapy using adeno-associated viral (AAV) vectors are an effective means of delivering transgenes encoding for these specific proteases which include neprilysin, endothelin converting enzyme, and insulin degrading enzyme to regions in the brain affected by AD pathology. In this study the convection enhanced delivery method using the step-design cannula (Bankiewicz, 2005) was used to deliver recombinant AAV (rAAV) vectors expressing either a native, membrane bound form of human neprilysin gene (NEP-n) or an engineered, secreted form of the neprilysin gene (NEP-s) into the right hippocampus and right frontal cortical regions of the mouse brain. The control group was treated with an rAAV vector expressing a mutant neprilysin gene (NEP-m) with a single amino acid substitution in the active site rendering it inactive. Six weeks after injection, immunohistochemistry for NEP revealed strong expression throughout the hippocampus in animals treated with the NEP-n and NEP-m vectors. Animals treated with the NEP-s showed expression in a smaller portion of the hippocampus compared to the NEP-n treated group. Immunohistochemistry for total Aβ was significantly decreased in animals receiving the NEP-n and NEP-s viral vectors when compared to control animals in both the hippocampus and cortex. Congo red staining followed a similar trend revealing significant decreases in the hippocampal fissure and CA1
regions and the cortex for NEP-n and NEP-s treatment groups compared to mice given control treatments. These data suggest that increasing the expression of certain endogenous β-amyloid degrading enzymes through gene therapy using the CED step cannula provides an efficient means of increasing transgene distribution and ultimately may provide a promising therapeutic avenue through which to treat AD.

INTRODUCTION

Alzheimer’s disease (AD) is the most common form of senile dementia that can occur sporadically or as a result of genetic mutations in the genes encoding presenilin 1, presenilin 2, or amyloid precursor protein (APP). These mutations result in the overproduction of Aβ peptides which ultimately lead to the formation of extracellular amyloid plaques and intracellular neurofibrillary tangles consisting of hyperphosphorylated tau. The AD cases with these autosomal dominant mutations, commonly referred to as early onset AD or familial AD (FAD) (Fidani and Goate1992), account for only 1-2% of all AD cases. Interestingly, the more common late onset AD cases do not seem to overproduce Aβ peptide species suggesting that there is a deficit in the clearance mechanisms which are normally involved in regulation of Aβ levels in the brain. Therefore, the accumulation of Aβ in late onset AD is attributed to an imbalance between its production and degradation/clearance.
Several endogenous proteases have been shown to degrade Aβ in the brain and other tissues both *in vivo* and *in vitro*. This family of zinc metalloproteases, include neprilysin (NEP), insulin degrading enzyme (IDE; insulysin), and endothelin converting enzymes (ECE-1 and ECE-2). Other proteases that appear to play a role in Aβ metabolism include matrix metalloproteinase-9 (Yan et al., 2006) and cathepsin B (Mueller-Steiner et al., 2006) and plasmin (Turner et al., 2002). Down-regulation of these degrading enzymes within the brain during aging could potentially contribute to Aβ accumulation eventually leading to development of AD pathology (Caccamo et al., 2005); (Yasojima et al., 2001).

NEP is a membrane bound a 92-kDa glycosylated ectoenzyme whose active site is oriented outside the cell. NEP belongs to the M13 sub family of neutral endopeptidases, and has a range of regulatory activities as it degrades numerous bioactive peptides (Turner et al., 2000), (Iwata et al., 2000a). Iwata et. al. in 2000 first demonstrated the ability of NEP to degrade Aβ peptides in the brain parenchyma and also illustrated that suppressing NEP would lead to an increase in Aβ deposition (Iwata et al., 2000a). NEP activity decreases with age in amyloid depositing regions, such as the cortex and hippocampus, yet remained unchanged in areas such as the striatum (Iwata et al., 2002), (Yasojima et al., 2001). NEP deficient mice showed a significant increase in amyloid deposition as opposed to their wild type counterparts indicating that NEP activity plays significantly impacts Aβ pathology (Iwata et al., 2000c), (Marr et al., 2004).
Recently, viral vector gene therapy has emerged as a viable approach to the delivery of potentially therapeutic genes. Several vectors and serotypes have been developed with improved efficiency, specificity, and safety (Thomas et al., 2003). In the present study we investigate the effects of upregulating the NEP enzyme by using a recombinant adeno-associated viral (rAAV) vector, serotype 5, on Aβ load in the brain using a novel convection enhanced delivery administration technique to further increase area of distribution and potentially increase transduction efficiency of the transgene (a limitation of viral vector gene therapy). rAAVs are paroviruses that require a helper virus, typically Adenovirus or herpes simplex virus, in order to support viral replication (Berns and Parrish, 1996). rAAV vectors are desirable candidates for gene therapy in the central nervous system because AAV is a nonpathogenic virus; it has low immunogenicity and it is deficient for replication due to the removal of all the viral encoded genes. Further, rAAV serotype 5 vectors are very efficient in infecting neuronal cells and maintaining long term expression (Burger et al., 2005), (Mandel et al., 2006). Therefore we have chosen to examine the efficacy of a rAAV serotype 5 vector to deliver the recombinant expressed NEP cDNA. The rAAV vectors used in this study express either a membrane bound native form of the NEP cDNA, or a modified form of the NEP cDNA that is secreted into the extracellular compartment. We observed a significant reduction in the levels of Aβ in the mice injected with the NEP viruses, both the native NEP and the secreted NEP appeared to reduce the levels of Aβ load significantly compared to the mutated NEP in both the hippocampus and the anterior cortex.
MATERIALS AND METHODS

*Generation of NEP Gene Constructs and rAAV Production:*

The NEP gene (GI:4503442) was cloned using polymerase chain reaction (PCR) from a GenePool cDNA library obtained from Invitrogen. The primers used for the full length NEP were

GAGGAATTCAACCGGTCCACCATGCGGGGCCTGCTTGGGCAGC (contains an Eco RI and Age I restriction sites, 5' primer) and

GAGATCGATTACCAGACTTCACACTTGTGAGGCGG (contains a Cla I and Ale I restriction sites, 3' primer). The PCR product was cloned into pBluescript and sequenced to confirm sequence identity. The NEP was then cloned into the vector called pTR5-MCS at the Eco RI and Cla I cloning sites. This vector contains the AAV terminal repeats for AAV virus production and the CBA promoter for NEP mRNA transcription. A Hemagglutinin (HA) tag was added to the C-terminus of NEP gene at the Ale I restriction site. The following oligonucleotides

GTGTGAAGTCTGGATGGCTTCTAGCTATCTTATGACGTGCCTGACTATGCCAGTGTAA and its compliment were annealed and ligated to the vector. Generating the soluble truncated form of NEP (232-2313 bp): The 5' primer that was used for this PCR was

GAGGAATTCAACCGGTGCAGGACTGGTGGCCTGCTTGGGCAGC, and the 3' primer was the same as above. The PCR product was cloned as described for the full length NEP including the addition of a HA-tag. To add the secretion signal sequence the following oligonucleotide and its compliment were annealed and
ligated at the Age I site of the construct

[CCGTCCACCATGAAGTTATGGGATGTCGTGGCTGTCTGCCTGGTGCTGC
TCCACACCGCGTCCG]. The sequence was confirmed to be in frame with the
NEP gene coding frame. This signal peptide sequence was derived from the
GDNF gene. The NEP-m was generated via site directed mutagenesis of the
NEP-n construct. The codon for glutamic acid 585, GAA, was changed to encode
for valine, GTA. The recombinant viruses were generated and purified using the
method of Zolotukhin et al. (2002). Infectious rAAV particles are expressed as
vector genomes (v/g)/mL. Vector genomes were quantitated using the dot plot
protocol, with a probe for the CBA promoter, as described by (Zolotukhin et al.,
2002).

Transgenic Mice

APP + PS1 mice (Holcomb et al., 1998) were acquired from the breeding
colonies at the University of South Florida. Multiple mice were housed together
whenever possible until the time of the experiment; mice were then singly-
housed 1 week before surgical procedures until the time of sacrifice. Study
animals were given water and food ad libitum and maintained on a twelve hour
light/dark cycle and standard vivarium conditions. Mice in this colony have been
interbred for the last 8 years and have had the retinal degeneration mutation
(rd1) removed by selective breeding.

In study 1, two cohorts of mice were used, the first cohort consisted of
APP + PS1 mice aged 6 months (n =32) and a second cohort 6 mo old APP +
PS1 mice (n =18) were treated at separate times. Animals in each cohort were
assigned to one of three groups. Group one received a control vector expressing mutated NEP (first cohort n=9; second cohort n=6). Group two received rAAV vector expressing native neprilysin transgene (first cohort n =12; second cohort n=6). Group three received the rAAV vector expressing the secreted neprilysin transgene (first cohort n = 11; second cohort n=6). All groups were sacrificed after six weeks post intracranial injection at 7.5 mo of age.

In study 2, APP + PS1 mice were 15 months of age at the time of treatment and were assigned to one of two groups. Group 1 received the NEP-s rAAV vector while group two received the control vector which was rAAV expressing GFP. Mice were sacrificed at 20 mo of age.

Surgical Procedure

Study 1

Immediately before surgery mice were weighed then anesthetized using isoflurane. Surgery was performed using a stereotaxic apparatus. The cranium was exposed using an incision through the skin along the median sagittal plane, and two holes were drilled through the cranium over the right anterior cortex injection site and the right hippocampal injection site. Previously determined coordinates for burr holes, taken from bregma were as follows; anterior cortex, anteroposterior, 1.5mm; lateral, -2.0mm, vertical, 3.0mm, hippocampus, anteroposterior, -2.7mm; lateral -2.5mm, vertical, 3.0mm. Burr holes were drilled using a dental drill bit (SSW HP-3, SSWhite Burs Inc., Lakewood, NJ). Injections of 2μl of total volume of each of the viral vectors in sterile PBS at a concentration of 1.5 X 10^{11} vg/ml were dispensed into hippocampus and cortex over a period of
2 min. using a 27 gauge step design cannula needle (see below) attached to a 10 µl syringe (Hamilton Co., Reno, NV). The incision was then cleaned and closed with surgical staples. Animals were recovered within 10 minutes and housed singly until time of sacrifice.

**Step Design Cannula**

The step design cannula was used for all intracranial surgeries. Fused silica tubing (polymicro technologies, Phoenix, AZ) was inserted into a 27 gauge Hamilton blunt ended needle and fixed in place with super glue (Krauze et al., 2005). The end of the silica tubing was cut leaving 1mm of tubing protruding from the end of the Hamilton needle.

**Study 2**

Immediately before surgery fifteen month old mice were weighed then anesthetized using isoflurane. Surgery was performed in a similar manner mentioned previously. Four holes were drilled through the cranium over the right and left cortex injection site and the right and left hippocampal injection sites with predetermined coordinates listed previously. NEP rAAV vectors were administered intracranially using a 26 gauge beveled Hamilton needle (Hamilton Co., Reno NV). Injections of 2 µl were given over a four min period at a rate of 0.5µl/min.

**Immunohistochemistry**

In study 1, 6 weeks post surgery; mice were weighed, overdosed with pentobarbital (200 mg/kg) and perfused with 25 ml of 0.9% normal saline solution then 50 ml of freshly prepared 4% paraformaldehyde. Brains were collected from
the animals immediately following perfusion and immersion fixed in 4% paraformaldehyde for 24 hrs. Mouse brains were cryoprotected in successive incubations in 10%, 20%, 30% solutions of sucrose; 24hrs in each solution. Subsequently, brains were frozen on a cold stage and sectioned in the horizontal plane (25 μm thickness) on a sliding microtome and stored in Dulbecco’s phosphate buffered saline (DPBS) with 0.2% sodium azide solution at 4˚C.

Eight sections 100 μm apart spanning the site of injection were chosen and free-floating immunochemical and histological analysis was performed to determine transgene expression using anti-HA biotinylated rabbit polyclonal antibody at a concentration of 1:1000 (Roche, Indianapolis, IN), total Aβ using a rabbit primary anti-Aβ serum at a concentration 1:10,000 and a secondary anti-rabbit antibody (Serotec, Raleigh, NC). Another series of sections were mounted on slides and stained with Congo red to assess compact congophilic positive plaque load. Immunohistochemical procedural methods are analogous to those described by Gordon et al. 2002, for each marker. Six to eight sections from each animal were placed in multisample staining tray and endogenous peroxidase was blocked (10% methanol, 3% H₂O₂, in PBS). Tissue samples were permeabilized (with 0.2% lysine, 1% Triton X-100 in PBS solution), and incubated overnight in appropriate primary antibody. Sections were washed in PBS then incubated in corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissue was again washed after a 2 h incubation period and incubated with Vectastin® Elite® ABC kit (Vector Laboratories, Burlingame, CA) for enzyme conjugation. Finally, sections were
stained using 0.05% diaminobenzidine and 0.3% H\textsubscript{2}O\textsubscript{2}. For anti-HA 0.5% nickelous ammonium sulfate was added for color enhancement. Tissue sections were then mounted onto slides, dehydrated, and coverslipped. Each immunochemical assay omitted some sections from primary antibody incubation period to evaluate nonspecific reaction of the secondary antibody.

Congo red histology was performed using sections that were premounted on slides then air dried for a minimum of 24 hours. The sections were rehydrated for 30 seconds before beginning the staining protocol. For Congo red, hydrated sections were incubated in an freshly prepared alkaline alcoholic saturated sodium chloride solution (2.5mM NaOH in 80% alcohol) for 20 min, then incubated in 0.2% Congo red in alkaline alcoholic saturated sodium chloride solution for 30 minutes. Slides with sections were rinsed through three changes of 100% ethanol, and cleared through three changes of xylene and finally coverslipped with DPX. Histological sections from control animals treated with rAAV expressing GFP were mounted on slides and dehydrated through a series of increasing concentrations of ethanol. The mounted sections were then cleared in three changes of histoclear and coverslipped with DPX.

Stained sections were imaged using an Evolution MP digital camera mounted on an Olympus BX51 microscope at 100X final magnification (10 X objective). Eight horizontal brain sections (100µm apart; every 4\textsuperscript{th} section) were taken from each animal and four nonoverlapping images near the site of injection from each of these sections were captured (32 measurements per mouse). All images were taken from the same location in all animals. Quantification of
positive staining product surrounding and including the injection sites in the right frontal cortex and the right hippocampus and the corresponding regions in the left hemisphere were determined using Image-Pro® Plus (Media Cybernetics®, Silver Springs, MD. Quantification of percent area of positive stain for cortex and hippocampus for both right and left hemispheres was determined. Data were analyzed using ANOVA statistical analysis followed by Fisher’s LSD test for individual means differences as recommended by the computer software program StatView® version 5.0.1 (SAS Institute, Raleigh, NC).

**Enzyme Activity Assay**

NEP activity of the three different NEP constructs was characterized and adapted from a previous fluorometric assay method (Johnson and Ahn, 2000) and the NEP ELISA kit (R&D Systems) for a 96 well plate format with slight modifications. HEK 293 cells were transfected with NEP-n, NEP-m, and NEP-s plasmids and control cells were transfected with a GFP plasmid using lipofectamine 2000 per invitrogen protocol. Cells were harvested after 72 hrs. and samples were centrifuged at 1,000 x g 4º C for 45 min. using Beckman J6-HC Centrifuge (Beckman Instruments, Inc., Palo Alto, CA) to obtain cell media fraction and a cell pellet containing the membrane fraction. The membrane fraction was resuspended in M-PER mammalian protein extraction reagent buffer (Thermo Scientific, Rockford, IL) to obtain a cell lysate. Protein concentration was determined from the cell media and cell lysate containing the membrane fractions from each sample using a general BCA assay (Per Pierce Protocol). Aliquots of the cell media and membrane fractions were (100 μg) were incubated
in NEP ELISA plate containing NEP capture antibody. The plate was washed with PBS buffer then incubated with 20 μM (final) of the fluorogenic peptide (MOCA- Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH), (R&D Systems) in Tris-HCL buffer (sodium phosphate pH 7.4 containing 0.1M NaCl) MOCA- Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH is efficiently quenched by resonance energy transfer to the dinitrophenyl group and the continuous fluorescent intensity is increased upon internal cleavage of the peptide (NEP cleavage between the Ala-Phe bond). The increased fluorescence produced from cleavage of the substrate was measured using a Molecular Devices fMax spectrofluorometer plate reader (MDS Analytical Technologies, Sunnyvale, CA) with a 60 min time point to normalize independent experiments. A standard curve of (7-methoxycoumarin-4-yl) acetyl (MOCA) was analyzed along with each assay. Values were calculated and expressed as RFU/ min/ ml protein. Data were analyzed using ANOVA statistical analysis test.

RESULTS

Three rAAV vectors expressing different versions of human NEP were developed. cDNAs were packaged in AAV vectors to generate either the native, membrane bound, form of the enzyme (denoted NEP-n), a secreted form of the enzyme (denoted NEP-s) or an enzymatically-deficient mutant enzyme (denoted NEP-m). The secreted form of the enzyme contained a signal peptide in place of the transmembrane domain of the native enzyme to direct the enzyme’s secretion into the extracellular compartment (Fig 1). The gene sequences are
under the control of the hybrid chicken β-actin cytomegalovirus (CBA) promoter sequence and were tagged with haemagglutinin (HA) peptide sequence for detection within the brain and discrimination from endogenous NEP. The control group received the rAAV expressing NEP-m cDNA, which contains a single point mutation in the active site, E585V, rendering the expressed enzyme inactive.

Prior to virus production the NEP constructs were tested in HEK 293 cells to evaluate the expression cassettes and the effectiveness of the signaling peptide in directing secretion of the NEP-s gene product. Cell lysate and conditioned media from transfected and untransfected cells were examined by Western blot analysis to determine NEP expression. Untransfected cell lysate and media were negative for NEP protein expression. NEP-n and NEP-m protein expression was only detected in the cell lysate fraction and not the media. In contrast, NEP-s protein was detected in both the cell lysate and the media indicating that the signaling peptide did effectively direct secretion of the NEP protein.

rAAV vectors for NEP-m, NEP-n and NEP-s were injected unilaterally into the right hippocampus and right anterior cortical regions of six month old APP + PS1 mice. Animals received 2 μl of virus at a flow rate of 2.5 μl/min and at a concentration of 1.5 x 10^{11} vg/mL. The transgene expression of NEP was evaluated six weeks after injection. Gene expression in the mice which received either the NEP-n or the NEP-s was compared to control animals which received NEP-m or were untreated.

Immunostaining of the tissue with an anti-haemagglutinin (HA) antibody revealed NEP expression in all animals for NEP-n, NEP-m and NEP-s. The gene
expression patterns filled virtually all of the hippocampus. The staining for NEP-n and NEP-m was greater in intensity than the staining for NEP-s. NEP-n was detected in CA4 neurons in the hilus and CA2 and CA3 neurons of the hippocampus pyramidal cell layer (Fig. 2A). Some expression of NEP-n was even noticeable in the entorhinal cortex. Interestingly, it appears that the cell bodies in the dentate granule cell layer were not stained as intensely as the corresponding neuropil, suggesting some dendritic localization of the transfected protein. NEP-s was detected in cell bodies of CA4 neurons in the hilus, some CA3 neurons as well as a few cells in the molecular layers of hippocampus. NEP-s expression was of noticeably less intense and limited in area of distribution compared to that of NEP-n and NEP-m expression (Fig 2C). It is uncertain the degree to which this reflects reduced expression, or diffusion of the soluble enzyme in vivo or during tissue processing. NEP-m staining pattern was very similar to the pattern with NEP-n (Fig. 2B) revealing expression throughout the hippocampus in the dentate gyrus as well as in all CA regions.

When the cortical regions were analyzed for expression, there was again a greater amount of NEP-n and NEP-m expression than NEP-s (greater intensity of HA staining; Fig 3). NEP-n as well as NEP-m positive expression was concentrated in the anterior cortex (Fig 3A and B), but was observed also in the striatum and corpus callosum and to small extent in the contralateral hemisphere. Again, NEP-m and NEP-n expression profiles were very similar, with expression largely confined to the neuropil and little staining of the neuronal somata. NEP-s cortical HA staining was less intense than that of NEP-n HA staining and was
confined to largely to somatic areas of neurons, conceivably in vesicular compartments in preparation for secretion (Fig. 3C). Overall, intensity of HA immunohistochemical staining in the cortical region of the brain appeared less intense than in the hippocampus possibly indicating a lower level of the gene expression of NEP in this region. Hippocampal and anterior cortical regions of uninjected animals showed no noticeable HA staining (Fig 2D and 3D).

The rAAV NEP-s, NEP-m and NEP-n vector constructs were tested for enzyme activity in vitro using HEK 293 cells. Cells were transfected using NEP-n, NEP-m and NEP-s plasmids and control cells were transfected with the GFP plasmid. All cells were harvested 72 hrs after transfection using lipofectamine 2000. The NEP specific activity was determined as RFU/min/ml protein. HEK 293 cells transfected with NEP-n had significantly higher NEP specific activity in the cell lysate containing the membrane fraction than the cells transfected with the NEP-m, NEP-s and the control GFP transfected cells (Fig 4). NEP specific activity of the lysate from NEP-s transfected cells was also significantly greater than NEP-m and GFP transfected cell lysates. In addition, the NEP-s specific activity in the cell media was significantly greater than the NEP-n, NEP-m, and GFP transfected cells (Fig 4).

Our next goal was to evaluate the effects of a single intracranial administration of a rAAV vector containing either the secreted or native form of the NEP gene in APP + PS1 transgenic mice to determine the effect of over expression of the transgene on amyloid deposition. In study 1 rAAV vectors were injected unilaterally into the right anterior cortex and hippocampus of six
month old mice. The control group was treated with rAAV containing a NEP-m gene. Total Aβ load was ascertained six weeks after intracranial injections by immunohistochemical methods. The regional Aβ distribution and density in APP + PS1 transgenic mice that were control animals were similar to aged matched untreated animals reported by us previously (Gordon et al., 2002).

Immunohistochemistry for Aβ revealed bothdarkly stained compact plaques and more lightly stained diffuse plaque deposits in the APP+PS1 animal tissue (Fig 5A and 5D). Plaque deposition was distributed throughout the cortical regions as well as in the hippocampus (although most concentrated in the molecular layers of the dentate gyrus and the CA1 region, surrounding the hippocampal fissure). Animals injected with the control rAAV-NEP-m showed Aβ immunohistochemical staining patterns throughout the cortex and hippocampus comparable to those of untreated APP transgenic mice of the same age. A notable decrease in the amount of hippocampal Aβ staining was observed in animals injected with either the rAAV expressing NEP-n or NEP-s six weeks after the time of injection when compared to animals injected with the control rAVV-NEP-m vector (Fig. 5A-5F).

The reductions in Aβ deposition were not only limited to the areas surrounding the cortical and hippocampal injection sites. Significant reductions were also notable in corresponding areas contralateral to the site of injection (although only a few cells showed very faint positive HA staining in the contralateral anterior cortex and hippocampus). ANOVA analysis of total Aβs in the ipsilateral hemisphere revealed significant decreases of, 78% and 65% in the anterior cortex and hippocampus respectively, in mice receiving NEP-n injections.
Decreases in total Aβ were also observed in mice receiving the NEP-s rAAV with significant declines of 60% in the anterior cortex and 56% in the hippocampus (Fig. 6A). Quantification of total Aβ in the contralateral hemisphere showed significant reductions of 56% and 36% in the anterior cortex and hippocampus respectively, of mice receiving NEP-n. Similarly, significant decreases of 53% and 44% in total Aβ of the contralateral anterior cortex and contralateral hippocampus respectively, were seen in mice receiving NEP-s (Fig. 6B).

Congophilic plaque load was analyzed following intracranial injections of rAAV vectors. The density of congophilic labeling was substantially less than Aβ immunohistochemistry, staining only fibrillar Aβ deposits as expected (Gordon et al., 2002). Animals injected with the control rAAV-NEP-m showed positive congophilic staining patterns throughout the cortex and hippocampus comparable to those of untreated APP transgenic mice of the same age (Fig. 7A and 7D). Figures 6B and 6E show that the presence of congophilic staining for the mice receiving the NEP-n vector was visibly less, especially in the hippocampus, compared to both the control animals and the animals that received the NEP-s vector (Fig 7C and 7F). ANOVA analysis revealed that animals receiving the NEP-n rAAV showed significant reductions in the hippocampal region (56%) and in the anterior cortex (56%; Fig 8A) ipsilateral to the site of injection. rAAV expressing NEP-s also had significant decreases in the compact plaque load in both the hippocampus (51%) and cortical regions (57%;Fig 8A). When compact plaque load was calculated in the contralateral hemisphere and compared to the control group, significant decreases were
observed only in the hippocampus (49%) of animals receiving the NEP-s vector treatment (Fig 8B). Animals receiving the NEP-n showed a decreased trend but no statistical difference in congophilic staining from the NEP-m. No significant decreases of Congo red staining were noted in the contralateral anterior cortex of animals receiving either treatment (Fig 8B).

Study 2

In study 1 we demonstrated that either NEP-s or NEP-n could reduce amyloid accumulation when tested in a prevention type of study. Study 2 was designed to test NEP-s, which appeared slightly superior to NEP-n at sites distant from the injection, in mice with large amounts of preexisting amyloid deposits, what some would term a therapeutic type of study design. Aged APP + PS1 transgenic mice (15 months of age) were injected into hippocampus and anterior cortex bilaterally. Tissues were collected 5 mo later when mice were 20 mo old.

Total Aβ load was reduced following intracranial administration of rAAV-NEP-s in aged mice. Panels A and C of fig. 10 show positive immunohistochemical staining of total Aβ in the hippocampus and cortex respectively of aged APP+PS1 mice treated with rAAV-NEP-s. Panels B and D of Fig. 9 showed positive Aβ staining in the hippocampus and cortical regions of untreated aged mice. Panel E shows quantification of Aβ immunostaining of NEP-s treated and untreated 20 mo old APP+PS1 mice. Total Aβ was reduced significantly in the left anterior cortex as well as in the left and right hippocampus following treatment with the NEP-s vector compared to control untreated animals.
Total congophilic staining is reduced following intracranial administration of rAAV-NEP-s in aged 20 month old mice. Panels A and C of Fig. 11 show total positive congophilic staining in the hippocampus and cortex respectively of APP+PS1 mice treated with rAAV-NEP-s. Panels B and D of Fig. 11 show positive congophilic staining of the hippocampus and cortex respectively of untreated 20 month old mice. Panel E of Fig. 11 shows quantification of congophilic staining in NEP-s and untreated 20 Mo old APP+PS1 mice. Total congophilic staining was significantly reduced in the left and right anterior cortex as well as the left and right hippocampal regions in animals receiving treatment with the NEP-s vector compared to untreated control animals.

DISCUSSION

Several recent findings have clearly implicated the important role of endogenous proteases such as neprilysin in the catabolism of Aβ peptides in the brain. Also known as enkephalinase and CD10, it is capable of cleaving enkephalins and terminating peptidergic neurotransmission. In addition to NEP, other endogenous Aβ degrading proteases maintain a conserved catalytic domain which includes a zinc binding motif, HEXXH (Turner et al., 2000). Other proteases in addition to the zinc metalloproteases that appear to play a role in Aβ metabolism include matrix metalloproteinase-9 (Yan et al., 2006) and cathepsin B (Mueller-Steiner et al., 2006) and plasmin (Turner et al., 2001). Down-regulation of these degrading enzymes within the brain during aging could
potentially contribute to Aβ accumulation eventually leading to development of AD pathology (Caccamo et al., 2005), (Yasojima et al., 2001).

Deficiency in the expression of NEP and other metallopeptidases in the aged brain and in animal knockout models correlates with plaque accumulation in a region specific manner (Iwata et al., 2000), (Eckman et al., 2001), (Fukami et al., 2002), (Yasojima et al., 2001b), (Farris et al., 2004), (Saito et al., 2003), (Caccamo et al., 2005), (Miners et al., 2006). Enzyme kinetic studies using specific NEP inhibitors have shown that neprilysin can efficiently degrade numerous peptides on the N-terminus of hydrophobic amino acid residues, which is essential for the efficient catabolism of the Aβ peptide (Marie-Claire et al., 1997), (Shimada et al., 1996), (Hoang et al., 1997), (Turner et al., 2001), (Leissring et al., 2003), (Hersh, 2003). Furthermore, in vivo studies show that an increase in the enzyme activity of amyloid degrading enzymes specifically neprilysin, endothelin converting enzyme, and insulin degrading enzyme, have a significant effect on both intracellular and extracellular Aβ levels in the brain (Hama et al., 2001), (Zou et al., 2006), (Eckman et al., 2006), (Guan et al., 2008). Most recently, Farris et al., demonstrated that when the expression of endogenous NEP is partially or completely inhibited both Aβ deposition and cerebral amyloid angiopathy are significantly exacerbated (Farris et al., 2007b). Subsequently, peptidases have become popular targets for Alzheimer’s disease therapies.

Previously, we examined the effects of the overexpression of ECE-1 gene on amyloid load in the APP + PS1 mouse model (Carty et al., 2006). In the
current study, we examine the effectiveness of rAAV to deliver NEP into hippocampus and anterior cortex of the mouse brain to determine its effects on amyloid burden. In particular, we are interested in developing a delivery method that increases the dissemination of therapeutic levels of NEP protein. Previous studies involving gene therapy approaches for the treatment of Alzheimer's pathology have used viral vectors to reduce Aβ deposition by overexpressing Aβ degrading enzymes, including NEP, ECE and IDE and more recently Cathepsin B, a cysteine protease also implicated in the lysosomal degradatory pathway of Aβ peptides (Marr et al., 2003), (Iwata et al., 2004), (Mueller-Steiner et al., 2006). A comparison of gene transfer studies indicate that overexpression of neprilysin appears to have the most pronounced effect on plaque load in the brain (Farris et al., 2007a), (Eckman et al., 2003), (Hersh et al, 2008), (Spencer et al., 2008). Marr et al (2003) used lentiviral vector and were successful in reducing amyloid levels in APP Tg mice but only moderately peptides (Marr et al., 2003). In addition, others have shown that the transgenic overexpression of NEP and IDE crossed with transgenic APP mice significantly reduces amyloid accumulation in the brain (Leissring et al., 2003), (Poirier et al., 2006), (Meilandt et al 2009). Iwata et al used AAV, as in this study, to express NEP-n and were also successful in reducing Aβ levels in the hippocampus of Tg2576 mice.

In this paper, we further explore the use of different rAAV expressing different forms of the NEP gene. Particularly, we examine a secreted form of the NEP in addition to using the CED delivery method to increase distribution and ultimately total area of gene expression. We also determine the effect of both the
native and secreted versions of NEP expression in the anterior cortex and hippocampus comparing the relative distribution and differences in NEP gene expression in specific cell types in the brain. Unlike previous gene transfer studies with these proteases we use the double transgenic APP + PSI mouse model which have a very aggressive amyloid deposition profile and exhibit memory deficits at a younger age than single transgenic amyloid depositing models. Recombinant AAV has become widely used for the transduction of neuronal cells. rAAV is nonpathogenic, has low immunogenicity, lacks all viral genes and is capable of long term expression in neurons. This profile makes rAAV a good candidate for the use as a gene therapy vector for neurological disorders (Mandel et al 2006), (Mandel and Burger 2004). One limitation with a gene therapy approach for a disease such as Alzheimer’s is that the therapeutic protein must be delivered to the whole brain. Unfortunately, the intracranial injections into the brain parenchyma using simple diffusion does not allow for efficient uptake of the transgene or significant dispersion of the AAV vectors to significantly large areas of the affected regions within the brain. Thus if only a small area of the brain is transduced by the viral vector, multiple injections would be required to cover the entire brain. Therefore, in an attempt to overcome this problem we engineered the NEP gene to contain a signal peptide sequence in the hopes of creating a nucleus from which the expressed protein could diffuse to greater regions of the brain; thus eliminating the requirement for a large number of injections. Additionally we implemented a novel infusion technique using the CED method allowing us to obtain a larger area of distribution of the rAAV
vectors upon delivery with a single injection in the brain parachyma both in the hippocampus and cortical regions of the brain. CED is a method of delivering clinically relevant volumes of therapeutic agents to significantly larger areas of the brain in comparison to simple diffusion methods. The CED technique is designed to utilize the phenomenon of bulk flow and positive pressure to distribute macromolecules to a large area within solid tissue. Macromolecules can therefore be administered to significantly larger areas of specific brain regions by utilizing the advantage of fluid convection within and throughout the interstitial space in the brain (Leiberman et al. 1995, Sanftner et al 2005). The greatest area of distribution is therefore achieved by using an increased and optimal pressure gradient, flow rate, and volume of liquid material (Sanftner et al., 2005). One of the mechanistic limitations of the CED method as well as the simple injection method is the reflux of the injected material up the injection tract. In 2002 Krauze et. al. developed a reflux free step cannula design which effectively minimizes reflux by placing silicone coated tubing within the stainless steel blunt end cannula creating a step that prevents the backflow of fluid. The optimization of more efficient cannula designs coupled with the encouraging results from studies showing enhanced gene transfer and distribution emphasizes the therapeutic potential of the CED method in helping overcome some of the mechanical disadvantages of gene delivery in regards to gene therapy (Krauze et al., 2005b).

Implementing these techniques, rAAV serotype-5 NEP vectors were injected unilaterally into the mouse hippocampus and anterior cortex.
Examination of the expression profile, using anti-HA immunohistochemistry, of the expressed NEP reveals that the rAAV constructs of both NEP-m and NEP-n can transduce several different neuronal and glial cell types within the mouse brain. Interestingly, it appears that the cell bodies in the dentate molecular layer were not stained as intensely as the corresponding axonal projections of these cells which were very darkly stained. This may suggest that the expressed NEP is processed in the endoplasmic reticulum and golgi apparatus of the cell body, then transported along the axon via vesicles to the synaptic terminal. These results are consistent with data published in 2006 by Huang et al. describing the normal neuronal metabolism of NEP (Huang et al, 2006) and by Iwata expressing NEP-n with rAAV (Iwata et al 2004). The expression profiles are also consistent with the previously published serological specificity of the AAV vectors (Alisky et al., 2000),(Burger et al., 2004),(Choi et al., 2005). The AAV 5 serotype has previously been shown to effectively transduce non-dividing neuronal cells as well as glial cells in the mouse brain.

The NEP-s expression profile revealed less intensity of HA staining when compared to the NEP-m and NEP-n staining patterns. Cells within the hilus of the molecular layer were stained as were as cells in the CA3 region of the hippocampus. We believe that more diffuse staining of the NEP-s construct does not necessarily mean that this vector had lower transduction efficiency. It is likely due to the diffusion of the NEP protease throughout the extracellular milieu, making it more difficult to detect using anti-HA immunohistochemistry compared to the membrane bound NEP-n protease. However, another explanation for the
decreased staining of the NEP-s protease may be that once the NEP-s is secreted the NEP protein or at least the HA-tag is more prone to degradation by other extracellular proteases. We plan to conduct further investigation of the expression and activity profile using in vitro studies to delineate the metabolism of both the NEP-n and NEP-s proteases. Additionally, the NEP-s staining was more intense in the cortical regions compared to the hippocampus which may indicate that the signal peptide is processed more effectively in the cortex compared to the hippocampus. Despite the differences in staining intensity of the different NEP proteins our in vitro data confirmed that both NEP-n and NEP-s proteases shown activity, while the NEP-m version did not. Additionally, only the NEP-s protein showed enzyme activity once secreted into the cell media. We also plan to re-examine the levels of soluble protease activity in the cortex compared to the hippocampus of injected animal to see if this is the case in vivo.

Our results demonstrate that the up-regulation of NEP through rAAV vectors can provide a viable method to decrease the total amyloid deposition in the brain of amyloid depositing Tg mice. NEP-n and NEP-s were able to significantly reduce total Aβ deposition in the anterior cortex and hippocampus at the site of injection. Similarly, both NEP-n and NEP-s significantly reduced the level of congophilic deposits. Interestingly, when the area of positive staining for Aβ was analyzed in the hippocampus and cortical regions of the contralateral hemisphere, both the NEP-s and NEP-n rAAV were able to decrease total Aβ (in anterior cortex) load compared to the control group. Only NEP-s treated animals showed significant reductions in total Aβ load in the contralateral hippocampus.
compared to control animals. However, examination of the congophilic deposits on the contralateral anterior cortex showed no reduction. Interestingly, on the contralateral hippocampal side there was a 40-50% reduction of congophilic staining, but only the reduction with NEP-s was statistically significant compared to the control group. The reduction on the contralateral hemisphere observed here is likely due to retrograde transport of the rAAV and anterograde transport of the NEP protein. Retrograde transport of rAAV has previously been reported (Burger et al 2004). The lower number of neuronal connections between the ipsilateral and contralateral sides in the anterior cortex compared to those present in the hippocampus could explain why there is a reduction in congophilic staining in the contralateral hippocampus and not the contralateral anterior cortex. Further, since the contralateral anterior cortex showed reduction of total A\(\beta\) load but not congophilic staining it would seem to suggest that a critical amount of NEP expression is required in order to reduce the insoluble A\(\beta\) plaque formation.

As previously mentioned our findings showed that by implementing the use of a secreted form of the NEP protease in addition to using the CED method for intracranial administration, we were able to successfully improve the area of gene expression to further reduce amyloid load in the brain. An alternative method which would potentially overcome the limitations of a direct intracranial injection is to attempt to express the NEP protease in a peripheral fashion.

Previous studies using anti-amyloid vaccines have clearly demonstrated that effective therapy does not have to cross the blood brain barrier to have a
significant effect on amyloid load in the brain. It is well established that both passive and active Aβ immunotherapy can effectively decrease amyloid load from the brain as well as improve cognitive impairment in transgenic animals (Morgan et al., 2000), (Janus et al., 2000), (Dodart et al., 2002), (Masliah et al., 2005), (Wilcock et al., 2004), (Solomon, 2005), (Maier et al., 2006). The mechanism underlying this phenomenon referred to as the ‘peripheral sink’ hypothesis asserts that amyloid is in a state of equilibrium throughout the body. Therefore, sequestering and removing amyloid in the periphery will change the equilibrium such that amyloid will move from the brain into the periphery (DeMattos et al., 2002), (Matsuoka et al., 2003), (Lemere et al., 2003), (Deane et al., 2005). Most recently, ex vivo gene transfer studies using cell mediated over expression of amyloid degrading peptidases have shown initial success and may provide a useful alternative to passive immunotherapy. Hemming et al in 2007 showed that over expression of secreted neprilysin in primary fibroblasts reintroduced peripherally into transgenic APP mice showed a robust reduction in plaque load (Hemming et al., 2007). Additionally, other methods implementing cell mediated gene expression using both leukocytes and erthrocytes have been used enhance Aβ degradation in the periphery and in the CNS (Guan et al., 2008), Lui et al 2008).

We also wanted to examine the effect of increasing NEP expression in aged animals where there is already significant deposition of Aβ. Evidence has shown that rAAV viral vectors can successfully transfet neurons in the CNS and result in sustained and stable long term gene expression in the brain (McCown et
Our results demonstrate that the rAAV NEP-s vector was able to significantly reduce both total Aβ load and fibrillar Aβ in aged animals. These data are consistent with evidence from Spencer et al (2008). As expected, the NEP-s showed strong expression in the brain 5 months following treatment in older animals 20 months of age (treatment was at 15 mo. of age) demonstrating persistence and stability of the transgene expression. Upon observation of congophilic positive plaque load in 20 mo. old animals compared to plaque load in 15 mo.old animals it appears that treatment with NEP in the old animals with a large amount of preexisting plaques simply halts the progression of plaque build up rather than actively removing compact plaques established well before treatment onset. Further investigation must be done to verify the aforementioned observation. Gordon et al. in 2001 showed that congophilic positive staining, primarily composed of Aβ40, increases until about 12 months of age in the double transgenic APP+PS1 mouse and remains relatively stable up until 18 months of age (Gordon et al, 2001).

Although we have yet to perform a detailed examination, initial observations showed no noticeable toxic effects in mice receiving the NEP vectors. No neuron loss or gross morphometric changes were observed in fixed brain tissue. No significant changes in total body weight were noted, indicating that up regulation of NEP did not appear to have adverse effects or cause general toxicity in the mouse model during our study period. NEP has been shown to have other endogenous peptide substrates, including atrial natriuretic
peptide, substance P, endothelin and enkephalin. It is uncertain whether interactions between NEP and these other potential substrates in the brain may cause potential problems although one recent study suggests that high levels of enkephalins can contribute to memory and cognitive deficits in amyloid producing mice, further implicating the importance of NEP activity in AD (Meilandt et al., 2008). Monitoring changes in these NEP peptide substrates between control and treatment groups may help identify any potentially harmful side effects resulting from increases in NEP activity. Previous studies have revealed that in the AD brain as well as in the animal model of AD, NEP are down regulated specifically in areas that are prone to plaque formation (Yasojima et al., 2001a), (Fukami et al 2002). Therefore, up-regulation of NEP to restore normal levels of this endogenous protease should have minimal adverse effects. In future studies, we could regulate the levels of NEP expression by using an inducible promoter system which would enable the levels of NEP to be managed as required per individual and potentially reduce unwanted side effects of continuous high levels of NEP expression. Future studies which implement a combination of peripheral and central gene transfer studies can also have additive effect on total amyloid load in the brain. Another therapeutic gene therapy technique could implement a cell mediated gene transfer technique in an in vivo fashion. Rivest et al in 2008 demonstrated that injection of lentivirus expressing the TLR2 gene resulted in successful expression of the gene in bone marrow cells. Additionally, these cells successfully migrated to the CNS of amyloid depositing transgenic mice ensuing in a decrease in amyloid pathology (Richard et al., 2008). These findings further
demonstrate that viral vectors and cell mediated therapy can be combined to provide a novel therapeutic technique in the treatment of neurological disease.

We have yet to determine if this reduction in the Aβ levels with our NEP-n or NEP-s rAAV vectors will lead to significant improvement in behavioral tests such as the Morris water maze in both the young and old populations of transgenic mice. However, these data are consistent with reports that NEP can degrade Aβ in vitro, and that partial knockdown of the NEP gene leads to more rapid accumulation of Aβ (Farris et al., 2007), (Eckman et al., 2003). The present work adds to the evidence that NEP plays an important role in Aβ deposition by demonstrating that local overexpression NEP enzyme activity can dramatically reduce the deposition of amyloid in the brains of APP transgenic mice. Thus, regulation of NEP through methods such as gene therapy may be used as a potential therapeutic target for the treatment of Alzheimer's disease warranting further study into the use of gene therapy techniques in a combinatorial fashion to increase effectiveness while reducing adverse events.

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FIGURE 1: DIAGRAMMATIC REPRESENTATION OF rAAV CONSTRUCTS EXPRESSING THE NEP GENE UNDER THE CONTROL OF THE CHICKEN $\beta$-ACTIN (CBA) PROMOTER. Note the C-terminal fusion HA tag in both sequences and the secretion signaling peptide in the NEPHA-s construct. Panel A shows a gene map of the recombinant AAV NEP-n and NEP-s constructs under control of the hybrid CBA chicken $\beta$-actin promoter. A hemagluttinin tag has been appended to the NEP encoding sequence to allow for easy detection.
**FIGURE 2: EXAMINATION OF NEP EXPRESSION IN TRANSDUCED CELLS OF THE HIPPOCAMPUS CONTRALATERAL AND IPSILATERAL TO VECTOR INJECTION.** Strong NEP-n and NEP-m expression is detected using anti-HA immunostaining in the right hippocampus following intracranial administration of NEP-n and NEP-m vector (panels A and B, respectively). Panel C shows less intense diffuse NEP-s expression in granule cells of the dentate gyrus in the right hippocampus following intracranial administration of NEP-s. Panels D shows no positive staining in the uninjected hippocampus of an untreated age matched animal. Magnification = 40X for all panels. dg = dentate gyrus; CA1 = Cajal Area 1; CA3 = Cajal area 3.
FIGURE 3: EXAMINATION OF EXPRESSION LEVELS OF NEP AFTER INTRACRANIAL ADMINISTRATION OF rAAV VECTORS INTO THE RIGHT ANTERIOR CORTEX OF MICE. Panels E and F show strong NEP-n and NEP-m expression as detected by anti-HA immunostaining in the anterior cortex and striatum ipsilateral to the injection site. Panel G shows slightly less intense diffuse NEP-s expression in the anterior cortex and striatum ipsilateral to the injection site. Panels H shows no positive staining in the uninjected cortex of untreated aged matched animals. Str = striatum; CC = corpus callosum; CX = cortex. Magnification = 40X.
**FIGURE 4: QUANTIFICATION OF NEP SPECIFIC ACTIVITY IN HEK293 TRANSFECTED CELLS.** Nephrilysin specific activity is significantly higher in the HEK293 cells transfected with NEP-n in the cell lysate (membrane fraction) compared to NEP-m, NEP-s, and GFP transfected cell lysates. NEP-s transfected cell lysates (cell media fraction) showed significantly higher NEP activity compared to NEP-n, NEP-m and GFP expressing cells. The asterisk (*) indicates significance with a p-value < 0.05. The number (#) indicates significance with a p-value < 0.001. Note differences in Y axis scaling for each panel.
**FIGURE 5:** TOTAL AMYLOID LOAD IS REDUCED FOLLOWING INTRACRANIAL ADMINISTRATION OF NEP-s OR NEP-n rAAV VECTORS.

Aβ immunostaining is observed in mice throughout both hippocampus (panels A, B and C) and anterior cortex (panel D, E and F). Aβ staining in the ipsilateral hippocampus of animals receiving intracranial injection NEP-n (panel B) or NEP-s (panel C) is reduced compared to control vector NEP-m (panel A). Aβ staining in the right anterior cortex of mice receiving intracranial injection of NEP-s (panel D) or NEP-n (panel F) is reduced compared to control vector NEP-m (panel B).

Scale bar = 120μm.
**FIGURE 6:** QUANTIFICATION TOTAL AMYLOID LOAD IN THE HIPPOCAMPUS AND CORTICAL REGIONS FOLLOWING INTRACRANIAL ADMINISTRATION OF rAAV. Panel A shows percent area of positive staining for total Abeta load of the right cortex and hippocampus ipsilateral to the injection site. Panel B shows quantification left cortical and hippocampal percent area of positive staining for total abeta load of the contralateral hemisphere to the injection site. The star (*) indicates significance with a p-value < 0.05; (**) indicates p-value <.001.
**FIGURE 7: CONGOPHILIC COMPACT PLAQUE LOAD IS REDUCED FOLLOWING INTRACRANIAL ADMINISTRATION OF NEP rAAV VECTORS.**

Total congophilic staining is observed in mice throughout both hippocampus (panels A, B and C) and anterior cortex (panel D, E and F). Positive congophilic staining in the ipsilateral hippocampus of animals receiving intracranial injection NEP-n (panel B) or NEP-s (panel C) is reduced compared to control vector NEP-m (panel A). Congophilic staining in the right anterior cortex of mice receiving intracranial injection of NEP-s (panel D) or NEP-n (panel F) is reduced compared to control vector NEP-m (panel B). Scale bar = 120μm.
FIGURE 8: QUANTIFICATION OF TOTAL CONGOPHILIC LOAD IN THE HIPPOCAMPUS AND CORTEX FOLLOWING INTRACRANIAL ADMINISTRATION OF rAAV. Panel A shows quantification of percent area of positive staining for total congophilic staining of the cortex and hippocampus ipsilateral to the injection site in the hippocampus and cortex. Panel B shows quantification of percent area of positive stain of total congophilic staining in the contralateral hemisphere to the injection site. The star (*) indicates significance with a p-value < 0.05
FIGURE 9: DISTRIBUTION OF NEP EXPRESSION 20 WEEKS AFTER rAAV NEP-S ADMINISTRATION IN AGED APP + PS1 MICE AT 15 MONTHS. Brains sections were immunostained for NEP with an antibody recognizing both rodent and human NEP. Panels A, C, and E show NEP expression from mice treated with rAAV-NEP-s. Panels B, D, and E show no NEP expression from control animals treated with rAAV-GFP.
**FIGURE 10:** TOTAL ABETA LOAD IS REDUCED FOLLOWING INTRACRANIAL ADMINISTRATION OF rAAV NEP-S IN AGED MICE. Panels A and C show positive immunohistochemical staining of total abeta in the hippocampus and cortex respectively of APP+PS1 mice treated with rAAV-NEP-s. Panels B and D show positive abeta staining of the hippocampus and cortex of untreated mice. Panel E shows quantification of Aβ immunostaining in NEP-s and untreated 20 Mo old APP+PS1 mice. Mice were injected at 15 mo of age in hippocampus and cortex of both hemispheres. RCX = right anterior cortex, LCX= left anterior cortex, RHPC = right hippocampus, LHPC=left hippocampus. Y axis is Aβ load (percent area occupied by reaction product). ** P < 0.01
**FIGURE 11: TOTAL CONGOPHILIC STAINING IS REDUCED FOLLOWING INTRACRANIAL ADMINISTRATION OF rAAV NEP-s IN AGED MICE.** Panels A and C show positive immunohistochemical staining of total congo in the hippocampus and cortex respectively of APP+PS1 mice treated with rAAV-NEP-s. Panels B and D show positive abeta staining of the hippocampus and cortex of untreated mice. Panel E shows quantification of Aβ immunostaining in NEP-s and untreated 20 Mo old APP+PS1 mice. Mice were injected at 15 mo of age in hippocampus and cortex of both hemispheres. RCX = right anterior cortex, LCX = left anterior cortex, RHPC = right hippocampus, LHPC = left hippocampus. Y axis is Aβ load (percent area occupied by reaction product). ** P < 0.01
REFERENCE LIST


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Marie-Claire C, Ruffet E, Antonczak S, Beaumont A, O'Donohue M, Roques BP, Fournie-Zaluski MC (1997) Evidence by site-directed mutagenesis that arginine 203 of thermolysin and arginine 717 of neprilysin (neutral endopeptidase) play
equivalent critical roles in substrate hydrolysis and inhibitor binding. Biochemistry 36:13938-13945.


PAPER 3:

CONVECTION-ENHANCED DELIVERY AND MANNITOL AS A METHOD TO INCREASE DISTRIBUTION OF AAV VECTORS 5, 8, AND 9 AND INCREASE GENE PRODUCT IN THE ADULT MOUSE BRAIN.

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ABSTRACT

The use of recombinant adeno-associated viral (rAAV) vectors as a means of gene delivery to the central nervous system has emerged as a viable method of gene therapy for the treatment of several types of degenerative brain diseases. A major disadvantage of typical intracranial injections into the brain parenchyma is a limited distribution of the rAAV macromolecules to all brain regions where therapies may be needed. Optimizing specific parameters of the administration techniques with the purpose of obtaining maximal gene distribution and gene uptake is an important obstacle to overcome for gene therapy studies. Convection enhanced delivery (CED) is a method of delivering clinically relevant volumes of therapeutic agents to significantly larger areas of the brain. The CED technique is designed to utilize the phenomenon of bulk flow and positive pressure to distribute macromolecules to a large area within solid tissue in a direct intracranial injection procedure. In the present study the CED method using the step-design cannula (Krauze et al., 2005a) was used to deliver AAV vector serotype 5 expressing GFP into the hippocampus and cortical regions of the mouse brain. Regions of the hippocampus and cortex receiving the CED injection showed significantly more robust expression of GFP and an
increased area of distribution than the simple diffusion method. We also compare transduction efficiency of AAV serotypes 5, 8, and 9 using CED administration. Following a single CED injection AAV 9 resulted in the largest area of distribution in the mouse CNS. Moreover, the CED method, in addition to systemic administration of mannitol, was used to deliver rAAV vectors (serotypes 5 and 9) expressing GFP into the hippocampus of 11 month old mice. Mannitol is a blood brain barrier interruptive reagent which also induces hyperosmolarity thereby reducing intracranial pressure and facilitating the movement of particles through the interstitial space. Mice were injected intracranially into the right hippocampus using the CED method with (n=8) or without (n=8) pre treatment with mannitol delivered intraperitoneally. Animals receiving systemic injections of mannitol followed by the intracranial CED injection of rAAV into the hippocampus showed an increased area of distribution compared to animals that did not receive pre treatment with mannitol. In addition, GFP expression was also detected in regions distant from the initial site of injection, in animals receiving the CED method with mannitol, including significant expression in the contralateral hemisphere to the injection site. These data suggest that by optimizing injection techniques such as CED in addition to mannitol induced hyperosmolarity, provides an efficient means of increasing viral vector distribution and transgene uptake.
INTRODUCTION

AAV (adeno associated viral) vectors have recently emerged as a promising and novel mean by which transgenes can be delivered to different tissue types successfully in a large range of animal species including humans. AAV have a unique profile with a number of advantageous characteristics which identify them as one of the most feasible gene transfer vectors in the treatment of a variety of diseases, including neurological diseases, compared to other viral vectors. AAVs are one of the smallest viruses, approximately 25 nm in diameter, and their DNA genome is less than 5 kb which contains two large open reading frames with inverted terminals repeats located at either end Thomas (Berns, 1990), (Thomas et al., 2003); (Wu et al., 2006). The most attractive characteristics of AAV is their lack if pathogenicity, persistence of the transgene as an episome, and long term gene expression. AAV vectors arrive in a variety of flavors otherwise known as serotypes. The AAV serotype refers to the efficiency by which the specific AAV can infect a particular cell type through attachment to specific cell surface receptors. A new AAV serotype is defined by the inability of an antibody that is reactive to the viral capsid protein of one serotype in neutralizing those of another serotype (Choi et al., 2005). The availability of different AAV serotypes is another major advantage that lends to its feasibility as a therapeutic in the treatment of neurodegenerative disease. Through the modification of serotype diversity, a variety different capsid proteins can be incorporated to create pseudotypes of recombinant AAV (mainly derived from AAV 2) which can ultimately mediate tropism and transduction efficiencies
targeted towards specified cell and tissue types throughout the body to optimize
gene therapy (Choi et al., 2005), (Akache et al., 2006), (Cearley and Wolfe,
2006), (Kwon and Schaffer, 2008).

Unfortunately, one of the major disadvantages of a single intracranial
injection of AAV vectors into the brain parenchyma using simple diffusion does
not allow for efficient uptake of the transgene or significant distribution of the AAV
macromolecules to significantly large areas of the affected regions within the
brain. Convection enhanced delivery (CED) is a method of delivering clinically
relevant volumes of therapeutic agents to significantly larger areas of the brain in
a direct intracranial injection procedure in comparison to simple diffusion
methods. The CED technique is designed to utilize the phenomenon of bulk flow
and positive pressure to distribute macromolecules to a large area within solid
tissue. The CED technique was originally proposed by scientists in the early
1990s as a method of delivering drugs, or macromolecules, directly to the
parenchyma that would not normally cross the blood brain barrier (Raghavan et
al., 2006). Due to the lack of approved drugs that can be directly intracranially
administered to the brain and the difficulty in predicting methods that ensure
delivery of the therapeutic agent to its target site, CED remains an experimental
procedure. Furthermore, research of CED delivery devices is under current
investigation by several researchers (Bankiewicz et al., 2000), (Raghavan et al.,
2006).

This CED method has been investigated in gene therapy studies as a way
to increase the distribution of AAV vectors in the brain. Studies conducted by
Bankiewicz et. al. in 2000 revealed that CED can significantly increase gene transfer and distribution of AAV expressing AADC in the striatum of MPTP-treated monkeys. The AAV vector was found to be safely distributed throughout the entire region of the striatum compared to the simple injection method where the distribution was severely limited (Bankiewicz et al., 2000). Similar results were replicated in the rat brain by Cunningham et. al. in 2000 with AAV2 expressing thymidine kinase (TK) where the CED method showed robust gene transfer and increased distribution area within the putamen. CED injections in the striatum were found distribute the AAV-TK throughout the striatum after a single injection into this region and TK immunoreactive cells were also found outside the striatum, in the globus pallidus, subthalamic nucleus, thalamus, and substantia nigra (Cunningham et al., 2000), (Hadaczek et al., 2006).

One of the mechanistic limitations of the CED method as well as the simple injection method is the reflux of the injected material from the injection hole upon the removal of the cannula. In 2002 Krauze et. al. developed a reflux free step cannula design which effectively eliminates reflux by placing silicone coated tubing within the cannula creating a step that prevents the backflow of fluid (Krauze et al., 2005a). The optimization of more efficient cannula designs coupled with the encouraging results from studies showing enhanced gene transfer and distribution emphasizes the therapeutic potential of the CED method in helping overcome some of the mechanical disadvantages of gene delivery in regards to gene therapy (Krauze et al., 2005b).
The use of osmotic agents such as mannitol is another method that can be used to increase the area of distribution of macromolecules throughout the CNS. Mannitol is a blood brain barrier interruptive reagent and is also known to temporarily increase vascular pressure subsequently reducing intracranial pressure. High concentrations of mannitol intravenously infused are currently used in patients with traumatic brain disease to reduce intracranial pressure. This osmotic agent pulls fluid from the CNS by increasing vascular osmotic pressure. Several studies have also shown that with intra-arterial infusion of mannitol the blood brain barrier can be opened to enhance the distribution of chemotherapeutics throughout the CNS in both rats and humans (Nilaver et al., 1995), (Rapoport, 2001), (Fu et al., 2003). In this particular study we anticipate that by optimization of these mechanistic strategies in addition to AAV serotype tissue specificity we can optimize transduction efficiency and increase distribution area of AAV in the brain.

MATERIALS AND METHODS

Animals

Non transgenic C57BL6 mice were acquired from the breeding colonies at the University of South Florida. Multiple mice are housed together whenever possible until the time of use for the study; mice were then singly housed just before surgical procedures until the time of sacrifice. Study animals were given water and food (ad libitum) and maintained on the twelve hour light/dark cycle
and standard vivarium conditions. Mice aged 9 to 11 months were used for all experimental procedures with an $n = 4 - 6$ for each experimental group.

**Step Design Cannula**

The step design cannula was used for all intracranial surgeries. Fused silica tubing (polymicro technologies, Phoenix, AZ) was inserted into a 27 gauge Hamilton blunt ended needle and fixed in place with super glue (Krauze et al., 2005a). The end of the silica tubing was cut leaving 1mm of tubing protruding from the end of the Hamilton needle.

**GFP Expression Using CED**

Part A; animals were assigned to one of two cohorts, group one received AAV vector expressing GFP ($n = 6$). These animals received a single intracranial injection of the AAV-GFP vector using the CED injection method (5ul/min) or the traditional injection method (0.5ul/min) into either the right or left hippocampus and into the right or left frontal cortex. Immediately before surgery mice were weighed then anesthetized using isoflurane. Surgery was performed on animals using a stereotaxic apparatus, injections using the CED method were used to inject into hippocampus and frontal cortex at a flow rate of 5ul/min over a total period of 2 min. (infusion time of 0.4min) using the CED method described earlier. The traditional injection method was used to inject into the hippocampus and frontal cortex at a flow rate of .5ul/min over a total period of 4 min. (infusion time of 2 min.) also described earlier. The surgical procedure was performed by exposing the cranium using an incision through the skin along the median sagittal plane, and two holes were drilled through the cranium over the right frontal cortex.
injection site and the right hippocampal injection site. Previously determined
coordinates for burr holes, taken from bregma were as follows; frontal cortex,
anteroposterior, 1.5mm; lateral, -2.0mm, vertical, 3.0mm, hippocampus,
anteroposterior, -2.7mm; lateral -2.5mm, vertical, 3.0mm. Burr holes were drilled
using a dental drill bit (SSW HP-3, SSWhite Burs Inc., Lakewood, NJ). Each
animal received both injection types in opposite hemispheres to control for
mouse to mouse variability in gene uptake and expression. Six weeks post
surgery mice were weighed, overdosed with pentobarbital (200 mg/kg) and
perfused with 25 ml of 0.9% normal saline solution then 50 ml of freshly prepared
4% paraformaldehyde. Brains were collected from the animals immediately
following perfusion and immersion fixed in 4% paraformaldehyde for 24hrs.
Mouse brains were cryoprotected in successive incubations in 10%, 20%, 30%
solutions of sucrose; 24hrs in each solution. Subsequently, brains were frozen
on a cold stage and sectioned in the horizontal plane (25 μm thickness) on a
sliding microtome and stored in Dulbecco’s phosphate buffered saline (DPBS)
with 0.2% sodium azide solution at 4°C.

Six to eight sections 100 μm apart spanning the site of injection were
chosen and free-floating immunochemical and histological analysis was
performed to determine gene expression using an anti-GFP antibody at a
concentration 1:3,000 (Chemicon; Temecula, CA). Immunohistochemical
procedural methods were analogous to those described by Gordon et al. 2002 for
each marker. Animal tissue was placed in multisample staining tray and
endogenous peroxidase blocked (10% methanol, 30% H₂O₂, in PBS). Tissue
samples were then permeabilized (with lysine 0.2%, 1% Triton X-100 in PBS solution), and incubated overnight in appropriate primary antibody. Sections were washed in PBS then incubated in corresponding biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissue was again washed after a 2hr. incubation period and incubated with Vectastin® Elite® ABC kit (Vector Laboratories, Burlingame, CA) for enzyme conjugation. Finally, sections were stained using 0.05% diaminobenzidine and 0.3% H2O2 (for CD45 and FcγR 0.5% nickelous ammonium sulfate was added for color enhancement). Tissue sections were mounted onto slides, dehydrated, and coverslipped.

Part B of the experiment was to assess the safety profile of the CED injection method immediately following the injection procedure. Animals received a single injection of saline (n =6) using either the CED method or the traditional injection method into the right or left hippocampus and frontal cortex. Each animal received both injection methods into opposite hemispheres to control for variations between animals with regard to microglial activation and neurotoxicity to determine the safety profile of the CED technique. Animals were sacrificed 4 days post surgery as described earlier to determine whether the CED technique causes an acute significant increase in microglial activation or mechanical tissue damage using an immunohistochemical procedure described earlier. Six sections 100 μm apart spanning the site of injection were chosen from each animal for part B of the experiment and free-floating immunohistochemical and histological analysis was performed for analysis of the safety profile of the CED method in comparison to the traditional injection method. A series of sections
from experiment part B were immunostained for CD45 to determine relative microglial activation. Each immunochemical assay omitted some sections from primary antibody incubation period to evaluate nonspecific reaction of the secondary antibody. Another series of sections from part B were mounted on slides and stained with fluoro-jade stain (0.001%, Sigma Aldrich, St. Louis, MO) to assess the degree of neurotoxicity. Fluoro-jade is an anionic fluorochrome that selectively stains degenerating neurons effectively detecting neuropathic lesions by fluorescent microscopy (Schmued et al., 1997). A subsequent series of sections from part B (6 per animal) were mounted on slides and stained with the Cresyl Violet (Nissl) stain (0.05%, Sigma Aldrich, St. Louis, MO). The cresyl violet stains all Nissl bodies of the rough endoplasmic reticulum and other acidic components in all neuronal cytoplasm therefore an absence of staining will indicate neuron loss. For experiments which were analyzed to determine acute effects of the CED technique all immunohistochemical staining for CD45 and fluoro-jade and cresyl violet (Nissl) staining all procedures followed the same protocol aforementioned 4 days post surgery rather than six weeks post surgery.

**GFP Expression with Serotypes AAV 5, 8, and 9**

Study animals were assigned to one of three treatment groups receiving AAV serotype 5, 8, or 9. All AAV vectors contain a coding sequence for green fluorescent protein (GFP). All animals received an intracranial injection into the right hippocampus and all injections were performed using the CED technique described earlier. Each group received a single intracranial injection of 2ul of an each respective AAV vector expressing GFP (1.5 x 10^{11}vg/ul) into the right
hippocampus. All intracranial surgeries were performed on a stereotaxic apparatus using predetermined hippocampal coordinates for the mouse hippocampus described earlier. Six weeks post injection animals were sacrificed and brain tissue was collected as described earlier.

**GFP Expression with Serotypes AAV 5 or 9 and Mannitol**

Animals were assigned to one of four groups. Group 1 and 2 received a single intracranial injection of 2ul of AAV serotypes 5 and 9 respectively expressing GFP (1.5 $\times$ 10$^{11}$vg/ul concentration) into the right frontal cortex and right hippocampus using the CED injection method. Group 3 and 4 also received a single intracranial 2ul injection of the AAV-GFP serotypes 5 and 9 (1.5 $\times$ 10$^{11}$vg/ul concentration) into the right frontal cortex and hippocampus in addition to a 200 ul single systemic intraperitoneal injection of 25% mannitol administered 15 minutes before the intracranial injection. The left untreated hemisphere in all animals remained untreated and used as an internal control. Surgery was again performed on animals using a stereotaxic apparatus using burr hole coordinates previously described. Six weeks post injection animals were sacrificed and brain tissue was collected as described earlier. Sections were immunohistochemically stained using anti-GFP antibody (1:3000; Chemicon; Temecula, CA) to test for gene distribution and expression in addition to the markers listed in part A using tissue animals in each group.

**Quantification and Statistical Analysis**

All immunostained sections were imaged using an Evolution MP digital camera mounted on an Olympus BX51 microscope at 100X final magnification
(10 X objective). Immunofluorescent double labeled images were taken using a confocal microscope. Six to eight horizontal brain sections (100µm apart; every 4th section) were taken from each animal and four nonoverlapping images near the site of injection from each of these sections were captured (24 measurements per mouse). All images were taken from the same location in all animals. Quantification of positive staining surrounding and including the injection sites in the right frontal cortex and the right hippocampus and the corresponding regions in the left hemisphere were determined using Image-Pro® Plus (Media Cybernetics®, Silver Springs, MD). ANOVA statistical analysis was performed using StatView® version 5.0.1 (SAS Institute, Raleigh, NC).

RESULTS

Non transgenic mice, 9 months old, were injected bilaterally into the hippocampus using either the CED injection method or the traditional injection method. The CED injection method was performed as previously stated using the step design cannula with a flow rate of 5µl/min with a total injection time of approximately 2 min. including the time the cannula remained in the injection site to further prevent backflow of the AAV vector material. The simple injection was done at a flow rate of 0.5µl/min with a total injection time of approximately 4 min. Each animal received both the CED method in one hemisphere and the traditional injection method in the opposite hemisphere to control for variations in gene uptake between individual animals. The animals were survived 4 weeks post surgery and histology was performed to assess gene distribution and
expression. Regions of the hippocampus and cortex receiving the CED injection showed more robust expression of GFP and an increased area of distribution than the traditional injection method (figure 1). In addition, GFP expression was also detected in regions distant from the initial site of injection in the hemisphere which received the CED injection method. Areas with positive GFP staining included the entorhinal cortex, as well as some neurons in the striatum and thalamus. The striatum and entorhinal cortex showed a significantly increased area of distribution in the animals receiving the CED injection as opposed to the traditional injection method. The hippocampal and cortical regions in which the CED method was implemented also showed an increased intensity of staining immediately surrounding the cannula tip and in some distal areas in regions surrounding the injection site. The difference in staining intensity appears to indicate an increase in gene product (GFP) and potentially indicate that the CED technique increases the transduction efficiency of the AAV vector by increasing the susceptibility of the cell to infection.

Part B of the experiment was to determine if safety profile of the CED method on mechanical damage of parenchymal brain tissue. Brain samples were analyzed for acute effects of the CED method in a second cohort of animals. Non transgenic mice (n=6) were bilaterally injected in the same manner described above (injections were of PBS) using both the CED and traditional injection methods. The mice were sacrificed and tissue was analyzed 4 days post injection and histology performed. Neurotoxicity was determined using the fluoro-jade stain which was only positive in a very small area in 2 animals which
was not significant. Microglial expression was also analyzed to assess the increase in microglial activation due to the mechanical technique. CD45 is a protein tyrosine phosphatase that is only expressed when microglial cells become activated, in this case, as a result of tissue damage (figure 2). Immunohistochemistry revealed that CD45 staining was increased but not significantly following the CED injections but this increase was limited to the area immediately surround the cannula tract (figure 2). In addition the tissue was also stained for cresyl violet or Nissl. The cresyl violet stains all Nissl bodies of the rough endoplasmic reticulum and other acidic components in all neuronal cytoplasm, therefore an absence of staining indicates neuron loss. The results from the tissue did not reveal any significant areas devoid of Nissl staining signifying that there was no appreciable neuron loss (Figure 2).

To examine whether AAV serotype was able to affect transduction efficiency as well as area of distribution three different vector serotypes were administered into right hippocampus of 11 month old non transgenic mice using the CED method. A comparison of area of distribution as well as a comparison of transduced neurons in the hippocampus following administration of AAV serotypes 5, 8 and 9 each expressing GFP revealed that serotype 9 was distributed to a significantly greater area than either 8 or 5. Six weeks post injection, serotypes 8 and 9 not only transduced neurons throughout a large region of the entire hippocampus but they were also successful in transducing neurons in the left hippocampus contralateral to the site of injection. Conversely, AAV serotype 5 was primarily expressed in the neurons of the hippocampus
ipsilateral to the injection site. GFP positive neurons were limited to the pyramidal and molecular cell layers dentate gyrus as well as cells in the hilus and CA1 regions. A few GFP positive cells were observed in the entorhinal cortex in addition to a few GFP positive cells in the lateral thalamic region directly adjacent to the medial hippocampal regions but these were insignificant in comparison to serotypes 8 and 9. AAV 8 and 9 serotypes revealed intense staining of GFP positive neurons in all three CA regions of the hippocampus as well as very intense staining of pyramidal cells in the molecular layer of the dentate gyurs and a large number of positive cells in the hilus in the ipsilateral hippocampus. A large number of intensely stained cells can also be observed in the entorhinal cortex of the right hemisphere. It appears that the majority of cells that have been transduced and are positive for GFP have intense staining not only of the cell body but the axonal and dendritic projections (although slightly less intense). These axonal projections are positive throughout the hippocampus and corpus callosum (which contains axonal connections between the right and left hemisphere). The GFP positive axons are clearly stained at midbrain level ventral from the top of the cortex. These axons are positive at the cells bodies from which they originate in the right contralateral hemisphere to the injections site, through the midline to the axonal termination site in the left hippocampus. Very few cells bodies were positively stained in the left uninjected hemispheres of mice receiving the AAV 8 and 9 serotypes. Observations of the left hemisphere contralateral to the injections revealed positive staining of the axon terminals of neurons in the right dentate gyrus and CA regions. Intensely stained
neurons can also be observed in the right entorhinal cortex for AAV 8 and 9 treatment groups, while the left entorhinal cortex was less intense and as in the left hippocampus, only axons were positively stained for GFP. Interestingly, GFP expression in animals receiving either AAV 8 or AAV 9 was observed not only in regions proximal to the right hippocampal injection site (as with AAV 5), or the left hippocampus resulting from retrograde axonal transport, but was also evident in neurons distal to the site of injection. Following a single injection of AAV 8 or AAV9 GFP positive neurons in these groups were observed in the anterior cortex, thalamic nuclei, striatum, septal nuclei, superior colliculus, subiculum and cerebellum of the ipsilateral hemisphere.

Quantification of the percent area of positive stain revealed that AAV9 showed a 5 fold significant increase in the area of distribution in the right (ipsilateral) hippocampus with the left (contralateral) hippocampus showed an approximate 6 fold increase in the area of distribution (figure 3). While, AAV serotype 8 was able to transduce a significantly greater area and number of neurons than AAV5, it was significantly less effective, therefore distributed to a significantly smaller region than AAV 9.

Finally, we analyzed whether the osmotic agent, mannitol, could improve total area of distribution thus enhancing transduction efficiencies of the different AAV serotypes in addition to the utilization of the CED method for the intracranial administration. AAV serotypes 5 and 9 (the highest and lowest transducing vectors in our study respectively) were given via direct intracranial injection using the CED method to 11 month old non transgenic animals with or without systemic
pre treatment with mannitol. Animals receiving a systemic injection of 25% mannitol followed by CED administration into the right hippocampus of AAV 5 showed significantly greater area of GFP expression 6 weeks post injection compared to animals only receiving AAV 5 administration alone. GFP expression in animals receiving systemic mannitol was throughout the entire right hippocampal region and was also observed in the left (contralateral) hippocampus (figure 4). All brain slices (25µm) in thickness through the hippocampus showed positive GFP staining in the mannitol treated group while the animals that did not receive mannitol did not have staining in the most ventral and dorsal portions of the hippocampus (Figure 4). A 3 fold significant increase in the percent area of positive GFP stain in the right hippocampus was observed compared to animals that did not receive mannitol. In the contralateral hemisphere we observed a 2 fold significant increase in GFP expression in mannitol treated animals compared to animals without mannitol pretreatment (figure 4).

All animals receiving AAV 9 administered using CED with or without systemic mannitol showed much higher GFP expression than animals receiving AAV 5 similar to our previous findings. In our comparison between animals receiving AAV 9 with systemic mannitol pretreatment and those that did not receive mannitol we did not observe significant differences of GFP expression in the right hippocampus (at the site of injection) or in the left hippocampus (contralateral to the injection site). GFP expression in the right hemisphere was within the cell bodies of pyramidal neurons in the CA regions as well as granule
cells in the dentate gyrus, mossy fibers and cells within the hilus. Axonal projections making up the perforant pathway of the hippocampus were also positive for GFP expression. All cell bodies and axonal projections in these regions were intensely stained revealing a significant amount of GFP expression. GFP positive neurons were observed in the subiculum and entorhinal cortex which complete the hippocampal circuit. GFP expression in the left hemisphere was limited to axonal projections which originate in the right hippocampal regions (the site of injection). In the left hippocampus a few cells in the hilus were positive for GFP expression, but most staining was observed in mossy fibers as well as axonal projections of CA regions presumably of those originating in the right hippocampus. The left entorhinal cortex was also positive for GFP expression but significantly less than the right entorhinal cortex (figure 5). This was also true for cells within the thalamus (figure 5). GFP positive neurons were also observed in the right thalamic nuclei which receives input from the subiculum of the hippocampus, and intense staining was observed in white matter tracts corpus collusum which connect the right and left hemispheres as well as the hippocampal commissure or commissure of fornix adjoining the right and left hippocampus. Neurons positive for GFP expression were also observed in the anterior cortex, caudate putamen, striatum, lateral and dorsal septal nuclei, superior colliculus, and cerebellum of the ipsilateral hemisphere in both groups receiving AAV9 independent of whether they received mannitol pretreatment.

To further characterize vector serotype tropism and whether tropism and transduction efficiency of AAV 5 and AAV9 was modified by mannitol the tissue
was double labeled using fluorescent immunostaining. Our results, following injection with either AAV5 or AAV9, showed no co-localization of astrocytes (GFAP which stains astrocytes) and GFP positive labelling in the dentate gyrus of the hippocampus (or in any other regions in the brain) (figure 8). This was noted in all animals receiving either serotype with or without mannitol pretreatment. GFP labeled cells only co-localized with Neu-N (which stains neurons) positive staining (figure 8). Positive labelling in the left uninjected hemisphere was in a few cells in the hilus of the DG but the majority of positive labelling was in the axons surrounding the cell bodies (figure 8, panel E-H).

DISCUSSION

The recent identification of different AAV serotypes has advanced the study of rAAV vectors which has quickly become a major dominant focus in the field of gene therapy. There are a number of studies aimed at characterizing the different AAV serotypes with respect to transduction efficiency, tissue tropism, cell surface receptors, intracellular processing, and capsid structure. AAV2 serotype was the first to be cloned into bacterial plasmids by Samulski et. al. in 1982 and to date it is the most characterized of all the serotypes of which a total of 11 have been discovered (Samulski et al., 1982). AAV5 was originally discovered in a human clinical sample in 1984 as a contaminant in adenovirus stock and it contains ITRs that are not unlike the structures of AAV2 ITRs and is the most divergent of all of the serotypes (Choi et al., 2005), (Wu et al., 2007). AAV8 was originally isolated from rhesus monkey tissue in 2002 by Gao and
colleagues (Gao et al., 2002). AAV9, like AAV5, was originally discovered in human tissue, while AAV10 and AAV11 are the most recently identified serotypes, initially isolated from cynomolgus monkey tissue in 2004 by Mori et. al., to date have not been fully characterized (Mori et al., 2006), (Wu et al., 2006), (Wu et al., 2007). Several recent studies have shown that different serotypes have distinct variations in their transduction efficiencies depending on the specific cell or tissue type, yet the molecular mechanisms which determine the preferred target cell for many serotypes remains unknown. Despite what is currently known concerning different AAV transduction profiles, the data regarding AAV serotype specific tissue tropism is subject to different interpretation due to variations in vector titer, promoters, and transgenes between studies. In the CNS, serotype characterization studies have revealed that AAV1, and 5 have higher transduction efficiencies than AAV2 throughout all the regions and cell types of the CNS (Alisky et al., 2000); (Burger et al., 2004), (Burger et al., 2005) while AAV4 will efficiently transduce specific cell types such as astrocytes within the subventricular zone (Davidson et al., 2000), (Weber et al., 2003), (Wu et al., 2006). Studies by Wolfe and colleagues also reveal that AAV7, 8, 9 and Rh10 expressing cDNA for lysosomal enzyme are also capable of transducing neurons within specific regions in the mouse brain. AAV9 and AAVRh10 appeared to have the highest transduction efficiencies and were found to undergo vector genome transport through axonal transport pathways (Cearley and Wolfe, 2006, 2007)(Cearley and Wolfe, 2006).
AAV2 vectors are the most extensively characterized and due to their well-established safety profile and range of infectivity, approximately 20 clinical trails have been conducted using the AAV2 vector serotypes in numerous patients (Herzog and Hagstrom, 2001), (Grieger and Samulski, 2005b), (Wu et al., 2006), (Cai et al., 2009). Phase I/II clinical trails have been initiated utilizing recombinant AAV 2 vectors for the treatment of human diseases such as cystic fibrosis, α-1 anti-trypsin deficiency, Parkinson’s disease, Batten’s disease, muscular dystrophy, hemophilia B, (Robbins and Ghivizzani, 1998) (Zhong et al., 2008). Unfortunately, one major disadvantage of using AAV in the treatment of brain disorders is the limitation in the dispersion of specific therapeutic gene or protein to relevant regions or the entire brain (Cearley and Wolfe, 2007). In this particular study we aim to characterize and compare transduction efficiencies in the mouse CNS for AAV serotypes 5, 8, and 9 after a single intracranial administration and improve distribution of the AAV as well as increase gene expression in brain regions distal to the injection site using optimal injection techniques in an innovative manner. Previous data from comparison studies of different AAV serotypes have shown AAV 2, 5, 7, 8 and 9 efficiently transduce neurons in the murine CNS (Fu et al., 2003) (Burger et al., 2004) (Burger et al., 2005) (Taymans et al., 2007). We hypothesized that we would be able to successfully transduce neurons in the mouse brain after a single intracranial injection of AAV 5, 8, or 9 (that express GFP) and we will be able to increase the area of vector distribution using a CED administration technique in addition to systemic mannitol treatment in a novel combinatory fashion.
Following a single intracranial injection we successfully demonstrated that AAV9 was the most efficient serotype in transducing a larger number of neurons in a larger area of the brain than both AAV 5 and AAV 8. AAV 8 was only slightly less efficient than AAV 9 and much more efficient that AAV5 at transducing neurons in the mouse CNS. Our data in consistent with others who have also shown that both AAV 8 and 9 can more efficiently transduce a larger region of brain than AAV 5 in a murine animal model and yet others have shown this is pattern is consistent in other species as well (Cearley and Wolfe, 2006). AAV 9 not only provides superior transduction compared to serotypes 5 and 8 in the CNS but in other organs such as heart, liver and lung as well as global transduction of several cell and tissue types in the periphery when systemically injected (Bish et al., 2008), (Zincarelli et al., 2008). Furthermore, studies have clearly shown AAV serotype 8 to have significantly superior transduction efficiency than AAV2 or 5 in murine and nonhuman primate animal models which is also consistent with our data showing serotype 8 to be more efficient at transducing neurons in the mouse CNS than serotype 5 (Davidoff et al., 2005).

There are several explanations which may account for the differences in transduction efficiencies between the different serotypes. AAV must first bind to the cell surface receptor in order to successfully transduce the neurons once injected into the brain (Cearley and Wolfe, 2006), (Kwon and Schaffer, 2008), (Daya and Berns, 2008). This is followed by a series of essential steps; viral uptake through endocytosis, intracellular trafficking and translocation of the particle to the nucleus, virion uncoating, synthesis of double stranded DNA and
finally viral gene expression (Rabinowitz et al., 2004), (Nash et al., 2007), (Kwon and Schaffer, 2008), (Nash et al., 2009); (Choi et al., 2005); (Cearley and Wolfe, 2006), (Cearley et al., 2008); (Daya and Berns, 2008). Each of these steps is essential to successful transgene expression and also determines the unique tropism of the AAV serotype. Although the cell surface binding receptor for AAV 1, 2, 3, 4, and 5 have been previously described, receptors for more recently discovered serotypes remains unclear. In 1998 Samulski et al determined that AAV 2 utilizes the heparin sulfate proteoglycan receptor as its binding target and also uses secondary binding co-receptor targets (including the αVβ5 integrin and fibroblast growth factor receptor) which help stabilize viral binding thereby enhancing transduction efficiency (Summerford and Samulski, 1998), (Summerford et al., 1999), (Qiao et al., 2002), (Qing et al., 2003). AAV 1 has been shown to bind to the same cell surface receptor and co-receptors as AAV 2, while AAV 5 has been shown to interact with the sailic acid receptor and platelet growth factor receptor and AAV 4, similar to AAV5, will bind to the sailic acid receptor but needs specific carbohydrate linkage to successfully bind to the cell surface (Walters et al., 2000), (Walters et al., 2001), (Kaludov et al., 2003), (Akache et al., 2006). Most recently, Akache et al. have demonstrated that AAV 8 uses the 37/67 kD laminin receptor (LamR) to bind to mammalian cells and overexpression of the LamR receptor results in increased binding of AAV 2, 3, 8 and 9 (expressing GFP) in vitro and a 2.5 fold increase in GFP expression (Akache et al., 2006). These data indicate that the differences in expression patterns between the different AAV serotypes used in the present study is
primarily mediated by the cells surface receptor that the AAV serotype binds to as well the co-receptors and the expression patterns of these receptors in the mouse CNS. Other factors such as those previously mentioned (intracellular trafficking and translocation of the particle to the nucleus, virion uncoating, and synthesis of double stranded DNA and gene expression) may play a secondary role in transduction efficiencies. Finally, the capacity to neutralize viral infection particles with neutralizing antibodies, generated from a previous exposure, also can affect the transduction profile of each serotype. Our findings as well as others confirm that AAV 9 has significantly higher transduction efficiency in neurons in the CNS compared to AAV 8 and AAV 5. The differences in cell specific transduction efficiencies are likely a consequence of the effective cellular entry through cell surface receptor modulation as well as un-packaging and post-entry properties of each serotype (Hauck et al., 2004), (Wang et al., 2007). We speculate that in this study AAV 9 was the most efficient likely due to its effective and stable binding to specific cell surface receptors possibly the LamR receptor or other potential receptors in addition to their expression profile within the murine CNS. AAV 9 may be taken up by the cell more efficiently followed by more efficient translocation and viral uncoating and un-packaging within the nucleus of the neuronal cell and efficient DNA replication and expression properties. The relative importance of each step in the process of transduction in the mouse CNS remains unclear and warrants more study which would lend to efforts in creating tailored recombinant AAV vectors with unique and disease modifying specificity.
Our next goal was to determine an optimal injection protocol aimed at improving distribution properties of the AAV vectors within the brain parenchyma. All of the intracranial injections into the hippocampus were performed using the CED technique with a modified version of the step design cannula (Bankiewicz et al., 2000) (Krauze et al., 2005a) which has previously been shown by us and others to further increase the area of distribution of material in the brain parenchyma in murine, canine, nonhuman primate as well as in humans (Bankiewicz et al., 2000), (Krauze et al., 2005b), (Szerlip et al., 2007), (Cunningham et al., 2008), (Dickinson et al., 2008), (Fiandaca et al., 2008). In the present study we successfully further increase the area of distribution and gene expression by combining the CED method with administration of mannitol systemically approximately 15 min prior to the intracranial injection of AAV. Our data are consistent with previous studies using AAV 2, in which mannitol further increased the area of distribution within the striatum of the rat brain (Burger et al., 2004). We clearly demonstrated that mannitol, by osmotically lowering intracranial pressure, could significantly increase the area of distribution of AAV 5 after a single CED administration into the DG of the right hippocampus. This distribution was evident throughout the entire right hippocampus allowing viral particles to travel to areas throughout the right hippocampus as well as other areas adjacent to the injection site such as the entorhinal cortex and thalamus (although the expression was limited to a few cells in these regions). In addition a significant amount of GFP gene expression was noted in the left uninjected hemisphere most likely due to axonal retrograde transport which was not as
apparent without mannitol pretreatment. The increase in GFP expression in the left hippocampus we attribute to greatly increasing the number of cells in the injected hemisphere that were exposed and therefore infected and expressing significantly more GFP that was then transported axonally to the left hemisphere. Mannitol also increased GFP gene expression in the AAV 9 treatment group in the entorhinal cortex and thalamus. We did not see significant changes in the hippocampus due to the preexisting high transduction efficiency of AAV 9 throughout the right and left hippocampi.

Despite the rapidly growing popularity of AAV as a method of gene delivery, much remains unknown with regard to cellular mechanisms that are responsible for different AAV characteristics. Although AAV demonstrate long-term gene expression in vivo and a good safety profile, potential risks due to the host response to these vectors need further study. Unlike adenovirus vectors which induce expression of chemokines and acute toxicity, AAV have not been previously associated with an inflammatory response or significant toxicity (Beuler et al., 1999), (Carter et al., 2000), (Muruve et al., 2004). Some studies have shown repeated administration of AAV in the periphery results in a humoral immune response, generated in the first administration, which dramatically lowers transduction efficiency upon second administration (Halbert et al., 1997),(Halbert et al., 1998). This problem can be overcome by host immunosuppression (Halbert et al., 1998), (Manning et al., 1998). The majority of the human population (an estimated 80%) has been naturally previously exposed to wt AAV 2 and demonstrates presence of neutralizing anticapsid antibodies.
(Chirmule et al., 1999), (Erles et al., 1999) which could be a potential problem for clinical success of AAV 2. Recently studies have demonstrated that generating recombinant AAV can effectively overcome wt AAV neutralizing antibodies (Mandell et al., 2004). Through modification of wt AAV and the creation of a variety of rAAV with uniquely tailored specificity, there is immeasurable potential for the use of AAV as a successful gene therapy technique.

In the present study we investigate not only differences in transduction profiles of specific AAV serotypes but also optimization of administration techniques with regard to AAV in the CNS. In recent years, scientists have conducted several studies aimed at improving transduction efficiencies of AAV vectors in different tissue types through modification of the AAV genome. The utilization of a “cross-packaging” strategy which essentially enables an unbiased direct comparison of the transduction rates without influence of ITRs on transgene expression has recently become a novel way to create recombinant AAV vectors (Rabinowitz and Samulski, 2000), (Rabinowitz et al., 2004), (Grieger and Samulski, 2005a), (Wu et al., 2006). To accomplish this the Cap genes of different serotypes are placed downstream of the AAV2 Rep genes which ultimately allows the generation of serotypes specific capsids while the packaged genomes within the capsids are identical, these are also referred to as hybrid viruses (Rabinowitz et al., 2002), (Wu et al., 2006). In addition to transcapsidation first described by Rabinowitz et. al. in 2002 there are several other techniques to accomplish the formation of a hybrid virus armed with specific modifications to enhance efficiency of gene uptake, transfer, and
expression in a specified therapeutic avenue. These include absorption modifications where the capsid surface is modified to carry a foreign antibody that will bind to the cell surface receptor of interest to increase absorption efficiency. Mosaic capsids are another method to increase transduction efficiency. This technique involves creating a mixture of viral capsid proteins from different serotypes at various ratios in order to combine tissue tropisms of interest (Xiao et al., 1999), (Rabinowitz and Samulski, 2000), (Kruger et al., 2008). Finally, chimeric capsids can also provide a means of increasing transduction of a particular specificity and this technique involves the packaging of capsid proteins with foreign peptide sequences, such as a hemagglutinin (HA) tag fused at either the N or C terminus of the capsid coding sequence to alter tissue tropism (Yang et al., 1999), (Bowles et al., 2003), (Wu et al., 2006).

Despite the rapidly growing body of knowledge in the field of AAV vector serology there is still much to be done. In addition to the 11 AAV serotypes that have been found, over 100 AAV variants have also been discovered. Delineating differences in transduction properties, and defining distinct characteristics of each virus is a burgeoning area of investigation. The technological advances in recombinant molecular biology have permitted investigators to construct a myriad of recombinant AAV vectors that can be customized to take advantage of differences in transduction profiles which can be targeted for several different cell types. In addition, optimization of administration techniques that increase distributive properties of AAV such as CED and mannitol have improved gene delivery capacity of these vectors suggesting near limitless potential they may
provide as a superior gene transfer vehicle through which to treat a variety of neurological diseases.

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**FIGURE 1: GFP EXPRESSION IS INCREASED FOLLOWING INTRACRANIAL ADMINISTRATION OF AAV5 USING CONVECTION ENHANCED DELIVERY.**

Percent positive area stained for anti-GFP is increased in the hippocampus, cortex and thalamus in 9 month old mice using CED method (5μl/min flow rate). The percent positive staining of GFP is significantly increased in the striatum and entorhinal cortex using the CED method compared to traditional injection method. The star (*) indicates significance with a p-value < 0.05. Magnification 100X on Olympus BX51.
**FIGURE 2: CED METHOD DOES NOT RESULT IN NEURON LOSS OR SIGNIFICANT INCREASE IN CD45 EXPRESSION.** Nissl staining showed no obvious loss of neurons in the hippocampus following either the CED injection method (panel B) or the traditional injection method (panel A). CD45 immunostaining is increased (panel E) but not significantly in animals receiving the CED injection (panel D) compared to animals receiving the traditional injection (panel C). Quantification of CD45 immunostaining is represented as % Area stain (panel E). Magnification 40X on Olympus BX51.
**FIGURE 3: COMPARISON OF AAV SEROTYPES 5, 8, AND 9 EXPRESSING GFP.**

Expression of GFP in the right and left hippocampus following a single CED intracranial injection into the right hippocampus of AAV5, 8, and 9 serotypes. AAV 9 was distributed to a significantly larger area of the hippocampus in both the left and right hippocampus (panels A and B) following treatment than both AAV serotype 8 and 5 (panel C-F). The star (*) indicates significance with a p-value < 0.05. Magnification 40X on Olympus BX51.
**FIGURE 4: GFP EXPRESSION IS INCREASED FOLLOWING ADMINISTRATION OF AAV5 USING CED AND SYSTEMIC MANNITOL.**

Percent positive area stained for anti-GFP is significantly increased in the right and left hippocampus in 11 month old mice following CED delivery of AAV5 and systemic mannitol pretreatment (panel C and D) compared to animals injected with AAV 5 and did not receive systemic mannitol (panel A and B). Panel E shows quantification of percent area positive stain for anti-GFP immunohistochemistry. The star (*) indicates significance with a p-value < 0.05. Magnification 40X on Olympus BX51 (panels A-D).
FIGURE 5: GFP EXPRESSION IS INCREASED IN THE THALAMUS AND ENTORINAL CORTEX FOLLOWING ADMINISTRATION OF AAV9 USING CED AND SYSTEMIC MANNITOL. Positive area stained for anti-GFP was intense throughout the entire hippocampus for all 11 month old mice following CED delivery of AAV9 with (panels A and B) or without systemic mannitol pretreatment (panels C and D). A larger area of positive area staining was evident in both the left and right entorhinal cortex and the right thalamus following CED delivery of AAV9 in animals pretreated with systemic mannitol (panels G and H) compared to animals that did not receive systemic mannitol (panels E and F). Magnification 40X on Olympus BX51 (panels A-H).
FIGURE 6: QUANTIFICATION OF ANTI-GFP STAINING FOLLOWING AAV 9 ADMINISTRATION. Quantification of Percent positive area stained for anti-GFP is significantly increased in the thalamus and entorhinal cortex in 11 month old mice following CED delivery of AAV9 and systemic mannitol pretreatment (panel B) No significant difference in anti-GFP staining was noted in the right or left hippocampus following AAV 9 administration and systemic mannitol pretreatment. The star (*) indicates significance with a p-value < 0.05.
FIGURE 7: TRANSDUCTION EFFICIENCY IN HIPPOCAMPUS FOLLOWING AAV5 AND AAV9 ADMINISTRATION. Transduction of GFP following AAV 5 and AAV9 administration into the hippocampus. GFP is expressed in the dentate gyrus granule and pyramidal cell bodies and axons in CA regions in the hippocampus primarily in the right hemisphere following a single intracranial injection of AAV 5 using CED method and systemic mannitol administration into the right hippocampus and in axonal projections in the left hippocampus (panels C and D; and E-H). GFP is expressed in neuronal cell bodies and axonal projections in all regions of right hippocampus following AAV 9 injections using CED and systemic mannitol administration (panels A and B). GFP expression is primarily in axonal projections eminating from the right hippocampus as well as in some neuronal cell bodies in the hilus of the dentate gyrus (panels I-K). Magnification 40X panels A-D; magnification 100X panels E-K.
FIGURE 8: TRANSDUCTION EFFICIENCY AND GFP EXPRESSION IN CELL TYPES FOLLOWING AAV 5 AND AAV9 ADMINISTRATION INTO HIPPOCAMPUS. GFP is expressed in the granule and pyramidal cell layers in the hippocampus as well as in neurons in entorhinal cortex primarily in the right hemisphere following a single intracranial injection into the right hippocampus. Panels A-D shows no co-localization (panel D) of astrocytic staining (GFAP) (panel B) and GFP positive labeling (panel A) in the dentate gyrus of the hippocampus (or in any other regions in the brain). This was noted in all animals receiving either serotype. Positive labelling in the left uninjected hemisphere was in a few cells in the hilus (panel E) of the DG. Co-localization of NeuN and GFP positive staining indicate that mainly neurons were transfected in this region (panel E-H). The majority of positive labelling was in the axons surrounding the cell bodies (panel E). Panels I-L show staining of the entorhinal cortex ipsilateral to the injection site. Co-localization of GFP positive cells and Neu-N (panel L) indicates that AAV transfec mainly neurons in this region. Magnification 100X.
Injected Side Dentate Gyrus

| A | GFP |
| B | GFAP |
| C | DAPI |
| D | MERGED |

Uninjected Side Dentate Gyrus

| E | GFP |
| F | NeuN |
| G | DAPI |
| H | MERGED |

Injected Side Entorhinal

| I | GFP |
| J | NeuN |
| K | DAPI |
| L | MERGED |
REFERENCE LIST


CONCLUSIONS

Alzheimer’s disease (AD) is the sixth leading cause of death in the United States and has been known as one of the most pervasive and devastating forms of dementia afflicting the aged population worldwide accounting for 70% of all dementia cases. Although the pathological etiology which causes a slow progressive decline in normal brain function is well characterized, we have yet to identify novel therapeutics that result in the cessation of the disease process and a marked overall significant and longterm improvement in cognitive function. Unfortunately, with the steady increase in life expectancy of our aged population, the number of individuals over the age of 65 affected with this disease is rapidly increasing and will continue to increase by approximatly 50% over the next two decades. Current estimations predict that 10 million baby boomers will develop AD in the coming years. AD causes emotional trauma to family members and caregivers in addition to generating a growing economic burden as healthcare costs continue to rise in accordance with the increasing number of individuals who suffer from the disease. Finding a novel therapeutic to delay onset of symptoms, improve cognitive function and potentially prevent further mental decline is essential to relieve both economic and emotional burdens which may plague many individuals in the near future.
The pathological disease process of AD that instigates the destruction of neurons in the CNS is thought to ultimately result in the progressive decline in mental function and cognitive abilities that manifest as a general loss of memory that is severe enough to interfere with daily life. This decline in memory can be an early clinical symptom of AD. Because there are several other types of dementia that have overlapping symptom patterns, a true diagnostic confirmation of AD is the presence of neuritic plaques composed of beta-amyloid (Aβ) and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein identified in post-mortem brain tissue.

The Aβ peptide which was first discovered in 1984 has for decades been identified as the central pathological feature from which all other neuropathological insults that occur in AD disease progression stem (Glenner et al., 1984). The Amyloid Cascade Hypothesis, first described by John Hardy and David Allsop circa 1991 suggests that it is the overproduction and ensuing accumulation of Aβ peptides, resulting from the aberrant processing of APP that is the fundamental event which then drives the formation of phosphorylated tau into neurofibrillary tangles which then result in disruption and disregulation of synaptic transmission, eventually causing the death of neurons and manifestation of dementia (Hardy and Allsop, 1991), (Selkoe, 1991a, 2001), (Tanzi, 2005). This theory has provided a roadmap of AD pathology for decades and is supported by a number of epidemiological genetic studies, molecular in vitro studies as well as in vivo studies using animal models of amyloid accumulation.
The role of APP protein metabolism in AD pathogenesis has been extensively studied, although its native biological role is still only speculative. Some studies suggest that APP is involved in synaptic formation and repair as well as neuronal plasticity (Wang et al., 2006), (Priller et al., 2006). Recent studies provide substantial evidence that APP expression is upregulated during neuronal differentiation following neural injury. Additionally, APP has been implicated to participate in cell signaling, long-term potentiation, and cell adhesion but only a limited number of studies have been done in this area to date (Zheng et al., 2006), (Priller et al., 2006). APP undergoes numerous post translation modifications (De Strooper et al., 2000) as well as many types of proteolytic processing. A number of genetic studies clearly illustrate that mutations in APP can cause the over production of Aβ peptides resulting in an increased ratio of the more fibrillogenic Aβ1-42/1-40 (Wang et al., 1999), (Ling et al., 2003). The APP gene has been localized to chromosome 21 and thus as a result AD-like neuropathology is consistently observed in Down’s syndrome or trisomy 21, due to the increased APP expression and thus higher Aβ levels (Glenner and Wong, 1984).

Unfortunately, the autosomal dominant genetic mutations that result in AD (originally identified during the 1990's) which include the APP mutations and the presenilin mutations (PS1 and PS2), occur infrequently and account for a miniscule percentage of the current population (approximately 1-2 %) which suffers from the disease (Selkoe, 1991b), (Nunan and Small, 2002), (Saito et al., 2003). These FAD (familial AD) mutations also result in very aggressive disease
progression and clinical symptoms can become apparent as early as 30 to 40 years of age but most commonly before the age of 65 (Selkoe, 1991c). Thus the overwhelming majority of AD cases occur in an age dependent manner at a rate that increases exponentially with age. Recently, a more thorough investigation into the cellular processes that mediate the sporadic late onset AD cases has become a dominant approach in the identification of specific targets in the treatment of AD.

The identification of the FAD mutations and the role they play in the production of the toxic Aβ peptides (due to aberrant changes in APP processing) gave rise to the first generation of Aβ overexpressing mouse models of AD. The PDAPP mouse model of AD was the first transgenic mouse containing the overexpressed mutant human APP and provided a new animal model through which to investigate mechanisms of amyloid deposition and removal (but did not contain tau pathology nor neuron loss) (Games et al., 1995). Other transgenic mouse models followed including the Tg2576 which was the first mouse model to show learning memory deficits that were closely correlated with age dependent amyloid deposition in a reliable fashion (Hsiao et al., 1996), (Morgan et al., 2000), (Ashe, 2006). Additional transgenic mouse models of AD include the double transgenic APP + PS1 mutations that harbor an increased aggressive pathology that begins at a younger age than that of the aforementioned single transgenic animals. These mice also show learning and memory deficits at an early age (which again correlated with paranchymal amyloid load) compared to
the mouse models with only the APP mutations (Borchelt et al., 1997), (Citron et al., 1998), (Holcomb et al., 1998), (Gordon et al., 2002).

The development of mouse models of Aβ accumulation and deposition paved the way for the development of several therapeutic target strategies that have evolved over recent years and yet unfortunately an effective therapeutic that can permanently reverse cognitive deficits suffered by AD patients remains elusive. Currently, there are two types of medications approved for the treatment of AD which include cholinesterase inhibitors and N–methyl–D-aspartate (NMDA) antagonist. Although some patients respond initially to these drugs they do not modify the disease process, only having a transient moderate effect on clinical symptoms with a host of unappealing side effects. The lack of disease modifying drugs for AD has fueled research efforts focused on development of novel therapeutics that have the potential to halt its progression and improve clinical symptoms.

The supposition that Aβ is the primary toxic pathogenic agent that accumulates subsequently inducing a number of other pathological abnormalities has provided the catalyst driving several therapeutic approaches aimed at lowering Aβ levels in the brain to treat AD (Hardy and Allsop, 1991), (Turner et al., 2004). Although Aβ toxicity has been extensively studied and is generally accepted, the specific form of Aβ aggregate that is the primary caustic moiety contributing to neuron dysfunction is still debated. Initial reports identified fibrillar Aβ as the major toxic species which subsequently resulted in decreased synaptic function and neuronal death. Current reports have refuted these initial findings
revealing that large Aβ deposits or plaques may in fact play a neuroprotective role. Their formation may provide a mechanism in which smaller soluble Aβ moieties are sequestered into larger insoluble aggregates preventing them from causing destruction. Specifically, the soluble oligomeric Aβ assemblies have been implicated as the driving factor in AD pathogenesis as they exhibit potent toxicity in their capacity to significantly decrease long term potentiation (LTP), contribute to learning and memory deficits in vivo and induce neuronal cell death in vitro (Lambert et al., 1998), (Naslund et al., 2000), (Klein, 2002), (Glabe, 2005), (Townsend et al., 2006), (Selkoe, 2008), (Varvel et al., 2008). Under normal physiological conditions Aβ is constitutively produced and catabolized in the brain which suggests that it may have a role as a physiological metabolite of APP processing. Furthermore, recent studies have shown that picomolar concentrations of Aβ containing both monomers and oligomers cause an increase in hippocampal long term potentiation and improved memory whereas higher concentrations in the nanomolar range, Aβ conversely causes a reduction in potentiation by modifying the activity of voltage-dependent Ca + channels and GTPase activity in neurons (Cirrito et al., 2003), (Puzzo et al., 2008); (Koudinov and Berezov, 2004). These results suggest the production of low levels of Aβ is a normal physiological process involved in modulating neurotransmission that occurs during learning and memory. Conversely, high levels of Aβ leads to deficits in neurotransmission eventually leading cognitive deficits manifested as dementia (Cirrito et al., 2003), (Haass and Selkoe, 2007), (Puzzo et al., 2008). The link between Aβ levels and its influence on learning and memory has been
supported by several in vivo studies involving APP knockout mice that show impairments in memory and LTP (Seabrook et al., 1999). These studies have provided one focus of disease modification strategies pertaining to drugs that can potentially inhibit Aβ formation, a process requiring β and γ secretase activity (Selkoe, 1991b), (Hardy and Selkoe, 2002). The identification of the essential enzyme BACE 1, a transmembrane aspartyl protease that is believed to contribute to the majority of β-secretase activity, required for the production of Aβ in the brain initially provided a novel therapeutic target for the treatment of AD. Thus finding effective inhibitors of BACE 1 was an early disease modification approach in the development of AD drug targets. The first BACE 1 knockout animal models were initially characterized in 2001, and fortuitously did not show any phenotypic deleterious consequence while showing a decrease in Aβ in the brain (Roberds et al., 2001), (Dominquez et al., 2001), (Turner et al., 2004). Unfortunately, BACE 1 inhibitors have shown little success which may be due to the fact that BACE 1 has recently been found to have several endogenous substrates. Upon further investigation it has been revealed that BACE 1 knockout mice have several deleterious phenotypes including premature death, cognitive deficits, and hypermyelination (Dominquez et al., 2001), (Turner et al., 2004).

Targeting γ-secretase activity provided yet another disease modifying therapeutic approach initially receiving a great deal of attention and support in the initial stages of developing drug targets for the treatment of AD. In vivo studies involving γ-secretase and PS1 knockout models reveal the essential role
it plays Notch signaling (an important transmembrane protein that is essential
during development) rendering these animals non viable (Shen, 1998). Although
γ-secretase inhibitors can successfully reduce Aβ synthesis, accumulation and
deposition, they have also been shown to improve cognition. Blocking BACE 1
activity also prevents Notch signaling and chronic dosing of this class of inhibitors
has been shown to have several deleterious effects including changes in the
spleen, thymus, and gastrointestinal changes (Searfoss et al., 2003), (Milano et
al., 2004), (Wong et al., 2004). The expression of both β and γ-secretase is
ubiquitous and evidence that they maintain important activity in several essential
physiological processes has limited their potential as viable therapeutic targets
for AD drug development.

Another therapeutic strategy that has recently gained more momentum in
the AD research community as a possible drug target aimed at modifying the AD
disease process, involves modifying mechanisms of Aβ degradation (as opposed
to targeting its synthesis through secretase inhibition). The normal synthesis and
catabolism of the Aβ peptide has been demonstrated to be a dynamic process
involving several endogenous β-amyloid degrading enzymes and include a family
of zinc metalloprotease expressed in the periphery and in the brain. In contrast
to inherited FAD, the accumulation of Aβ in late onset sporadic AD, has been
implicated to be the result of an imbalance between Aβ production and removal.
This deficiency in the removal process has been attributed to abnormal
accumulation and formation of Aβ deposits in the brain (Selkoe, 2001), (Saito et
al., 2003), (Turner et al., 2004). Deficits in the normal Aβ removal is mediated
through several contributing cellular processes, including drainage by diffusion of Aβ into the extracellular matrix, low-density lipoprotein related receptor protein 1 (LRP-1) mediated transport across blood vessels into the circulation acting as a peripheral sink (Sagare et al., 2007), (Deane et al., 2009), and enzymatic degradation through endogenously expressed proteases (Iwata et al., 2001), (Saito et al., 2001), (Saito et al., 2003). Although a growing number of endogenous proteases have been implicated in several studies as having amyloid degrading capabilities in vitro, only a small minority of these have been identified as major contributing factors in the pathological accumulation of Aβ peptides in the brain. Under normal physiologic conditions the catabolism of Aβ is mediated through the activation of neprilysin, endothelin converting enzyme, insulin degrading enzyme, angiotensin converting enzyme and matrix metalloproteases 2, 6 and 9, all of which belong to a family of zinc metalloproteinases (Mentlein et al., 1998), (Carson and Turner, 2002), (Mukherjee and Hersh, 2002), (Saito et al., 2003), (Turner et al., 2004), (Mouri et al., 2006), (Turner and Nalivaeva, 2007), (Miners et al., 2008). More recently, additional proteases that have been shown to have several cleavage locations along the Aβ peptide thus may contribute to maintaining homeostatic Aβ levels, include plasmin, mitochondrial peptidasome (PreP) and cathepsins B and D (Turner and Nalivaeva, 2007).

A growing body of evidence from both in vivo and in vitro data, including data presented in papers 1 and 2, support the importance of these proteases in preventing the aberrant accumulation of Aβ in the brain. A series of in vitro
studies first identified insulin degrading enzyme and neutral endopeptidase (later recognized as neprilysin) as proteolytic enzymes capable of cleaving the Aβ peptide at multiple sites. Subsequently, a series of studies published in 2001 and 2002 by Saido and Iwata et al clearly established NEP as a major modulator of Aβ metabolism in the brain (Iwata et al., 2000), (Iwata et al., 2001), (Saito et al., 2001), (Iwata et al., 2002). Furthermore, post mortem analysis of human AD brain tissue showed a distinct correlation between increases in Aβ deposition and decreases in NEP mRNA levels as well as NEP and IDE protein levels in a region specific manner compared to control individuals (Yasojima et al., 2001b), (Yasojima et al., 2001a), (Caccamo et al., 2005). Further investigation into the link between NEP and Aβ under normal physiological conditions has attempted to identify cellular mechanisms that may modify NEP gene expression in the AD brain environment. Pardossi-Piquard et al, in 2005, proposed that NEP gene transcription under normal physiological conditions can be upregulated by the βAPP intracellular domain (ACID), the cytosolic fragment generated following cleavage of APP by γ-secretase (Pardossi-Piquard et al., 2005). These data support the speculation that Aβ production/degradation may be closely linked but does not explain decreased NEP expression in the post mortem AD tissue or the net accumulation and deposition of amyloid in late onset AD cases. Interestingly, Jiang and colleagues in 2008 provide additional molecular mechanisms that contribute to Aβ metabolism, reporting that ApoE, a known risk factor in late onset AD, can bind Aβ and promote its degradation (Jiang et al., 2008). Once bound to Aβ it is postulated that the ApoE can acts as a chaperone allowing
efflux out of the brain across the blood brain barrier through specific receptors LRP1 or VLDL (Poirier, 2000), (Deane et al., 2004), (Zlokovic et al., 2005), (Deane and Zlokovic, 2007) or facilitate its degradation by NEP and IDE in the late endosome of microglia (Jiang et al., 2008). The efficiency with which ApoE binds soluble Aβ, either facilitating or slowing its degradation, is dependent on the lipidation state and specific isoform. Unfortunately the underlying molecular mechanisms modulating this process have yet to be clarified. The overall accumulation and deposition of Aβ, particularly in late onset sporadic AD, is likely the result of the additive effects of multiple deleterious events occurring over time which influence production, accumulation and fibrillogenesis, and degradation.

These data are supported by a large number of in vivo studies with transgenic animal models to further understand the roles of specific enzymes in the catabolism of Aβ. Initial reports of mice deficient of these enzymes, specifically NEP, ECE, and IDE demonstrated significant increases in steady state levels of Aβ in the brain. Eckman and colleagues reported that the dual knockdown of NEP and ECE resulted in an additive cumulative effect on amyloid load in the brain (Eckman et al., 2006). Additional studies using the direct transduction of neurons resulting in the increased expression in a variety of β-amyloid degrading enzymes including NEP, ECE, IDE, MMP-2 and 9, plasmin and cathepsin B were accompanied by the significant reductions in the overall Aβ in vitro (Marr et al., 2004), (Mueller-Steiner et al., 2006), (Yan et al., 2006). In vivo studies involving gene transfer methods for the treatment of Alzheimer’s pathology using viral vectors have had some success in reducing Aβ deposition
by overexpressing Aβ degrading enzymes, including NEP, ECE and IDE and more recently Cathepsin B, a cysteine protease also implicated in the lysosomal degradatory pathway of Aβ peptides (Marr et al., 2004), (Mueller-Steiner et al., 2006). The degree to which each enzyme contributes to the overall catabolism of Aβ is not entirely delineated. Some reports identify neprilysin as having the most pronounced effect on plaque load in the brain, while others identify MMP-9 as the most efficient proteolytic enzyme in the degradation of Aβ, compared to others, due to its ability to degrade both fibrillar and monomeric forms of Aβ in vitro (Eckman et al., 2003), (Yan et al., 2006), (Farris et al., 2007), (Hersh and Rodgers, 2008), (Spencer et al., 2008).

In paper 1 we investigate the effectiveness of overexpessing ECE on amyloid load in the more aggressive model of Aβ deposition double transgenic APP+PS1 mice using rAAV vectors as a method of gene therapy. Recombinant AAV has become widely used for the transduction of neuronal cells. AAV is nonpathogenic, has low immunogenicity, lacks all viral genes and is capable of long term expression in neurons. Thus making rAAV an attractive method for the use as a gene therapy vector for neurological disorders (Burger et al., 2005), (Mandel et al., 2006). Moreover, rAAV is currently being examined in a number of neurological clinical trials (Mandel and Burger, 2004). Six weeks post intracranial injection of rAAV-ECEHA serotype 5, into the right hippocampus and anterior cortical regions, we demonstrated successful transduction of several different neuronal cell types in the mouse CNS including CA4 neurons in the hilus and CA3 neurons of the hippocampus pyramidal cell layer (paper 1, figure
We also were able to show transduction of neurons in the dentate molecular layer, as well as a number of round cells possibly oligodendrocytes. The positive transduction of cells in the CNS although concentrated at the injection sites was also evident in regions distal from these regions and was detected in the striatum, entorhinal cortex, subiculum, and corpus callosum along the midline of the ipsilateral hemisphere. Additionally, some positive neurons in the contralateral left hippocampus were also detected. These findings are consistent with Burger and colleagues, and are believed to result from retrograde transport of the virus along axonal projections originating in the right hippocampus and synapsing in the left hippocampus (Burger et al., 2004). Positive transduction of neurons expressing ECE were evidenced by anti HA immunohistochemistry. Not only were we able to show an increase ECE expression following rAAV treatment, but were successfully demonstrate that the transduced neurons show a 70% increase in ECE activity compared to control rAAV-GFP animals. Furthermore, this increase in ECE activity resulted in significant decreases in total amyloid load and congophilic plaque load in these regions (paper 1, figure 5 and 6). Our data are consistent with reports that ECE can degrade Aβ in vitro, and that partial knockdown of the ECE gene leads to more rapid accumulation of Aβ in transgenic mouse models (Eckman and Eckman, 2005).

Our results from paper 1 showed that modification of ECE expression through the use of rAAV vectors contributes to degradation of Aβ, ultimately decreasing both total Aβ and congophilic load in the brain. In paper 2 we investigate whether overexpression of neprilysin will have an equal or greater
effect on Aβ levels. In vivo data demonstrating NEP contributes to Aβ
degradation, has been proven by results that show an increase in endogenous
Aβ levels in a gene dose dependent manner. Decreased NEP protein
expression thus contributes to plaque formation and increase CAA levels in NEP
knockout animal studies (Farris et al., 2007). Similar results were obtained with
NEP specific inhibitors that dramatically decrease NEP activity also resulting in
significant increases in endogenous Aβ levels in mice (Iwata et al., 2001),
(Eckman and Eckman, 2005), (Iwata et al. 2001), (Eckman et al. 2005).
Previously reported gene transfer studies demonstrated that treatment of amyloid
depositing APP transgenic mice using lenti virus and AAV to overexpress NEP
could significantly decelerate/decrease amyloid accumulation (Marr et al., 2003),
(Iwata et al., 2004); (Iwata et al 2004), (Marr et al 2003).

In Paper 2 we investigate both the effectiveness of rAAV to deliver NEP to
neurons in the mouse brain as well as novel means of increasing dispersion of
the virus and gene product to therapeutically relevent regions within the mouse
CNS. Specifically, we examine transduction efficiency and area of distribution of
two different NEP constructs (a membrane bound or an engineered secreted
form; NEP-n and NEP-s respectively) in the hippocampus and anterior cortex.
Conclusively, we compared the effects of overexpression of both NEP enzymes
on amyloid burden. Unlike previous gene transfer studies aimed at modification
of AD pathology, we administered rAAV by convection enhanced delivery
method, initially described by Krauze and collegues, in an effort to increase the
dispersion of the rAAV to larger regions in the brain parenchyma to improve gene
transduction efficiency and increase levels of NEP protein expression to obtain a larger global effect on total amyloid burden.

Six weeks post injections of rAAV vectors administration into the right hippocampal and anterior cortical regions, we were able to obtain successful gene transfer NEP constructs which include those previously mentioned, NEP-n and NEP-s as well as an inactive mutant NEP (NEP-m) construct. We were able to transduce a variety of neuronal cell types similar to those reported in paper 1 due to the fact that we used the same AAV serotype 5. Positive NEP expression revealed by anti-HA immunohistochemistry showed staining concentrated in areas at the site of injection in the hippocampus and anterior cortex as well as in sites distal from the injection site. NEP protein expression was evident throughout the entire hippocampus and some staining was observed in the entorhinal cortex in the right hemisphere (ipsilateral to the injection site). Minimal staining was also observed in the contralateral hippocampus of animals receiving the NEP-n and NEP-m rAAV. Staining was also seen in the striatum and corpus callosum and to small extent in the ipsilateral hemisphere along the midline. The staining for NEP-n and NEP-m was greater in intensity covering a larger region both in the hippocampus and anterior cortex compared to the staining for NEP-s (Paper 2, Fig. 2A and 2C). The NEP-s construct was engineered, through modification of the membrane binding sequence to instead encoding a signal peptide sequence in the hopes of creating a nucleus from which the expressed protein could diffuse to greater regions of the brain; thus eliminating the requirement for a large number of injections. The NEP-s expression profile
revealed less intensity of HA staining when compared to the NEP-m and NEP-n staining patterns. Cells within the hilus of the molecular layer were stained as were as cells in the CA3 region of the hippocampus. Although the NEP-s showed less intense staining this may have been due to the diffusion of the NEP protease throughout the extracellular matrix. Diffuse secreted NEP protein may not be concentrated in a specified region, making it more difficult to detect using anti-HA immunohistochemistry compared to the membrane bound NEP-n protease. An alternative explanation for the NEP-s staining pattern may be that once the NEP-s is secreted the NEP protein or possibly the HA-tag is more prone to degradation by other extracellular proteases that is would not otherwise be exposed to upon secretion not necessarily that this vector had lower transduction efficiency.

Although NEP-s staining was significantly less intense (compared to the other constructs), the anterior cortex showed significantly more intense staining compared to the hippocampus. This result may indicate that the signal peptide is processed more effectively in the cortex compared to the hippocampus. Despite the differences in staining intensity of the different NEP proteins, in vitro data confirmed that both NEP-n and NEP-s proteases showed significant levels of enzyme activity in the cell pellet, while the NEP-m version did not. Additionally, only the media from NEP-s transfected cells showed significant enzyme activity. The in vitro activity data was further corroborated with results from our examination of amyloid burden in APP+PS1 mice following treatment with the rAAV NEP constructs. NEP-n and NEP-s were able to significantly
reduce total Aβ deposition and congophilic deposits in the anterior cortex and hippocampus at the site of injection. Examination of Aβ staining in the contralateral hemisphere revealed that NEP-s rAAV significantly decreased total Aβ load in hippocampus and cortex compared to the control group. NEP-n treated animals showed significant decreases in total Aβ in the contralateral anterior cortex but not the hippocampus. More, interestingly, examination of congophilic staining on the contralateral hippocampal side revealed there was approximately 40% and 50% reductions with the NEP-n and NEP-s treatments respectively, but only the reduction with NEP-s was statistically significant compared to the control group. No reductions in congophilic staining were observed in the contralateral anterior cortex with either NEP constructs. The reduction on the contralateral hemisphere observed here is likely due to retrograde transport of the rAAV which has been previously reported (Burger et al., 2004; Iwata et al., 2004) and anterograde transport of the NEP protein. No expression was detected in the contralateral cortex which may be due a decreased number of neuronal connections between the right and left cortices compared to those in the hippocampus. This may explain our results which show a reduction in congophilic staining in the contralateral hippocampus and not the contralateral anterior cortex. We were also able to demonstrate decreases in amyloid load in aged 20 month old APP+PS1 mice following overexpression of NEP-s for five months (injections were administered bilaterally at 15 months). Total Aβ was reduced significantly in the left anterior cortex as well as in the left and right hippocampus following treatment with the NEP-s vector compared to
control untreated animals. Total congophilic staining was also significantly reduced in both contralateral and ipsilateral hemisphere in the hippocampi and cortices following intracranial administration of rAAV-NEP-s. Our results reported here in paper 2 are consistent with data previously reported by Iwata et al in 2004 using rAAV to overexpress NEP in young and old APP transgenic mice (Iwata et al., 2004). Our results also revealed that we were able to show more substantial decreases in Aβ load in both the ipsilateral and contralateral hemisphere's following NEP overexpression in the more aggressive amyloid depositing double transgenic mouse model in both young and old animals. Additionally, we demonstrate reductions were not limited to soluble forms of Aβ but significant reductions in congophilic load as well. An explanation for decreases in congophilic staining may be that NEP degrades oligomeric and monomeric soluble Aβ aggregates (Kanemitsu et al., 2003), (Yan et al., 2006), generally believed to be the toxic precursors to fibrillar insoluble aggregates preventing accumulation rather than actively degrading existing deposits. Ultimately, the engineered NEP-s construct was more efficient in decreasing fibrillar Aβ load in the contralateral hemisphere compared to the NEP-n and control vectors, while the NEP-n more efficiently decreased total Aβ load, in the ipsilateral hemisphere. As previously mentioned our findings showed that by implementing the use of a secreted form of the NEP protease in addition to using the CED method for intracranial administration, we were able to successfully improve the area of gene expression and further reduce amyloid load in the brain. We speculate that these findings result from the ability of NEP-s to diffuse
readily throughout the extracellular matrix upon secretion therefore obtaining access (more readily than membrane bound NEP-n) to both intracellular and extracellular pools of Aβ aggregates.

The AAV 5 serotype has previously been shown to effectively transduce non-dividing neuronal cells as well as glial cells in the mouse brain. Our data support this finding, demonstrating that by using rAAV serotype 5 expressing different human gene constructs of NEP or ECE; we can transduce several different neuronal and glial cell types. The expression profiles for the gene transfer experiments in papers 1 and 2 are also consistent with the previously published serological specificity of the AAV vectors (Alisky et al., 2000), (Burger et al., 2004), (Choi et al., 2005). Although the transduction of specific cell types were similar between study animals receiving ECE constructs and NEP constructs the staining patterns within the neurons were different. Unlike ECE, staining patterns for NEP revealed less intense staining in the cell bodies in the dentate molecular layer compared to the corresponding axonal projections of these cells which were very darkly stained. These results are consistent with data published by Huang et al. and Iwata et al., describing the normal neuronal metabolism of NEP processing first in the endoplasmic reticulum then in golgi apparatus of the cell body and finally transported along the axon via vesicles to the synaptic terminal (Iwata et al., 2004); (Iwata et al 2004), (Huang et al., 2006). Although we demonstrate that both ECE and NEP significantly decrease amyloid, due to the differences in injection methods we cannot directly compare the efficiency by which ECE and NEP decrease Aβ load in the APP+PS1 aminal
model. Despite this caveat, we can conclude that by implementing novel the CED delivery method in addition to modification of the NEP construct directing its secretion allowed significantly increased area of vector distribution thus exposing more cells to viral infection and ultimately enhancing gene expression levels following a single intracranial injection. Due to the differences in localization of endogenous amyloid degrading enzymes, a list which is continually growing, it is likely that they each contribute to the normal catabolism of Aβ maintaining its basal levels in the brain. In vitro studies have identified that each of the proteases have distinctive Aβ cleavage sites that yield unique fragments (Iwata et al., 2001)(Yan et al., 2006), (Nalivaeva et al., 2008). Additionally, some proteases, including NEP, IDE, ECE and ACE have been shown to only degrade monomeric soluble Aβ. NEP can also degrade toxic oligomer forms and still others including plasmin and MMP-9 have been shown to degrade fibrillar Aβ. It is therefore likely that the different proteases play an additive role in maintaining homeostatic endogenous levels of Aβ in the brain degrading different pools of Aβ aggregates ultimately preventing deposition.

Although we have identified rAAV as a potential gene transfer method through which we can upregulate amyloid degrading enzymes to modify AD pathology in vivo, there are limitations and obstacle that must be overcome to make gene therapy a more attractive therapeutic approach in the clinic. One of the current limitations of gene therapy is obtaining reliable gene transfer techniques that will provide therapeutically relevant levels of gene product. Specifically, with the treatment of neurological disorders, methods involving a
direct intracranial injection are limited due to gene transduction and expression
being confined to a very small region primarily at the site of injection. This
limitation would require numerous injections to overcome the site specific
expression of the gene of interest adding to potential complications. We address
this issue in paper 3 through the investigation of several novel mechanisms
aimed at enhancing gene transduction efficiency and improving distribution of
viral vectors within the parachyma to increase gene product. Specifically, we
investigated the different transduction characteristics of three different serotypes,
AAV5, AAV8, and AAV 9 each of which express GFP (green fluorescent protein)
in the mouse CNS. Additionally, we implemented a novel “convection enhanced
delivery technique” using a slightly modified reflux free cannula design (Krauze et
al., 2005a) to dispense AAV directly into the brain parenchyma. Finally, we
attempt to optimize the CED injection method and distribution parameters by
using a reflux free cannula design in addition to pre-treatment with hyperosmotic
mannitol, an agent that decreases intracranial pressure, administered by
intraperitoneal injection. Previous reports have demonstrated CED, which
employs positive pressure and a high flow rate, to dramatically facilitate
widespread distribution of AAV within the parachyma following a single
intracranial injection (Bankiewicz et al., 2000), (Cunningham et al., 2000). In
addition we have modified the CED cannula using a step design (Krauze et al.,
2005a) which we have customized to provide a more efficient method to prevent
reflux of AAV suspension following administration (paper 3, materials and
methods). A comparison of AAV vectors revealed that each AAV serotype
showed distinctly different transduction patterns with AAV 9 being the most efficient transducer of neurons in several brain regions in the ipsilateral hemisphere as well as the contralateral hemisphere (paper 3; figure 2). AAV 5 was the least efficient and showed the smallest region of vector distribution and GFP expression, while AAV 8 showed similar patterns of transduction to AAV 9 but showed a decreased amount of GFP expression. Our findings as well as others confirm that AAV 9 has significantly higher transduction efficiency in neurons in the CNS compared to AAV 8 and AAV 5 (Cearley and Wolfe, 2006). The differences in transduction patterns may be a result of different binding affinity to specific cell surface receptors as well as un-packaging and post-entry properties of each serotype (Hauck et al., 2004), (Wang et al., 2007). We speculate that in this study the efficiency of AAV 9 transduction of neurons may be due to its effective and stable binding to cell surface receptors possibly the LamR receptor or other potential receptors. It may also be that specific capsid proteins allow AAV 9 to be taken up by the cell more efficiently followed by more efficient translocation and viral uncoating and un-packaging within the nucleus of the neuronal cell and efficient DNA replication and expression properties. Additionally, anti-GFP immunohistochemistry revealed that AAV 9 treated animals had the most intense darkly stained pattern of both cell bodies and axonal projections, compared to animals treated with AAV 5 or 8. These results may indcicate that cells transduced with AAV9 may be producing more GFP which is later transported via vesicles within the cell body and along axons to regions that have connectivity with the injection site in both the ipsilateral and
contralateral hemispheres. Another possible explanation for the high transduction efficiency of AAV 9 recently proposed by several research groups, may be that more than one AAV 9 virion can transduce a single cell and that once a viral particle gains entry into the cell it may be easily transported axonally to other regions of the brain (Passini et al., 2002), (Burger et al., 2004), (Cearley and Wolfe, 2006). Despite differences in vector distribution and GFP expression, all three serotypes appear to primarily transduce neurons, which was proven by co-localization of double labeled NeuN, a neuronal marker, and GFP in the mouse CNS. Double labeling of GFP and GFAP, a marker for astrocytes, showed no co-localization of the two proteins indicating that AAV did not transduce astrocytes. These data are consistent with previous reports of AAV 5, 8 and 9, transduction patterns in the murine CNS (Burger et al., 2004), (Davidoff et al., 2005), (Broekman et al., 2006), (Cearley and Wolfe, 2006). A clear understanding of AAV characteristics that mediate cellular mechanisms underlying viral infectivity of specific cell and tissue type would advance efforts to create tailored recombinant AAV vectors with disease modifying specificity.

Once AAV 9 was identified in our study as having the highest transduction efficiency in the mouse CNS following a single CED intracranial injection, we investigated the effects that mannitol had on vector distribution. We determined that systemic pre-treatment with hyperosmotic mannitol significantly improved vector distribution of AAV. Previous reports have demonstrated improved distribution of AAV 2 following direct intracranial injection into the striatum (Burger et al., 2004) and thalamus (Fu et al., 2003) with intravascular
administration of mannitol or co-administration with rAAV into the parachyma (Mastakov et al., 2002). The effects of hyperosmotic mannitol in previous studies has been reported to cause an efflux of water out of surrounding tissue or brain cells and increasing the porosity of the extracellular matrix (Rapoport, 2001), (Neeves et al., 2007). Additionally, in 2002, Sandberg and colleagues investigated the affects of mannitol administration on CED delivery in the rat brain stem and did not find significant differences or improved distribution parameters for CED with either systemic administration or co-administration of mannitol. We assert that this may have been due to the slow infusion rate, 0.1µl/min, or the large molecule size of the molecule delivered (FITC-dextran) (Sandberg et al., 2002). In contrast, we use a high flow rate (2.5 µl/min) to administer very small AAV vector particles to the hippocampus resulting in widespread distribution of AAV throughout the entire hippocampal region and to regions distal to the site of injection in both the ipsilateral and contralateral hemispheres (paper 3; figures 3 & 4).

The efficacy and safety profile of the CED technique has been well established in both animal studies and in human trials (Laske et al., 1997), (Chen et al., 1999), (Bankiewicz et al., 2000). In recent years the CED method has gained acceptance and popularity as an extremely effective method to enhance delivery of an expanding list of different molecules with wide range molecular sizes including therapeutics agents, proteins, macromolecules, viral vectors, and nanoparticles in the brain (Cunningham et al., 2008), (Kikuchi et al., 2008), (Perlstein et al., 2008), (Song and Lonser, 2008), (Patel et al., 2009). Most
recently, this novel technique has been implemented in several clinical trials to
delivery therapeutics agents for the treatment of Parkinson’s disease and
glioblastoma multiform (Lopez et al., 2006), (Vogelbaum, 2007), (Vogelbaum et
al., 2007). Despite its clinical efficacy, the parameters under which CED can be
optimized providing further distribution are still under investigation. Our data are
consistent with previous reports describing significantly improved distribution of
rAAV in the parenchyma. We also demonstrate that by combining the CED with
mannitol further enhanced distribution parameters for CED administration of AAV
particles. In addition to CED and mannitol, investigators have used co-infusions
of heparin with rAAV 2 (Mastakov et al., 2002) (Nguyen et al., 2001) (Hadaczek
et al., 2006) or bFGF (basic fibroblast growth factor) (Hadaczek et al., 2004) to
significantly increase viral distribution in the CNS. Presumably, these molecules
increase viral spread by preventing or limiting the interaction between the virion
particle and its cellular receptor or co-receptor thus masking the a significant
portion of the potential viral receptors, thereby increasing the number of cells that
can potentially be infected by decreasing the cells that undergo infection by
multiple virion particles (Mastakov et al., 2002). Unfortunately, due to the
potential risks of these agents they may have little clinical appeal but identifying
an alternative method of improving viral spread in the CNS by mediating cell
surface receptor interactions is a viable approach to improving rAAV efficiency.

Overall we provide convincing evidence that Aβ levels can be modified
through regulation of endogenous β-amyloid degrading enzymes in the brain,
which suggests that these enzymes contribute to the normal catabolism of Aβ
peptides preventing its accumulation and deposition. Moreover, we show that significant up-regulation of these enzymes specifically NEP and ECE can be achieved through gene therapy using rAAV vectors that provide reliable, stable gene expression and viable gene product with significant enzyme activity. Furthermore, we demonstrate the potential for the use of rAAV as a clinical therapeutic in the treatment of AD by providing novel methods that improve viral distribution to larger regions of the brain parenchyma, a major obstacle that must be overcome in the field of gene therapy. We demonstrate that optimal injection techniques in addition to serotype specific AAV characteristics can significantly enhance transduction efficacy and overall disease modifying capacity. Further investigation must be done to fully understand and characterize cellular mechanisms underlying viral infectivity of AAV thus unlocking the true potential of their use in the treatment of neurological diseases.
REFERENCES CITED


contributes to efficient clearance of amyloid-beta peptide in mouse brain. JNeurosci 24:991-998.


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