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# Novel Amyloid Beta-Protein Degrading Enzymes - Membrane Type 1 Matrix-Metalloproteinase and Myelin Basic Protein

A Dissertation Presented

by

**Mei-Chen Liao**

to

The Graduate School

in Partial Fulfillment of the

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for the Degree of

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in

**Molecular and Cellular Biology**

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Abstract of the Dissertation

# Novel Amyloid Beta-Protein Degrading Enzymes - Membrane Type 1 Matrix-Metalloproteinase and Myelin Basic Protein

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The progressive accumulation of β-amyloid (Aβ) in senile plaques and in the cerebral vasculature is the hallmark of Alzheimer's disease (AD) and related disorders. Several major pathways for Aß clearance include receptor-mediated cellular uptake, blood-brain barrier transport and direct proteolytic degradation. My thesis focused on two novel Aß degrading enzymes- membrane type 1 matrix-metalloproteinase (MT1-MMP) and myelin basic protein (MBP).

Matrix metalloproteinase 2 (MMP2) was shown to be expressed in reactive astrocytes surrounding amyloid plaques and may contribute to Aß degradation. MT1-MMP is the physiological activator for the zymogen pro-MMP2. In addition to MMP2, its activator MT1-MMP was also expressed in reactive astrocytes surrounding amyloid deposits in transgenic mice. Using a cell-based system MT1-MMP overexpression can degrade exogenous Aß peptides. Purified MT1-MMP degraded both soluble and fibrillar

Aß peptides and this activity was blocked by specific MMP inhibitors. Mass spectrometry analysis identified multiple cleavage sites on Aß. Furthermore, *in situ* experiments showed that purified MT1-MMP degraded parenchymal fibrillar amyloid plaques that form in the brains of transgenic mice. Together, these findings indicate that MT1-MMP possesses Aß degrading activity *in vitro*.

MBP is the major structural protein component of myelin sheath. MBP possesses endogenous serine proteinase activity and can undergo autolysis. Recently, our lab showed that MBP binds Aß and inhibits Aß fibril formation. Aß peptides were degraded by purified human brain MBP and recombinant human MBP, but not an MBP fragment without autolytic activity. Similarly, cells expressing MBP degraded exogenous Aß peptides. In addition, purified MBP also degraded assembled fibrillar Aß. Mass spectrometry analysis identified multiple cleavage sites on Aß. Further, *in situ* experiments showed that purified MBP can degrade parenchymal amyloid plaques and cerebral vascular amyloid that form in the brains of transgenic mice. Lastly, series of C-terminal deletion MBP proteins were tested for autolysis and Aß degradation activities to identify the responsible region. Together, these findings indicate that purified MBP possesses Aß degrading activity *in-vitro.*

To conclude, I characterized two novel Aβ degrading enzymes *in-vitro*. Further *in vivo* experiments are needed to investigate the role of these two Aβ degrading enzymes in the pathology of AD.

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# CHAPTER 1-Introduction

## 1.1 – Amyloid β-Protein and Alzheimer's disease

Alzheimer's disease (AD) is the most common cause of senile dementia and increases in frequency with age. Clinically, progressive memory loss results from neural and synaptic loss in the cortex and limb structures, including hippocampus and amygdale. In later stages of disease, damage in the cortical and subcortical regions results in loss of cognitive abilities including speech and praxis and impaired motor abilities (*1*). The progressive accumulation of β-amyloid (Aβ) in senile/ neuritic plaque and the cerebral vasculature is the hallmark of AD and widely used in the pathological diagnosis of AD. Aβ is generated by proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase (*2-3*). The main species of Aβ are 40 and 42 amino acids in length. Aβ42 is much more amyloidogenic than Aβ40 because of its two additional hydrophobic amino acids at the carboxyl terminal end and it deposited first (*4*). The Aβ42 peptide is predominant in the senile plaque, forming a β-sheet structure, which is insoluble and resistant to proteolysis. Mutations in APP are found in certain familial forms of AD. Most mutations occur near the β- and γ-cleavage sites and promote the generation of Aβ40 and Aβ42 (*5-6*). There are other mutations within the Aβ sequence resulting in so-called familial forms of cerebral amyloid angiopathy (CAA), which results from diffuse Aβ deposition in the neuropil and leads to recurrent and often fatal hemorrhagic episodes at mid-life (*7*). These mutations usually result in altering the fibrillogenic property of Aβ. For example, the APP E693Q (Dutch) (*8*)and APP D694N

(Iowa) (*9*) mutations promote amyloid fibril formation due to loss of a negative charge at their respective sites (*10*). Pathologically, these mutations are characterized by severe cerebral vascular fibrillar amyloid deposition, but only diffuse parenchymal Aβ deposits with the absence of fibrillar plaques (*11-13*).

## 1.2 – Amyloid β-Protein Clearance

The mechanisms of the pathogenesis of AD remain unclear and are debated. Research on early-onset familial Alzheimer's disease (FAD) showed that mutations in the genes encoding APP and presenilins 1 and 2 cause increases in the level of Aβ42. Increasing the production of Aβ plays an important role in FAD pathology. However, the Aβ42 elevation is rare in the sporadic form of AD (SAD), which account for 95% of all disease cases (*14*). Aβ is constantly produced and maintained at very low levels in the normal physiological condition in the senile plaque-free brain (*15-16*). The steady-state level of Aβ is controlled by a balance between production and clearance (*17*). Studies revealed that Aβ has been turned over rapidly in human central nervous system, approximately 8% per hour (*18*). Therefore, it has been hypothesized that SAD may be caused by the impaired clearance of Aβ peptides. Several major pathways for Aβ clearance have been investigated as showed in Figure 1.1 (*1*) including receptor-mediated cellular uptake such as binding to receptor for advanced glycation end products (RAGE) in endothelial cells (*19-20*) also cellular uptake through low-density lipoprotein receptor-related protein-1 (LRP-1) (*21-22*), transportation cross the blood-brain barrier via the P-glycoprotein (PgP/MDR1/ABCB1) efflux pump (*23-24*) and direct proteolytic degradation.

It has been published that several proteinases or peptideases are capable of degrading Aβ including neprilysin (NEP) (*25-26*), insulin-degrading enzyme (IDE) (*27*), uPA/tPA-plasmin system (*28*), endothelin-converting enzyme (ECE) (*29*), angiotensin-converting enzyme (ACE) (*30*), gelatinase A (defined as MMP2) (*31-32*), gelatinase B (MMP9) (*33*), Prep (*34*) and Cathepsin B (*35*). Each of these enzymes has multiple cleavage sites in the Aβ peptide (*1*). These Aβ-degrading enzymes are present in multiple cellular compartments, for example, NEP, ECE, MMP2, and MMP9 are secreted into the extracellular space or expressed at the cellular surface such as IDE. And presequence proteinase (PreP) and IDE can be found in mitochondria involved in mitochondrial Aβ clearance (*36-37*). Essentially all Aβ-degrading enzymes can degrade monomeric Aβ, whereas aggregated forms can only be degraded by MMP9 (*38*), plasmin (*39*), and Cathepsin B (*35*). However, only NEP, IDE and ECE and MMP9 have been directly shown to have a significant role in the regulation of Aβ levels in the brains of experimental animal models (*26, 40-41*).

## 1.3 – Membrane Type 1 - Matrix Metalloproteinase

Matrix metalloproteinases (MMPs) are $Ca^{2+}$- and $Zn^{2+}$-dependent enzymes involving in degradation and remodeling of the extracellular matrix (ECM) (*42*). All MMPs are synthesized as prepro-enzymes and secreted as inactive pro-MMPs. MMPs have conserved unique sequence PRCG(V/N)PD in the prepeptide domain and zinc binding motif HEXXHXXGXXH in the catalytic domain and they share conserved overall structure (*43*). They are regulated at the transcriptional and post-transcriptional level, and their activity is also controlled via specific activators and inhibitors (*44*). The

MMPs are synthesized as latent or inactive proeznymes or zymogens due to an intramolecular complex formed between the single cysteine residue in their pro-peptide domain and the essential zinc atom in the catalytic domain which blocks the active site. The activation of MMPs requires the disruption of the cysteine-zinc switch by proteolytic removal of the propeptide domain or by ectopic perturbation of the cysteine-zinc interaction, which is referred as the “cysteine-switch” mechanism (*45*).

MT1-MMP is the first MMP to be identified as an integral membrane protein with a single transmembrane domain and a short cytoplasmic C-terminal tail (*46*). MT-MMPs contain an RXK/RR furin-like enzyme recognition motif between their pro-peptide and catalytic domains, and are activated by furin before they reach the cell surface (*47*). MT1-MMP was demonstrated to be a primary activator of pro-MMP2. MT1-MMP is inhibited by the endogenous tissue inhibitors of MMPs (TIMP-2) and recruits pro-MMP2 forming a ternary complex. Then, adjacent uninhibited MT-1 cleaves and the tethered pro-MMP2 (*48*).

MT1-MMP is expressed in lung, placenta, kidney, ovaries, intestine, prostate and spleen (*49*), and mesenchymal tissues of embryos such as bone, muscle and fibroblastic tissues (*46*). In human brain, MT1-MMP was found in human glial cells in white matter (*50*). MT1-MMP is involved in the breakdown of various ECM components including collagens type Ⅰ, Ⅱ, and Ⅲ, laminin 1 and 5, fibronectin, fibrin, and proteoglycans (*44*). This function allows it to play multiple functions in normal biological processes, such as embryonic development, blastocyst implantation, ovulation, bone remodeling, reproduction and apoptosis (*51*) or in pathological processes, such as rheumatoid arthritis,

cardiovascular disease, cancer and tumor invasion and metastasis, would healing, cell migration, invasion and cancer metastasis and angiogenesis (*52*).

MMP2 expression and activity is induced by pathogenic Aβ in human cerebrovascular smooth muscle cells, which have been used as an *in vitro* CAA model system (*53*). In astrocytes the activity of MMP2 is also increased in the presence of Aβ (*54-57*). Activated astrocytes, which are found surrounding the amyloid plaques in the human AD brain and APPsw (Tg-2576) transgenic mice, which over-express human Swedish mutant AβPP and produce high levels of wild-type Aβ, have shown to be involved in the Aβ degradation in the extracellular space (*57-60*). Expression of MT1-MMP is induced in U87 human glioma cells in response to β-amyloid peptide (*60*). It has been reported that membrane-type matrix matelloproteinases induce cleavage and shedding of the APP ectodomain and that one of its cleavages sites is on the Aβ peptide (*61*). However, any role of MT1-MMP in the degradation of Aβ peptides and the pathology of AD is unknown.

## 1.4 – Myelin Basic Protein

The "classic" myelin basic proteins (MBPs) are major structural components of myelin sheaths accounting for 30% of total myelin protein. There are four different major isoforms- 17.3, 18.5 20.2 and 21.5 kDa generated from alternative splicing. The 18.5 kDa variant is abundant in mature myelin and is well-studied (*62*). The 18.5 kDa variant is composed of 180 amino acids including many basic residues- 19 Arg and 12 Lys. MBP has no cysteine residue to form disulfide bonds and results in a flexible structure to allow adaptation to environmental conditions. It is predominantly found to

have a random-coil structure in aqueous solutions but gains much more secondary structure such as β-sheet or α-helix in the presence of lipid (*63*). Residues 85-96 can form an amphipathic α-helix responsible for membrane anchoring (*64-65*). MBP has numerous post-translational modifications including N-terminal acylation, methylation of arginine, phosphorylation, deamination and deimination. These modifications can change the net charge of the protein, which results in a family of microheteromers or charge isoforms (*66*). Through chromatography, 8 isoforms (C1-C8) have been resolved. C1 is the most positively charged while C8 is the least charged. One of major functions of MBP is to hold together the cytoplasmic leaflets of myelin membranes in order to maintain proper compaction of the myelin sheath through the electrostatic interaction between the positive arginine and lysine residues of MBP with the negatively charged phosphate groups of membrane lipid (*67*). MBP plays an important role in the pathology of mutiple sclerosis (MS), which is an autoimmune disease characterized by demyelination within white matter (*68*). There are different theories for the pathology of MS such as the sensitizing of T cells in the periphery through molecular mimicry of MBP or the aberrant modification of MBP, which may destabilize myelin membrane structure (*69*). Recently it has been reported that MBP has autocleavage activity, which may be responsible for generating an immunodominant peptide that sensitizes T cells to attack MBP in the myelin sheath resulting in myelin breakdown. In this study, serine residue 151 was identified as the active serine residue involved in this autocatalysis (*70*). Besides mediating its autolysis no specific substrate has been reported for MBP.

In the early stages of AD, significant and diffuse myelin breakdown in the white matter is evident (*71*). Also, in the white matter regions there are much fewer fibrillar

amyloid deposits than are commonly found in grey matter region. Recently our lab has shown that MBP strongly interacts with Aβ peptides and prevents their assembly into mature amyloid fibrils (*72-73*). Through the course of these studies we found that upon longer incubations the levels of Aβ peptides were reduced upon treatment with MBP. In light of this observation, coupled with the report that MBP possesses proteolytic activity, it may be possible that Aβ is a substrate for MBP due to their strong interaction.

## 1.5 – Figures

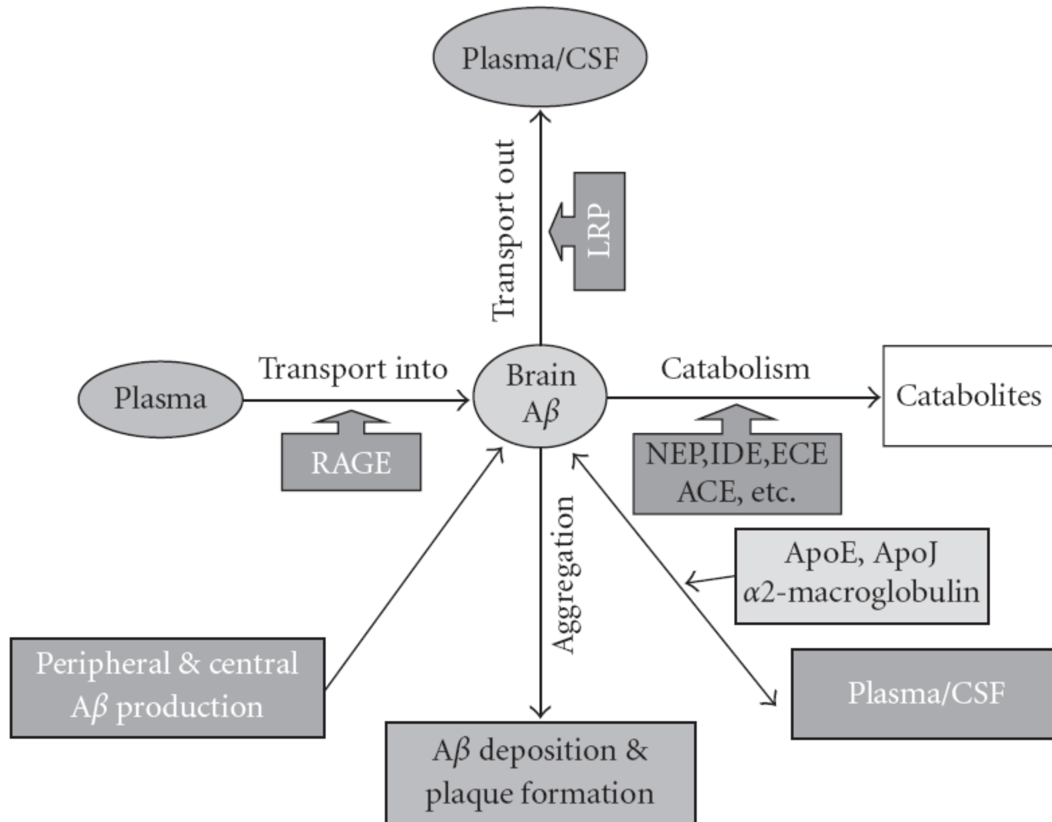



**Figure 1.1 Brian Aβ clearance mechanisms.** The steady-state level of Aβ is controlled by a balance between production and clearance. Several major pathways for Aβ clearance have been proposed including receptor-mediated cellular uptake, blood-brain barrier transport into the circulation, and direct proteolytic degradation (*1*).

# CHAPTER 2 - Degradation of Soluble and Fibrillar Amyloid β-Protein by Matrix Metalloproteinase (MT1-MMP) *in vitro*

## 2.1 - Summary

The progressive accumulation of β-amyloid (Aβ) in senile plaques and in the cerebral vasculature is the hallmark of Alzheimer's disease and related disorders. Degradation of Aβ by specific proteolytic enzymes is an important process that regulates its levels in brain. Matrix metalloproteinase 2 (MMP2) was shown to be expressed in reactive astrocytes surrounding amyloid plaques and may contribute to Aβ degradation. Membrane type-1 (MT1)-MMP is the physiological activator for the zymogen pro-MMP2. Here, we show that in addition to MMP2, its activator MT1-MMP is also expressed in reactive astrocytes in regions with amyloid deposits in transgenic mice. Using a Cos-1 cell expression system, we demonstrated that MT1-MMP can degrade exogenous Aβ40 and Aβ42. Purified soluble form of MT1-MMP degraded both soluble and fibrillar Aβ peptides in a time dependent manner, yielding specific degradation products. Mass spectrometry analysis identified multiple MT1-MMP cleavage sites on soluble Aβ40 and Aβ42. MT1-MMP-mediated Aβ degradation was inhibited with the general MMP inhibitor GM6001 or the specific MT1-MMP inhibitor tissue inhibitor of metalloproteinases-2. Furthermore, *in situ* experiments showed that purified MT1-MMP degraded parenchymal fibrillar amyloid plaques that form in the brains of Aβ precursor

protein transgenic mice. Together, these findings indicate that MT1-MMP possesses Aβ degrading activity *in vitro*.

## 2.2 – Introduction

A key pathological feature of Alzheimer's disease (AD) is the progressive accumulation of β-amyloid (Aβ) in senile plaques and the cerebral vasculature. Aβ is derived from amyloidogenic processing of the amyloid precursor protein (AβPP), which involves sequential cleavage by β-secretase and γ-secretase (*2-3*). The steady-state level of Aβ peptides in the brain is controlled by a balance between production and clearance (*17*). Impaired clearance of Aβ peptides is likely important in the pathogenesis of AD, especially in the more common sporadic form. Several major pathways for Aβ clearance have been identified including receptor-mediated cellular uptake, blood-brain barrier transport (*21-22*), and direct proteolytic degradation.

Several proteinases/peptidases which can degrade Aβ have been reported including neprilysin (NEP) (*25-26*), insulin-degrading enzyme (IDE) (*27*), plasmin (*28*), endothelin-converting enzyme (ECE) (*29*), angiotensin-converting enzyme (ACE) (*30*), myelin basic protein (*74*), matrix metalloproteinase (MMP) 2 (*31-32*), and MMP9 (*33*). Regarding MMP2, it has been reported to cleave Aβ peptides at several sites (*32*). MMP2 expression and activity are induced in cultured human cerebrovascular smooth muscle cells in response to pathogenic Aβ (*53*). Also, in astrocytes the activity of MMP2 is increased in the presence of Aβ (*54-57*). Reactive astrocytes are found in regions with fibrillar amyloid deposits in brain tissue of human AD subjects and of APPsw (Tg-2576)

transgenic mice and have been shown to participate in the Aβ degradation in the extracellular space (*57-60*).

MMP2 is released in a latent form (pro-MMP2) that requires activation by membrane-type 1 (MT1)-MMP (*75*). MT1-MMP was the first MMP to be identified as an integral membrane protein with a single transmembrane domain and a short cytoplasmic C-terminal tail (*46*). MT1-MMP is inhibited by the endogenous tissue inhibitor of MMPs 2 (TIMP-2) and recruits pro-MMP2 forming a ternary complex. Then, adjacent uninhibited MT1-MMP cleaves the tethered pro-MMP2 (*48*). MT1-MMP is expressed in a variety of tissues including brain (*50*). In addition to activating pro-MMP2, MT1-MMP is involved in the breakdown of various extracellular matrix components including collagens, laminins, fibronectin, and proteoglycans (*44*). This function enables it to participate in numerous normal biological processes, such as reproduction, embryonic development, wound healing, angiogenesis, and apoptosis (*51*) or in pathological processes, such as rheumatoid arthritis, cardiovascular disease, tumor invasion and metastasis (*52*).

Expression of MT1-MMP can be induced in human glioma cells and human cerebrovascular smooth muscle cells in response to Aβ (*60*). It was reported that MT-MMPs induce cleavage and shedding of the AβPP ectodomain and that one of these cleavage sites is within the Aβ peptide region (*61*). However, any role for MT1-MMP in the degradation of Aβ peptides and the pathology of AD is unknown. In the present study, it shows that, like MMP2, MT1-MMP is expressed in reactive astrocytes in regions with fibrillar microvascular amyloid deposits in a human AßPP transgenic mouse model. Subsequently, it shows that MT1-MMP expressed in Cos-1 cells is capable of degrading

soluble Aβ40 and Aβ42 peptides. A purified soluble truncated form of MT1-MMP also degraded soluble and fibrillar Aβ *in vitro*. Mass spectrometry analysis identified multiple MT1-MMP cleavage sites on soluble Aβ40 and Aβ42. Furthermore, *in situ* experiments show that purified soluble MT1-MMP can degrade parenchymal fibrillar amyloid plaques that form in the brains of AβPP transgenic mice. Together, these data indicate that MT1-MMP possesses Aβ degrading activity.

## 2.3 – Materials and Methods

*Reagents and Chemicals* - Synthetic Aβ40 and Aβ42 peptides were synthesized by solid-phase Fmoc (9-fluorenylmethoxycarbonyl) amino acid chemistry, purified by reverse phase high performance liquid chromatography, and structurally characterized as previously described (*76*). Thioflavin-S (Th-S), Thioflavin-T (Th-T) and TIMP2 were purchased from Sigma-Aldrich (St. Louis, MO). The general MMP inhibitor GM6001 was purchased from Calbiochem (La Jolla, CA).

*AβPP Transgenic Mice* - Generation of Tg-SwDI transgenic mice on a pure C57BL/6 background was previously described (*77*). These mice express low levels of human Swedish/Dutch/Iowa mutant AβPP in neurons under control of the mouse Thy1.2 promoter. Tg-SwDI mice accumulate extensive cerebral microvascular fibrillar amyloid. Brain tissues from homozygous 24 months old Tg-SwDI and similarly aged control non-transgenic mice were used in this study. In other experiments brain tissues from 18 months old Tg2576 mice, a model of AD-like parenchymal fibrillar amyloid plaques, was used (44).

*Immunofluorescent Labeling* - Immunofluorescent stainings were performed on paraffin sections as recently described (*77*). The following primary antibodies were used for immunostaining: monoclonal antibody 66.1 (1:300), which recognizes residues 1 to 5 of human Aβ (*19*); rabbit polyclonal antibody to collagen type Ⅳ (1:100; Research Diagnostics Inc., Flanders, NJ); mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) for identification of astrocytes (1:300, Chemicon); mouse monoclonal anti-keratan sulfate antibody for the detection of activated microglia (clone: 5D4, 1:200, Seikagaku Corporation, Japan); rabbit polyclonal antibody to MT1-MMP (1:100; Triple Point Biologics Inc., Forest Grove, OR); rabbit polyclonal antibody to MMP2 (1:100; Sigma). Primary antibodies were detected with goat anti-rabbit IgG (Alex 594; 1:2500; Molecular Probes Inc., Eugene, OR) or/and donkey anti-mouse IgG (Alex 488; 1:2500; Molecular Probes, Inc., Eugene, OR). Th-S staining for fibrillar amyloid was performed as described (*78*).

*Gelatin Substrate Zymography* - Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA). Full-length MT1-MMP and pro-MMP2 in pcDNA3.1 plasmids were the kind gifts of Dr. Jian Cao (Department of Medicine, Stony Brook University, NY, USA). Triplicate near confluent cultures were transfected with plasmids for expression of pro-MMP2, MT1-MMP or both pro-MMP2 and MT1-MMP using FuGENE 6 (Roche, Indianapolis, IN). Transfected cells were incubated in serum-free culture media and 72 h. The conditioned culture media samples were collected and

aliquots were electrophoresed on 8% SDS-polyacrylamide gels containing 0.1% gelatin at 100 V for 2 h at 22℃. The gels were removed and incubated in rinse buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 2.5% Triton-X 100) for 3 h with several changes, washed 3 x 10 min with $ddH_2O$, then incubated in assay buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$) overnight at 37℃, washed 3 x 10 min with $ddH_2O$, stained with 0.25% Coomassie Brilliant Blue R-250 and then destained. Gelatinolytic MMP activity was observed as clear zones of lysis in the gels.

*Aβ Degradation in Cos-1 Cells Expressing Human MT1-MMP* - Triplicate near confluent cultures were transfected with purified empty pcDNA3.1 plasmid DNA or full-length MT1-MMP in pcDNA3.1 DNA by FuGENE 6 treatment (Roche, Indianapolis, IN), followed by addition of 2 μg/ml of Aβ40 or Aβ42 in serum-free media for 48 h. The culture media samples were collected and cell lysates were prepared. Aβ in the cell culture media samples was quantitatively analyzed by immunoblotting and sandwich ELISA analysis as described above.

*Quantitative Immunoblotting* - Samples containing MT1-MMP or Aβ were added directly into SDS-PAGE sample buffer, and stored at –70℃. Aliquots were loaded onto 12% or 10-20% polyacrylamide gels, electrophoresed and transferred onto Hybond-ECL nitrocellulose membranes (Amersham, Arlinton Heights, IL) at 100 V for 1.5 hour at RT. Membranes were blocked in 5% milk/PBS/0.05% Tween20 (PBS-T) for 1 h at RT. Primary antibodies were added (RP1-MMP14 for MT1-MMP; mAb20.1 for Aß) for 1 h at RT, washed 3 x 5 min with PBS-T. Horseradish peroxidase-conjugated mouse sheep

anti-rabbit or anti-mouse IgG (1:5000 Amersham-Pharmacia, Piscataway, NJ), and washed 3 x 5 min with PBS-T. Bands were visualized using the ECL detection method (Amersham-Pharmacia, Piscataway, NJ). Quantitation of MT1-MMP or Aβ bands was performed using a VersaDoc Imaging System (BioRad, Hercules, CA) and the manufacturer's Quantity Oneton software.

*Aβ ELISA Analysis* - The levels of soluble Aβ40 and Aβ42 peptides were measured using a quantitative sandwich ELISA as previously described (*77*).

*Purification of MT1dTM Protein* - The cDNA for a soluble, truncated form of MT1-MMP encoding residues $Met^1$-$Gly^{535}$ that lack the carboxyl-terminal transmembrane and cytosolic domains of full length MT1-MMP (MT1dTM) in pSG5 expression vector was the kind gift of Dr. Jian Cao (Department of Medicine, Stony Brook University, NY, USA). Two hundred ml of serum-free conditioned media from Cos-1 cells overexpressing soluble MT1-MMP were passed through gelatin-agarose (Sigma-Aldrich, St. Louis, MO) to remove any gelatinases and then concentrated using an Amicon ultrafiltration unit (NMWL 5000 membrane) (Millipore, Bedford, MA). The enzymatic activity of purified MT1dTM was determined using the specific substrate- Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg (Bachem, California, CA).

*In vitro Soluble Aβ Degradation* - Synthetic Aβ40 or Aβ42 were first dissolved in dimethyl sulfoxide to a concentration of 1 mg/ml. 40 nM of purified MT1dTM were incubated with 1 μM of synthetic Aβ40 or Aβ42 in zymogen buffer (50 mM Tris-HCl,

pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$) at 37℃ for specific lengths of time. The Aβ levels were measured in the samples by SDS-PAGE on 10-20% polyacrylamide Tris-Tricine gels and subsequent quantitative immunoblotting (as described above). In some experiments the selective MMP inhibitors GM6001 (100 μM; Calbiochem) or TIMP2 (40 nM; Sigma) were added.

To visualize MT1-MMP generated Aβ cleavage products N-terminal biotin labeled Aβ40 or Aβ42 were incubated with purified MT1dTM for 24 h at 37 C. The samples were diluted in the sample buffer containing 9M urea/ 5% acetic acid and methyl green. For analysis an acid/urea 22% polyacrylamide gel was prepared and pre run anode to cathode at 250 V for 30 min at 4℃ in 5% glacial acetic acid running buffer (*79*). Following the pre run, the samples were loaded on the gel and electrophoresed at 4 ℃ from anode to cathode with increasing the voltage every 15 min as follows: 25, 50, 100, 200 volts and then 275 volts for 15 h until the end of the run. Prior to transfer, the acid/urea gel was neutralized in a glass tray by washing 5x with Tris-HCl/glycine transfer buffer on a rocking platform for 15 min. Then the gel was transferred to PVDF membranes be electroblotting for 2.5 hr (80V) at 4℃. After transfer, the membrane was boiled in PBS for 5 min in a glass dish and was cooled down in PBS. The membrane was blocked in 5% milk/PBS/0.05% Tween20 (PBS-T) for 1 h at RT. The membrane was incubated with streptavidin-horseradish peroxidase (1:5000 dilution) for 1 h at RT, and washed 3 x 5 min with PBS-T. Bands were visualized using the ECL detection method as described above.

*Mass Spectrometry* - 40 nM purified MT1dTM was incubated with 1 μM synthetic Aβ40 or Aβ42 in zymogen buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$) at 37℃ for 2 days. After incubation, the samples were dried in a rotary evaporator (Savant, Farmingdale NY), suspended in 20 μl of 0.1% TFA, ZipTipped using μC18 tips (Millipore, Milford, MA) and then eluted to the target. The addition of 1 μl of matrix consisting of acetonitrile/0.1% trifluoroacetic acid containing ∝-cyano-4-hydroxy cinammic acid (CHCA, 5 mg/ml) was dried on the sample plate. Samples were run on a *Voyager-DE STR* (Applied Biosystems, Framingham, MA) using a matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometer system operated in the reflector mode unless otherwise indicated. The mass scale (*m/z* 500-5000) was calibrated with a mixture of peptides or internal calibration was performed using a matrix ion at *m/z* 568.1330 and Aβ42 peptide amino acid 1-13 *m/z* 1561.6672. For samples acquired in the linear mode, 1 μl was dissolved in 10 μl of a 50% solution of acetonitrile/0.3% trifluoroacetic acid containing sinapinic acid (10 mg/ml) and dried on the sample plate. The mass scale (*m/z* 1000-25000) was calibrated with myoglobin (400 fM/μl).

*In vitro Fibril Aβ Degradation* - To prepare amyloid fibrils, 5 mM Aβ42 in DMSO was diluted in PBS to 100 μM, vortexed for 30 sec, and incubated at 37 ℃ for 5 days (*80*). Triplicate samples of 10 μM of aged fibrillar Aβ was then incubated with 100 nM of purified MT1dTM in absence or presence of 100 μM GM6001 at 37 ℃ for 5 days. After digestion, the remaining fibrillar Aβ was quantitated using a Th-T fluorescence binding assay. Briefly, 5 μl of 100 μM Th-T was added to 100 μl of sample, mixed and

incubated at RT in the dark for 10 min. Th-T fluorescence, an indicator of fibril Aβ binding, was measured at $\lambda_{ex}$ of 446 nm and $\lambda_{em}$ of 490 nm.

*Transmission Electron Microscopy* - Sample aliquots were deposited onto carbon-coated copper mesh grids. Sample grids were allowed to stand for 60 sec, and excess solution was wicked away. Sample grids were then negatively stained with 2% (w/v) uranyl acetate and allowed to dry. The samples were viewed with an FEI Tecnai 12 BioTwin transmission electron microscope at 80 kV, and digital images were taken with an Advanced Microscopy Techniques camera.

*In-Situ Fibrillar Amyloid Plaque Degradation* - For this analysis the well-characterized Tg2576 (APPsw) mouse model of AD that develops abundant fibrillar amyloid pathology (*81*) was used. Brains were removed from anesthetized 18 months old Tg2576 mice after perfusion with cold saline and snap-frozen on dry ice. Five-μm cryostat sections were collected on slides. Every other section was flipped 180° so that identical faces of adjacent sections were exposed (*80*). Paired adjacent sections (one incubated with zymogen buffer, the other with 100 nM purified MT1dTM in absence or presence of 100 μM GM6001) in triplicate were incubated at 37℃ for 5 days, stained with thioflavin-S (ThS), and then imaged with fluorescence microspcopy. The parenchymal plaque amyloid area of ThS fluorescence was determined using image analysis software (Image J). Fractional area was compared between paired sections.

*Statistical Analysis* - Data were analyzed by Student's t-test at the 0.05 significance level.

## 2.4 – Results

**MT1-MMP and MMP2 Are Expressed in Brain Regions with Prominent Cerebral Microvascular Fibrillar Aß Deposits in Tg-SwDI Mice**

Previously, MMP2 was found increased in reactive astrocytes adjacent to parenchymal amyloid plaques in aged AβPP transgenic mouse brain (38). MMP2 expressed by reactive astrocytes is implicated in extracelluar Aβ catabolism (38). We have generated the Tg-SwDI mouse model, which develops early-onset and progressive accumulation of regional cerebral microvascular fibrillar amyloid deposition (*77*). Th-S staining of brain sections of aged Tg-SwDI mice revealed extensive fibrillar Aβ accumulation in the microvessels of the thalamus, but not in the cortex (Figure 2.1 A,B). MMP2 is also expressed in cells in the thalamus where fibrillar microvascular Aß accumulates, but not the cortex (Figure 2.1 C,D). MMP2 is expressed as an inactive zymogen (pro-MMP2) requiring proteolytic activation by MT1-MMP. Labeling for MT1-MMP also showed strong expression by cells in the thalamus where fibrillar microvascular Aβ accumulates, but not the cortex (Figure 2.1 E,F).

**MT1-MMP and MMP2 are Selectively Expressed in Reactive Astrocytes in Brain Regions with Microvascular Fibrillar Amyloid Deposits in Aged Tg-SwDI Mice**

To identify the cell type that expresses MMP2 and MT1-MMP near microvascular amyloid deposits the Tg-SwDI mouse brain sections were double immunolabeled for GFAP to detect reactive astrocytes and either MMP2 or MT1-MMP (Figure 2.2). Immunolabeling for MMP2 and its activator MT1-MMP strongly co-

localized with GFAP-positive cells. Immunolabeling for activated microglia in these tissue sections failed to show co-localization with MMP2 or MT1-MMP (data not shown). These data show that like MMP2, its activator MT1-MMP is selectively expressed in reactive astrocytes near cerebral microvascular fibrillar amyloid deposits in aged Tg-SwDI mouse brain.

### Exogenous Aβ40 and Aβ42 Degradation in Cos-1 Cells Expressing Human MT1-MMP

We next determined if MT1-MMP, like MMP2, could play a role in Aβ degradation using a cell culture expression system. Cos-1 cells were chosen since they do not normally express either MT1-MMP or MMP2. Therefore, Cos-1 cells were transfected to express pro-MMP2 alone, MT1-MMP alone or both pro-MMP2 and MT1-MMP. Post transfection, the cells were incubated with serum-free media for an additional 48 h. The cell lysates were collected and analyzed by immunoblotting using an anti-MT1-MMP antibody demonstrating protein expression in the cells transfected with the MT1-MMP plasmid (Figure 2.3 A). The culture media samples were collected and subjected to gelatin zymography to assay for MMP2 activities (Figure 2.3 B). The zymography assayed showed that pro-MMP2 was only expressed in the Cos-1 cells transfected with the pro-MMP2 plasmid. Whereas pro-MMP2 alone migrated at ≈72 kDa the co-transfection with pro-MMP2 and MT1-MMP exhibited activated MMP2 which migrated as a doublet at ≈66 kDa. These experiments demonstrated that MT1-MMP expressed in Cos-1 cells was enzymatically active.

To determine if MT1-MMP expressed in Cos-1 cells could degrade Aβ, the cells were transfected with either empty plasmid vector (pcDNA3.1) or the MT1-MMP plasmid vector. Post transfection; the cells were incubated with 2 μg/ml of freshly prepared soluble Aβ40 or Aβ42 in serum-free media for an additional 48 h. The cell lysates were collected and analyzed by immunoblotting using the anti-MT1-MMP antibody confirming MT1-MMP protein expression in the transfected cells (Figure 2.4 A). Although small amounts of Aβ peptides were found associated with the cells present in the cell lysates, there was no difference in the amounts between control and MT1-MMP expressing Cos-1 cells (data not shown). The media samples were collected and analyzed for Aβ40 and Aβ42 peptide levels by immunoblotting using monoclonal anti-Aβ (Figure 2.4 B,D, respectively) and by quantitative ELISA measurements (Figure 2.4 C,E, respectively). These results indicate that both Aβ40 and Aβ42 were strongly reduced by about 50% and 70%, respectively, in MT1-MMP transfected Cos-1 cells.

### ***In Vitro* Aβ40 and Aβ42 Degradation by Purified Soluble MT1-MMP**

MT1-MMP is normally expressed as a membrane bound enzyme. However, a soluble transmembrane domain-lacking form of MT1-MMP (MT1dTM) can be used to study the proteolytic function of the enzyme in solution. Therefore, we used purified MT1dTM protein to investigate if Aβ peptides could be degraded *in vitro*. Aβ40 or Aβ42 (1 μM) was incubated at 37℃ in the presence or absence of purified MT1dTM (40 nM) up to 24 h. At designated time points, samples were collected and analyzed for Aβ levels by quantitative immunoblotting using the anti-Aβ mAb. As shown in Figure 2.5,

Aβ40 and Aβ42 were degraded by purified MT1dTM *in vitro* in a time-dependent manner with ≈40% reduction in the levels of both peptides in 24 h.

To confirm that the enzymatic activity of purified MT1dTM was required for the Aβ degradation *in vitro,* we used the general MMP inhibitor GM6001 and specific MT1-MMP inhibitor TIMP; Aβ40 was incubated with purified MT1dTM at 37℃ in the presence or absence of GM6001 or TIMP2 for 24 h. The immunoblotting data presented in Figure 2.6 show that MT1dTM mediated Aβ40 degradation was blocked by GM6001 and TIMP2, indicating that the proteolytic activity of MT1dTM was responsible for the observed Aβ degradation.

The data above demonstrate that MT1-MMP exhibits proteolytic activity towards Aβ40 or Aβ42 *in vitro* or in Cos-1 cells expressing MT1-MMP. However, these analyses only show loss of intact Aβ peptides based on immunoblotting or ELISA analysis. To identify MT1-MMP-mediated Aβ cleavage products, we performed acid/urea gel analysis, a technique that can resolve low molecular mass peptides. For this analysis soluble amino-terminal, biotinylated Aβ40 or Aβ42 peptides were incubated with purified MT1dTM for 48 h. Following incubation, the samples were electrophoresed on a 22% polyacrylamide acid/urea gels, transferred to membranes, and analyzed for biotin-labeled intact Aβ and amino-terminal fragments using a streptavidin-horseradish peroxidase conjugate. As shown in Figure 2.7, the levels of intact biotin-labeled Aβ40 and Aβ42 were markedly reduced by digestion with MT1dTM and several biotin-labeled amino-terminal fragments of each Aβ peptide were observed. These data further confirm that MT1dTM degrades soluble Aβ *in vitro*.

To identify specific cleavage products, synthetic Aβ40 or Aβ42 was digested with purified MT1dTM and analyzed by MALDI-TOF mass spectrometry (Figure 2.8). The major fragments generated from proteolytic cleavage of Aβ40 were similar to those generated from Aβ42. Several cleavage sites were identified mainly around V12 through L17, generating major fragments of 1-14 to 1-17, which were consistent with the amino terminal major cleavage products shown in the acid/urea gels (Figure 2.7).

### ***In Vitro* Fibrillar Aβ Degradation by Purified Soluble MT1-MMP**

Aβ peptides largely accumulate in the AD brain in the form of fibrillar amyloid deposits. To determine whether MT1-MMP could degrade fibrillar Aβ, we prepared aged fibrillar Aβ42, subsequently incubated it with purified MT1dTM at 37℃ for 5 days and measured the remaining fibrillar Aβ using a Th-T fluorescence binding assay. Figure 2.9 A shows a >50% reduction in the Th-T fluorescence signal in the fibrillar Aβ sample treated with MT1dTM. Importantly, the MMP inhibitor GM6001 largely blocked MT1dTM mediated fibrillar Aβ degradation, indicating the loss of fibrillar Aβ was dependent on the enzymatic activity of MT1dTM. To further confirm this finding at the ultrastructural level fibrillar Aβ was incubated in the absence or presence of purified MT1dTM for 5 days, and then TEM analysis was performed to visualize the extent fibrillar Aβ structure (Figure 2.9 B). Fibrillar Aβ incubated with MT1dTM showed a marked reduction in the number and length of amyloid fibrils. Together, these data indicate that MT1dTM is also capable of degrading the assembled fibrillar form of Aβ.

### **MT1dTM Degrades Parenchymal Fibrillar Aβ Plaques *in Situ***

The above data showed that purified MT1dTM was capable of degrading soluble and fibrillar synthetic Aβ peptides *in vitro*. We next determined if purified MT1dTM could degrade actual amyloid deposits that form in the brains of AβPP transgenic mice. To do this, adjacent brain slices of aged Tg2576 mice, which contain abundant amyloid plaques, were incubated at 37℃ for 5 days with buffer alone or purified MT1dTM in the presence or absence of the MMP inhibitor GM6001. After incubation the sections were stained with Th-S, and the area of fluorescence between matching fibrillar plaque deposits from adjacent sections was measured. The area of Th-S fluorescence of adjacent brain sections did not show a difference when incubated with buffer alone, while the area of parenchymal amyloid plaque deposits was significantly decreased ($p < 0.001$) in the brain sections incubated with purified MT1dTM (Figure 2.10). Importantly, amyloid plaque degradation by purified MT1dTM was effectively blocked with the MMP inhibitor GM6001. These results suggest that purified MT1dTM is capable of degrading fibrillar amyloid plaques in brain tissue.

## 2.5 – Discussion

In the present study we show that MT1-MMP, the physiological activator of pro-MMP2, can degrade soluble and fibrillar forms of Aβ *in vitro*. The MMP superfamily consists of secreted and membrane types of metalloproteinases largely involved in degradation and remodeling of the extracellular matrix. It was previously shown that MMP2 and MMP9 are produced by reactive astrocytes surrounding amyloid plaques in aged human AβPP transgenic mice (*38, 54-57*). Similarly, we found that MMP2 and its activator MT1-MMP are expressed in reactive astrocytes in the brain regions with

microvascular amyloid deposits in aged Tg-SwDI mice (Figure 1 and 2). Previously, we reported that pathogenic Aβ stimulates the expression and activation of MT1-MMP and MMP2 in the cultured human cerebrovascular smooth muscle cells (*53, 82*). Consistent with these earlier *in vitro* findings, in aged Tg-SwDI mice we also found MMP2 and MT1-MMP expression in the smooth muscle cell medial layer of meningeal vessels that occasionally contained fibrillar Aβ deposits (data not shown).

MT1-MMP was shown to degrade Aβ peptides in both its natural transmembrane form expressed in Cos-1 cells and as a purified soluble form lacking the carboxyl-terminal transmembrane region (MT1dTM). However, more robust degradation of Aβ peptides was observed when MT1-MMP was expressed in Cos-1 cells compared to using the soluble MT1dTM form *in vitro*. This disparity may reflect different levels of MT1-MMP present in each type of experiment or, more likely, is a consequence of the soluble MT1dTM exhibiting less enzymatic activity than its natural transmembrane counterpart (*83*). In any case, soluble MT1dTM provided a useful tool to demonstrate degradation of soluble and fibrillar Aβ in *in vitro* and *in-situ* experiments.

Most of the well-known Aβ-degrading enzymes such as endothelin converting enzyme, insulin degrading enzyme, and neprilysin largely show degradative activity toward soluble forms of Aβ but not on fibrillar Aβ. However, plasmin and MMP9 are two Aβ-degrading enzymes shown to be capable of degrading fibrillar Aβ *in vitro* (*39, 80*). In the initial experiments of the present study we mainly focused on the degradation of the monomer form of soluble Aβ in the *in vitro* assay or in Cos-1 cells. However, in Figure 2.4 above the prominent Aβ monomer a faint Aβ dimer band was observed which was also degraded in Cos-1 cells expressing MT1-MMP. Although we did not

investigate the specific degradation of other soluble forms of Aβ such as trimers, tetramers, or higher order oligomers our experiments showed that fibrillar Aβ was degraded by soluble MT1dTM. On the basis of this latter finding we predict that other soluble oligomeric forms of Aβ are likely degraded by MT1-MMP although this will need to be confirmed.

Several structural models of amyloid fibrils have been proposed (*84*). The common feature is the β-pleated sheet structure perpendicular to the fibril axis with a hairpin loop at the C-terminus. The conversion of soluble Aβ to fibrillar amyloid is accompanied by an increased resistance to proteolytic degradation (*85*). In this regard it is noteworthy that purified soluble MT1dTM can similarly degrade fibrillar Aβ *in vitro* and fibrillar amyloid deposits in brain tissue sections of human AβPP transgenic mice. MMP9, like MT1-MMP, cleaves between residues $Ala^{30}$-$Ile^{31}$ (*80*). This site is exposed on the surface of Aβ fibrils allowing access for cleavage by MMP9 and MT1-MMP (*80*). In contrast, Aβ fibrils were observed to be more resistant to degradation by MMP2. It was proposed that the major MMP2 cleavage site of $Leu^{34}$-$Met^{35}$ within the hydrophobic domain of Aβ would be inaccessible within an amyloid structure (*85*). Collectively, these findings suggest that the various Aβ-degrading enzymes likely work at different sites in the brain for Aβ catabolism. For example, secreted Aβ-degrading enzymes such as IDE, MMP2, and MMP9 may effectively target soluble forms of Aβ in interstitial fluid whereas membrane-bound Aβ-degrading enzymes such as neprilysin and MT1-MMP are better suited for deposited fibrillar Aβ or Aβ associated with cell surfaces.

MT1-MMP appears to be highly expressed in brain regions exhibiting amyloid pathology and neuroinflammation (Figure 2.1 and 2.2). On the other hand, in normal brain or in the absence of amyloid pathology little, if any, expression of MT1-MMP is observed. This suggests that under normal conditions MT1-MMP likely has little involvement in regulating basal brain Aβ levels compared with other Aβ-degrading enzymes that are constitutively expressed. However, when amyloid deposition and neuroinflammation occur, as in AD, reactive astrocytes and vascular smooth muscle cells markedly increase their expression of MT1-MMP which may then play a significant role degrading soluble and deposited Aβ peptides. This increased expression in response to amyloid deposition implies that MT1-MMP may be an opportunistic Aβ-degrading enzyme. Future experimentation will be needed to determine if MT1-MMP does indeed contribute to Aβ degradation *in vivo* under pathological conditions when it is likely expressed.

Various members of the MMP superfamily may play some role regulating the levels of Aβ in the CNS. For example, MMP2, MMP9, and MT1-MMP possess Aβ-degrading activity (*33, 86*). MMP2, MMP3, MMP9 and MT1-MMP exhibit increased expression in response to Aβ (*53, 60, 87*). However, the protein levels and activity of MMP2, MMP3, and MMP9 showed no difference in the frontal cortex of AD patients compared with control patients (*88*). This may reflect a very limited, focal expression in specific cells that was not discerned in this study. It was reported that MT1-MMP, MT3-MMP and MT5-MMP have α secretase-like shedding activity on AβPP which would preclude Aβ formation (*61*). More specifically, recombinant MT3-MMP showed multiple cleavage sites on AβPP within the Aβ domain. Since the shedding pattern for

MT1-MMP and MT3-MMP are very similar, MT1-MMP may also cleave AβPP within the same sites. Here, the mass spectrometry data showed an MT1-MMP cleavage site at the $His^{14}$-$Gln^{15}$, which is the same as an MT3-MMP shedding site on AβPP (30). However, Aβ peptide was not degraded by recombinant MT3-MMP or by cells expressing MT3-MMP. Therefore, regarding MT-MMPs the Aβ degradation activity appears specific to MT1-MMP.

In conclusion, we have demonstrated that MT1-MMP is selectively expressed in reactive astrocytes near fibrillar amyloid deposits in human AβPP transgenic mouse brain. MT1-MMP was found to degrade soluble Aβ40 and Aβ42 as well as fibrillar amyloid. Together, these data suggest MT1-MMP could function as an opportunistic Aβ degrading enzyme when expressed by reactive astrocytes adjacent to fibrillar amyloid deposits. Future *in vivo* studies are needed to determine its role in relation to other well known Aβ degrading enzymes in regulating Aβ levels in brain.

## 2.6 – Figures

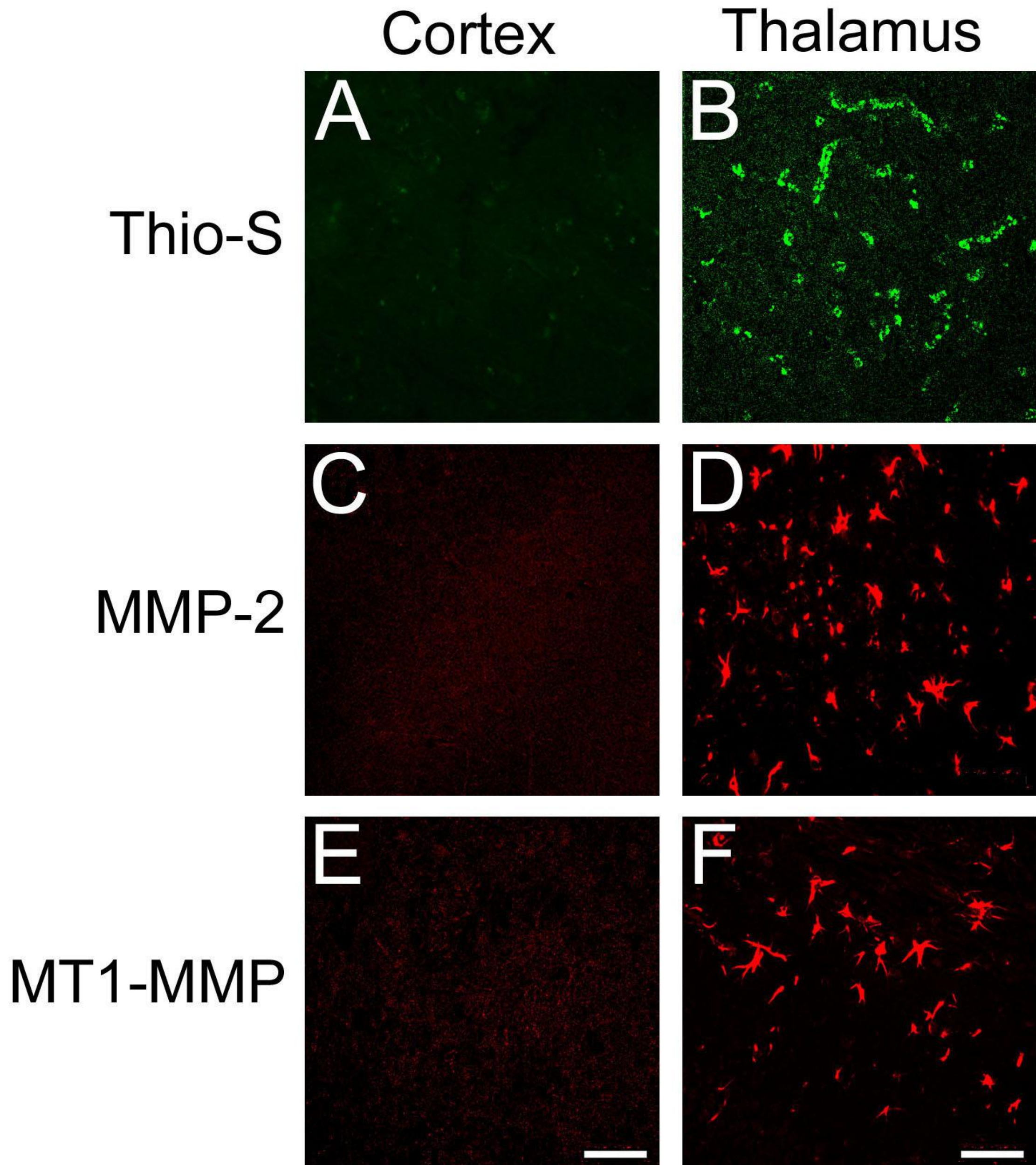



**Figure 2.1 MT1-MMP and MMP2 are expressed in regions of fibrillar Aβ accumulation in Tg-SwDI mouse brain.**
Brain sections from 24-month-old Tg-SwDI mice were labeled for fibrillar Aß using Th-S (green) showing that the cortex (A) lacks appreciable fibrillar amyloid whereas the thalamic region (B) contains extensive microvascular amyloid accumulations. Immunolabeling for MMP2 or MT1-MMP (red) in adjacent brains sections shows weak expression in the cortex (C and E, respectively) but strong expression in the

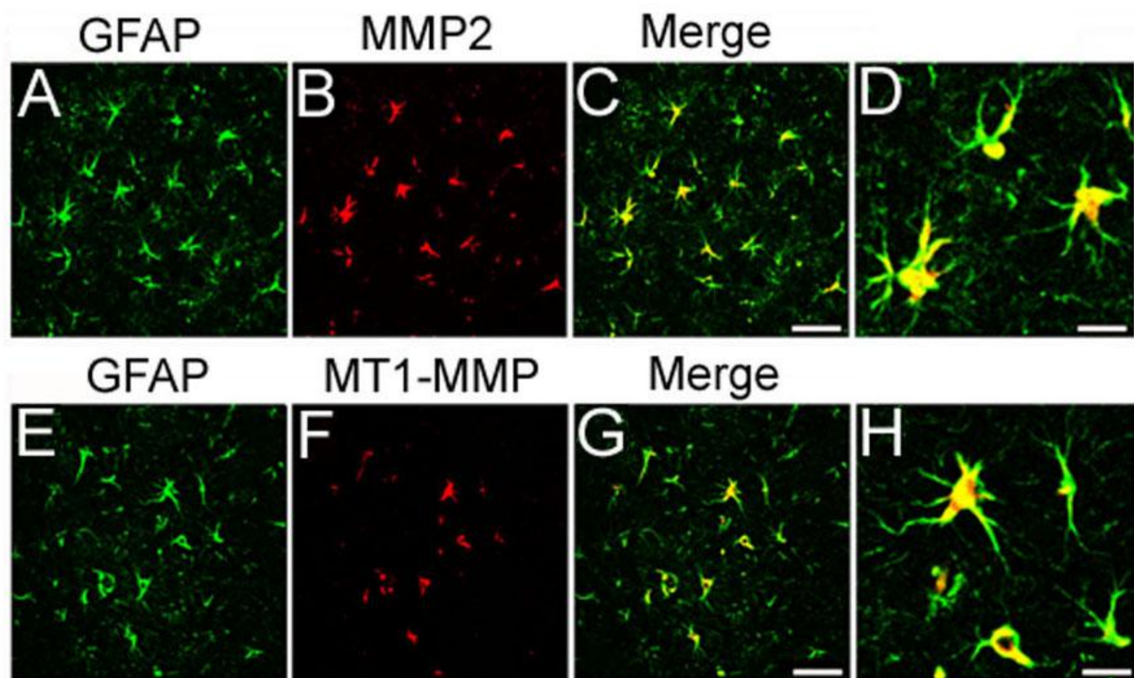



**Figure 2.2 MT1-MMP and MMP2 are expressed in reactive astrocytes near fibrillar microvascular amyloid deposits in Tg-SwDI mouse brain.**
Brain sections from 24-month-old Tg-SwDI mice were double immunolabeled for GFAP to identify astrocytes (green) and MMP2 or MT1-MMP (red). The thalamic region, which contains extensive microvascular fibrillar amyloid, is shown. Numerous reactive astrocytes were observed (A and E) as well as strong immunolabeling for MMP2 (B) and MT1-MMP (F). Merging of the images showed strong co-localization of GFAP and MMP2 (C) or MT1-MMP (G). Scale bars = 50 μm. Higher magnifications of the merged images are shown in (D) and (H), respectively. Scale bars = 10 μm

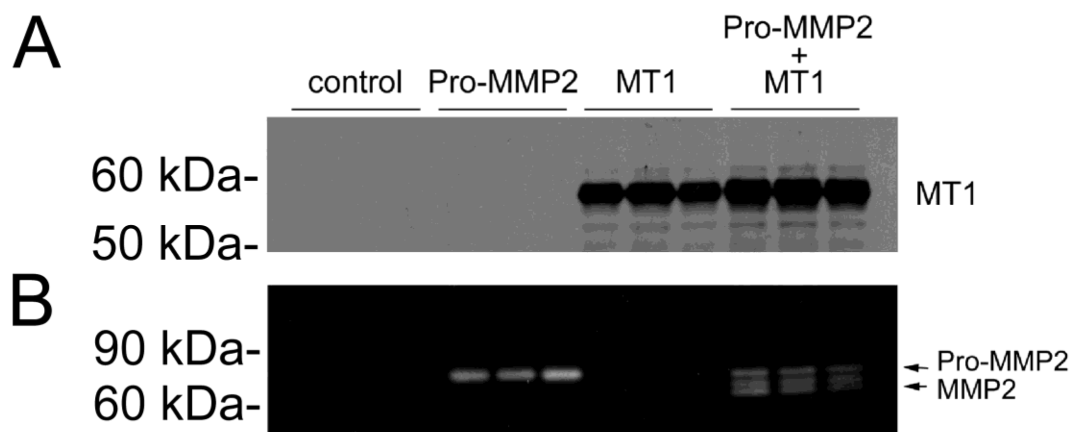



**Figure 2.3 Activation of pro-MMP2 by MT1-MMP expressed in Cos-1 cells.** Triplicate cultures of Cos-1 cells were transfected with empty plasmid vector (pcDNA3.1), Pro-MMP2 vector alone, MT1-MMP vector alone, or both Pro-MMP2 vector and MT1-MMP vector. Twenty four hours post transfection, the cells were incubated in serum-free medium for an additional 48 h. (**A**) The cell lysates were collected and analyzed by immunoblotting by using anti-MT1-MMP**.** (**B**) The culture media samples were collected and analyzed by gelatin zymography. Co-expression of Pro-MMP2 and MT1-MMP led to conversion of pro-MMP2 to MMP2 demonstrating the MT1-MMP was proteolytically active.

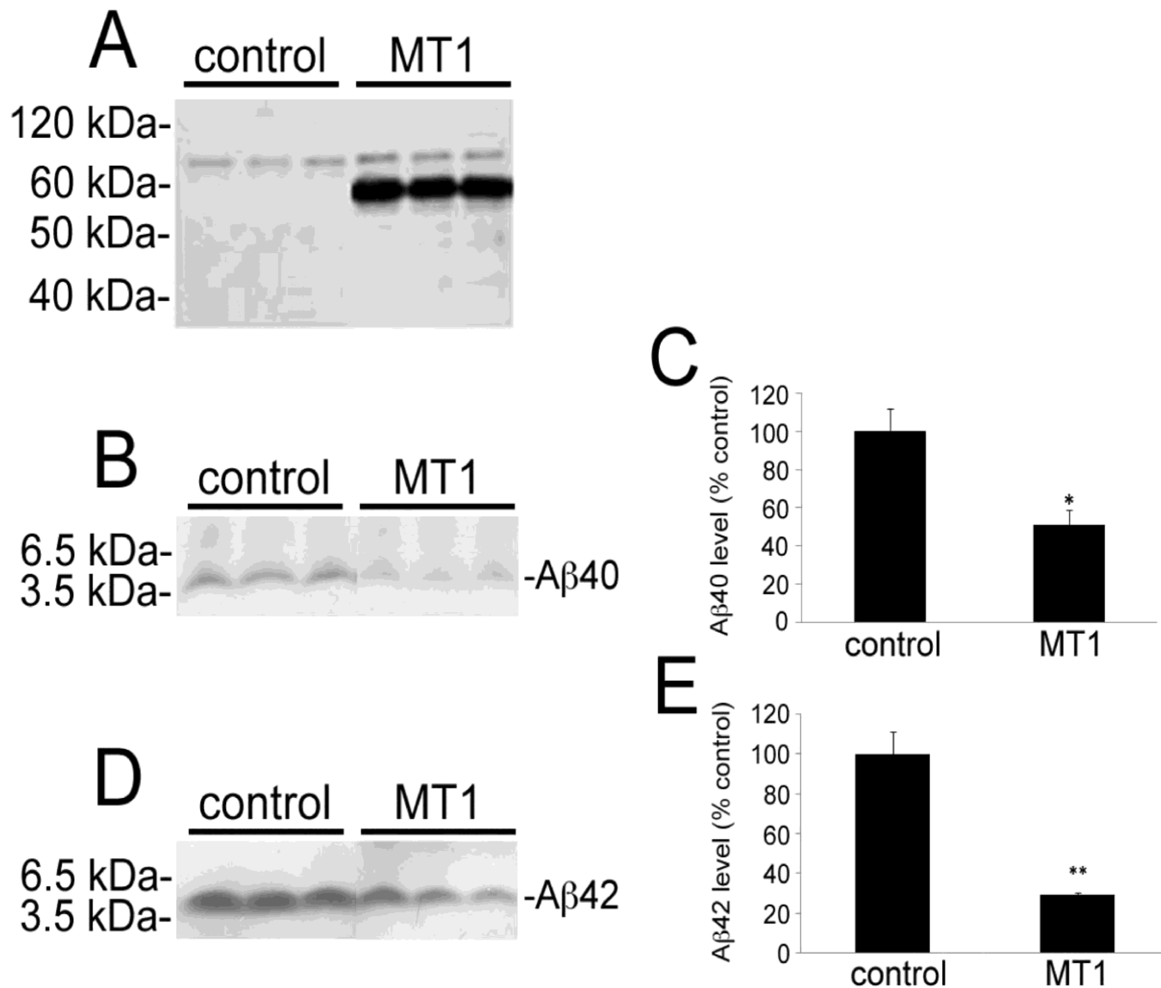



**Figure 2.4 Aβ40 and Aβ42 are degraded by MT1-MMP expressed in Cos-1 cells.** Triplicate cultures of Cos-1 cells were transfected with empty plasmid vector (pcDNA3.1) or MT1-MMP vector. Twenty four hours post transfection, the cells were incubated with 2 μg/ml of freshly solublized Aβ40 or Aβ42 in serum-free media for an additional 48 h. (**A**) The cell lysates were collected and analyzed by immunoblotting using anti-MT1-MMP. The culture media samples were collected and analyzed for Aβ40 and Aβ42 peptides levels by immunoblotting using anti-Aβ (**B** and **D,** respectively) and by ELISA (**C** and **E**, respectively). The data shown are the mean ± S.D. (n=3). *, $p < 0.05$; **, $p < 0.01$.

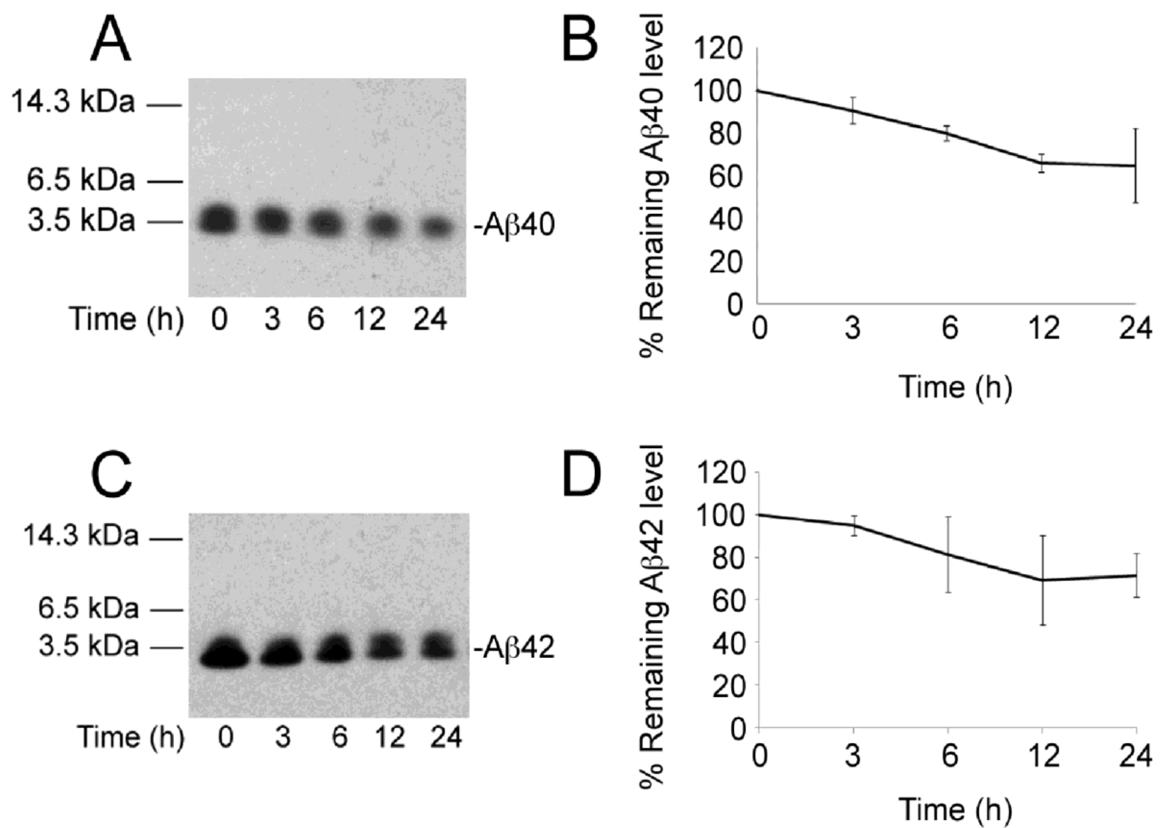
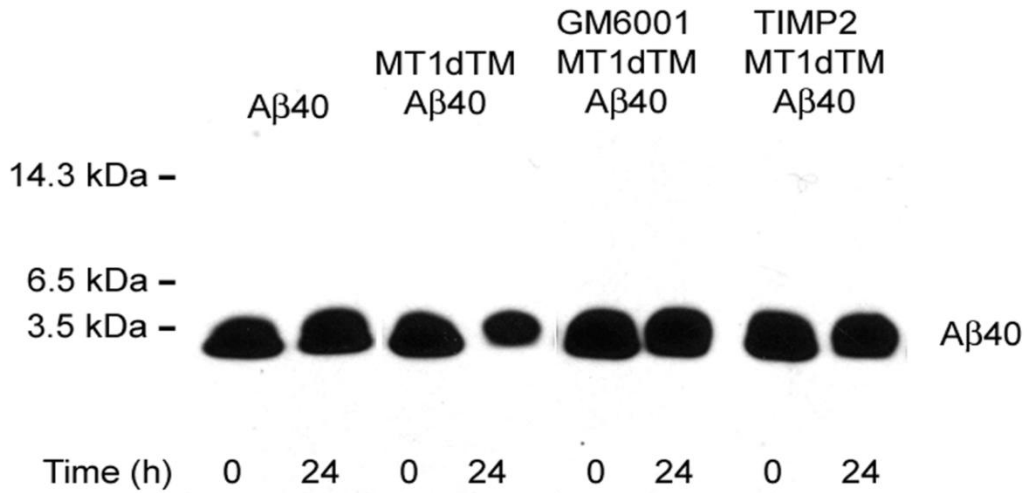



**Figure 2.5 Aβ40 and Aβ42 degradation by soluble MT1dTM.**
Aβ40 (**A** and **B**) or Aβ42 (**C** and **D**) was incubated at 37℃ in the presence or absence of purified 40 nM of MT1dTM. At each time point, samples were collected and analyzed for Aβ level by quantitative immunoblotting using anti-Aβ mAb. The data shown are the mean ± SD of three separate determinations.



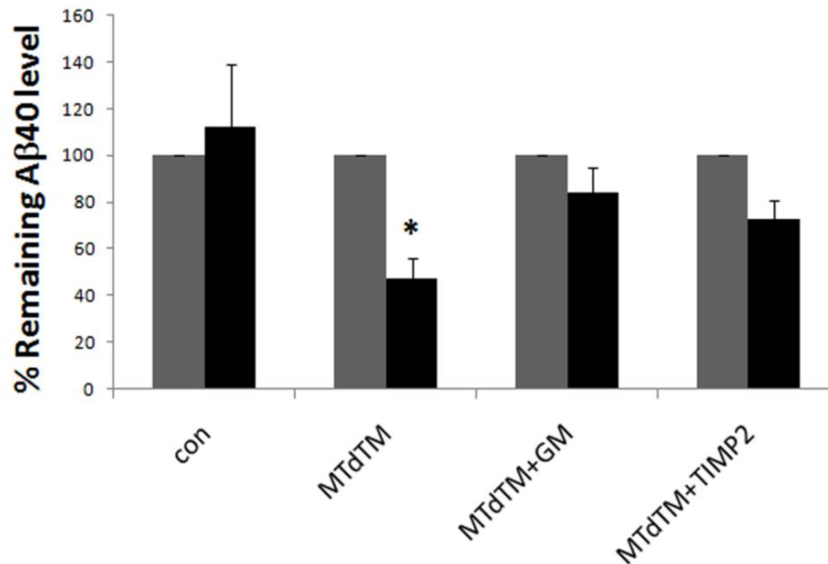




**Figure 2.6 Aβ40 degradation by soluble MT1dTM is inhibited by GM6001 and TIMP2.**
Aβ40 was incubated with purified soluble MTdTM at 37℃ in the presence or absence of the general MMP inhibitior GM6001 (10 μM) or the specific MT1-MMP inhibitor TIMP2 (40 nM) for 24 h. Following incubation, the samples were collected and analyzed for Aβ levels by quantitative immunoblotting using anti-Aβ (**A**). The data shown are the mean ± SD of three separate determinations (**B**). *, $p < 0.05$, paired *t* test.

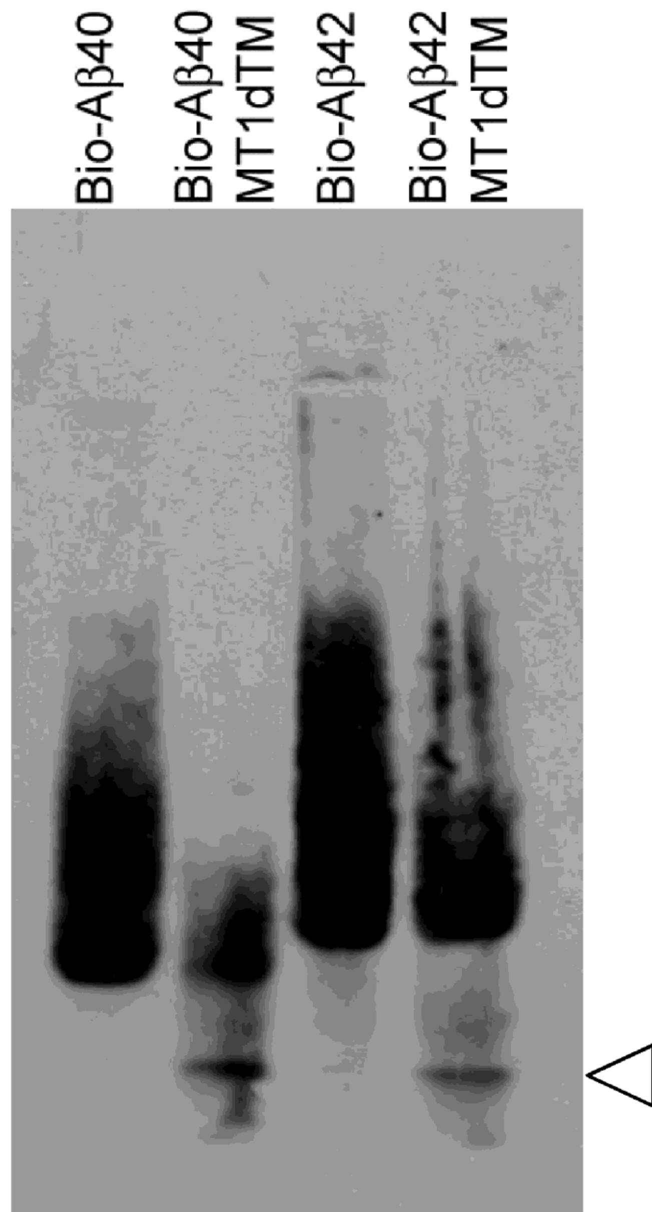



**Figure 2.7 Analysis of MT1dTM-mediated Aβ cleavage fragments on acid/urea gel.**

Soluble amino terminal, biotinylated Aβ40 or Aβ42 was incubated with purified soluble MT1dTM for 48 h. Following incubation, samples were separated on 22% polyacrylamide acid/urea gels, transferred to membranes, and analyzed for Aβ products by using a streptavidin-horseradish peroxidase conjugate to detect biotinylated peptides and fragments. Lane 1, biotinylated-Aβ40; lane 2, biotinylated-Aβ40 + MT1dTM; lane 3, biotinylated-Aβ42; and lane 4, biotinylated-Aß42 + MT1dTM. The brackets denote amino terminal cleavage products common to Aß40 and Aß42.

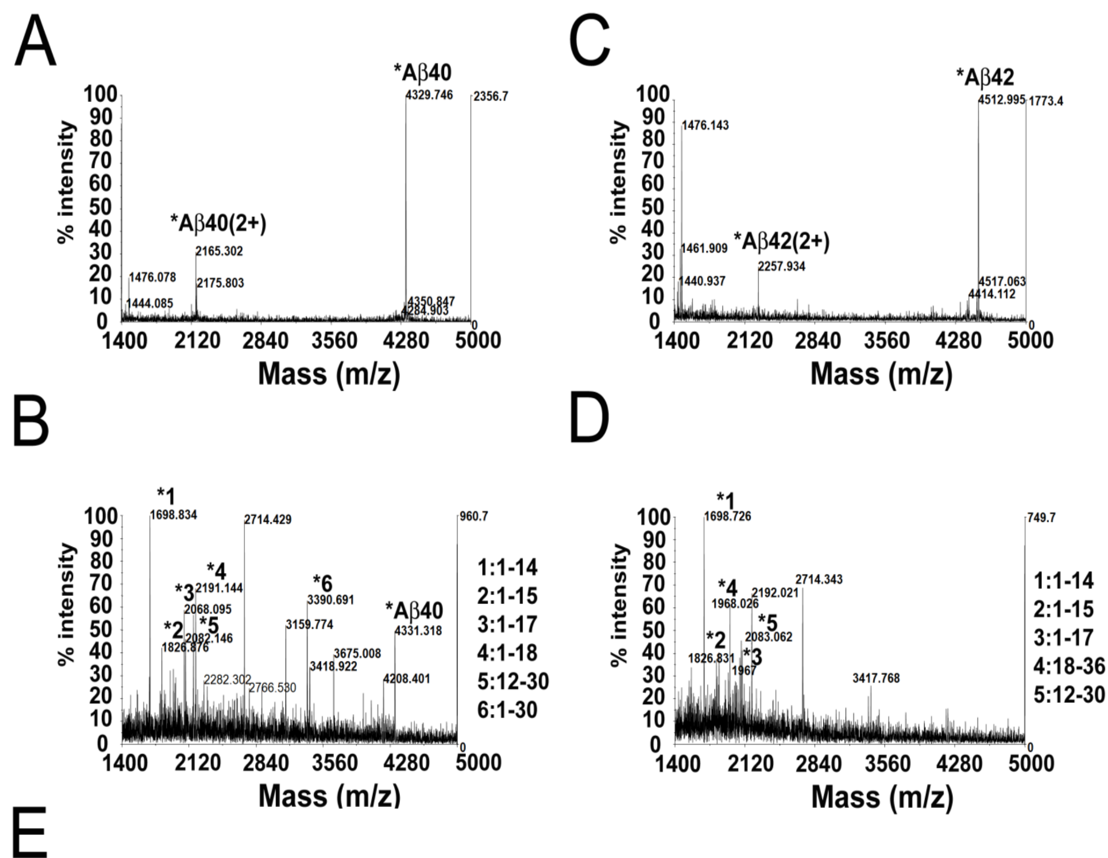



**Figure 2.8 MALDI-TOF MS analysis of Aβ fragments released by purified MT1dTM.**

Synthetic Aβ40 alone (**A**), Aβ42 alone (**C**), Aβ40 and purified MT1dTM (**B**) or Aβ42 and purified MTdTM (**D**) were incubated with 37℃ for 2 days. After incubation the samples were analyzed by MALDI-TOF mass spectrometry. Comparing with Aβ40 or Aβ42 alone, several specific peaks were identified as Aβ fragments (reflector mode). (**E**) Summary of the MT1dTM cleavage sites on Aβ (▼).

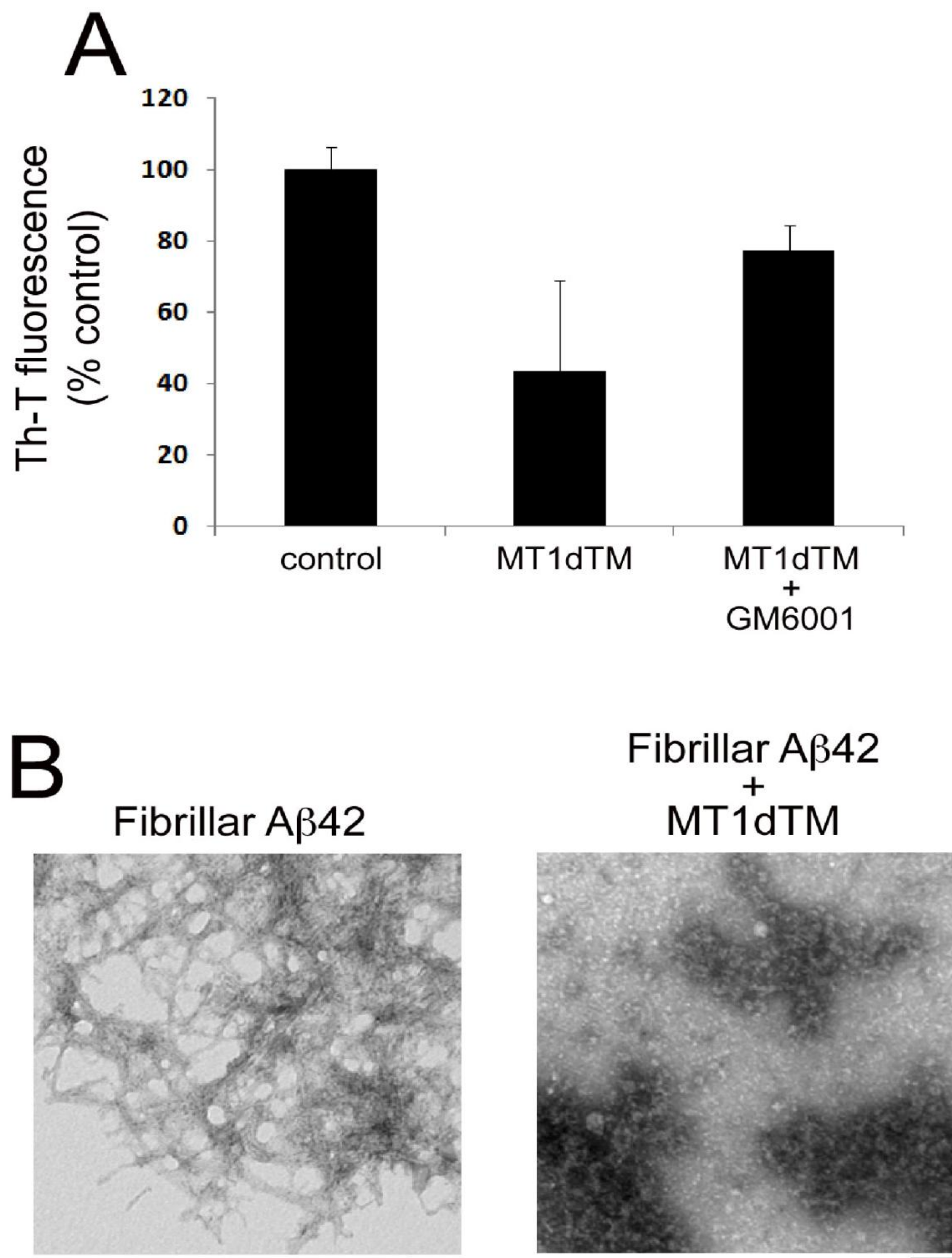



**Figure 2.9 Fibrillar Aβ degradation by soluble MT1dTM.**
(**A**) Fibrillar Aβ42 was incubated alone or with MT1dTM in the presence or absence of the MMP inhibitor GM6001 for 37℃ for 5 days. The remaining fibrillar Aβ was quantitated using a Th-T binding fluorescence assay. The data shown are the mean ± SD of three separate determinations. (**B**) Fibrillar Aβ42 was incubated alone or with purified MT1dTM at 37℃ for 5 days. The samples were collected and analyzed by TEM. Scale bars = 100 nm.

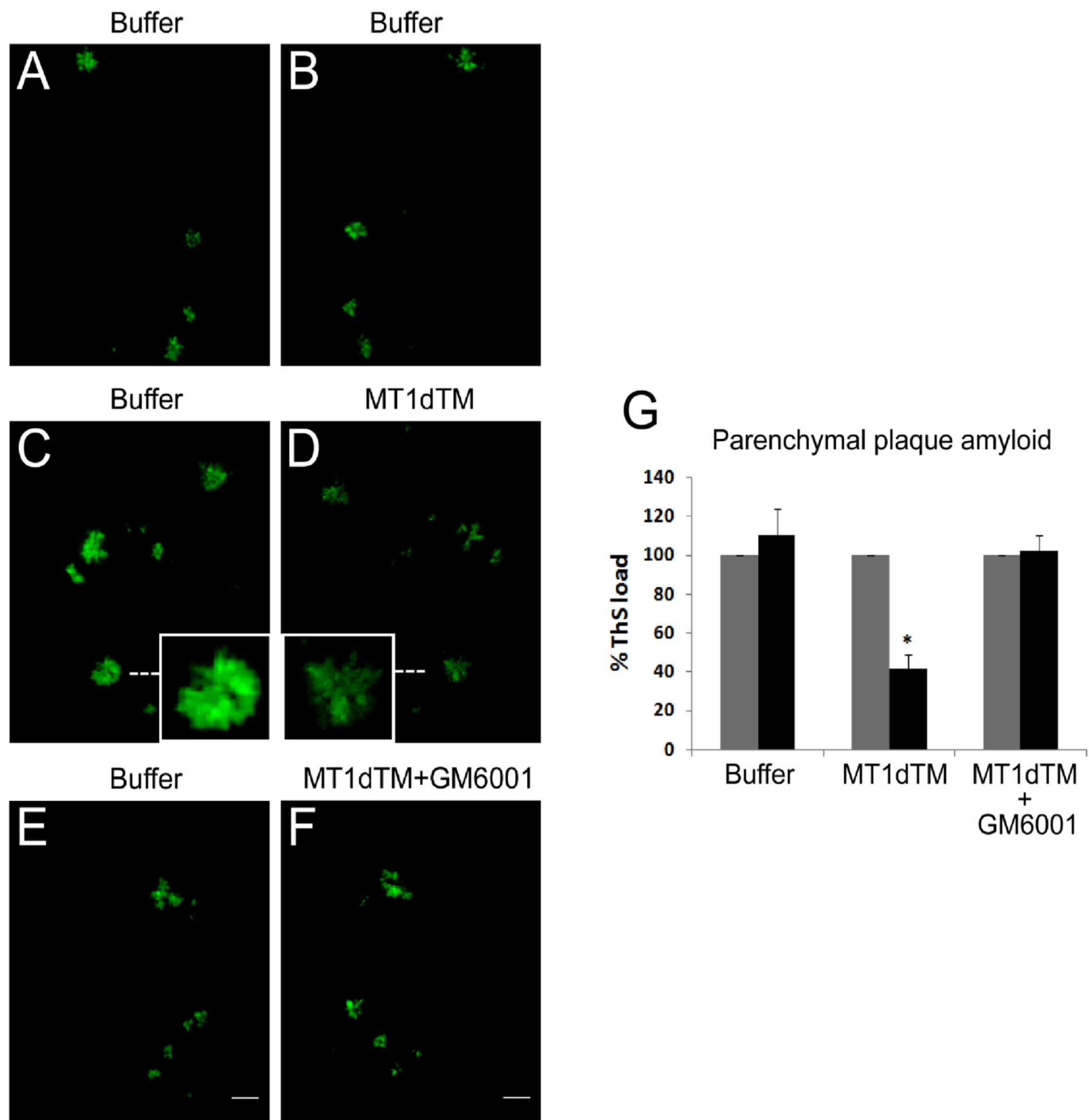



**Figure 2.10** ***In situ*** **brain fibrillar amyloid plaque degradation by soluble MT1dTM.**
Adjacent 5 µm fresh frozen brain sections from 18 months old Tg2576 mice were incubated alone (**A,B,C,E**) or with purified MTIdTM (**D**) or GM6001-treated MT1dTM (**F**) at 37℃ for 5 days. The sections were then fixed, and stained with Th-S. Insets show parallel representative plaques enlarged. Scale bars = 50 µm. (**G**) The parallel cortical fibrillar amyloid plaque areas were quantified in the treated and untreated sections and expressed as percent remaining Th-S area. The data presented are the mean ± S.D. of n = 25 plaques (buffer alone); n = 37 plaques (incubated with MT1dTM); n = 27 plaques (incubated with GM6001-treated MT1dTM). *, $p < 0.001$, paired *t* test.

# CHAPTER 3 - Degradation of Amyloid β-Protein by Purified Myelin Basic Protein

## 3.1 – Summary

The progressive accumulation of β-amyloid (Aβ) in senile plaques and in the cerebral vasculature is the hallmark of AD and related disorders. Impaired clearance of Aβ from brain likely contributes to the prevalent sporadic form of AD. Several major pathways for Aβ clearance include receptor-mediated cellular uptake, blood-brain barrier transport and direct proteolytic degradation. Myelin basic protein (MBP) is the major structural protein component of myelin and plays a functional role in the formation and maintenance of the myelin sheath. MBP possesses endogenous serine proteinase activity and can undergo autocatalytic cleavage liberating distinct fragments. Recently, we showed that MBP binds Aβ and inhibits Aβ fibril formation (*73, 89*). Here we show that Aβ40 and Aβ42 peptides are degraded by purified human brain MBP. MBP-mediated Aβ degradation is inhibited by serine proteinase inhibitors. Similarly, Cos-1 cells expressing MBP degrade exogenous Aβ40 and Aβ42. In addition, we demonstrate that purified MBP also degrades assembled fibrillar Aβ *in vitro*. Mass spectrometry analysis identified distinct degradation products generated from Aβ digestion by MBP. Lastly, we demonstrate *in situ* that purified MBP can degrade parenchymal amyloid plaques as well as cerebral vascular amyloid that form in brain tissue of Aβ precursor protein transgenic

mice. Together, these findings indicate that purified MBP possesses Aβ degrading activity *in vitro.*

## 3.2 – Introduction

The progressive accumulation of β-amyloid (Aβ) in senile/neuritic plaques and the cerebral vasculature is the hallmark of Alzheimer's disease (AD) and widely used in the pathological diagnosis of the disease. Aβ is generated by proteolytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase (*2-3*). The main species of Aβ are 40 and 42 amino acids in length. Aβ42 is much more amyloidogenic than Aβ40 because of its two additional hydrophobic amino acids at the carboxyl terminal end of the peptide (*4*). The Aβ42 peptide is the predominant form in senile plaques, forming a β-sheet structure, which is insoluble and resistant to proteolysis.

Although increased production of Aβ has been implicated in the onset of familial forms of AD, it has been hypothesized that the more common sporadic forms of AD may be caused by the impaired clearance of Aβ peptides from the CNS. Several major pathways for Aβ clearance have been proposed including receptor-mediated cellular uptake, blood-brain barrier transport into the circulation, and direct proteolytic degradation (*1, 19, 90*). In the latter case, several proteinases or peptidases have been identified that are capable of degrading Aβ, including neprilysin (NEP) (*25-26*), insulin-degrading enzyme (IDE) (*27*), the uPA/tPA-plasmin system (*28*), endothelin-converting enzyme (ECE) (*29*), angiotensin-converting enzyme (ACE) (*30*), gelatinase A (MMP-2) (*31-32*), gelatinase B (MMP-9) (*33*) and acyl peptide hydrolase (*91*). Each of these

enzymes has been shown to cleave Aβ peptides at multiple sites (*1*). However, only NEP, IDE, ECE and MMP-9 have been shown to have a significant role in regulating Aβ levels in the brains of experimental animal models (*26, 40-41*).

The "classic" myelin basic proteins (MBPs) are major structural components of myelin sheaths accounting for 30% of total myelin protein. There are four different major isoforms generated from alternative splicing with molecular weights of 17.3, 18.5 20.2 and 21.5 kDa. The 18.5 kDa variant, composed of 180 amino acids including 19 Arg and 12 Lys basic residues, is most abundant in mature myelin (*62*). One of the major functions of MBP is to hold together the cytoplasmic leaflets of myelin membranes in order to maintain proper compaction of the myelin sheath through the electrostatic interaction between the positive Arg and Lys residues of MBP and the negatively charged phosphate groups of the membrane lipid (*67*). MBP plays an important role in the pathology of multiple sclerosis, which is an autoimmune disease characterized by demyelination within white matter (*68*). Recently, it was reported that purified MBP exhibits autocleavage activity generating distinct peptide fragments (*70*). In this study, serine 151 was reported as the active site serine residue involved in autocatalysis.

In the early stages of AD, appreciable and diffuse myelin breakdown in the white matter is observed (*71*). Also, in white matter regions there are much fewer fibrillar amyloid deposits than are commonly found in grey matter regions. Recently, our laboratory has shown that MBP strongly interacts with Aβ peptides and prevents their assembly into mature amyloid fibrils (*73, 89*). Through the course of these studies we observed that upon longer incubations the levels of Aβ peptides were reduced upon treatment with MBP. In light of this observation, coupled with the report that MBP

possesses proteolytic activity, we hypothesized that MBP may degrade Aβ peptides. In the present study, we show that purified human brain MBP can degrade soluble Aβ40 and Aβ42 peptides *in vitro*. Purified MBP also degraded fibrillar Aβ *in vitro*. Mass spectrometry analysis identified distinct degradation products generated from soluble and fibrillar Aβ digestion by MBP. Furthermore, purified MBP degraded parenchymal and vascular fibrillar amyloid deposits *in situ* in the brain tissue of APP transgenic mice. Together, these findings indicate that purified MBP possesses Aβ degrading activity *in vitro*.

## 3.3 – Materials and Methods

*Reagents and Chemicals* – Synthetic naïve or amino-terminal biotinylated Aβ40 and Aβ42 peptides were synthesized by solid-phase Fmoc (9-fluorenylmethoxycarbonyl) amino acid chemistry, purified by reverse phase high performance liquid chromatography, and structurally characterized as previously described (*76*). Phenylmethane sulfonylfluoride (PMSF), thioflavin-T (ThT), and thioflavin-S (ThS) were purchased from Sigma-Aldrich (St. Louis, MO). Normal human brain white matter tissue was provided by Dr. Annemieke Rozemuller, Dept. of Neuropathology, Free University Medical Center, Amsterdam, The Netherlands. The cDNA for 18.5 kDa MBP was cloned into the adenoviral vector plasmid, pacAd5 CMV K-NpA. After verifying its integrity in this plasmid, it was then sent to the University of Iowa Gene Transfer Vector Core for viral packaging and propagation. The completed Ad-MBP was at a titer of ≈ 5.5 x $10^7$ pfu/μl.

*Isolation and Purification of MBP from Normal Human Brain White Matter* - MBP was purified from normal human white matter as described previously (*92*). The predominant 18.5 kDa MBP exists as a family of charge isomers that differ in net charge and result from various posttranslational modifications. To isolate the individual charge isomers of MBP, the brain homogenates were loaded onto a CM52 cation exchange column, and the components were eluted with a 0-0.2 M NaCl gradient. Component 8 was found in the void volume while the more cationic components (C5, C4, C3, C2, and C1) eluted with an increasing salt gradient. The components were dialyzed against water, lyophilized, and stored at -80 ℃. The most abundant MBP C1 component was used in all subsequent studies.

*Quantitative Immunoblotting* - Samples containing MBP or Aβ were added directly into SDS-PAGE sample buffer, and stored at –70℃. Aliquots were loaded onto 12% or 10-20% polyacrylamide gels, electrophoresed and transferred onto Hybond-ECL nitrocellulose membranes (Amersham, Arlington Heights, IL) at 100 V for 1.5 hour at RT. Membranes were blocked in 5% milk/PBS/0.05% Tween20 (PBS-T) for 1 h at RT. Primary antibodies were added (mAb22 for MBP, Serotec, Raleigh, NC; mAb20.1 for Aβ) for 1 h at RT, washed 3 x 5 min with PBS-T. Secondary horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies were then added to the membranes (1:5000 Amersham-Pharmacia, Piscataway, NJ), which were then washed 3 x 5 min with PBS-T. Bands were visualized using the ECL detection method (Amersham-Pharmacia, Piscataway, NJ). Quantitation of MBP or Aβ bands was performed using a VersaDoc

Imaging System (BioRad, Hercules, CA) and the manufacturer's Quantity Oneton software.

*In Vitro Aβ Degradation* - Aβ peptides were initially prepared in hexafluoroisopropanol, dried, and resuspended in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml. 1 μM Aβ40 or Aβ42 was incubated in the absence of presence of 250 nM of purified MBP, hisMBP or hisMBP1 in PBS buffer with 1 mg/ml of BSA at 37 ℃ for 24 h. The Aβ samples were then analyzed by quantitative immunoblotting as described above.

*Aβ Degradation in Cos-1 Cells Expressing Human MBP* - Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-products, Woodland, CA) in 24-well tissue culture plates. Triplicate near confluent cultures were infected with Ad-MBP (MOI: 250), followed by addition of 2 μg/ml of amino-terminal biotinylated Aβ40 or Aβ42 in serum-free media for 48 h. The culture media samples were collected and cell lysates were prepared. The level in biotinylated-Aβ peptides in the cell culture media or cell lysate samples was quantitatively analyzed by immunoblotting using streptavidin HRP. The cell culture lysates were analyzed for MBP expression by immunoblotting as described above.

*In Vitro Fibril Aβ Degradation* - To prepare amyloid fibrils, 5 mM Aβ42 in DMSO was diluted in PBS to 100 μM, vortexed for 30 s, and incubated at 37 ℃ for 5 days (*80*). Triplicate samples of 10 μM of aged fibrillar Aβ were incubated with purified MBP in PBS containing 1 mg/ml BSA at 37 ℃ for 48 h. After digestion, the remaining fibrillar

Aβ was quantified using a ThT fluorescence assay. Briefly, 5 µl of 100 µM ThT was added to 100 µl of sample, mixed and incubated at RT in the dark for 10 min. ThT fluorescence was measured at $\lambda_{ex}$ of 446 nm and $\lambda_{em}$ of 490 nm. Loss of ThT fluorescence corresponded to loss of amyloid fibrils. Alternatively, FITC-labeled fibrillar amyloid was prepared by mixing unlabeled Aβ42 and FITC-labeled Aβ42 (9:1 ratio) to 100 µM in PBS, vortexed for 30 s, and incubated at 37 ℃ for 5 days. Triplicate samples of 10 µM FITC-labeled fibrillar Aβ were incubated with purified MBP in PBS containing 1 mg/ml BSA at 37 ℃ for 48 h. After incubation, the samples were centrifuged at 14,000 x *g* for 30 min, the supernatants were removed and the pellets were dissolved in 100 µl of PBS. Fluorescence in the pellet, corresponding to remaining fibrillar Aβ, was measured at $\lambda_{ex}$ of 480 nm and $\lambda_{em}$ of 515 nm.

*Electron Microscopy* - Sample mixtures were deposited onto carbon-coated copper mesh grids and negatively stained with 2% (w/v) uranyl acetate. The samples were viewed with a FEI Tecnai 12 BioTwin transmission electron microscope, and digital images were taken with an Advanced Microscopy Techniques camera.

*Mass Spectrometry* – Purified soluble MBP or aged fibrillar Aβ42 (10 µM) were incubated in the absence or presence of purified MBP in PBS at 37 ℃ for 5 days. After incubation, the samples were centrifuged at 14,000 x *g* for 30 min, the supernatants were removed and dried in a rotary evaporator (Savant, Farmingdale NY), suspended in 20 µl of 0.1% TFA, ZipTipped using µC18 tips (Millipore, Milford, MA) and then eluted to the target. The addition of 1 µl of matrix consisting of acetonitrile/0.1% trifluoroacetic acid

containing ∝-cyano-4-hydroxy cinammic acid (CHCA, 5 mg/ml) was dried on the sample plate. Samples were run on a *Voyager-DE STR* (Applied Biosystems, Framingham, MA) using a matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometer system operated in the reflector mode unless otherwise indicated. The mass scale (*m/z* 500-5000) was calibrated with a mixture of peptides or internal calibration was performed using a matrix ion at *m/z* 568.1330 and Aβ42 peptide amino acid 1-13 *m/z* 1561.6672. For samples acquired in the linear mode, 1 μl was dissolved in 10 μl of a 50% solution of acetonitrile/0.3% trifluoroacetic acid containing sinapinic acid (10 mg/ml) and dried on the sample plate. The mass scale (*m/z* 1000-25000) was calibrated with myoglobin (400 fM/μl).

*In-Situ Fibrillar Amyloid Plaque Degradation* - For this analysis the well-characterized Tg2576 (APPsw) mouse model of AD that develops abundant fibrillar amyloid pathology (*93*) was used. Brains were removed from anesthetized 18 months old Tg2576 mice after perfusion with cold saline and snap-frozen on dry ice. Five-μm cryostat sections were collected on slides. Every other section was flipped 180° so that identical faces of adjacent sections were exposed (*80*). Paired adjacent sections (one incubated with PBS containing 1 mg/ml BSA, the other with 5 μM purified MBP in absence or presence of 1 mM PMSF) in triplicate were incubated at 37℃ for 2 days, stained with thioflavin-S, and then imaged with fluorescence microscopy. The parenchymal plaque amyloid or vascular amyloid area of ThS fluorescence was determined using image analysis software (Image J). Fractional area was compared between paired sections.

*Statistical Analysis* - Data were analyzed by Student's t-test at the 0.05 significance level.

## 3.4 – Results

### MBP autolysis involves serine proteinase activity

Recently, purified MBP was reported to possess serine proteinase autolytic activity (*70*). To confirm this finding, MBP was purified from normal human white matter. A typical elution profile of an acid extract of chloroform/methanol-delipidated human brain homogenates through CM52 cation exchange column is illustrated in Figure 3.1. For the most abundant MBP C1 component, a high degree of purity was obtained yielding a single protein species of ≈18.5 kDa as detected by SDS-PAGE and silver staining (Figure 3.2A) and by mass spectrometry (Figure 3.2B). To confirm the previously reported serine proteinase autolytic activity, purified human MBP was incubated at 37℃ in the absence or presence of the serine proteinase inhibitors PMSF for 72 h, aliquots were removed and analyzed by immunoblotting using the anti-MBP antibody mAB22. As shown in Figure 3.2C, MBP exhibited autolysis, which was completely inhibited by treatment with PMSF. These experiments confirmed that purified human brain MBP exhibits serine proteinase autolytic activity. Other purified MBP isoforms, C2 and C3, also exhibited autolytic activity as shown for C1 (data not shown). The MBP C1 isoform was used in all subsequent experiments.

### *In vitro* Aβ40 and Aβ42 degradation by purified MBP

We have recently shown that MBP strongly binds Aβ peptides and inhibits Aβ fibril formation (*89*). In light of the endogenous proteinase activity of MBP, we sought

to determine if it could proteolytically degrade Aβ peptides *in vitro*. Quantitative immunoblotting analysis presented in Figure 3.3 shows that incubation with purified MBP resulted in degradation of soluble synthetic Aβ40 and Aβ42 peptides. One nM MBP degraded ≈ 2 nM Aβ40 and ≈ 3 nM Aβ42 per hour. Although the rate of Aβ hydrolysis was relatively slow Aβ42 was degraded faster than Aβ40 in the same time period.

**Exogenous Aβ40 and Aβ42 degradation in Cos-1 cells expressing human MBP**

We next determined if MBP expressed in a cell-based system could degrade soluble Aβ peptides. To do this, we used an adenoviral vector (Ad-MBP) to express MBP in Cos-1 cells, a cell type that does not normally express MBP protein. Immunoblotting confirmed that Cos-1 cells infected with Ad-MBP express MBP while uninfected and Ad-GFP infected Cos-1 cells showed no MBP expression (Figure 3.4A, upper panel). To analyze exogenous Aβ degradation in this system we used amino terminal-biotinylated Aβ peptides to increase the detection sensitivity. Post infection, 2 μg/ml of soluble biotinylated-Aβ40 or -Aβ42 was added into the culture media and then incubated for 48 h. Detection of Aβ peptides in the culture media samples using streptavidin-HRP showed that MBP expression in Cos-1 cells resulted in loss of both Aβ40 and Aβ42 (Figure 3.4A, middle and lower panels, respectively). Quantitation of biotinylated-Aβ40 or -Aβ42 levels in the culture media of Ad-MBP infected cells showed that they were significantly decreased compared with uninfected or Ad-GFP infected Cos-1 cells (Figure 3.4B and C). Essentially the same results were obtained using unlabeled Aβ40 and Aβ42 peptides that were detected by quantitative immunoblotting or

ELISA (data not shown). Finally, quantitation of cell-associated biotinylated-Aβ showed no difference in the levels of biotinylated-Aβ40 or -Aβ42 with the Cos-1 cells under any condition (Figure 4D, E and F). Together, these results suggest that MBP expressed in Cos-1 cells can promote Aβ degradation.

***In vitro* Aβ fibril degradation**

We next determined whether MBP could degrade fibrillar Aβ. As a first approach, 10 μM of aged fibrillar Aβ42 was incubated with or without 1 μM purified MBP at 37℃ for 48 h and the remaining fibrillar Aβ was measured using the ThT fluorescence binding assay. A shown in Figure 3.5A, the remaining ThT fluorescence signal was decreased by nearly 50% in the presence of purified MBP. As an alternative approach, fibrillar Aβ was prepared by aging FITC-labeled Aβ42 with unlabeled Aβ42 (at a 1:9 molar ratio) and then incubated with or without 1 μM purified MBP at 37℃ for 48 h. The remaining amyloid fibrils were collected by centrifugation and measured by fluorescence spectroscopy. Similar to the ThT fluorescence binding assay, when incubated with purified MBP fibrillar Aβ decreased by nearly 50% (Figure 3.5B). Finally, fibrillar Aβ was incubated with or without purified MBP for 48 h and then TEM was performed to visualize the remaining fibrillar Aβ at the ultrastructural level. Incubation with purified MBP markedly reduced the extent of fibrillar Aβ (Figure 3.5C). Together, these data strongly suggest that MBP can degrade fibrillar Aβ.

**MBP-Generated Cleavage Products of Aβ**

Fibrillar Aβ42 was digested with purified MBP to determine specific cleavage products. Aβ fragments were isolated and analyzed by MALDI-TOF mass spectrometry. Nine specific Aβ degradation fragments (Figure 3.6C) were observed that were not present in the supernatant of the post incubation fibrillar Aβ preparation alone (Figure 3.6A). Similarly, these nine specific Aβ fragments were not present in the post incubation of purified MBP alone, although MBP-specific fragments were observed resulting from MBP autolysis (Figure 3.6B). Figure 3.6D shows the multiple cleavage sites on fibrillar Aβ for digestion with MBP. We also determined soluble Aβ42 degradation products by MBP and analyzed by MALDI-TOF. For soluble Aβ42, four of the same specific fibrillar Aβ42 degradation peaks were observed, however the signals obtained were much lower (data not shown).

**MBP degrades parenchymal and vascular amyloid deposits *in situ***

The above data showed that purified MBP was capable of degrading soluble and fibrillar synthetic Aβ peptides *in vitro*. We next determined if purified MBP could degrade actual amyloid deposits that form in the brains of APP transgenic mice. To do this, adjacent brain slices of aged Tg2576 mice were incubated at 37℃ for 48 h with buffer alone or purified MBP treated or untreated with PMSF. After incubation the sections were stained with ThS and the area of fluorescence between matching plaques or vascular amyloid deposits from adjacent sections was measured. The area of ThS fluorescence of adjacent brain sections did not show a difference when incubated with buffer alone, while the area of parenchymal and cerebral vascular amyloid deposits was significantly decreased in the brain sections incubated with purified MBP (Figures 3.7

and 3.8, respectively). However, the fibrillar amyloid degradation by purified human MBP was completely inhibited by pretreatment with PMSF. These results demonstrate that purified MBP is capable of degrading fibrillar amyloid deposits in brain and that serine proteinase activity of MBP was involved.

## 3.5 – DISCUSSION

In the present study, we have identified a novel function of purified MBP as an Aβ peptide degrading enzyme *in vitro*. First, we confirmed that highly purified MBP from normal human white matter exhibit serine proteinase autolytic activity. Subsequent experiments revealed that both soluble Aβ40 and Aβ42 peptides were degraded by purified MBP *in vitro* and in Cos-1 cells expressing MBP. Further, we demonstrated by several means that purified MBP could degrade fibrillar Aβ as well. Mass spectrometry analysis identified multiple MBP cleavage sites on soluble and fibrillar Aβ. Lastly, we showed that purified human MBP could degrade parenchymal and fibrillar amyloid deposits in brain tissue sections of APP transgenic mice.

Several lines of evidence argue against the possibility of a contaminating proteinase activity in our purified MBP preparations. First, we purified MBP from normal human white matter with a very high degree of purity as assessed by mass spectrometry and by the observation of a single protein band by HPLC analysis using SDS-PAGE and silver staining (Figure 3.2A and B). Second, MBP purified from a variety of highly diverse sources including human and mouse brain tissues, and Ad-MBP infected Cos-1 cells all exhibited similar autolytic and Aβ-degrading activity. Taken together, these data

strongly suggest that contamination of purified MBP with another Aβ-degrading enzyme is highly unlikely.

Compared with other known Aβ-degrading enzymes such as IDE, NEP, ECE and plasmin, the Aβ-degrading activity of MBP *in vitro* appears to be quite modest. For example, it was reported that 25 μM Aβ40 was degraded by 50 nM plasmin within 4 h (*94*). In comparison, we observed that 250 nM MBP could degrade 1 μM Aβ40 or Aβ42 in 24 h (Figure 3.3). Perhaps the conditions used in our *in vitro* Aβ degradation assays do not reflect the optimum environment for MBP to exert its proteolytic activity. MBP is one of the major components of CNS myelin produced by oligodendrocytes in brain (*95*). Although the total amount of MBP in brain is orders of magnitude higher than other Aβ-degrading enzymes whether MBP exhibits Aβ-degrading activity as a component of myelin is presently unknown. However, when demyelination occurs, as in AD and other neurodegenerative conditions, this opportunity may release soluble MBP that is capable of degrading Aβ.

Fibrillar Aβ in plaques and vascular deposits is highly resistant to proteolysis due to its inherent structural nature and modifications that occur such as oxidation and crosslinking (*96*). Many Aβ-degrading enzymes such as ECE, IDE, and NEP exhibit little or no degradative activity towards fibrillar Aβ. In contrast, plasmin and MMP-9 have been shown to degrade Aβ fibrils *in vitro* and amyloid plaques *in-situ* (*80*). Likewise, we demonstrate that purified MBP also exhibits fibrillar Aβ degradative activity and *in-situ* plaque degradation. Moreover, MALDI-TOF mass spectrometry analysis identified numerous fibrillar Aβ degradation fragments generated by purified

MBP consistent with other known Aβ-degrading enzymes that exhibit multiple cleavage sites on Aβ.

MBP is the main structural protein of the myelin sheath and is associated with myelin membrane through interaction between the positive Arg and Lys residues and the negatively charged phosphate groups of the membrane phospholipids (*97*). It was reported that MBP still undergoes autolysis in the presence of lipid (*70*). Here we show that MBP expressed in Cos-1 cells could still degrade Aβ40 and Aβ42. Together, these findings suggest that MBP still retains its proteolytic activity in association with lipids or cell membranes, although the detailed enzymatic activity of MBP *in vivo* needs to be further investigated.

AD is largely considered as a disease of the brain gray matter (*98*). However, widespread and diffuse myelin breakdown has been reported in AD patients and white matter deficits are observed in the early stage of the disease (*99*). It has been reported that MBP levels were significantly decreased in the white matter of AD patients (*100-101*). It is noteworthy that the levels of Aβ40 and Aβ42 were increased in the AD white matter exhibiting decreased levels of MBP (*100*) and Aβ deposits were also observed (*102*). Our present results that demonstrate the Aβ-degrading activity of purified MBP, coupled with our recent reports that MBP can potently inhibit the fibrillar assembly of Aβ peptides (*73, 89*), are consistent with these findings. It has also been proposed that MBP could counteract the surface structure of Aβ fibril mediated cytotoxicity (*103*). In any case, future *in vivo* studies are warranted to further understand the potential multiple functions of MBP in inhibition of Aβ fibril assembly, Aβ degradation and modulation of Aβ mediated cytotoxicity that may play a role in the pathogenesis of amyloid deposition.

## 3.6 – FIGURES

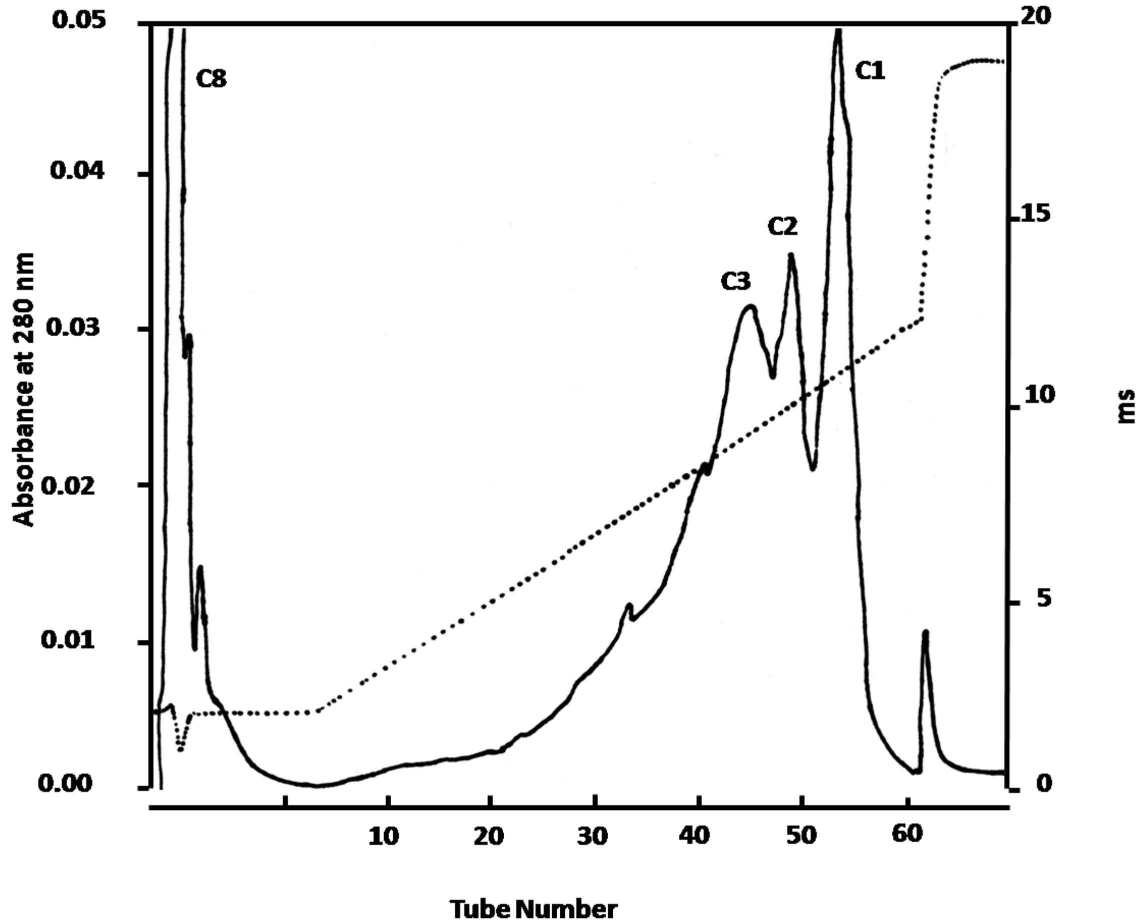



**Figure 3.1 Elution profile of an acid extract of human white matter on carboxymethylcellulose.** Human brain white matter was defatted in chloroform/methanol and extracted in 0.2 N $H_2SO_4$. The acid extract was applied to the CM52 column, equilibrated in 6M Urea/80 mM Glycine, pH 10.5. The column was washed with the starting buffer. Then a linear salt gradient was begun consisting of 0 to 0.2 M NaCl/80 mM Glycine, pH 10.5. Fractions were collected every 3ml and absorbance was measured at 280 nm.

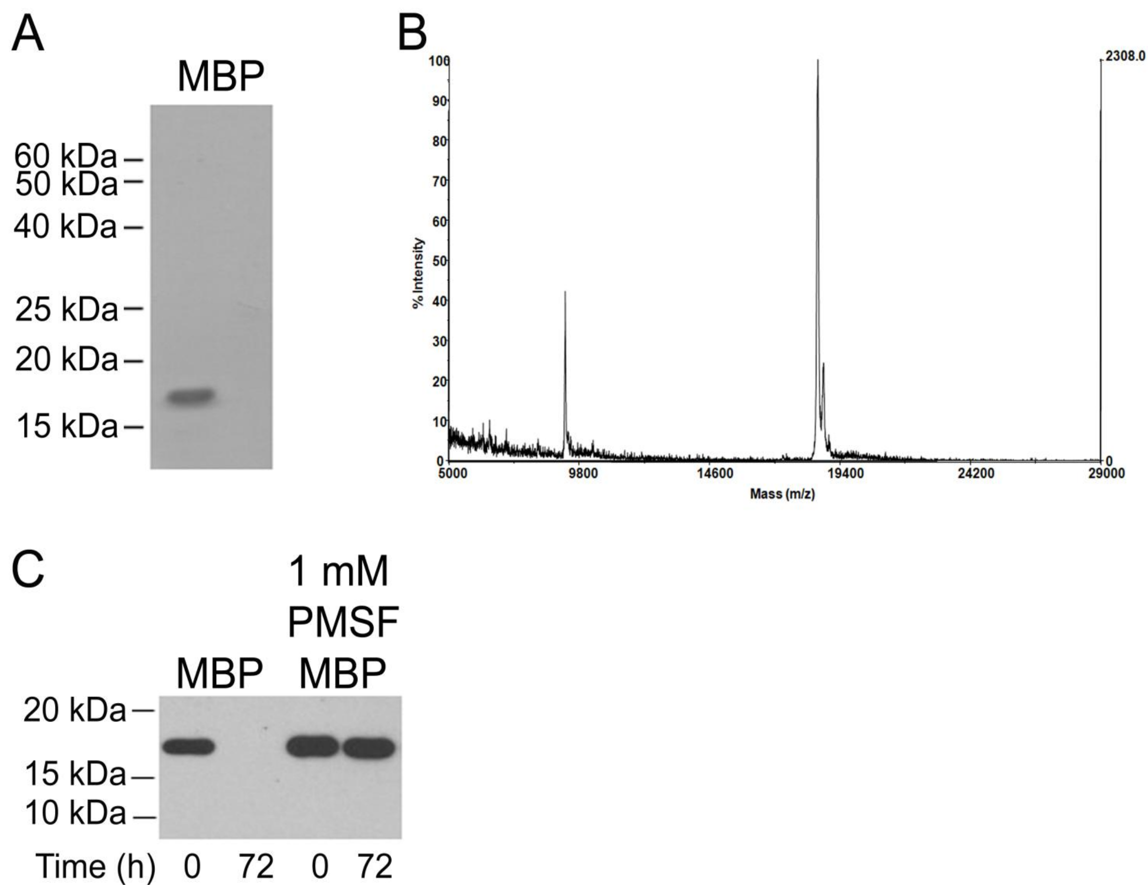



**Figure 3.2 Purified MBP exhibits autolytic activity.**
**(A)** Purified human MBP was analyzed by SDS-PAGE and silver staining. **(B)** Mass spectrometry analysis of purified human MBP (linear mode). **(C)** Purified human MBP was incubated at 37℃ in the absence or presence of 1 mM PMSF for 72 h. Aliquots were analyzed by immunoblotting using the anti-MBP mAb22.

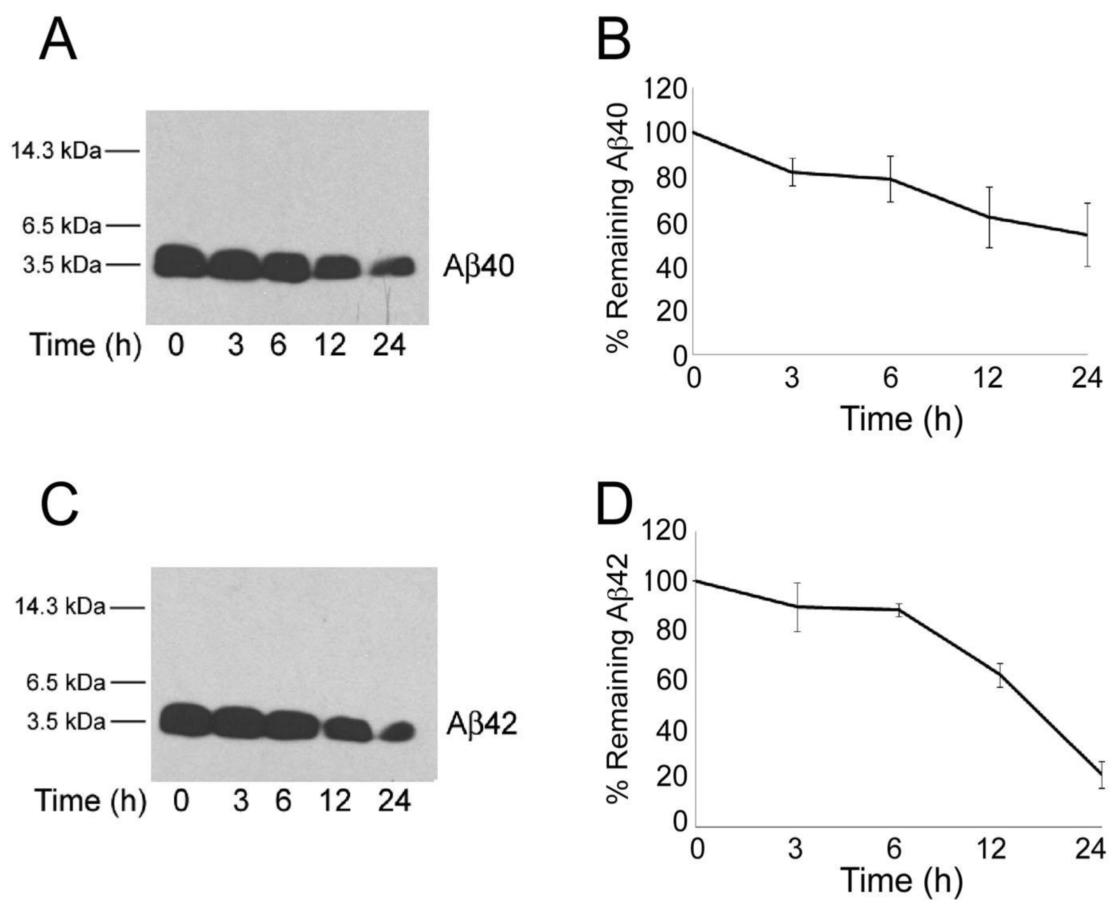



**Figure 3.3** ***In vitro*** **time-dependent degradation of Aβ40 and Aβ42 by purified MBP.**

Samples of 1 μM Aβ40 (**A**) or Aβ42 (**C**) were incubated at 37℃ in the presence or absence of 250 nM of purified MBP for increasing lengths of time. At each time point, samples were collected and analyzed for remaining Aβ by quantitative immunoblotting using the anti-Aβ mAb20.1. Quantitation of Aβ40 (**B**) and Aβ42 (**D**). The data shown are the mean ± SD of three separate determinations.

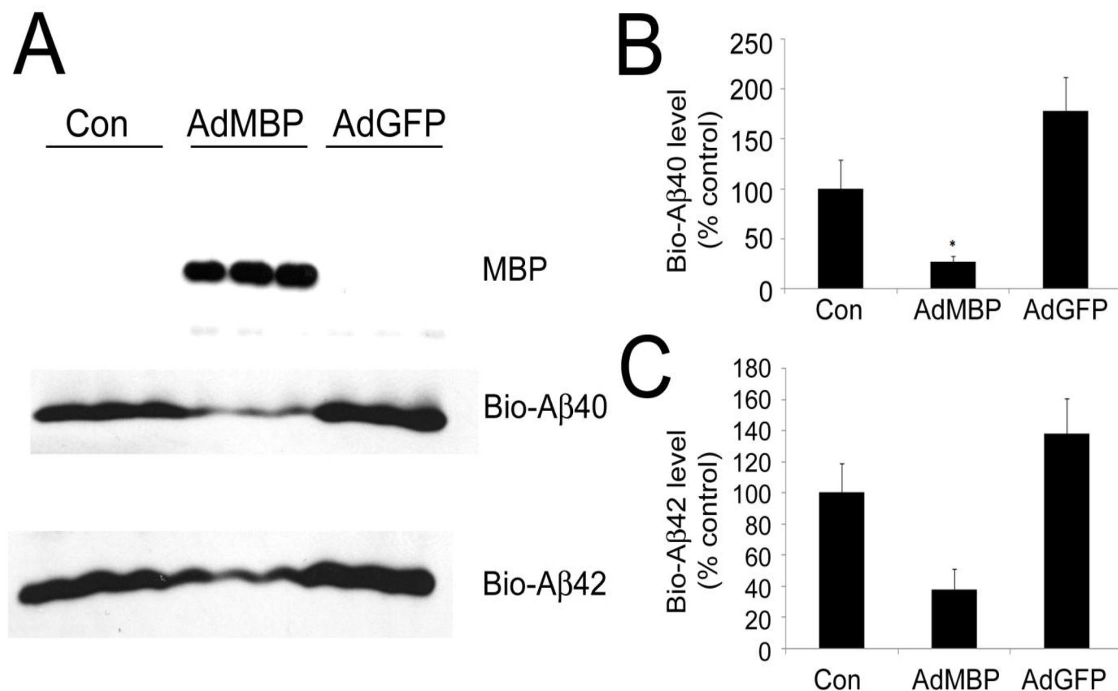



**Figure 3.4 A-C Exogenous biotinylated-Aβ40 and -Aβ42 degradation by MBP expression in Cos-1 cells.**

Near confluent cultures of Cos-1 cells were uninfected or infected with Ad-MBP or Ad-GFP as a control at a MOI 250. Twenty four hours post infection, the cells were incubated with 2 μg/ml of biotinylated-Aβ40 or -Aβ42 in serum-free media for an additional 48 h. **(A)** The cell lysates were collected and analyzed by immunoblotting using the anti-MBP mAb22 to confirm MBP expression. The cell culture media samples were collected and analyzed for Aβ levels by blotting using streptavidin HRP. Quantitation of the levels of biotinylated-Aβ40 (**B**) and biotinylated-Aβ42 **(C)** in the collected cell culture media samples. The data presented are the mean ± S.D. of three separate determinations. Exogenous Aβ40 and Aβ42 peptides in the MBP-infected Cos-1 cells were significantly decreased by MBP (*, $p < 0.05$).

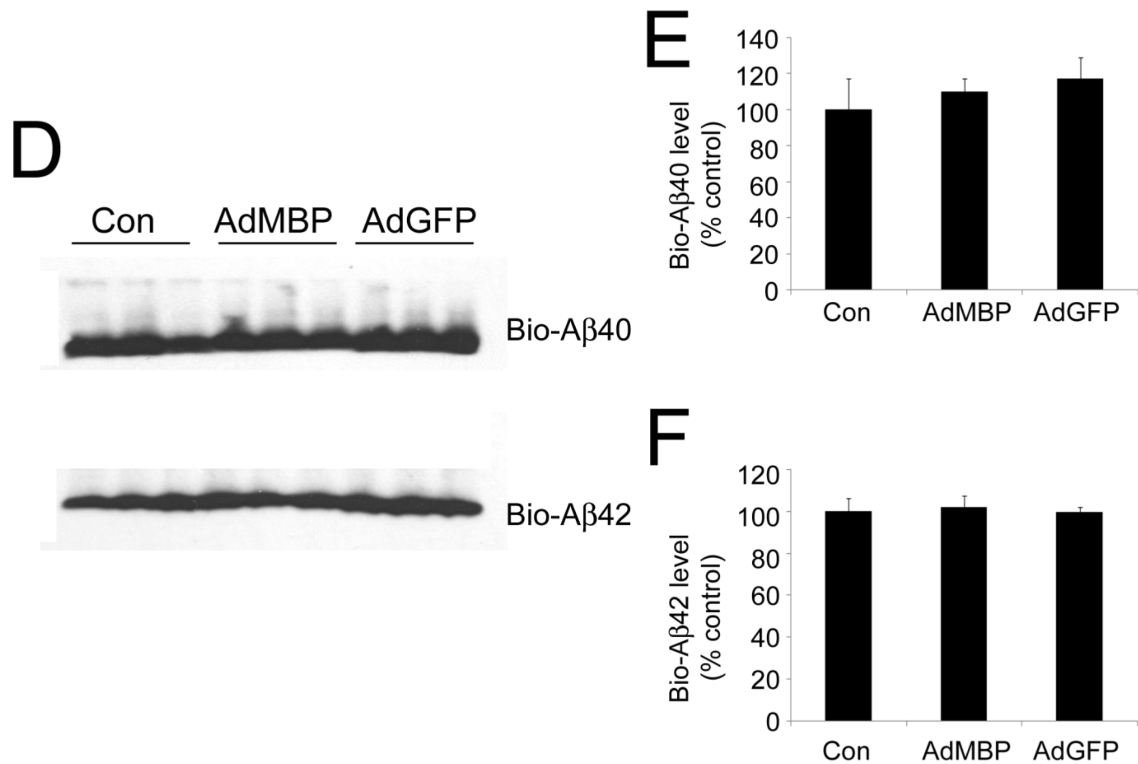



**Figure 3.4 D-F Exogenous biotinylated-Aβ40 and -Aβ42 degradation by MBP expression in Cos-1 cells.**
Near confluent cultures of Cos-1 cells were uninfected or infected with Ad-MBP or Ad-GFP as a control at a MOI 250. Twenty four hours post infection, the cells were incubated with 2 μg/ml of biotinylated-Aβ40 or -Aβ42 in serum-free media for an additional 48 h. **D)** The cell lysate samples were collected and analyzed for Aβ levels by blotting using streptavidin HRP. Quantitation of the levels of biotinylated-Aβ40 (**E**) and biotinylated-Aβ42 (**F**) in the collected cell lysate samples. The data presented are the mean ± S.D. of three separate determinations. Exogenous Aβ40 and Aβ42 peptides in the MBP-infected Cos-1 cells were significantly decreased by MBP (*, $p < 0.05$).

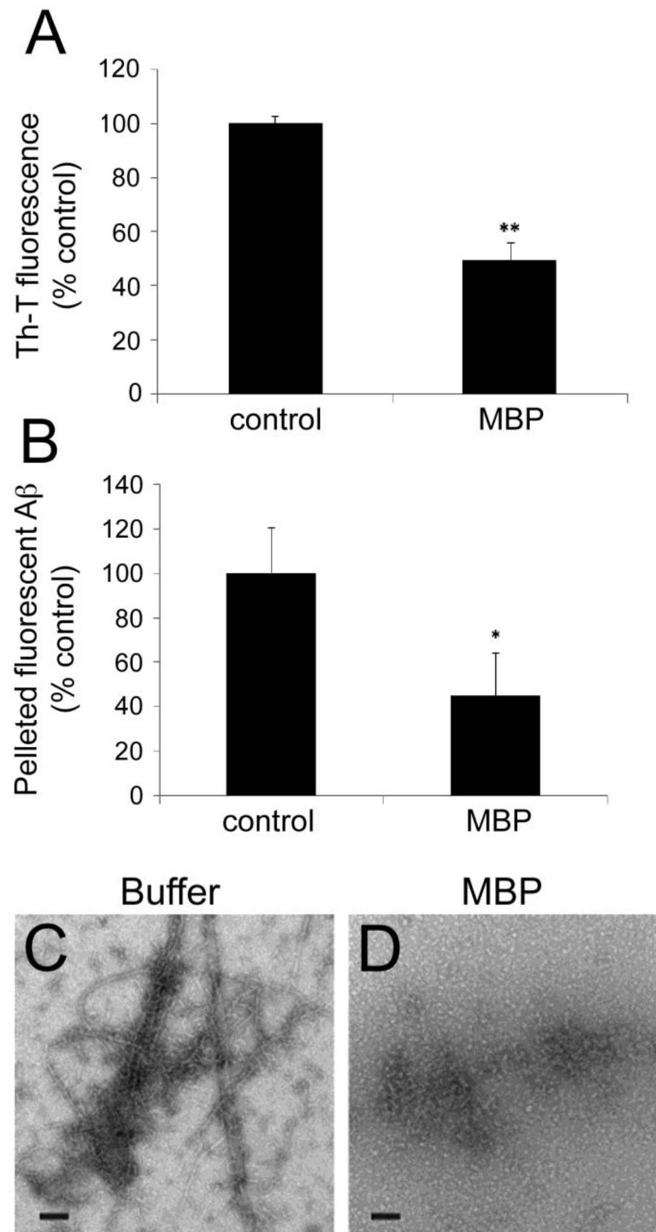



**Figure 3.5** ***In vitro* fibrillar Aβ degradation by purified MBP.**
**(A)** Fibrillar Aβ42 was incubated in the absence or presence of purified MBP at 37℃ for 48 h. The remaining fibrillar in the samples was quantitated using a Th-T fluorescence assay. The data shown are the mean ± SD of three separate determinations. **, $p < 0.01$. **(B)** The fluorescence in the pellet of FITC-labeled fibrillar Aβ42 was prepared and incubated in the absence or presence of purified MBP. The remaining fluorescent fibrillar Aβ was pelleted by centrifugation and measured in a fluorimeter. The data shown are the mean ± SD of three separate determinations. *, $p < 0.05$. Fibrillar Aβ42 was incubated in the absence (**C**) or presence (**D**) of purified MBP at 37℃ for 48 h. The samples were then visualized by TEM. Scale bars = 100 nm.

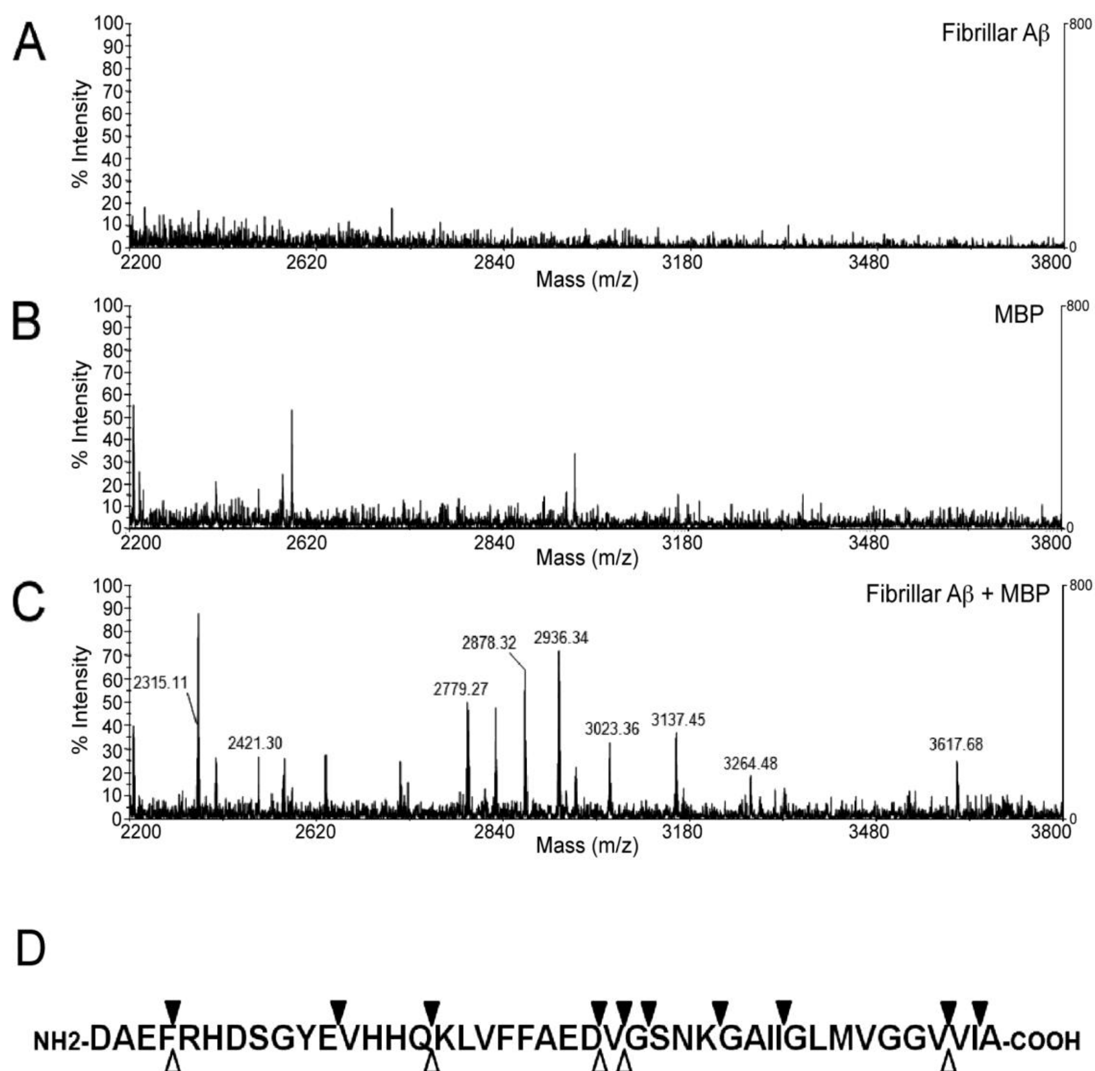



**Figure 3.6 MALDI-TOF MS analysis of Aβ fragments released from fibrillar Aβ digestion by purified MBP.**

Fibrillar Aβ42 alone (**A**), purified MBP alone (**B**), or fibrillar Aβ42 and MBP together (**C**) were incubated 37℃ for 5 days. After incubation the supernatants from the samples were anlayzed by MALDI-TOF mass spectrometry. Comparing with fibrillar Aβ42 alone or MBP alone, nine specific peaks were identified as Aβ fragments (reflector mode). **(D)** Summary of the MBP cleavage sites on Aβ42. ▼, cleavage sites identified on fibrillar Aβ42; Δ, cleavage sites identified on soluble Aβ42.

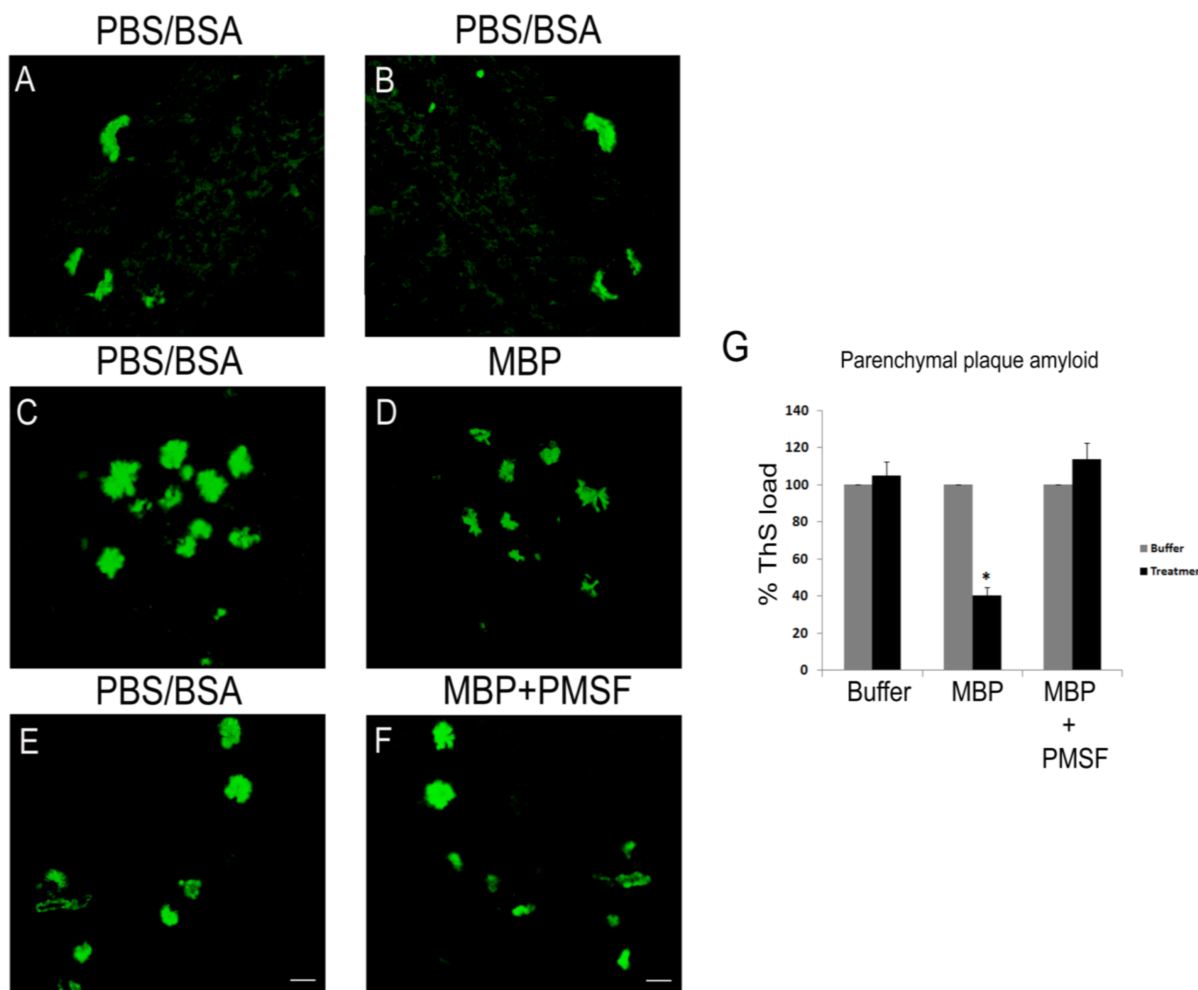



**Figure 3.7** ***In situ* degradation of brain parenchymal fibrillar Aβ plaques by purified MBP.**

Adjacent 5 µm fresh frozen brain sections from 18 months old Tg2576 mice were incubated alone (**A,B,C,E**) or with purified MBP (**D**) or PMSF-treated MBP (**F**) at 37℃ for 2 days. The sections were then fixed, and stained with ThS. Scale bars = 50 µm. (**G**) The parallel cortical fibrillar amyloid plaque areas were quantified in the treated (gray bars) and untreated (black bars) sections and expressed as percent remaining ThS area. The data presented are the mean ± S.D. of n = 25 plaques (buffer alone); n = 37 plaques (incubated with MBP); n = 29 plaques (incubated with PMSF-treated MBP). *, p < 0.001, paired *t* test.

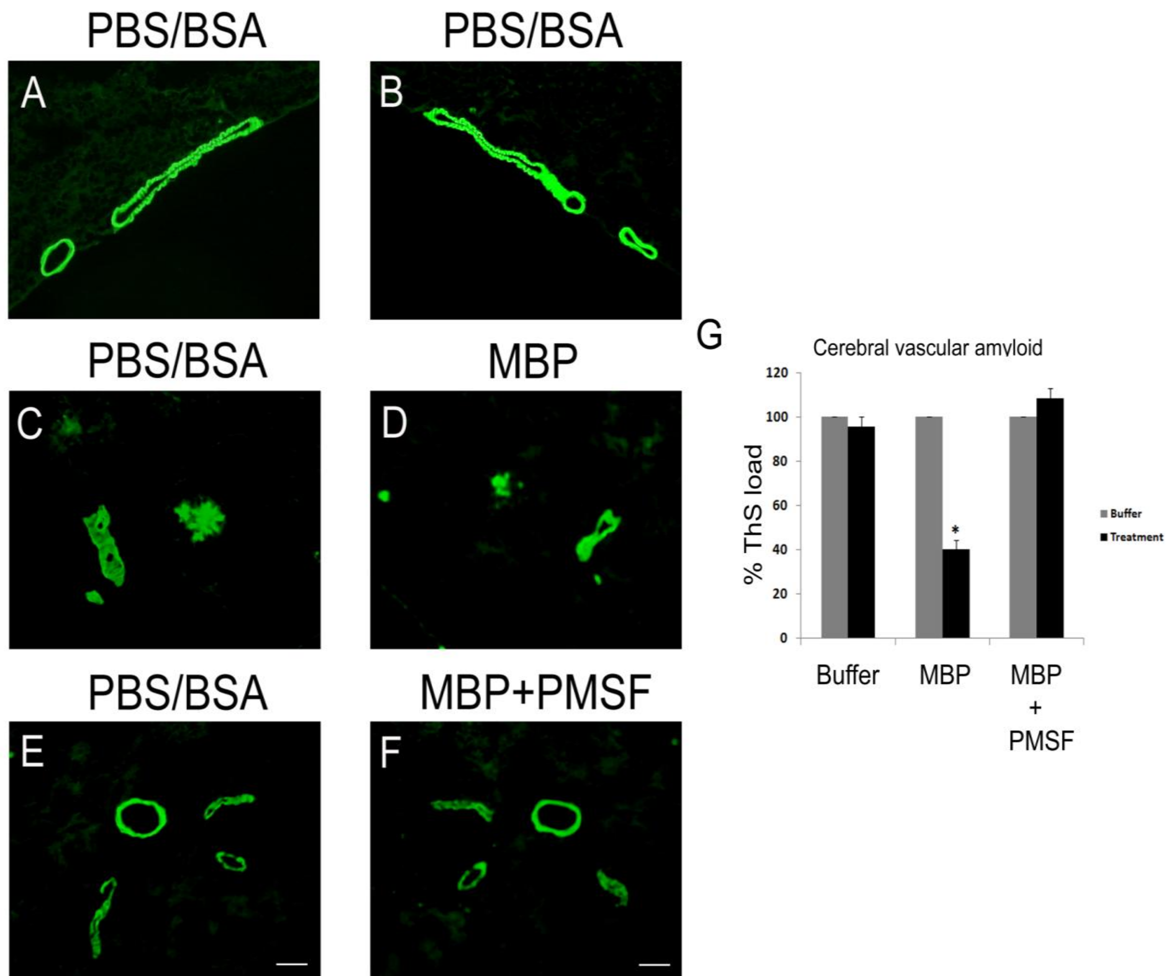



**Figure 3.8** ***In-situ*** **degradation of cerebral vascular fibrillar Aβ deposits by purified MBP.**
Adjacent 5 µm fresh frozen brain sections from 18 months old Tg2576 mice were incubated alone (**A,B,C,E**) or with purified MBP (**D**) or PMSF-treated MBP (**F**) at 37℃ for 2 days. The sections were then fixed, and stained with ThS. Scale bars = 50 µm. (**G**) The parallel cerebral vascular fibrillar amyloid deposit areas were quantified in the treated (gray bars) and untreated (black bars) sections and expressed as percent remaining ThS area. The data presented are the mean ± S.D. of n = 16 vascular deposits (buffer alone); n = 18 vascular deposits (incubated with MBP); n = 15 vascular deposits (incubated with PMSF-treated MBP). *, $p < 0.001$, paired *t* test.

# CHAPTER 4 – Enzymatic Activity of Purified Myelin Basic Protein

## 4.1 – Summary

**MBP is the major structural component of myelin sheath, and was reported to possess serine protease activity. In the previous chapter, we also demonstrate that MBP possesses Aβ-degrading activity. It has been proposed that serine 151 was the active serine residue. However, several lines of evidence in the present study show that serine 151 is not the precise proteolytic active site. To further define the mechanism, a series of C-terminal deletion mutants were expressed, purified and tested for protoelytic activity to aid in the identification the responsible region. Also, purified mouse brain MBP or recombinant mouse MBP also exhibit similar proteolytic activities suggesting these activities are conserved between mice and humans. Future *in vivo* experiments will be designed in human AßPP transgenic mouse models to understand the role of MBP in the pathology of AD.**

## 4.2 – Introduction

The "classic" myelin basic proteins (MBPs) are major structural components of myelin sheaths accounting for 30% of total myelin protein. There are four different major isoforms generated from alternative splicing with molecular weights of 17.3, 18.5 20.2 and 21.5 kDa. The 18.5 kDa variant, composed of 180 amino acids including 19 Arg and 12 Lys basic residues, is most abundant in mature myelin (*62*). One of the major

functions of MBP is to hold together the cytoplasmic leaflets of myelin membranes in order to maintain proper compaction of the myelin sheath through the electrostatic interaction between the positive Arg and Lys residues of MBP and the negatively charged phosphate groups of the membrane lipid (*67*). MBP plays an important role in the pathology of multiple sclerosis, which is an autoimmune disease characterized by demyelination within white matter (*68*). Recently, it was reported that purified MBP exhibits autocleavage activity generating distinct peptide fragments (*70*). In this study, serine 151 was reported as the active site serine residue involved in autocatalysis.

In chapter 3 of this thesis studies, we show that purified MBP from human white matter could degrade soluble Aβ40, Aβ42 peptides and fibrillar Aβ *in vitro*. Furthermore, purified MBP degraded parenchymal and vascular fibrillar amyloid deposits *in situ* in the brain tissue of APP transgenic mice. These findings indicate that purified MBP possesses Aβ degrading activity *in vitro*. Although Serine 151 was reported as the active serine residue for MBP autolysis activity this finding lacked confirmation by additional analysis (*70*).

In the present study we show that unmodified human MBP purified from bacterial recombinant protein expression exhibits essentially the same autolysis and Aβ-degrading activities of purified human brain MBP indicating that post-translational modifications are not necessary for these activities. Similarly, both brain purified and recombinant mouse MBP proteins exhibit a behavior similar to human MBP with regards to autolysis and Aβ degradation. A purified recombinant $MBP^{S151A}$ mutant still exhibits autolysis and Aβ-degradaing activity. In addition, in Cos-1 cells overexpressed $MBP^{S151A}$ degrades exogenous Aβ. Finally, $MBP^{S151A}$ purified from infected Cos-1 cells also exhibits

autolysis and Aβ-degrading activities. Collectively, these lines of evidence strongly suggest that serine 151 is not the proteolytic active site of MBP. Subsequently, a series of C-terminal deletion MBP proteins were prepared and tested for autolysis and Aß degradation activities to further define the responsible region.

## 4.3 – Materials and Methods

*Reagents and Chemicals* – Synthetic naïve or amino-terminal biotinylated Aβ40 and Aβ42 peptides were synthesized by solid-phase Fmoc (9-fluorenylmethoxycarbonyl) amino acid chemistry, purified by reverse phase high performance liquid chromatography, and structurally characterized as previously described (*76*). IPTG was purchased from Sigma-Aldrich (St. Louis, MO).

*Construction of Recombinant Human and mouse MBP and Series Serine-Alanine Mutants* - The cDNA encoding the 18.5-kDa isoform of human MBP and the 14-kDa isoform of mouse MBP were subcloned into the vector pPROEXHT (Life technologies, Grand Island, NY). This clone allowed production of recombinant human MBP with an amino-terminal 6x-His tag (6x-hisMBP). To produce serine-alanine human MBP mutant series of oligoneucleotide primers were designed as shown in Table 4.1 and site-directed mutagenesis were performed by PCR either following the protocol from QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) or Change-IT Multiple Mutation Site-Directed Mutagenesis Kit (cleveland, Ohio). Serine-alanine mutation was confirmed by DNA sequencing.

*Construction of Recombinant Carboxyl Terminal Deletion Human MBP Mutants* - The cDNAs encoding series of carboxyl terminal deletion human MBP mutants were amplified by polymerase chain reaction (PCR) using series primers shown in Table 4-2. The fragments were subcloned into the vector pPROEXHT (Life technologies, Grand Island, NY). The integrity of these fragments was confirmed by DNA sequencing.

*Recombinant Human and mouse MBP Expression and Purification* – Following transformation of the recombinant human MBP DNAs into *E. coli* BL21 (DE3) pLysS (Novagen), 5 ml cultures were grown in LB broth containing 100 μg/ml ampicillin at 37℃ for overnight and then used to inoculate 1 L cultures for larger scale production. Expression was induced at $A_{600}$ nm = 0.5 by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to 0.5 mM, and then growth was allowed to proceed for an additional 3 h. For recombinant MBP protein purification, the bacterial lysate was redissolved in 20 ml of a buffer containing 6 M urea, 5 mM imidazol, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, by stirring for 16 h at 4℃. This material was centrifuged at 6,000 x *g* for 30 min and then applied to a column containing 1 ml of HisBind resin (Invitrogen). Proteins that were nonspecifically bound were removed by washing with 10 ml of lysate buffer and lysate buffer containing 20 mM imidazole sequentially. Bound recombinant hisMBP proteins were eluted with 1 M imidazole in lysate buffer. The 6x-hisMBP-containing fractions were then dialysis against with buffer of 6M Urea and 80 mM Glycine, pH 10.5 or 9 for full-length MBP or MBP deletion mutants, respectively, loaded onto a Whatman CM52 column and eluted with 80 mM glycine buffer, pH 10.5 or

9, containing 200 mM NaCl. Protein refolding was achieved by removal of denaturant and salt by slow dialysis into distilled water.

*Isolation and Purification of MBP from Mouse Brain* — MBP was purified from normal human white matter or pooled mouse brains following previously described procedures (*104*). The predominant mouse 14.3 kDa MBP exists as a family of charge isomers that differ in net charge and result from various posttranslational modifications. To isolate the individual charge isomers of MBP, the mouse brain homogenates were loaded onto a CM52 cation exchange column, and the components were eluted with a 0-0.2M NaCl gradient. Component 8 was found in the void volume while the more cationic components (C5, C4, C3, C2, and C1) eluted with an increasing salt gradient. The components were dialyzed against water, lyophilized, and stored at -80°C. The most abundant MBP C1 component was used in all subsequent studies. Yields were typically ≈500 µg of MBP from 20 g of pooled mouse brains.

*Quantitative Immunoblotting* - Samples containing MBP or Aβ were added directly into SDS-PAGE sample buffer, and stored at –70℃. Aliquots were loaded onto 12% or 10-20% polyacrylamide gels, electrophoresed and transferred onto Hybond-ECL nitrocellulose membranes (Amersham, Arlington Heights, IL) at 100 V for 1.5 hour at RT. Membranes were blocked in 5% milk/PBS/0.05% Tween20 (PBS-T) for 1 h at RT. Primary antibodies were added (mAb61B for MBP; mAb20.1 for Aβ) for 1 h at RT, washed 3 x 5 min with PBS-T. Secondary horseradish peroxidase-conjugated sheep anti-mouse IgG antibodies were then added to the membranes (1:5000 Amersham-Pharmacia,

Piscataway, NJ), which were then washed 3 x 5 min with PBS-T. Bands were visualized using the ECL detection method (Amersham-Pharmacia, Piscataway, NJ). Quantitation of MBP or Aβ bands was performed using a VersaDoc Imaging System (BioRad, Hercules, CA) and the manufacturer's Quantity Oneton software.

*In Vitro Aβ Degradation* - Aβ peptides were initially prepared in hexafluoroisopropanol, dried, and resuspended in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/ml. 1 μM Aβ40 or Aβ42 was incubated in the absence of presence of 250 nM of purified MBP, hisMBP or hisMBP mutants in PBS buffer with 1 mg/ml of BSA at 37 ℃ for 24 h. The Aβ samples were then analyzed by quantitative immunoblotting as described above.

*Aβ Degradation in Cos-1 Cells Expressing Human MBP* - Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-products, Woodland, CA) in 24-well tissue culture plates. Triplicate near confluent cultures were infected with Ad-MBP (MOI: 250), followed by addition of 2 μg/ml of Aβ40 in serum-free media for 48 h. The culture media samples were collected and cell lysates were prepared. The level in Aβ peptides in the cell culture media or cell lysate samples was quantitatively analyzed by immunoblotting against Aβ mAb20.1. The cell culture lysates were analyzed for MBP expression by immunoblotting as described above.

*MBP purification from Cos-1 Cells Expressing Human MBP or $MBP^{S151A}$* - Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

fetal bovine serum (Gemini Bio-products, Woodland, CA) in P150 detri-dish. Confluent cultures were infected with Ad-MBP or Ad-MBP$^{S151A}$ (MOI: 500) for 48 h. The cell lysates were collected and then purified MBP or MBP$^{S151A}$ mutant protein by CM52 cation exchange column following same protocol as described above in the Chapter 3.

*Statistical Analysis* - Data were analyzed by Student's t-test at the 0.05 significance level.

## 4.4 – RESULTS

**Proteolytic activity and Aβ degradation activity of recombinant MBP**

MBP has numerous post-translational modifications including N-terminal acylation, methylation of arginine, phosphorylation, deamination and deimination. These modifications can change the net charge of the protein, which results in a family of microheteromers or charge isoforms (*66*). To test if recombinantly expressed MBP exhibits behavior similar to brain MBP with regards to autolysis and Aβ degradation, the cDNA of human MBP was cloned into the PROEX-HT system to generate N-terminal 6x-his tagged MBP. Previously, our laboratory showed that MBP strongly interacts with Aβ peptides and prevents their assembly into mature amyloid fibrils (*73, 89*). More recent work demonstrated that the amino terminal 64 amino acids of MBP harbors the Aβ binding domain and a stable peptide (MBP1) comprised of these residues is sufficient to inhibit Aβ fibrillogenesis. The purified recombinant 6x-hisMBP wild type (hisMBP) exhibited similar autolysis and Aβ-degrading activity as purified human brain MBP (Figure 4.1). However, the recombinant amino terminal fragment of MBP containing residues 1-64 (hisMBP1) possesses neither autolytic nor Aβ-degrading activities.

### Recombinant 6x-hisMBP$^{S151A}$ mutant exhibits autocleavage

The serine 151 in MBP was reported to be the active site serine involved in its autolysis (*70*). To confirm that serine 151 is the active site involved in autolysis as well as in the Aβ degradation we produced recombinant wild-type and S151A mutant human 6x-hisMBP. However, Figure 4.2 shows that recombinant 6x-hisMBP$^{S151A}$ possesses similar autolysis and Aβ-degrading activities suggesting that serine 151 is not the proteolytic active site.

### Exogenous Aβ40 degradation in Cos-1 cells expressing human MBP$^{S151A}$

In Chapter 3, we showed that exogenous Aβ peptides were degraded in Cos-1 cells overexpressing human MBP. To further evaluate if serine 151 is the proteolytic active site of MBP, we examined exogenous Aβ degradation in Cos-1 cells overexpressing MBP$^{S151A}$ or wild-type MBP. Immunoblotting confirmed that Cos-1 cells infected with Ad-MBP or Ad-MBP$^{S151A}$ express their respective proteins (Figure 4.3A). Quantitative immunoblotting or ELISA analysis showed that Aβ40 levels were similarly decreased in the culture media of Ad-MBP and Ad-MBP$^{S151A}$ compared to uninfected or Ad-GFP infected cells as shown in Figure 4.3B and C. These results suggest that MBP$^{S151A}$ mutant expressed in Cos-1 cells can still degrade Aβ.

### Purified MBP$^{S151A}$ protein from infected Cos-1 cells exhibits autolysis and Aβ-degrading activities *in vitro*

Subsequently, we purified MBP wild type and $MBP^{S151A}$ mutant from adenovirus infected Cos-1 cells to further test autolysis and Aβ-degrading activities. Figure 4.4 A shows that both wild-type and mutant MBP proteins exhibit autocleavage activity *in vitro*. Further, purified $MBP^{S151A}$ mutant still possesses Aβ-degrading activity as shown in Figure 4.4 B. Collectively, our findings strongly indicate that serine 151 is not the proteolytic active site of MBP.

**Site-directed mutational analysis of each serine residue in MBP still exhibited autolytic activity.**

To define the precise active serine site for MBP enzymatic activity, each single serine residue was individually mutated to alanine by site directed mutagenesis and was expressed in the bacterial PROEX-HT system as described in Materials & Methods. Surprisingly, each of these serine mutants still exhibited autocleavage as shown in Figure 4.5 suggesting that none of these serine residues was the active site.

**Characterization of autolysis and Aβ-degrading activities of serial carboxyl terminal deletion MBP mutants**

None of the individual serine residues was found to be responsible for MBP proteolytic activity. Therefore, to further understand the enzymatic mechanism of MBP, a series of carboxyl terminal deletion mutants were constructed in 6x-his tagged MBP cDNA. Following expression and purification, we tested the autolysis activity of these purified deletion mutants as shown in Figure 4.6 MBPd150, which lacks the serine 151, still possessed autolysis activity further confirming that serine 151 is not the proteolytic

active site for MBP. On the other hand, shorter deletion mutants such as MBPd120 or MBPd110 did not exhibit autocleavage activity. *In vitro* Aβ degradation activity of these carboxyl deletion mutants was also tested showing MBPd150, but not MBPd130, also could degrade Aβ40 (Fig. 4.7). These data suggests that the region containing amino acids 120-140 is necessary for MBP enzymatic activity.

**Proteolytic and Aβ-degrading activities of mouse brain MBP and recombinant mouse MBP**

We demonstrated that human brain MBP and recombinant human MBP possessed autolysis and Aβ-degrading activities. APP transgenic mice are commonly used to model Alzheimer's disease –like amyloid deposition. Since MBP is expressed in mouse brain it may influence amyloid assembly and deposition in these models. Therefore it is important to know if mouse MBP exhibits similar behavior to human MBP wih regards to autolysis and Aβ degradation. Since a mouse brain is very small and contains relatively little white matter compared to human brain we need to pool ≈45 mouse brains to obtain 20 g of starting material for the purification. This is sufficient to yield ≈500 µg of highly purified mouse MBP for analysis. The main species in the C1 fraction is the 14-kDa isoform, and the C2 or C3 contain 14-kDa and 18-kDa mixtures. Figure 4.8 shows that mouse brain MBP-C1 isoform exhibits autolysis and Aβ-degrading activities. Besides the cDNA of mouse 14-kDa MBP is also introduced into *E.Coli* PROEX-HT system as described in Materials & Methods, and purified recombinant mouse MBP also possesses autolysis and Aβ degradation. These data indicate that mouse MBP behavior similar to human MBP with regards to autolysis and Aβ degradation.

recombinant 6x-his mouse MBP 14-kDa all possessed autolysis and Aβ-degrading activities.

## 4.5 – DISCUSSION

In this study, purified $MBP^{S151A}$ mutant from different sources such as recombinant 6x-his-$MBP^{S151A}$, or from Ad-$MBP^{S151A}$ infected Cos-1 system still exhibits autolysis and Aβ-degrading activities. And Cos-1 cells overexpressed MBPS151A still could degrade exogenous Aβ strongly support that serine151 is not the active site for MBP. To further define the enzymatic mechanism, a sequential series of C-terminal deletion mutant was tested for autolysis and Aβ-degrading activities showing the region consist with amino acid 120-140 are important. However, the N-terminal 64 amino acid of MBP (MBP1), which is sufficient for interaction with Aβ, possessed neither autolysis nor Aβ-degrading activities. Lastly, mouse brain and recombinant mouse MBP similar to human MBP exhibited autolysis and Aβ-degrading activities.

In the previous study of MBP autolysis, it was proposed that serine 151 may be the active site (*70*). In this report, the authors labeled myelin basic protein with radiolabeled diisopropyl fluorophosphate (DFP), which selectively and covalently binds to active serine residues in proteolytic enzymes. Using mass spectrometry they concluded that serine 151 was the active site since only this single serine residue was labeled in the peptide that consisted of residues 140-152. However, purified serine 151 alanine mutants expressed in different system such as recombinant expressed in *E.Coli* or overexpressed in Cos-1 cells still exhibit autolysis and Aβ-degradation. Besides, the

shorter C-terminal deletion mutants such as d150, d140 still are active, also supporting that serine 151 is not the active site for MBP.

MBP has numerous post-traslational modifications resulting in different charge isoforms. The different charge isoform proteins purified from brain tissues (data not shown) and the recombinant purified MBP still exhibit similar autocatalytic and Aβ-degrading activities suggesting that post-translation modifications are not necessary for it's enzymatic activity. This recombinant MBP protein expression system is a useful system for us to define the precise active site for MBP. There are 19 serine residues in MBP, however, all of single serine mutants were still active suggesting either multiple serine residues may be required or a serine residue is not the active site for proteolytic activity. To further define this, a series of C-terminal deletion mutants were produced to test for activity and our results show that the region between amino acids 120 through 140 are necessary for MBP proteolytic function. Further efforts are needed to further define the precise nature of the proteolytic activity of MBP.

Recently in our lab we showed that the binding site for Aβ is located in the N-terminal 64 amino acids of MBP and that a stable peptide comprised of these residues (MBP1) is sufficient to inhibit the fibrillogenesis of Aβ. However, MBP1 possessed neither autolysis nor Aβ-degrading activities.

MBP purified from diverse sources including human and mouse brain tissues, and Ad-MBP infected Cos-1 cells all exhibit similar autolytic and Aβ-degrading activities. In adult mouse brain the 14.3 kDa MBP isoform predominates whereas in adult human brain it is the 18.5 kDa MBP isoform (*105*). The major difference in these two isoforms resides downstream from the first 64 amino acids of either MBP. Mouse MBP also

exhibits autolysis and Aβ-degrading activities also supporting that serine 151 is not the active site since there is no serine 151 residue in mouse MBP. Besides, mouse MBP behaves similar to human MBP in enzymatic activities, interaction with Aβ and inhibition of Aβ fibril formation (data not shown). Because it is popular to use human amyloid precursor protein transgenic mice model to study the Aβ deposition, it is worth to investigate the role of mouse MBP on Aβ clearance and deposition *in vivo* in these amyloid depositon model.

In summary, lots of evidence such as recombinant $MBP^{S151A}$ or $MBP^{S151A}$ overexpressed in Cos-1 cells showed that serine 151 is not the precise active site for MBP. A serial of C-terminal deletion mutant are tested to identify the region of amino acid 120-140 is required for MBP activity. Lastly, mouse MBP behaves similar to human MBP suggesting the autolysis and Aβ-degrading activities are conserved.

## 4.6 – TABLES & FIGURES

**TABLE 4.1**

**Oligonucleotide primers for serine-alanine site-directed mutagenesis.**

| Target serine residue | Sequence (5' to 3') |
|---|---|
| **Ser2Ala** | F: CGGAATTCCATGGCGGCACAGAAGAGACCCTCC |
| | R: GGAGGGTCTCTTCTGTGCCGCCATGGAATTCCG |
| **Ser7Ala** | F: TCACAGAAGAGACCCGCCCAGAGGCACGGATCC |
| | R: GGATCCGTGCCTCTGGGCGGGTCTCTTCTGTGA |
| **Ser12Ala** | F: TCCCAGAGGCACGGAGCCAAGTACCTGGCCACA |
| | R: TGTGGCCAGGTACTTGGCTCCGTGCCTCTGGGA |
| **Ser19Ala** | F: TACCTGGCCACAGCAGCTACCATGGACCATGCC |
| | R: GGCATGGTCCATGGTAGCTGCTGTGGCCAGGTA |
| **Ser40Ala** | F: ACGGGCATCCTTGACGCCATCGGGCGCTTCTTTG |
| | R: CAAAGAAGCGCCCGATGGCGTCAAGGATGCCCGT |
| **Ser56Ala** | F: GCGCCCAAGCGGGGCGCTGGCAAGGACTCACAC |
| | R: GTGTGAGTCCTTGCCAGCGCCCCGCTTGGGCGC |
| **Ser60Ala** | F: GGCTCTGGCAAGGACGCACACCACCCGGCAAGAA |
| | R: TTCTTGCCGGGTGGTGTGCGTCCTTGCCAGAGCC |
| **Ser71Ala** | F: ACTGCTCACTACGGCGCCCTGCCCCAGAAGTCAC |
| | R: GTGACTTCTGGGGCAGGGCGCCGTAGTGAGCAGT |
| **Ser76Ala** | F: TCCCTGCCCCAGAAGGCACACGGCCGGACCCAAG |
| | R: CTTGGGTCCGGCCGTGTGCCTTCTGGGGCAGGGA |
| **Ser102Ala** | F: TCGCACACCACCCCCGGCGCAGGGAAAGGGGAGA |
| | R: TCTCCCCTTTCCCTGCGCCGGGGGTGGTGTGCGA |
| **Ser132Ala** | F: CTACGGAGGCAGAGCGGCCGACTATAAATCGGCT |
| | R: AGCCGATTTATAGTCGGCCGCTCTGCCTCCGTAG |
| **Ser136Ala** | F: GCGTCCGACTATAAAGCGGCTCACAAGGGATTCA |
| | R: TGAATCCCTTGTGAGCCGCTTTATAGTCGGACGC |
| **Ser151Ala** | F: CCAGCTTAAAAATTTTGGCAAGCGTGCCCTGGGC |
| | R:GCCCAGGGCACGCTTGCCAAAATTTTTAAGCTGG |
| **Ser111-113-115Ala** | **5'phos-** AAGGGGAGAGGACTGGCCCTGAGGAGATTTAGGTGGGGGGC CGAAGGC |

**TABLE 4.2**

**Oligonucleotide primers for series of carboxyl-terminal deletion mutagenesis.**

| C-terminal deletion mutant | Sequence (5' to 3') |
|---|---|
| **full lengh** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCAGCGTCTAGCCATGGG** |
| **deletion 160** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCATCTTCCTCCCAGCTTAAAAATTTTG** |
| **deletion 150** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCACGTGCCCTGGGCATCGACT** |
| **deletion 140** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCACTTGTGAGCCGATTTATAGTCGGACGC** |
| **deletion 130** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCAGCCTCCGTAGCCAAATCCTGGTCT** |
| **deletion 120** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCATTCGGCCCCCCAGCTAAATCTGCT** |
| **deletion 110** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCACAGTCCTCTCCCCTTTCCCTGCGA** |
| **MBP1 (a.a. 1-64)** | **F: GGAATTCCATGGCGTCACAGAAGAGACC** |
| | **R: GACTAGTCTCACGGGTGGTGTGAGTCCTTGCCAGA** |

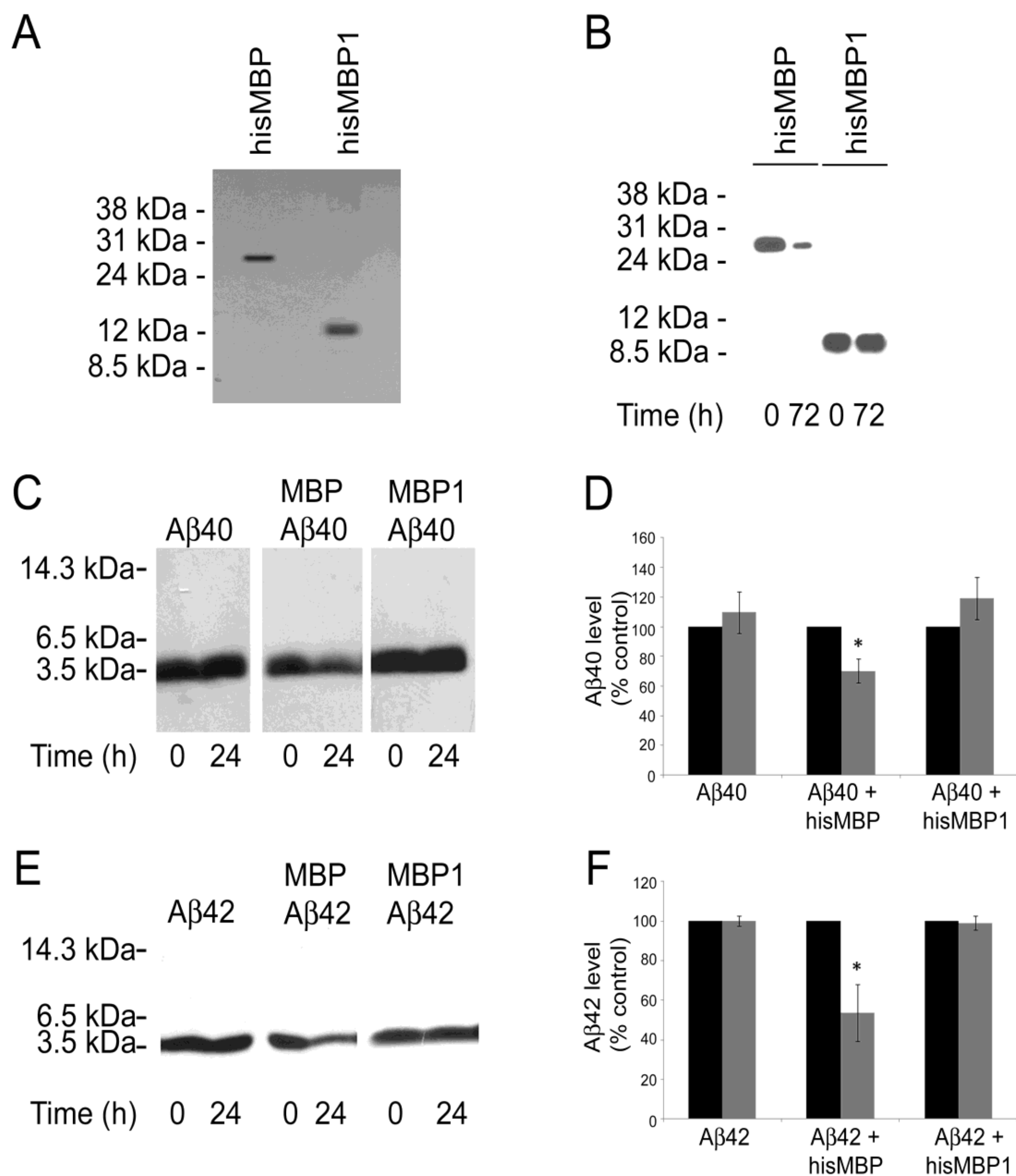



**Figure 4.1 Autolytic activity of recombinant hisMBP and hisMBP1 and *in vitro* Aβ degradation.**
**(A)** Purified recombinant hisMBP and hisMBP1 were analyzed by SDS-PAGE and Coomassie blue staining. **(B)** Purified recombinant hisMBP and hisMBP1 were incubated at 37℃ for 72 h. Aliquots were analyzed by immunoblotting using the anti-MBP50-64. Samples of 1 μM Aβ40 (**C**) or Aβ42 (**E**) were incubated at 37℃ in the presence or absence of 250 nM of purified MBP for 24 h and samples were analyzed for remaining Aβ by quantitative immunoblotting using the anti-Aβ mAb20.1. Quantitation of Aβ40 (**D**) and Aβ42 (**F**) The data presented are the mean ± S.D. of six separate determinations. (*, $p < 0.003$), paired *t* test.

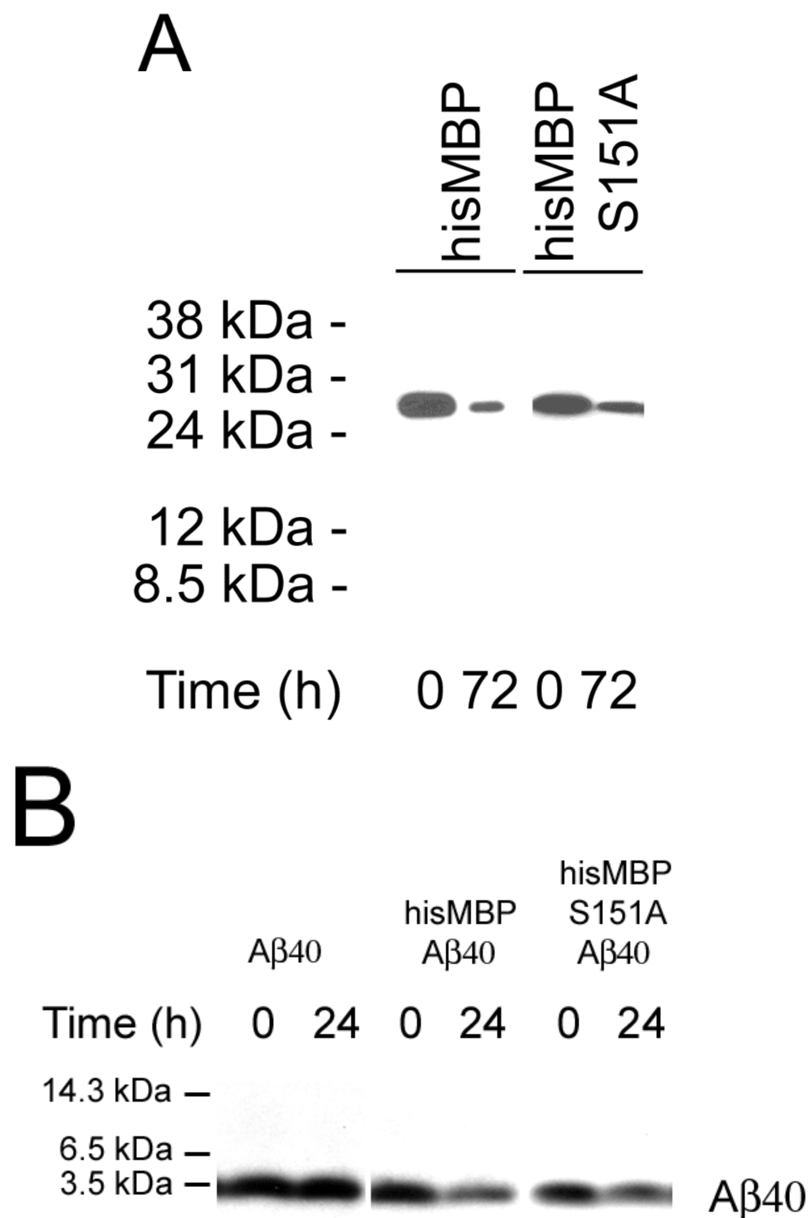
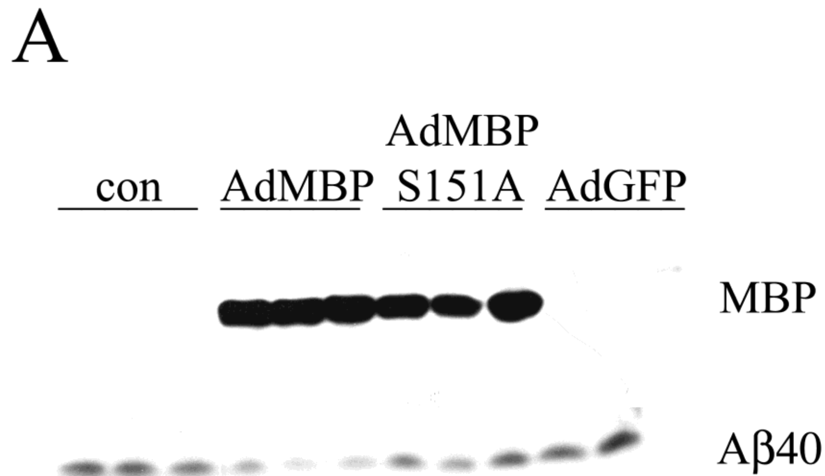



**Figure 4.2 Autolytic activity of recombinant hisMBP and hisMBPS151A.**
**(A)** Purified recombinant hisMBP and hisMBPS151A were incubated at 37℃ for 72 h. Aliquots were analyzed by immunoblotting using the anti-MBP50-64. (B) Samples of 1 μM Aβ40 were incubated at 37℃ in the presence or absence of 250 nM of purified MBP for 24 h and samples were analyzed for remaining Aβ by immunoblotting using the anti-Aβ mAb20.1.

A




B

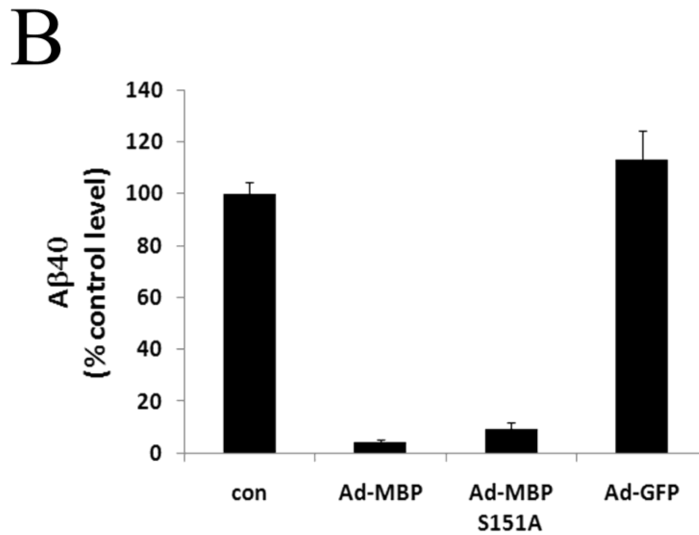
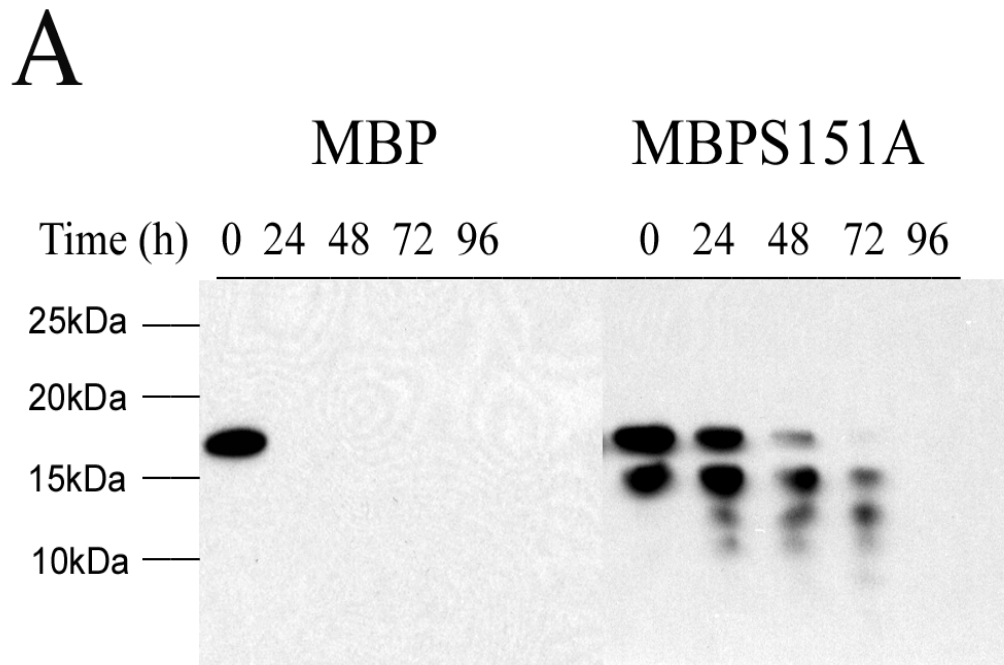



**Figure 4.3 Exogenous Aβ40 degradation by MBP wild type and MBPS151A expressed in Cos-1 cells.**
Near confluent cultures of Cos-1 cells were uninfected or infected with Ad-MBP, Ad-MBPS151A or Ad-GFP as a control at a MOI 250. Twenty four hours post infection, the cells were incubated with 2 μg/ml of Aβ40 in serum-free media for an additional 48 h. **(A)** The cell lysates were collected and analyzed by immunoblotting using the anti-MBP mAb22 to confirm MBP expression. The cell culture media samples were collected and analyzed for Aβ levels by blotting using mAbA20.1. **(B)** Quantitation of the levels of Aβ40 in the collected cell culture media samples. The data presented are the mean ± S.D. of three separate determinations. Exogenous Aβ40 and Aβ42 peptides in the MBP-infected Cos-1 cells were significantly decreased by MBP.



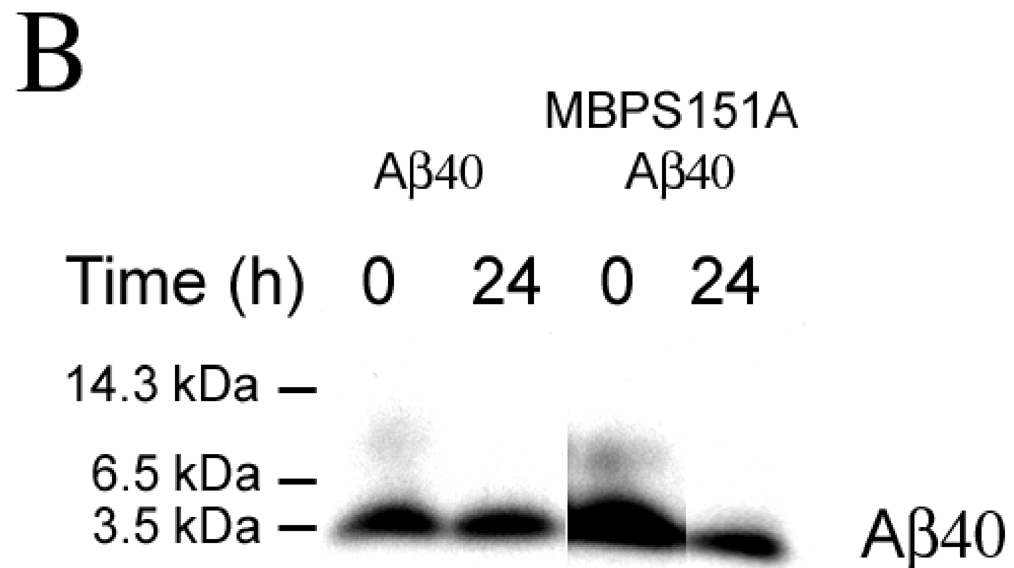




**Figure 4.4 Autolytic activity and Aβ-degrading activity of purified MBP and MBPS151A from Adenovirus infected Cos-1 cells.**

**(A)** Purified recombinant MBP and MBPS151A from infected Cos-1 cells were incubated at 37℃ for 72 h. Aliquots were analyzed by immunoblotting using the anti-MBP50-64. (B) Samples of 1 μM Aβ40 were incubated at 37℃ in the presence or absence of 250 nM of purified MBP for 24 h and samples were analyzed for remaining Aβ by immunoblotting using the anti-Aβ mAb20.1.

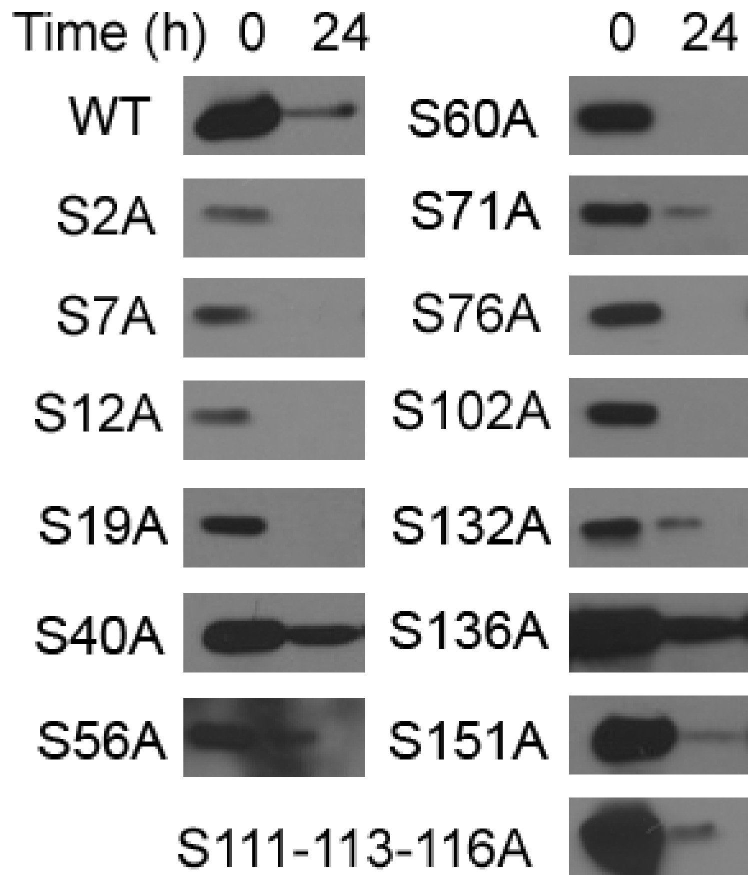



**Figure 4.5 Autolytic activity of recombinant hisMBP and hisMBP Ser/Ala mutants.**

Purified recombinant hisMBP and hisMBPSer/Ala mutants were incubated at 37℃ for 24 h. Aliquots were analyzed by immunoblotting using the anti-MBP50-64.

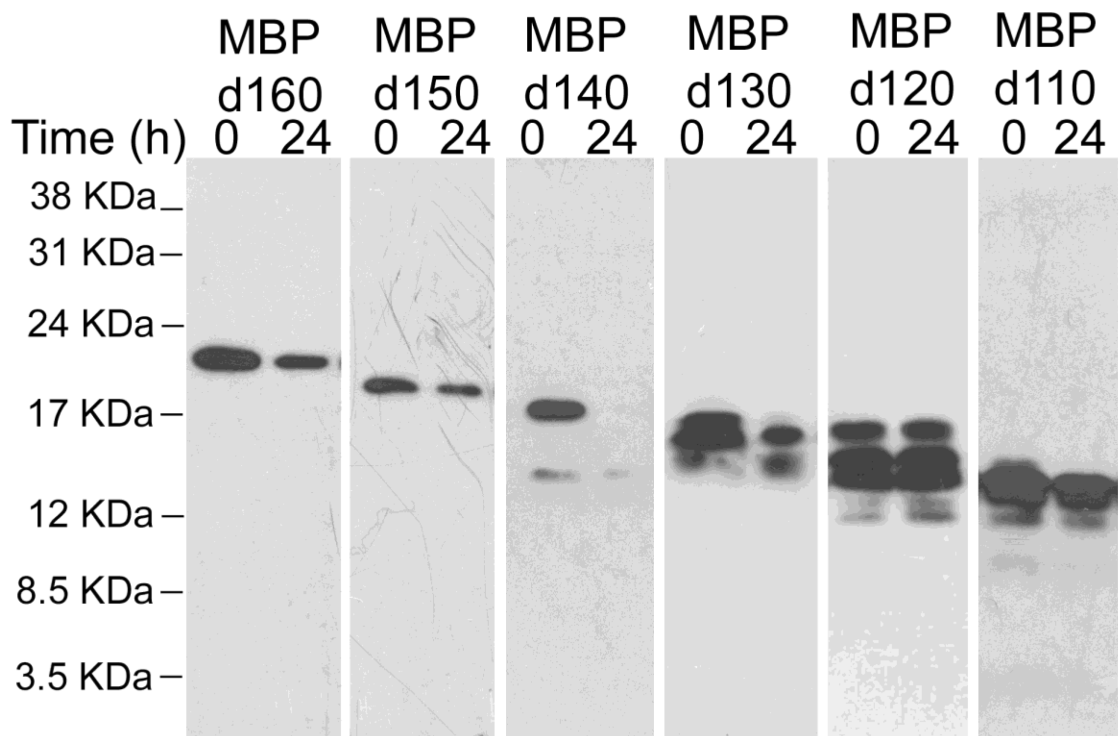



**Figure 4.6 Autolytic activity of recombinant carboxyl-terminal deletion his-tagged MBP mutants.**
Purified recombinant his-tagged carboxyl terminal deletion MBP mutants were incubated at 37℃ for 24 h. Aliquots were analyzed by immunoblotting using the anti-MBP1-64.

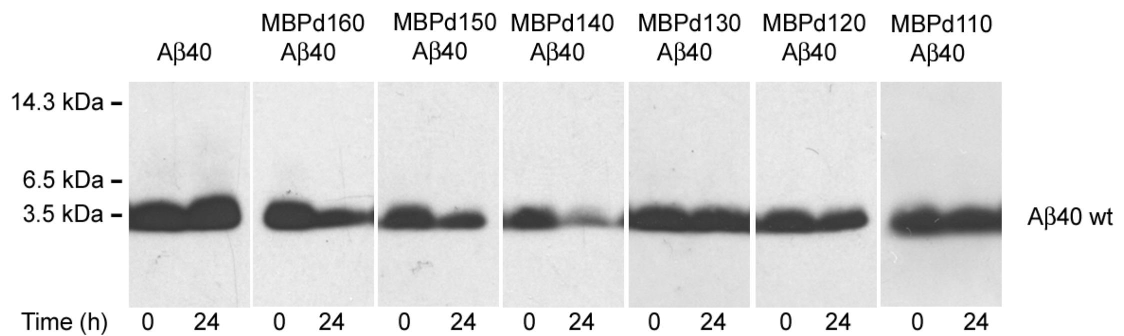
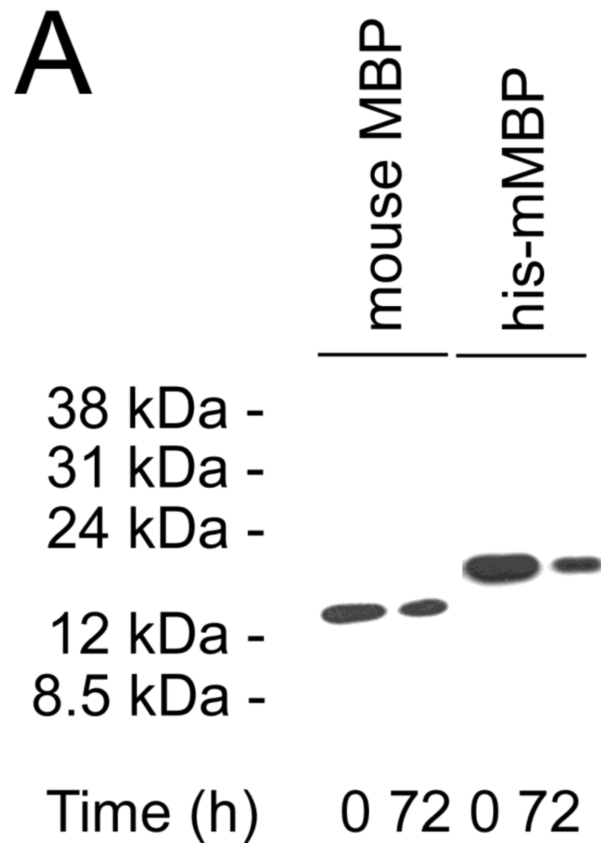



**Figure 4.7 *In vitro* Aβ degradation activity of recombinant carboxyl-terminal deletion his-tagged MBP mutants.**

Samples of 1 μM Aβ40 were incubated at 37℃ in the presence or absence of 250 nM of purified his-tagged carboxyl terminal deletion MBP mutants for 24 h and samples were analyzed for remaining Aβ by immunoblotting using the anti-Aβ mAb20.1.



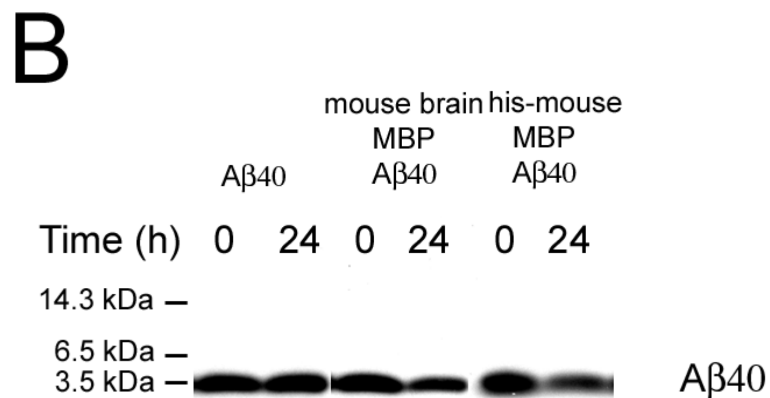




**Figure 4.8 Autolytic activity and Aβ-degrading activity of purified mouse brain MBP and recombinant mouse MBP.**
**(A)** Purified mouse brain MBP and recombinant 6x-his mouse MBP cells were incubated at 37℃ for 72 h. Aliquots were analyzed by immunoblotting using the anti-MBP50-64. (B) Samples of 1 μM Aβ40 were incubated at 37℃ in the presence or absence of 250 nM of purified mouse MBP for 24 h and samples were analyzed for remaining Aβ by immunoblotting using the anti-Aβ mAb20.1.

# CHAPTER 5 – Conclusions

## 5.1 – Summary of Results

The research in the past decade supports inefficient clearance of Aβ being largely responsible for progressive accumulation of Aβ, particularly in sporadic forms of AD and CAA. Enzymatic degradation of Aβ is one means to clear soluble monomeric and oligomeric forms, as well as insoluble fibrillar aggregates of Aβ. In this study, we used biochemical methods to characterize two Aβ-degrading enzymes in parallel – MT1-MMP and MBP.

Previusly, it was shown that MMP2 is selectively expressed in the reactive astrocytes surrounding amyloid plaques in APP-Sw transgenic mice and is involved in Aβ degrading for astrocytes. MT1-MMP, which is the physiological activator of MMP2, also colocalizes with astrocytes nearby vascular amyloid deposits in the APP-SwDI mice. MT1-MMP overexpressed in Cos-1 cells, which is active in pro-MMP2 activation, was capable of degrading exogenous Aβ. *In vitro* a purified soluble form of MT1-MMP, which is devoid of the transmembrane domain, degraded soluble and even fibrilar Aβ, and this activity was inhibited by the broad MMP inhibitor- GM6001 or the specific inhibitor- TIMP2. Furthermore, we could visualize the degradation products by using acid-urea gel, and identify multiple cleavage sites on Aβ for MT1-MMP by mass spectrometry. Lastly, purified soluble MT1-MMP could also degrade parenchymal fibrillar amyloid plaques that form in the brain of APP-Sw transgenic mice. Together, these findings suggest that MT1-MMP possesses Aβ-degrading activity *in vitro*.

MBP is the major structural protein component of myelin and plays a functional role in the formation and maintenance of the myelin sheath. MBP possesses endogenous serine proteinase activity and can undergo autocatalytic cleavage liberating distinct fragments. MBP was identified in brain homogenates as a prominent factor to bind and inhibit CAA mutant Aβ fibril formation. In chapters 3 and 4, I showed that purified MBP from a variety sources including human and mouse brain or recombinant human and mouse MBP could degrade soluble and fibrillar Aβ *in vitro*. Also, overexpressed MBP in cultured cells could degrade exogenous Aβ. Mass spectrometry analysis identified multiple cleavage sites on soluble or fibrillar Aβ. Furthermore, purified MBP could degrade parenchymal amyloid deposits as well as vascular amyloid that form in the brain of APP-Sw transgenic mice. Although it was proposed that serine 151 is the active site for MBP we found that a S151A mutant form of this protein obtained from different sources was devoid of proteolytic activity indicating that serine 151 is not the precise active site. Lastly, serial C-terminal deletion mutants of MBP were investigated to help resolve the region responsible for its proteolytic activity.

## 5.2 – Open Questions

## MT1-MMP

### The *in vivo* role of MT1-MMP in the pathology of AD

In this study, we demonstrate that purified MT1-MMP or overexpression of it in cells is capable of degrading Aβ *in vitro*. However, it remains unknown if MT1-MMP plays a significant role in regulating the amyloid pathology of AD *in vivo*. In transgenic mouse models of amyloid deposition, MMP2, MMP9 and MT1-MMP are selectively

expressed in reactive astrocytes surrounding the amyloid plaques in APPsw mice (*38*) or APP-TgSwDI mice (*77*). How much each of these enzymes contributes to the clearance of Aβ by astrocytes? In the study of APPsw mice, the authors analyzed MMP2 and MMP9 expression levels from isolated reactive astrocytes surrounding amyloid plaques by laser capture microdissection (LCM), and showed that the expression of these two enzymes were increased in similar level comparing to resting astrocytes. Previously, I tried to perform LCM to isolate reactive astrocytes from APP-SwDI mice, however, the amplification of RNA samples from LCM–captured cells was inconclusive. In addition to measuring expression levels, approaches to determine the specific contribution of each Aβ-degrading enzyme in pathological conditions are even more challenging and require more sophisticated experiments in the future.

Our study suggested that MT1-MMP can degrade Aβ *in vitro*. Analysis of proteinase gene knock-out mice is widely regarded as a viable method of determining whether or not a given proteinase is relevant to Aß degradation *in vivo*. Unfortunately, MT1-MMP deficient mice are developmentally abnormal and die early (*106-107*). Alternatively, conditional MT1-MMP gene knock-out mice could be used to cross with amyloid depositing transgenic mice to investigate the *in vivo* role of MT1-MMP. An alternative approach would be to use small inference RNA targeting the expression of MT1-MMP to decrease MT1-MMP levels in the amyloid depositing transgenic mice. On the other hand, we could over-express MT1-MMP in the brain of APP-overexpressing mice to determine if it influences the amount, form, or location of Aß accumulation in the brain.

**The potential role of MT1-MMP and MMP2 in regulating Aß metabolism**

MT1-MMP possesses dual effects regarding the regulation of Aß levels including direct proteolysis of Aß and activation of MMP2 resulting in more Aß degradation. For the first case, MT1-MMP could degrade directly Aß which are nearby cell membrane. For the latter case, MT1-MMP could active MMP2 and the secret active MMP2 would degrade more Aß, not only nearby cell surface but also the Aß more distant. In this situation, MT1-MMP indirectly regulates Aß level and magnifies Aß degradation.

## MBP

**The potential role of MBP interactions with Aß in the brain**

MBP is a flexible C-shaped molecule with a ß-sheet core structure (*108-109*). It has been observed that MBP is extended and flat within the myelin sheath. The residues 85-96 can form an amphipathic α-helix responsible for lipid bilayer anchoring (*65, 110*). Recently there is no direct evidence showing MBP in the compact myelin sheath possess proteolytic activity. However, I propose there is some possibility of MBP showing enzymatic activities with regarding autolysis or Aβ degradation in the *in vivo* environment. First, MBP still exhibited autolysis in the presence of lipid (*70*) implying the possibility of being active in the compact myelin sheath. Second, the C8 isoform which exhibits a more open conformation showed faster cleavage (*70, 111*). In multiple sclerosis, the levels of the C8 isoform can increase due to posttranslational modification resulting in destabilization and loose compaction (*112-114*). Similarly, it would be interesting to investigate the changes of post-translational modification on MBP in AD pathology. An other possibility for a MBP:Aβ interaction during AD and related

disorders could happen upon the release of MBP from damaged myelin. The pathology of AD includes the involvement of whiter matter lesions (*115-117*) and this change contributes directly to cognitive decline (*117*). Aβ is produced from amyloid precursor protein processing primarily in neuronal cells. It has shown that MBP is present in the neuronal fraction preparation from the affected cortex in Alzheimer's disease (*118*). Therefore, this piece of evidence increases the chance of MBP interacting with Aβ in the pathological condition. However, further work is needed to further investigate if MBP and Aβ co-localize in the brain tissue sections.

**MBP may still have effect on Aβ degradation or deposition even it is labile**

In pathological conditions, upon release from myelin sheath, MBP is subjected to degradation into several smaller peptides due to autolysis or by other proteinases (*70, 119-120*). For example, MMPs play a significant role in the fragmentation of MBP and demyelination (*121-122*). Nevertheless, MBP may still have an effect on Aβ degradation or inhibition of Aβ fibril formation. For example, some C-terminal deletion mutants still possessed Aβ-degrading activity as shown in chapter 4. Besides, the fragments of MBP, including the first 64 amino acids, could still inhibit Aβ fibril formation. This suggests that MBP fragments released during pathological conditions could still affect Aβ with respect to degradation or inhibition of fibril formation.

**To investigate the effect of MBP in Aβ clearance and deposition *in vivo***

Our lab showed that MBP could bind to and inhibit Aβ fibril formation (*73, 89*) and then MBP also degraded Aβ *in vitro* from this study. It is important to further

evaluate the physiological and pathological significance of MBP in Alzheimer's disease. One approach is the removal of MBP by using MBP gene knock-out mice - the shiverer mouse strain name C3Fe.SWV-*Mbp*$^{shi}$/J in which exons Ⅲ through Ⅶ of the MBP gene have been deleted (*123*). However, these shiverer mice die early at around 4 months of age. Therefore an early-onset model of Aβ deposition would be required. Crossing of the shiverer mice with 5x FAD mice, an early-onset model of AD plaque formation (*124*), or TgSwDI mice, an early-onset model of CAA will be needed to further evaluate the changes of Aβ depositions due to the absence of MBP in the brain. An alternative approach would be to use viral injection to over-express MBP protein or its biologically active fragments (e.g. MBP1), which are effective to interact with and inhibit Aβ fibril, into the above AD and CAA mouse models to further understand the effect of proteolysis and inhibition of fibril formation of MBP *in vivo*.

**Possible model for the role of MBP in these two transgenic mice –Tg2576 and Tg-SwDI**

Our initial MBP studies arose from the observation of different amyloid deposition patterns in the familial CAA transgenic mice TgSwDI compared to APP-Sw (Tg2576) transgenic mice where, in the latter, senile plaques mainly accumulated in the parenchymal region. The TgSwDI mice only show diffuse parenchymal Aβ deposits and robust cerebral microvascular accumulation of fibrillar amyloid. This deposition pattern occurs despite.CAA mutant Aβ exhibiting a high rate of fibrillogenesis *in vitro* comparing to wild type Aβ. We showed that MBP could inhibit CAA mutant Aβ fibril formation *in vitro*, and MBP has higher affinity toward Aβ40DI or Aβ42wt comparing to

Aβ40wt. In this study, we showed that MBP possessed Aβ-degrading activity. I propose the following hypothesis to explain the conflicting findings of Aβ deposition patterns in these two different transgenic mouse models. MBP released from damaged myelin degrades or inhibits Aβ fibril formation, however, MBP is very unstable since it possesses autolysis activity and is also susceptible to degradation by other proteasinases such as MMPs (*121-122*) or cathepsins (*125*). Then MBP degradation fragments, including the N-terminal 64 amino acid still could inhibit Aβ fibril formation. In Tg2576 mice, released MBP has lower binding affinity to wild-type Aβ peptide and a lower degradation rate of Aβ by MBP. In addition, the fibrillization rate for wilde type Aβ peptides is slower than CAA mutant peptides. The fibrillization rate may be slower than the rate of MBP degradation. Over time, small amounts of Aβ40wt or Aβ42wt form fibrils slowly and finally deposit in this region due to loss of MBP. However, in TgSwDI mice, CAA mutant peptides are degraded and fibril formation is inhibited in the parenchymal region due to higher binding affinity to MBP. CAA mutant peptides preferentially affect smooth muscle cells and this would cause more myelin degeneration because of hypoxic – ischemic damage to oligodendrocytes. During ischemic damage more MBP is released into parenchymal region (*126*). The presence of more parenchymal MBP could result in less CAA mutant deposits in the parenchymal region. However, this hypothesis may be an oversimplification to describe amyloid deposition in APP transgenic mice and is largely based on our *in vitro* work. In the pathological condition of the human of brain condition, there are many factors involved in the formation of parenchymal plaques including apolipoprotein E, heparin sulfate

proteoglycans, anti-chymotrypsin, etc. In the future it will be important to determine the role of MBP in the pathology of AD *in vivo*.

**Are there other substrates for MBP besides itself and Aβ?**

It is now known that MBP exhibited autolysis to cleave itself and also degrades Aβ from this study; however, the proteolytic activity of this protein remains still poorly understood. A common feature is that MBP digests itself or Aβ at multiple sites, lacking the specificity of typical serine proteinases, such as trypsin, which cleave on arginine and lysine of substrate. It is known that MBP interacts with many different ligands including lipids (*104, 127*), calmodulin (*128*), divalent cations (*129*), GTP (*130*) and cytoskeletal proteins such as tubulin (*131*) and actin (*132-133*) in physiological condition or interacts with heat shock protein 70 (*134*). It is possible, but currently unknown, that MBP also could degrade any of these interactive proteins. The other highly interesting candidate is amyloid precursor protein (APP) since MBP could degrade Aβ on multiple sites. If MBP could degrade APP, it may play a more significant role in the Aβ pathology by possibly further downregulating the synthesis of Aβ although this needs to be investigated.

**How to regulate MBP enzymatic activity?**

MBP possesses autolytic and Aβ-degrading activities. Earlier work suggested that MBP exhibits serine proteinase activity. Previously, we investigated if purified serine proteinase inhibitors that are normally found in the central nervous system, including α1-proteinase inhibitor, α2-macroglobulin, C1 inhibitor, and α1-anticymotrypsin (ACT), could block MBP autolysis activity. We found that only ACT

inhibited MBP autolysis suggesting a chymotrypsin-like specificity of this activity. ACT is an acute phase inflammation sensitive protein and the level of ACT is increased in the cerebrospinal fluid (CSF) and plasma in AD patients (*135*). Alao, it is known that ACT co-deposits with Aβ within senile plaques of AD brains and also promoted amyloid plaques depositions in AD transgenic mice (*136-137*). Further studies are needed to evaluate the relationship of ACT inhibition on MBP-mediated Aβ degradation and Aβ deposition.

## Therapeutic potential

Decreasing brain Aβ levels is an emerging therapeutic approach for preventing or treating AD. Several Aβ-degrading enzymes, which have been well studied in the transgenic mice, have this therapeutic potential. For example, viral-medicated delivery of human NEP in a transgenic mouse model of AD resulted in reduction of Aβ levels (*138-140*). With respect to treatment strategies, *ex vivo* gene delivery of MBP, BHT-3009, encodes full-length, human MBP, has been studied in different models of multiple sclerosis and even tested in a phase II clinical trial. It would be useful to employ a similar gene delivery into a mouse model of AD to test the therapeutic effect of MBP.

The family of Aβ-degrading enzymes is thought to play an important role in controlling the steady state level of Aβ in brain. Some of these enzymes are likely part of the normal regulatory process acting at several stages after Aβ synthesis. On the other hand, others are likely involved in acting on Aβ in the pathological condition. For example, the levels of MMP2 (*53, 60*), MMP9 (*87*), MT1-MMP (*141*), IDE (*142*) and

plasminogen activators (*143-144*) are increased in neuronal, microglial and vascular smooth muscle cells sitimulated by Aβ. It is likely that multiple proteinases act cooperatively in different compartments to regulate Aβ level. Also, reduction in the level or activity of an Aβ-degrading enzyme may be compensated for, by consequent up-regulation of other Aβ-degrading enzymes. However, aging, environmental factors, genetic factors, or disease could affect the efficiencies of Aβ-clearance pathways, impart in the activities of Aβ-degrading enzymes, resulting in higher Aβ levels and deposition. In this study, I used similar strategy such as purified proteins or cell-based systems to demonstrate Aβ-degrading activity of MT1-MMP and MBP *in vitro*. These two proteins have different properties; for example, when expressed in different cell types. For example, MT1-MMP is expressed in the reactive astrocytes and MBP is mainly expressed in oligodentrocytesand. They are found in different subcellular compartments. Whereas MT1-MMP is integral membrane protein, MBP is a membrane associated cytosolic proteins. The common feature is that these two proteins may play a more significant role in regulating Aβ level in the pathological condition not in maintaining the basal level of Aβ. MT1-MMP is only induced in the reactive astrocytes surrounding Aβ deposits and MBP may be released from damaged myelin under the early stage of disease development according our assumptions. The present work suggests that they could degrade Aβ *in vitro*, however, further studies are needed to determine the role of these two proteins in the pathology of AD.

# REFERENCES


1. Wang, D. S., Dickson, D. W., and Malter, J. S. (2006) beta-Amyloid Degradation and Alzheimer's Disease, *J Biomed Biotechnol 2006*, 58406.
2. Mattson, M. P. (2004) Pathways towards and away from Alzheimer's disease, *Nature 430*, 631-639.
3. Evin, G., and Weidemann, A. (2002) Biogenesis and metabolism of Alzheimer's disease Abeta amyloid peptides, *Peptides 23*, 1285-1297.
4. Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43), *Neuron 13*, 45-53.
5. Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992) Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production, *Nature 360*, 672-674.
6. Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos, L., Jr., Eckman, C., Golde, T. E., and Younkin, S. G. (1994) An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants, *Science 264*, 1336-1340.
7. Pardridge, W. M., Vinters, H. V., Yang, J., Eisenberg, J., Choi, T. B., Tourtellotte, W. W., Huebner, V., and Shively, J. E. (1987) Amyloid angiopathy of Alzheimer's disease: amino acid composition and partial sequence of a 4,200-dalton peptide isolated from cortical microvessels, *J Neurochem 49*, 1394-1401.
8. Vinters, H. V., and Farag, E. S. (2003) Amyloidosis of cerebral arteries, *Adv Neurol 92*, 105-112.
9. Kawai, M., Kalaria, R. N., Cras, P., Siedlak, S. L., Velasco, M. E., Shelton, E. R., Chan, H. W., Greenberg, B. D., and Perry, G. (1993) Degeneration of vascular muscle cells in cerebral amyloid angiopathy of Alzheimer disease, *Brain Res 623*, 142-146.
10. Van Nostrand, W. E., Melchor, J. P., Cho, H. S., Greenberg, S. M., and Rebeck, G. W. (2001) Pathogenic effects of D23N Iowa mutant amyloid beta -protein, *The Journal of biological chemistry 276*, 32860-32866.
11. Wisniewski, T., Ghiso, J., and Frangione, B. (1991) Peptides homologous to the amyloid protein of Alzheimer's disease containing a glutamine for glutamic acid substitution have accelerated amyloid fibril formation, *Biochem Biophys Res Commun 179*, 1247-1254.
12. Haass, C., and Steiner, H. (2001) Protofibrils, the unifying toxic molecule of neurodegenerative disorders?, *Nat Neurosci 4*, 859-860.
13. Goedert, M., and Spillantini, M. G. (2006) A century of Alzheimer's disease, *Science 314*, 777-781.
14. Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L.,

Selkoe, D., and Younkin, S. (1996) Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease.[see comment], *Nature medicine 2*, 864-870.

15. Gravina, S. A., Ho, L., Eckman, C. B., Long, K. E., Otvos, L., Jr., Younkin, L. H., Suzuki, N., and Younkin, S. G. (1995) Amyloid beta protein (A beta) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43), *Journal of Biological Chemistry 270*, 7013-7016.
16. Haass, C., and Selkoe, D. J. (1993) Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide, *Cell 75*, 1039-1042.
17. Saido, T. C. (1998) Alzheimer's disease as proteolytic disorders: anabolism and catabolism of beta-amyloid, *Neurobiol Aging 19*, S69-75.
18. Bateman, R. J., Munsell, L. Y., Morris, J. C., Swarm, R., Yarasheski, K. E., and Holtzman, D. M. (2006) Human amyloid-beta synthesis and clearance rates as measured in cerebrospinal fluid in vivo, *Nature medicine 12*, 856-861.
19. Deane, R., Du Yan, S., Submamaryan, R. K., LaRue, B., Jovanovic, S., Hogg, E., Welch, D., Manness, L., Lin, C., Yu, J., Zhu, H., Ghiso, J., Frangione, B., Stern, A., Schmidt, A. M., Armstrong, D. L., Arnold, B., Liliensiek, B., Nawroth, P., Hofman, F., Kindy, M., Stern, D., and Zlokovic, B. (2003) RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain, *Nature medicine 9*, 907-913.
20. Mackic, J. B., Stins, M., McComb, J. G., Calero, M., Ghiso, J., Kim, K. S., Yan, S. D., Stern, D., Schmidt, A. M., Frangione, B., and Zlokovic, B. V. (1998) Human blood-brain barrier receptors for Alzheimer's amyloid-beta 1- 40. Asymmetrical binding, endocytosis, and transcytosis at the apical side of brain microvascular endothelial cell monolayer, *Journal of Clinical Investigation 102*, 734-743.
21. Shibata, M., Yamada, S., Kumar, S. R., Calero, M., Bading, J., Frangione, B., Holtzman, D. M., Miller, C. A., Strickland, D. K., Ghiso, J., and Zlokovic, B. V. (2000) Clearance of Alzheimer's amyloid-ss(1-40) peptide from brain by LDL receptor-related protein-1 at the blood-brain barrier, *The Journal of clinical investigation 106*, 1489-1499.
22. Zlokovic, B. V. (2004) Clearing amyloid through the blood-brain barrier, *J Neurochem 89*, 807-811.
23. Kuhnke, D., Jedlitschky, G., Grube, M., Krohn, M., Jucker, M., Mosyagin, I., Cascorbi, I., Walker, L. C., Kroemer, H. K., Warzok, R. W., and Vogelgesang, S. (2007) MDR1-P-Glycoprotein (ABCB1) Mediates Transport of Alzheimer's amyloid-beta peptides--implications for the mechanisms of Abeta clearance at the blood-brain barrier, *Brain Pathology 17*, 347-353.
24. Lam, F. C., Liu, R., Lu, P., Shapiro, A. B., Renoir, J. M., Sharom, F. J., and Reiner, P. B. (2001) beta-Amyloid efflux mediated by p-glycoprotein, *Journal of Neurochemistry 76*, 1121-1128.
25. Iwata, N., Tsubuki, S., Takaki, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., Kawashima-Morishima, M., Lee, H. J., Hama, E., Sekine-Aizawa, Y., and Saido, T. C. (2000) Identification of the major Abeta1-42-degrading catabolic pathway

in brain parenchyma: suppression leads to biochemical and pathological deposition, *Nature medicine 6*, 143-150.

26. Iwata, N., Tsubuki, S., Takaki, Y., Shirotani, K., Lu, B., Gerard, N. P., Gerard, C., Hama, E., Lee, H. J., and Saido, T. C. (2001) Metabolic regulation of brain Abeta by neprilysin, *Science 292*, 1550-1552.
27. Qiu, W. Q., Walsh, D. M., Ye, Z., Vekrellis, K., Zhang, J., Podlisny, M. B., Rosner, M. R., Safavi, A., Hersh, L. B., and Selkoe, D. J. (1998) Insulin-degrading enzyme regulates extracellular levels of amyloid beta-protein by degradation, *The Journal of biological chemistry 273*, 32730-32738.
28. Ledesma, M. D., Da Silva, J. S., Crassaerts, K., Delacourte, A., De Strooper, B., and Dotti, C. G. (2000) Brain plasmin enhances APP alpha-cleavage and Abeta degradation and is reduced in Alzheimer's disease brains, *EMBO reports 1*, 530-535.
29. Eckman, E. A., Reed, D. K., and Eckman, C. B. (2001) Degradation of the Alzheimer's amyloid beta peptide by endothelin-converting enzyme, *The Journal of biological chemistry 276*, 24540-24548.
30. Hu, J., Igarashi, A., Kamata, M., and Nakagawa, H. (2001) Angiotensin-converting enzyme degrades Alzheimer amyloid beta-peptide (A beta ); retards A beta aggregation, deposition, fibril formation; and inhibits cytotoxicity, *The Journal of biological chemistry 276*, 47863-47868.
31. Yamada, T., Miyazaki, K., Koshikawa, N., Takahashi, M., Akatsu, H., and Yamamoto, T. (1995) Selective localization of gelatinase A, an enzyme degrading beta-amyloid protein, in white matter microglia and in Schwann cells, *Acta neuropathologica 89*, 199-203.
32. Roher, A. E., Kasunic, T. C., Woods, A. S., Cotter, R. J., Ball, M. J., and Fridman, R. (1994) Proteolysis of A beta peptide from Alzheimer disease brain by gelatinase A, *Biochem Biophys Res Commun 205*, 1755-1761.
33. Backstrom, J. R., Lim, G. P., Cullen, M. J., and Tokes, Z. A. (1996) Matrix metalloproteinase-9 (MMP-9) is synthesized in neurons of the human hippocampus and is capable of degrading the amyloid-beta peptide (1-40), *J Neurosci 16*, 7910-7919.
34. Falkevall, A., Alikhani, N., Bhushan, S., Pavlov, P. F., Busch, K., Johnson, K. A., Eneqvist, T., Tjernberg, L., Ankarcrona, M., and Glaser, E. (2006) Degradation of the amyloid beta-protein by the novel mitochondrial peptidasome, PreP, *Journal of Biological Chemistry 281*, 29096-29104.
35. Mueller-Steiner, S., Zhou, Y., Arai, H., Roberson, E. D., Sun, B., Chen, J., Wang, X., Yu, G., Esposito, L., Mucke, L., and Gan, L. (2006) Antiamyloidogenic and neuroprotective functions of cathepsin B: implications for Alzheimer's disease.[see comment], *Neuron 51*, 703-714.
36. Leissring, M. A., Farris, W., Wu, X., Christodoulou, D. C., Haigis, M. C., Guarente, L., and Selkoe, D. J. (2004) Alternative translation initiation generates a novel isoform of insulin-degrading enzyme targeted to mitochondria, *Biochemical Journal 383*, 439-446.
37. Stahl, A., Nilsson, S., Lundberg, P., Bhushan, S., Biverstahl, H., Moberg, P., Morisset, M., Vener, A., Maler, L., Langel, U., and Glaser, E. (2005) Two novel

targeting peptide degrading proteases, PrePs, in mitochondria and chloroplasts, so similar and still different, *Journal of Molecular Biology 349*, 847-860.

38. Yin, K. J., Cirrito, J. R., Yan, P., Hu, X., Xiao, Q., Pan, X., Bateman, R., Song, H., Hsu, F. F., Turk, J., Xu, J., Hsu, C. Y., Mills, J. C., Holtzman, D. M., and Lee, J. M. (2006) Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-beta peptide catabolism, *J Neurosci 26*, 10939-10948.
39. Tucker, H. M., Kihiko, M., Caldwell, J. N., Wright, S., Kawarabayashi, T., Price, D., Walker, D., Scheff, S., McGillis, J. P., Rydel, R. E., and Estus, S. (2000) The plasmin system is induced by and degrades amyloid-beta aggregates, *Journal of Neuroscience 20*, 3937-3946.
40. Eckman, E. A., Watson, M., Marlow, L., Sambamurti, K., and Eckman, C. B. (2003) Alzheimer's disease beta-amyloid peptide is increased in mice deficient in endothelin-converting enzyme, *The Journal of biological chemistry 278*, 2081-2084.
41. Farris, W., Mansourian, S., Chang, Y., Lindsley, L., Eckman, E. A., Frosch, M. P., Eckman, C. B., Tanzi, R. E., Selkoe, D. J., and Guenette, S. (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain in vivo, *Proc Natl Acad Sci U S A 100*, 4162-4167.
42. Woessner, J. F., Jr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodeling, *Faseb J 5*, 2145-2154.
43. Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B., and Bode, W. (1995) The metzincins--topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases, *Protein Sci 4*, 823-840.
44. Sternlicht, M. D., and Werb, Z. (2001) How matrix metalloproteinases regulate cell behavior, *Annual review of cell and developmental biology 17*, 463-516.
45. Van Wart, H. E., and Birkedal-Hansen, H. (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family, *Proc Natl Acad Sci U S A 87*, 5578-5582.
46. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. (1994) A matrix metalloproteinase expressed on the surface of invasive tumour cells, *Nature 370*, 61-65.
47. Pei, D., and Weiss, S. J. (1995) Furin-dependent intracellular activation of the human stromelysin-3 zymogen, *Nature 375*, 244-247.
48. Deryugina, E. I., Ratnikov, B., Monosov, E., Postnova, T. I., DiScipio, R., Smith, J. W., and Strongin, A. Y. (2001) MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells, *Experimental cell research 263*, 209-223.
49. Okada, A., Tomasetto, C., Lutz, Y., Bellocq, J. P., Rio, M. C., and Basset, P. (1997) Expression of matrix metalloproteinases during rat skin wound healing: evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A, *The Journal of cell biology 137*, 67-77.
50. Yamada, T., Yoshiyama, Y., Sato, H., Seiki, M., Shinagawa, A., and Takahashi, M. (1995) White matter microglia produce membrane-type matrix

metalloprotease, an activator of gelatinase A, in human brain tissues, *Acta neuropathologica 90*, 421-424.

51. McCawley, L. J., and Matrisian, L. M. (2001) Matrix metalloproteinases: they're not just for matrix anymore!, *Current opinion in cell biology 13*, 534-540.
52. Leppert, D., Lindberg, R. L., Kappos, L., and Leib, S. L. (2001) Matrix metalloproteinases: multifunctional effectors of inflammation in multiple sclerosis and bacterial meningitis, *Brain Res Brain Res Rev 36*, 249-257.
53. Jung, S. S., Zhang, W., and Van Nostrand, W. E. (2003) Pathogenic A beta induces the expression and activation of matrix metalloproteinase-2 in human cerebrovascular smooth muscle cells, *J Neurochem 85*, 1208-1215.
54. Gottschall, P. E., Yu, X., and Bing, B. (1995) Increased production of gelatinase B (matrix metalloproteinase-9) and interleukin-6 by activated rat microglia in culture, *Journal of neuroscience research 42*, 335-342.
55. Muir, E. M., Adcock, K. H., Morgenstern, D. A., Clayton, R., von Stillfried, N., Rhodes, K., Ellis, C., Fawcett, J. W., and Rogers, J. H. (2002) Matrix metalloproteases and their inhibitors are produced by overlapping populations of activated astrocytes, *Brain research 100*, 103-117.
56. Deb, S., and Gottschall, P. E. (1996) Increased production of matrix metalloproteinases in enriched astrocyte and mixed hippocampal cultures treated with beta-amyloid peptides, *J Neurochem 66*, 1641-1647.
57. Deb, S., Wenjun Zhang, J., and Gottschall, P. E. (2003) Beta-amyloid induces the production of active, matrix-degrading proteases in cultured rat astrocytes, *Brain Res 970*, 205-213.
58. Vehmas, A. K., Kawas, C. H., Stewart, W. F., and Troncoso, J. C. (2003) Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease, *Neurobiol Aging 24*, 321-331.
59. Wyss-Coray, T., Loike, J. D., Brionne, T. C., Lu, E., Anankov, R., Yan, F., Silverstein, S. C., and Husemann, J. (2003) Adult mouse astrocytes degrade amyloid-beta in vitro and in situ, *Nature medicine 9*, 453-457.
60. Deb, S., Zhang, J. W., and Gottschall, P. E. (1999) Activated isoforms of MMP-2 are induced in U87 human glioma cells in response to beta-amyloid peptide, *Journal of neuroscience research 55*, 44-53.
61. Ahmad, M., Takino, T., Miyamori, H., Yoshizaki, T., Furukawa, M., and Sato, H. (2006) Cleavage of amyloid-beta precursor protein (APP) by membrane-type matrix metalloproteinases, *Journal of biochemistry 139*, 517-526.
62. Givogri, M. I., Bongarzone, E. R., and Campagnoni, A. T. (2000) New insights on the biology of myelin basic protein gene: the neural-immune connection, *Journal of neuroscience research 59*, 153-159.
63. Polverini, E., Fasano, A., Zito, F., Riccio, P., and Cavatorta, P. (1999) Conformation of bovine myelin basic protein purified with bound lipids, *Eur Biophys J 28*, 351-355.
64. Bates, I. R., Feix, J. B., Boggs, J. M., and Harauz, G. (2004) An immunodominant epitope of myelin basic protein is an amphipathic alpha-helix, *The Journal of biological chemistry 279*, 5757-5764.
65. Bates, I. R., Boggs, J. M., Feix, J. B., and Harauz, G. (2003) Membrane-anchoring and charge effects in the interaction of myelin basic protein with lipid

bilayers studied by site-directed spin labeling, *The Journal of biological chemistry 278*, 29041-29047.

66. Chou, F. C., Chou, C. H., Shapira, R., and Kibler, R. F. (1976) Basis of microheterogeneity of myelin basic protein, *The Journal of biological chemistry 251*, 2671-2679.
67. Staugaitis, S. M., Colman, D. R., and Pedraza, L. (1996) Membrane adhesion and other functions for the myelin basic proteins, *Bioessays 18*, 13-18.
68. Kornek, B., and Lassmann, H. (2003) Neuropathology of multiple sclerosis-new concepts, *Brain research bulletin 61*, 321-326.
69. Moscarello, M. A., Mastronardi, F. G., and Wood, D. D. (2007) The role of citrullinated proteins suggests a novel mechanism in the pathogenesis of multiple sclerosis, *Neurochemical research 32*, 251-256.
70. D'Souza, C. A., Wood, D. D., She, Y. M., and Moscarello, M. A. (2005) Autocatalytic cleavage of myelin basic protein: an alternative to molecular mimicry, *Biochemistry 44*, 12905-12913.
71. Bartzokis, G., Cummings, J. L., Sultzer, D., Henderson, V. W., Nuechterlein, K. H., and Mintz, J. (2003) White matter structural integrity in healthy aging adults and patients with Alzheimer disease: a magnetic resonance imaging study, *Archives of neurology 60*, 393-398.
72. Hoos, M. D., Ahmed, M., Smith, S. O., and Van Nostrand, W. E. (2007) Inhibition of familial cerebral amyloid angiopathy mutant amyloid beta -protein fibril assembly by myelin basic protein, *The Journal of biological chemistry*.
73. Hoos, M. D., Ahmed, M., Smith, S. O., and Van Nostrand, W. E. (2009) Myelin basic protein binds to and inhibits the fibrillar assembly of Abeta42 in vitro, *Biochemistry 48*, 4720-4727.
74. Liao, M. C., Ahmed, M., Smith, S. O., and Van Nostrand, W. E. (2009) Degradation of amyloid beta protein by purified myelin basic protein, *The Journal of biological chemistry 284*, 28917-28925.
75. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. (1995) Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease, *The Journal of biological chemistry 270*, 5331-5338.
76. Burdick, D., Soreghan, B., Kwon, M., Kosmoski, J., Knauer, M., Henschen, A., Yates, J., Cotman, C., and Glabe, C. (1992) Assembly and aggregation properties of synthetic Alzheimer's A4/beta amyloid peptide analogs, *Journal of Biological Chemistry 267*, 546-554.
77. Davis, J., Xu, F., Deane, R., Romanov, G., Previti, M. L., Zeigler, K., Zlokovic, B. V., and Van Nostrand, W. E. (2004) Early-onset and robust cerebral microvascular accumulation of amyloid beta-protein in transgenic mice expressing low levels of a vasculotropic Dutch/Iowa mutant form of amyloid beta-protein precursor, *The Journal of biological chemistry 279*, 20296-20306.
78. Dickson, D. W., Wertkin, A., Mattiace, L. A., Fier, E., Kress, Y., Davies, P., and Yen, S. H. (1990) Ubiquitin immunoelectron microscopy of dystrophic neurites in cerebellar senile plaques of Alzheimer's disease, *Acta neuropathologica 79*, 486-493.

79. DeMattos, R. B., Bales, K. R., Cummins, D. J., Dodart, J. C., Paul, S. M., and Holtzman, D. M. (2001) Peripheral anti-A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease.[see comment], *Proceedings of the National Academy of Sciences of the United States of America 98*, 8850-8855.
80. Yan, P., Hu, X., Song, H., Yin, K., Bateman, R. J., Cirrito, J. R., Xiao, Q., Hsu, F. F., Turk, J. W., Xu, J., Hsu, C. Y., Holtzman, D. M., and Lee, J. M. (2006) Matrix metalloproteinase-9 degrades amyloid-beta fibrils in vitro and compact plaques in situ, *Journal of Biological Chemistry 281*, 24566-24574.
81. Ross, S. A., Cunningham, R. T., Johnston, C. F., and Rowlands, B. J. (1996) Neuron-specific enolase as an aid to outcome prediction in head injury, *British Journal of Neurosurgery 10*, 471-476.
82. Davis, J., Cribbs, D. H., Cotman, C. W., and Van Nostrand, W. E. (1999) Pathogenic amyloid beta-protein induces apoptosis in cultured human cerebrovascular smooth muscle cells, *Amyloid 6*, 157-164.
83. Cao, J., Sato, H., Takino, T., and Seiki, M. (1995) The C-terminal region of membrane type matrix metalloproteinase is a functional transmembrane domain required for pro-gelatinase A activation, *Journal of Biological Chemistry 270*, 801-805.
84. Wetzel, R. (2002) Ideas of order for amyloid fibril structure.[see comment], *Structure 10*, 1031-1036.
85. Crouch, P. J., Tew, D. J., Du, T., Nguyen, D. N., Caragounis, A., Filiz, G., Blake, R. E., Trounce, I. A., Soon, C. P., Laughton, K., Perez, K. A., Li, Q. X., Cherny, R. A., Masters, C. L., Barnham, K. J., and White, A. R. (2009) Restored degradation of the Alzheimer's amyloid-beta peptide by targeting amyloid formation, *Journal of Neurochemistry 108*, 1198-1207.
86. Roher, A. E., Kasunic, T. C., Woods, A. S., Cotter, R. J., Ball, M. J., and Fridman, R. (1994) Proteolysis of A beta peptide from Alzheimer disease brain by gelatinase A, *Biochemical & Biophysical Research Communications 205*, 1755-1761.
87. Lee, J. M., Yin, K. J., Hsin, I., Chen, S., Fryer, J. D., Holtzman, D. M., Hsu, C. Y., and Xu, J. (2003) Matrix metalloproteinase-9 and spontaneous hemorrhage in an animal model of cerebral amyloid angiopathy, *Annals of Neurology 54*, 379-382.
88. Baig, S., Kehoe, P. G., and Love, S. (2008) MMP-2, -3 and -9 levels and activity are not related to Abeta load in the frontal cortex in Alzheimer's disease, *Neuropathology & Applied Neurobiology 34*, 205-215.
89. Hoos, M. D., Ahmed, M., Smith, S. O., and Van Nostrand, W. E. (2007) Inhibition of familial cerebral amyloid angiopathy mutant amyloid beta-protein fibril assembly by myelin basic protein, *Journal of Biological Chemistry 282*, 9952-9961.
90. Deane, R., Wu, Z., Sagare, A., Davis, J., Du Yan, S., Hamm, K., Xu, F., Parisi, M., LaRue, B., Hu, H. W., Spijkers, P., Guo, H., Song, X., Lenting, P. J., Van Nostrand, W. E., and Zlokovic, B. V. (2004) LRP/amyloid beta-peptide interaction mediates differential brain efflux of Abeta isoforms, *Neuron 43*, 333-344.

91. Yamin, R., Bagchi, S., Hildebrant, R., Scaloni, A., Widom, R. L., and Abraham, C. R. (2007) Acyl peptide hydrolase, a serine proteinase isolated from conditioned medium of neuroblastoma cells, degrades the amyloid-beta peptide, *Journal of Neurochemistry 100*, 458-467.
92. Wood, D. D., and Moscarello, M. A. (1989) The isolation, characterization, and lipid-aggregating properties of a citrulline containing myelin basic protein, *Journal of Biological Chemistry 264*, 5121-5127.
93. Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996) Correlative memory deficits, Abeta elevation, and amyloid plaques in transgenic mice.[see comment], *Science 274*, 99-102.
94. Van Nostrand, W. E., and Porter, M. (1999) Plasmin cleavage of the amyloid beta-protein: alteration of secondary structure and stimulation of tissue plasminogen activator activity, *Biochemistry 38*, 11570-11576.
95. Omlin, F. X., Webster, H. D., Palkovits, C. G., and Cohen, S. R. (1982) Immunocytochemical localization of basic protein in major dense line regions of central and peripheral myelin, *Journal of Cell Biology 95*, 242-248.
96. Atwood, C. S., Martins, R. N., Smith, M. A., and Perry, G. (2002) Senile plaque composition and posttranslational modification of amyloid-beta peptide and associated proteins, *Peptides 23*, 1343-1350.
97. Cheifetz, S., and Moscarello, M. A. (1985) Effect of bovine basic protein charge microheterogeneity on protein-induced aggregation of unilamellar vesicles containing a mixture of acidic and neutral phospholipids, *Biochemistry 24*, 1909-1914.
98. Stout, J. C., Jernigan, T. L., Archibald, S. L., and Salmon, D. P. (1996) Association of dementia severity with cortical gray matter and abnormal white matter volumes in dementia of the Alzheimer type, *Archives of neurology 53*, 742-749.
99. Bartzokis, G., Cummings, J. L., Sultzer, D., Henderson, V. W., Nuechterlein, K. H., and Mintz, J. (2003) White matter structural integrity in healthy aging adults and patients with Alzheimer disease: a magnetic resonance imaging study, *Archives of neurology 60*, 393-398.
100. Roher, A. E., Weiss, N., Kokjohn, T. A., Kuo, Y. M., Kalback, W., Anthony, J., Watson, D., Luehrs, D. C., Sue, L., Walker, D., Emmerling, M., Goux, W., and Beach, T. (2002) Increased A beta peptides and reduced cholesterol and myelin proteins characterize white matter degeneration in Alzheimer's disease, *Biochemistry 41*, 11080-11090.
101. Wang, D. S., Bennett, D. A., Mufson, E. J., Mattila, P., Cochran, E., and Dickson, D. W. (2004) Contribution of changes in ubiquitin and myelin basic protein to age-related cognitive decline, *Neuroscience Research 48*, 93-100.
102. Wisniewski, H. M., Bancher, C., Barcikowska, M., Wen, G. Y., and Currie, J. (1989) Spectrum of morphological appearance of amyloid deposits in Alzheimer's disease, *Acta neuropathologica 78*, 337-347.
103. Yoshiike, Y., Akagi, T., and Takashima, A. (2007) Surface structure of amyloid-beta fibrils contributes to cytotoxicity, *Biochemistry 46*, 9805-9812.

104. Wood, D. D., and Moscarello, M. A. (1989) The isolation, characterization, and lipid-aggregating properties of a citrulline containing myelin basic protein, *J Biol Chem 264*, 5121-5127.
105. Fannon, A. M., and Moscarello, M. A. (1991) Characterization of myelin basic protein charge isomers from adult mouse brain, *Neuroreport 2*, 135-138.
106. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S. A., Mankani, M., Robey, P. G., Poole, A. R., Pidoux, I., Ward, J. M., and Birkedal-Hansen, H. (1999) MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover, *Cell 99*, 81-92.
107. Zhou, Z., Apte, S. S., Soininen, R., Cao, R., Baaklini, G. Y., Rauser, R. W., Wang, J., Cao, Y., and Tryggvason, K. (2000) Impaired endochondral ossification and angiogenesis in mice deficient in membrane-type matrix metalloproteinase I, *Proc Natl Acad Sci U S A 97*, 4052-4057.
108. Beniac, D. R., Luckevich, M. D., Czarnota, G. J., Tompkins, T. A., Ridsdale, R. A., Ottensmeyer, F. P., Moscarello, M. A., and Harauz, G. (1997) Three-dimensional structure of myelin basic protein. I. Reconstruction via angular reconstitution of randomly oriented single particles, *The Journal of biological chemistry 272*, 4261-4268.
109. Ridsdale, R. A., Beniac, D. R., Tompkins, T. A., Moscarello, M. A., and Harauz, G. (1997) Three-dimensional structure of myelin basic protein. II. Molecular modeling and considerations of predicted structures in multiple sclerosis, *The Journal of biological chemistry 272*, 4269-4275.
110. Bates, I. R., Matharu, P., Ishiyama, N., Rochon, D., Wood, D. D., Polverini, E., Moscarello, M. A., Viner, N. J., and Harauz, G. (2000) Characterization of a recombinant murine 18.5-kDa myelin basic protein, *Protein Expression & Purification 20*, 285-299.
111. Bates, I. R., Libich, D. S., Wood, D. D., Moscarello, M. A., and Harauz, G. (2002) An Arg/Lys-->Gln mutant of recombinant murine myelin basic protein as a mimic of the deiminated form implicated in multiple sclerosis, *Protein Expr Purif 25*, 330-341.
112. Cao, L., Goodin, R., Wood, D., Moscarello, M. A., and Whitaker, J. N. (1999) Rapid release and unusual stability of immunodominant peptide 45-89 from citrullinated myelin basic protein, *Biochemistry 38*, 6157-6163.
113. Ramwani, J. J., Epand, R. M., and Moscarello, M. A. (1989) Secondary structure of charge isomers of myelin basic protein before and after phosphorylation, *Biochemistry 28*, 6538-6543.
114. Beniac, D. R., Wood, D. D., Palaniyar, N., Ottensmeyer, F. P., Moscarello, M. A., and Harauz, G. (1999) Marburg's variant of multiple sclerosis correlates with a less compact structure of myelin basic protein, *Mol Cell Biol Res Commun 1*, 48-51.
115. Chalmers, K., Wilcock, G., and Love, S. (2005) Contributors to white matter damage in the frontal lobe in Alzheimer's disease, *Neuropathol Appl Neurobiol 31*, 623-631.

116. Duan, J. H., Wang, H. Q., Xu, J., Lin, X., Chen, S. Q., Kang, Z., and Yao, Z. B. (2006) White matter damage of patients with Alzheimer's disease correlated with the decreased cognitive function, *Surg Radiol Anat 28*, 150-156.
117. Wang, D. S., Bennett, D. A., Mufson, E. J., Mattila, P., Cochran, E., and Dickson, D. W. (2004) Contribution of changes in ubiquitin and myelin basic protein to age-related cognitive decline, *Neurosci Res 48*, 93-100.
118. Selkoe, D. J., Brown, B. A., Salazar, F. J., and Marotta, C. A. (1981) Myelin basic protein in Alzheimer disease neuronal fractions and mammalian neurofilament preparations, *Ann Neurol 10*, 429-436.
119. Ottens, A. K., Golden, E. C., Bustamante, L., Hayes, R. L., Denslow, N. D., and Wang, K. K. (2008) Proteolysis of multiple myelin basic protein isoforms after neurotrauma: characterization by mass spectrometry, *J Neurochem 104*, 1404-1414.
120. Liu, M. C., Akle, V., Zheng, W., Kitlen, J., O'Steen, B., Larner, S. F., Dave, J. R., Tortella, F. C., Hayes, R. L., and Wang, K. K. (2006) Extensive degradation of myelin basic protein isoforms by calpain following traumatic brain injury, *J Neurochem 98*, 700-712.
121. Shiryaev, S. A., Savinov, A. Y., Cieplak, P., Ratnikov, B. I., Motamedchaboki, K., Smith, J. W., and Strongin, A. Y. (2009) Matrix metalloproteinase proteolysis of the myelin basic protein isoforms is a source of immunogenic peptides in autoimmune multiple sclerosis, *PLoS One 4*, e4952.
122. D'Souza, C. A., and Moscarello, M. A. (2006) Differences in susceptibility of MBP charge isomers to digestion by stromelysin-1 (MMP-3) and release of an immunodominant epitope, *Neurochem Res 31*, 1045-1054.
123. Chernoff, G. F. (1981) Shiverer: an autosomal recessive mutant mouse with myelin deficiency, *The Journal of heredity 72*, 128.
124. Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., Guillozet-Bongaarts, A., Ohno, M., Disterhoft, J., Van Eldik, L., Berry, R., and Vassar, R. (2006) Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation, *J Neurosci 26*, 10129-10140.
125. Beck, H., Schwarz, G., Schroter, C. J., Deeg, M., Baier, D., Stevanovic, S., Weber, E., Driessen, C., and Kalbacher, H. (2001) Cathepsin S and an asparagine-specific endoprotease dominate the proteolytic processing of human myelin basic protein in vitro, *Eur J Immunol 31*, 3726-3736.
126. Ihara, M., Polvikoski, T. M., Hall, R., Slade, J. Y., Perry, R. H., Oakley, A. E., Englund, E., O'Brien, J. T., Ince, P. G., and Kalaria, R. N. (2010) Quantification of myelin loss in frontal lobe white matter in vascular dementia, Alzheimer's disease, and dementia with Lewy bodies, *Acta Neuropathol*.
127. Smith, R. (1992) The basic protein of CNS myelin: its structure and ligand binding, *J Neurochem 59*, 1589-1608.
128. Polverini, E., Boggs, J. M., Bates, I. R., Harauz, G., and Cavatorta, P. (2004) Electron paramagnetic resonance spectroscopy and molecular modelling of the interaction of myelin basic protein (MBP) with calmodulin (CaM)-diversity and conformational adaptability of MBP CaM-targets, *J Struct Biol 148*, 353-369.

129. Berlet, H. H., Bischoff, H., and Weinhardt, F. (1994) Divalent metals of myelin and their differential binding by myelin basic protein of bovine central nervous system, *Neurosci Lett 179*, 75-78.
130. Chan, C. K., Ramwani, J., and Moscarello, M. A. (1988) Myelin basic protein binds GTP at a single site in the N-terminus, *Biochem Biophys Res Commun 152*, 1468-1473.
131. Pirollet, F., Margolis, R. L., and Job, D. (1992) Ca(2+)-calmodulin regulated effectors of microtubule stability in neuronal tissues, *Biochim Biophys Acta 1160*, 113-119.
132. Hill, C. M., Libich, D. S., and Harauz, G. (2005) Assembly of tubulin by classic myelin basic protein isoforms and regulation by post-translational modification, *Biochemistry 44*, 16672-16683.
133. Boggs, J. M., Rangaraj, G., Hill, C. M., Bates, I. R., Heng, Y. M., and Harauz, G. (2005) Effect of arginine loss in myelin basic protein, as occurs in its deiminated charge isoform, on mediation of actin polymerization and actin binding to a lipid membrane in vitro, *Biochemistry 44*, 3524-3534.
134. Cwiklinska, H., Mycko, M. P., Luvsannorov, O., Walkowiak, B., Brosnan, C. F., Raine, C. S., and Selmaj, K. W. (2003) Heat shock protein 70 associations with myelin basic protein and proteolipid protein in multiple sclerosis brains, *Int Immunol 15*, 241-249.
135. Porcellini, E., Davis, E. J., Chiappelli, M., Ianni, E., Di Stefano, G., Forti, P., Ravaglia, G., and Licastro, F. (2008) Elevated plasma levels of alpha-1-anti-chymotrypsin in age-related cognitive decline and Alzheimer's disease: a potential therapeutic target, *Curr Pharm Des 14*, 2659-2664.
136. Nilsson, L. N., Bales, K. R., DiCarlo, G., Gordon, M. N., Morgan, D., Paul, S. M., and Potter, H. (2001) Alpha-1-antichymotrypsin promotes beta-sheet amyloid plaque deposition in a transgenic mouse model of Alzheimer's disease, *J Neurosci 21*, 1444-1451.
137. Rozemuller, J. M., Abbink, J. J., Kamp, A. M., Stam, F. C., Hack, C. E., and Eikelenboom, P. (1991) Distribution pattern and functional state of alpha 1-antichymotrypsin in plaques and vascular amyloid in Alzheimer's disease. A immunohistochemical study with monoclonal antibodies against native and inactivated alpha 1-antichymotrypsin, *Acta Neuropathol 82*, 200-207.
138. Hemming, M. L., Patterson, M., Reske-Nielsen, C., Lin, L., Isacson, O., and Selkoe, D. J. (2007) Reducing amyloid plaque burden via ex vivo gene delivery of an Abeta-degrading protease: a novel therapeutic approach to Alzheimer disease, *PLoS Med 4*, e262.
139. Hemming, M. L., Selkoe, D. J., and Farris, W. (2007) Effects of prolonged angiotensin-converting enzyme inhibitor treatment on amyloid beta-protein metabolism in mouse models of Alzheimer disease, *Neurobiol Dis 26*, 273-281.
140. Iwata, N., Mizukami, H., Shirotani, K., Takaki, Y., Muramatsu, S., Lu, B., Gerard, N. P., Gerard, C., Ozawa, K., and Saido, T. C. (2004) Presynaptic localization of neprilysin contributes to efficient clearance of amyloid-beta peptide in mouse brain, *J Neurosci 24*, 991-998.

141. Liao, M. C., and Van Nostrand, W. E. (2010) Degradation of soluble and fibrillar amyloid beta-protein by matrix metalloproteinase (MT1-MMP) in vitro, *Biochemistry 49*, 1127-1136.
142. Leal, M. C., Dorfman, V. B., Gamba, A. F., Frangione, B., Wisniewski, T., Castano, E. M., Sigurdsson, E. M., and Morelli, L. (2006) Plaque-associated overexpression of insulin-degrading enzyme in the cerebral cortex of aged transgenic tg2576 mice with Alzheimer pathology, *J Neuropathol Exp Neurol 65*, 976-987.
143. Tucker, H. M., Kihiko, M., Caldwell, J. N., Wright, S., Kawarabayashi, T., Price, D., Walker, D., Scheff, S., McGillis, J. P., Rydel, R. E., and Estus, S. (2000) The plasmin system is induced by and degrades amyloid-beta aggregates, *J Neurosci 20*, 3937-3946.
144. Tucker, H. M., Kihiko-Ehmann, M., Wright, S., Rydel, R. E., and Estus, S. (2000) Tissue plasminogen activator requires plasminogen to modulate amyloid-beta neurotoxicity and deposition, *J Neurochem 75*, 2172-2177.