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## The Effects of Calpain on the Degradation of Amyloid Beta (A $\beta$ ) Protein

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To the Graduate Council:

I am submitting herewith a thesis written by Xin Lu entitled "The Effects of Calpain on the Degradation of Amyloid Beta (A $\beta$ ) Protein." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Xuemin Xu, Major Professor

We have read this thesis and recommend its acceptance:

Meizhen Cui, Hildegard Schuller, Karla Matteson

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Graduate School

**The Effects of Calpain on the  
Degradation of Beta-Amyloid (A $\beta$ )  
Protein**

**A Thesis**

**Presented for the**

**Master of Science Degree**

**The University of Tennessee, Knoxville**

**Xin Lu**

**August 2008**

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## Abstract

Abnormal accumulation of amyloid beta-peptide (A-beta) is believed to be the primary event in the pathogenesis of Alzheimer's disease (AD). Two cellular mechanisms could contribute to the abnormal accumulation of A $\beta$  in the brain: over production and/or failure in clearance of this A-beta peptide. In an effort to identify the cellular system that is involved in A-beta clearance, we conducted experiments to investigate the effects of calpain inhibitors on the production of secreted A-beta and the intracellular accumulated derivatives of APP, using a culture cell model. Our results revealed that at low concentration, calpain inhibitors caused an increase in the accumulation of both A-beta 40 and A-beta 42. At high concentration, calpain inhibitors led to a decline in A-beta accumulation toward the basal level and an increase in intracellular accumulation of C-terminal fragments, including CTF-beta, CTF-alpha, generated by beta- and alpha-cleavage, respectively. These results suggest that calpain enzymes, which are a highly conserved superfamily of calcium dependent papain-like cysteine proteases, are involved in the metabolism of APP and the formation and accumulation of A-beta. To further identify the enzyme(s) that is responsible for calpain inhibitor-regulated A-beta formation and accumulation, we employed the small interference RNA (siRNA) approach to investigate the effect of knockdown of calpains on the formation and accumulation of A-beta. Our results suggest that different isoforms of calpain enzymes may function differently in A-beta

production and accumulation. This information may lead to a better understanding of the mechanism underlying the abnormal accumulation of A-beta peptide in the Alzheimer's disease brain and provide new insight into the pathogenesis of this disease.

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## **List of Abbreviations**

AD, Alzheimer's disease

A $\beta$ , amyloid  $\beta$ -peptide

APP,  $\beta$ -amyloid precursor protein

AICD, APP intracellular domain

CTF, carboxyl-terminal fragment

NCT, nicastrin

NFT, neurofibrillary tangles

PHF, paired helical filaments

PS, presenilin

RNAi, RNA interference

siRNA, small interfering RNA

TGN, trans-Golgi network

## Introduction

### **Alzheimer's disease and its two pathological hallmarks**

Alzheimer's disease (AD), also called Alzheimer disease or simply Alzheimer's, is the most common cause of dementia. Alzheimer's is a degenerative and terminal disease for which there is no known cure. In its most common form, it afflicts individuals over 65 years old, although a less prevalent early-onset form also exists. Only 10% of early-onset AD are due to rare, fully penetrant (autosomal dominant) mutations in 3 genes: A $\beta$  precursor protein (APP) on chromosome 21[32], presenilin 1 (PS1) on chromosome 14[33], and presenilin 2 (PS2) on chromosome 1[34-35]. On the other hand, most cases of AD are later in onset ( $\geq$  65 years of age), are nonfamilial, and are probably due to highly prevalent genetic variants with low penetrance. [36]

In 1906 at a meeting in Munich, a German psychiatrist, Alois Alzheimer first came up with the definition of a clinicopathological syndrome, which was later on named after his name as Alzheimer's disease (AD). The characteristics of this disease include progressive memory impairment; disordered cognitive function; altered behavior including paranoia, delusions and loss of social appropriateness; and a progressive decline in language function. He also discovered two distinct lesions in AD patients' brains: intracellular neurofibrillary tangles (NFT) and extracellular amyloid plaques (also known as senile plaques or neuritic plaques). Clinical science did not make any progress in defining the pathogenesis of this

disease until half a century later. Thanks to the advent of electron microscopy, in 1960s Michael Kidd in England and Robert Terry in the United States were able to describe the remarkable ultrastructural changes associated with the two distinct lesions which Alzheimer had described. In the mid 1970s, researchers found that neurons synthesizing and releasing acetylcholine underwent variable but usually severe degeneration. Consequently, pharmacological research made great efforts to increase acetylcholine levels in the synaptic cleft, mainly through inhibiting the degradative enzyme. However, these cholinergic drugs more often ended up with a lack of robust clinical benefit in most patients. This lack of clinical benefit was most likely due to the degeneration involving highly heterogeneous classes of neurons which was discovered later in the early 1980s. Since then, attention focused on trying to identify the underlying mechanisms for the synaptic dysfunction and perikaryal degeneration, by studying the two characteristic neuropathological lesions, senile plaques and neurofibrillary tangles, which have been regarded as two hallmarks of AD. [1]

Neuritic plaques are approximately spherical, extracellular deposits of  $\beta$ -amyloid proteins ( $A\beta$ ) fibrils closely surrounded by dystrophic axons and dendrites [2]. The majority of  $A\beta$  is in the form ending at amino acid 40 ( $A\beta_{40}$ ). A small portion of  $A\beta$  ends at amino acid 42 ( $A\beta_{42}$ ) [1,9].  $A\beta_{42}$ , which is slightly longer than  $A\beta_{40}$ , is a more hydrophobic form that is particularly prone to aggregation. [3] Evidence was found that in an AD-specific process. The initial deposition of  $A\beta_{42}$  is followed by growth of the amyloid with precipitation of soluble  $A\beta_{40}$ . The

resulting neuritic plaques become increasingly associated with activated microglia and reactive astrocytes. Those microglia and astrocytes produce a number of molecules such as cytokines, reactive oxygen and nitrogen intermediates and proteases that may be toxic to neuronal processes near neuritic plaques. They also generate factors that result in reciprocal activation and growth, which possibly triggers a local inflammatory cascade. [32]

The neurofibrillary tangles are made up of a protein called tau, which is an abnormally phosphorylated protein component of paired helical filaments (PHF) and interacts with neuronal microtubules. [4]. According to recent biochemical and animal model studies, the affinity of tau for microtubules is weakened by abnormal phosphorylation, which leads to the aggregation of tau into filaments and tangles. Early steps of this process show toxicity to the neuron, which might be exacerbated by tangle formation. [5, 6]

### **Hypotheses on the underlying mechanism of AD**

The tau tangle hypothesis focuses on the role of tau tangle in the pathogenesis of AD, as there is good correlation between NFT numbers and the severity of the disease. However, the later appearance of NFTs in the brains of AD patients and the evidence that transgenic mice over-expressing mutant tau did not induce amyloid plaque formation substantially weaken this point. [8] Now tau tangles are more commonly accepted as a downstream factor rather than the origin of AD.

The amyloid hypothesis (also known as amyloid cascade hypothesis or A $\beta$  hypothesis) was first proposed by D.J.Selkoe and J.A.Hardy in early 1990s, [12, 13] based on the facts that the gene encoding APP was mapped to chromosome 21.[14-16] and Down's syndrome patients with triple chromosome 21 always develop the neuropathology of AD[17]. The amyloid hypothesis states that abnormal accumulation of A $\beta$  is the primary causative event in the pathogenesis of AD [10, 18, 32]

Although this hypothesis has not been universally accepted, there is substantial evidence supporting this theory. 1) APP mutations at or near the  $\beta$ -,  $\gamma$ -, or  $\alpha$ -cleavage sites enhance either total A $\beta$  production or the increase in the more toxic form of A $\beta$ <sub>42</sub> [19-21]; 2) mutations in presenilin 1 and 2 that lead to AD also promote A $\beta$ <sub>42</sub> production [23] ; 3) Genetic variability in A $\beta$  catabolism may increase the risk of late-onset AD [28]

Therefore, the process of AD probably follows an "amyloid accumulation and deposition—tau hyperphosphorylation—tangles formation—neuron death" outline. Great efforts aimed at reducing A $\beta$  level are being carried out by pharmacological research [33] based on this theory.

## **The processing of amyloid precursor protein and A $\beta$ production**

### APP

Amyloid precursor protein is a large type I transmembrane protein. APP gene has been cloned and localized in chromosome 21 [14]. Processed by sequential enzymatic hydrolysis at  $\beta$ -,  $\epsilon$ -,  $\zeta$ - and  $\gamma$ - cleavage sites, APP could generate A $\beta$ 40/42 whose abnormal accumulation would induce AD. There are two mutations immediately before the  $\beta$ -cleavage site, call Swedish mutation, which tends to favor  $\beta$ -secretase cleavage, thus promote the production of A $\beta$  peptides.

### $\gamma$ -secretase complex

Presenilin, nicastrin (NCT), APH-1, and PEN-2 form an active  $\gamma$ -secretase complex. Presenilin 1 (PS1) undergoes endo proteolytic processing within its large cytosolic loop between TM6 and TM7, generating NTF and CTF which remain bound together forming a stable, heterodimeric PS complex. Primary neurons from PS1 deficient mice showed significantly decrease in A $\beta$  production [27]

### Amyloidogenic and non amyloidogenic pathways

APP can be processed in two pathways [Fig.1]. One is non-amyloidogenic through  $\alpha$ -secretase cleavage, producing membrane-anchored C terminal fragment (CTF $\alpha$ ) and Soluble APP $\alpha$  (sAPP $\alpha$ ). The other is amyloidogenic through  $\beta$ -secretase cleavage, producing sAPP $\beta$  and CTF $\beta$ . For both pathways there would be involvement of  $\gamma$ -secretase, which cleaves CTF $\alpha$  into APP

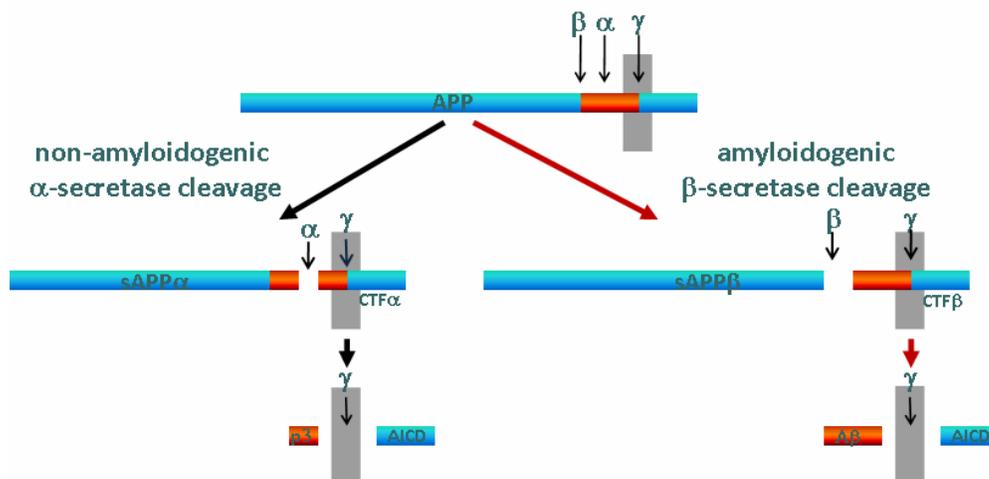
Intracellular Domain (AICD, also known as CTF $\epsilon$ ) and p3, and cleaves CTF $\beta$  into A $\beta$  peptides and CTF $\epsilon$ . [1]

The majority of A $\beta$  produced by  $\beta$ - and  $\gamma$ -secretase are A $\beta$ 40 and A $\beta$ 42.

Sequence analysis reveals that there are 7 to 9 amino acids' distance between the N-terminus of CTF $\epsilon$  (also known as AICD) and the C terminus of A $\beta$ 40/42.

This results in the identification on a novel cleavage site of APP between A $\beta$ 49 and A $\beta$ 50, a  $\epsilon$ -cleavage site. Currently, the cleavage at A $\beta$ 40/42 has been specifically referred to as  $\gamma$ -cleavage site [7,15,17]

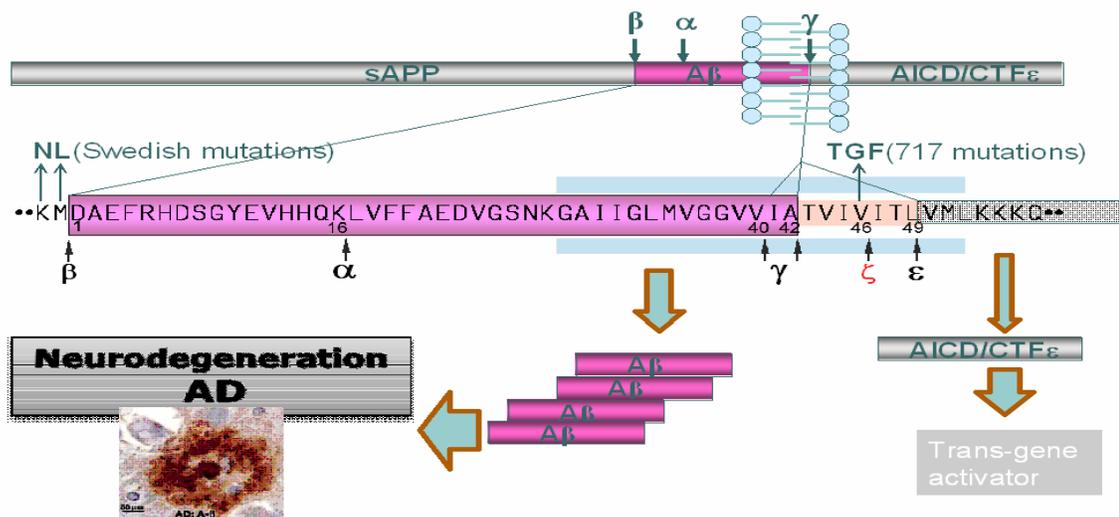
Four years ago, a new intracellular long A $\beta$  containing residues 1-46 (A $\beta$ 46) was identified and known to be produced from the hydrolysis at a novel  $\zeta$ -cleavage



**Figure 1: APP processing mediated by  $\alpha$ ,  $\beta$  and  $\gamma$  secretases**

AICD is also known as CTF $\epsilon$

site, between the known  $\gamma$ - and  $\epsilon$ - cleavage sites [18]. Through applying a differential inhibition method, A $\beta$ 46 has been known to be an intermediate precursor of secreted A $\beta$ 40/42. Moreover, another long A $\beta$  species, probably A $\beta$ 49 generated by  $\epsilon$ -cleavage, has also been identified. Further analysis showed that  $\gamma$ - cleavage is dependent on  $\zeta$ -cleavage during the proteolytic processing of APP. Therefore, a conclusion was reached that a series of sequential cleavages together generate secreted A $\beta$  from APP [Fig.2]. A $\beta$ 46 could be produced by  $\zeta$ -cleavage from  $\epsilon$ -cleavage generated A $\beta$ 49, and then could be cleaved at the  $\gamma$ -cleavage site, releasing A $\beta$ 40/42 [20]. This proposed pathway provides us explanation on how  $\gamma$ - cleavage occurs in a sequence found in the middle of a membrane, where normal enzymatic hydrolysis is impossible due to the extreme hydrophobic environment.



**Figure 2: sequential cleavages in APP processing**

## **Calpains and A $\beta$ accumulation**

Calpains are a highly conserved superfamily of calcium dependent papain-like cysteine proteases, with two 'classical' members, calpain 1 and 2. These two proteins are also known as  $\mu$ -calpain and m-calpain respectively, after their  $\text{Ca}^{2+}$  sensitivity in vitro (the former being activated at levels of micromoles per litre and the latter by millimoles per litre of  $\text{Ca}^{2+}$ ) [22]. Both calpains share similar mechanism of activation, with binding of multiple  $\text{Ca}^{2+}$  ions disrupting the salt bridges that keep the catalytic domain II in an open conformation [23]. Once disrupted by  $\text{Ca}^{2+}$ , the previously open conformation closes up, thereby initiating proteolytic activity. Shortly after substrate cleavage, there is also autolytic activity, which results in loss of calpain enzymatic activity after only a few minutes.

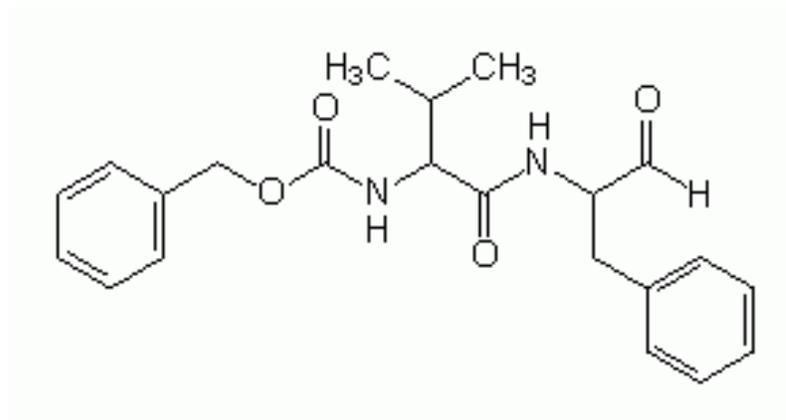
It is reported that calpains target tau and amyloid precursor protein. In a transgenic model of AD, normal cognition and synaptic transmission has been shown to be restored through calpain inhibition [24]. Moreover, an increased amount of calpain 2 has been found in extracts from AD patients' brains, localizing to the cytosolic fraction and in neurofibrillary tangles [25]. It has been suggested that some of the calpain inhibitors can modulate the production of A $\beta$ , possibly through influence on  $\gamma$ -secretase mediated processing of APP [26]. However, the reported observations of the effects of the calpain inhibitor on the formation of A $\beta$  are still controversial [28, 29, 30]

Here we propose that the calpain enzyme is involved in metabolism of APP, especially in the process of degradation on secreted A $\beta$  and the intracellular APP derivatives.

## Materials and Methods

### Reagents.

MDL28710 (carbobenzoxy-valinyl-phenylalaninal also known as calpain inhibitor III) [Fig.3], was obtained from Calbiochem and dissolved in dimethyl sulfoxide. A $\beta$ 40 and A $\beta$ 42 were purchased from American Peptide. siRNAs and HiPerFect Transfection Reagent were ordered from Qiagen, dissolved in siRNA suspension Buffer. Monoclonal antibody 6E10 (Signet Laboratories, Inc.) recognizes residues 1-17 of the A $\beta$  sequence. Monoclonal APP N-terminal-specific antibody 22C11 was from Boehringer. Monoclonal anti-actin antibody was from Sigma. Polyclonal antibody C15 raised against the C-terminal 15 residues of human APP has been described previously[18]



**Figure 3: Molecular formula of MDL28710**

MDL28710 is a membrane-permeable cysteine protease inhibitor which potently inhibits calpain. This compound has been shown to be neuroprotective in vivo and in vitro models of ischemic and excitotoxic injury.

### **RNA interference**

RNA interference (RNAi) is a mechanism that inhibits gene expression at the stage of translation or by hindering the transcription of specific genes. Small interfering RNA strands (siRNA) are keys to the RNAi process, and have complementary nucleotide sequences to the targeted RNA strand. Specific RNAi pathway proteins are guided by the siRNA to the targeted messenger RNA (mRNA), where they "cleave" the target, breaking it down into smaller portions that can no longer be translated into protein.

### **Cell culture**

N2a cells, expressing wild-type presenilin 1 (PS1wt) and Swedish mutant APP (APPsw), were kindly provided by Drs. Sangram S. Sisodia and Seong-Hun Kim (University of Chicago) and maintained as described previously [31]. Opti-MEM1 media were applied, with enhanced growth factor. Sixteen hours after splitting, the old Opti-MEM1 medium containing 10% FBS was replaced with fresh Opti-MEM1 containing 0.5% FBS. Twenty-four hours after that, the cells were either treated with or without inhibitors for 12 hours, or splitted again and treated with or without siRNA complex for the time periods indicated.

### **Immunoprecipitation and Western blot analysis**

Immunoprecipitation and Western blot analyses were carried out as described previously [18]. To determine the intracellular APP derivatives, cells were harvested and lysed in Western blot lysis buffer (50mM Tris-HCl, pH6.8, 8Murea, 5%  $\beta$ -mercaptoethanol, 2% SDS, and protease inhibitors). Secreted A $\beta$  was immunoprecipitated from conditioned media using a monoclonal A $\beta$ -specific antibody 6E10 (Signet Laboratories, Inc.). Both cell lysates and immunoprecipitates were analyzed by 11% Bicine/urea SDS-PAGE, or 10%-16% regular SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore). The membranes were then probed with specific antibodies, and the immunoreactivity bands were visualized using ECL-Plus (Amersham Biosciences).

## Results

### Part I: Treatment with inhibitors

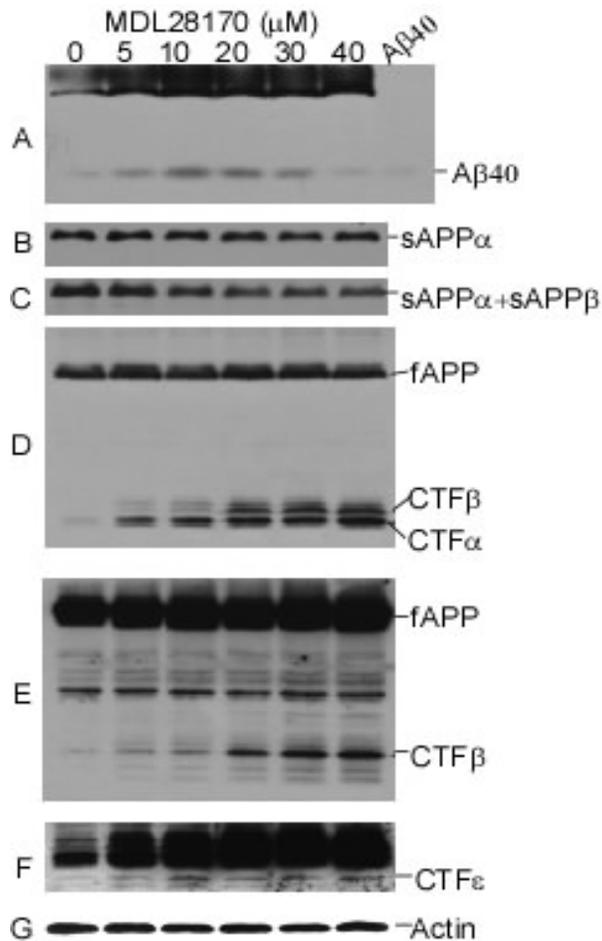
N2a cells stably expressing both wild-type PS1(PS1wt) and myc-tagged Swedish mutant APP (APP<sup>sw</sup>), which have been used in previous studies were treated with MDL28170 or MG132 at various concentrations for 12 h in Opti-MEM containing 0.5% FBS. After that, conditioned media and cell lysates were analyzed by SDS-PAGE.

MDL28170 caused an increase in amyloid- $\beta$  peptides at low concentrations and a decrease at high concentrations.

The conditioned media was processed by immunoprecipitation. Then was analyzed by 11% urea-SDS-PAGE (fig.4\_A). At low concentrations (up to 10 $\mu$ M), MDL28170 caused a dose-dependent increase in secreted A $\beta$ 40. At high concentrations (from 20 to 40 $\mu$ M), the increased A $\beta$ 40 showed a declining trend, back to its basal level.

MDL28170 caused intracellular accumulations of C-terminal fragments

Cell lysates were analyzed by regular 10-16% SDS-PAGE, probed with C15 (fig.4\_D, F) and 6E10 (fig.4\_E) antibodies. At low concentration, MDL28170 caused slight increase in the level of CTF $\alpha$  and CTF $\beta$ . At high concentration, MDL28170 caused a mark increase in the level of both CTF $\alpha$  and CTF $\beta$ . For CTF $\epsilon$ , an increase was observed at low concentration of MDL28170. However, at



**Figure 4: The effects of MDL28170 on APP derivatives**

Western blot results: A) Secreted A $\beta$ 40 immunoprecipitated from conditioned media analyzed by 11% urea-SDS-PAGE and probed by 6E10; B) Conditioned media analyzed by 10% SDS-PAGE and probed by 6E10; C) Conditioned media analyzed by 10% SDS-PAGE and probed by 22C11; D) Cell lysates analyzed by 10-16% SDS-PAGE and probed with C15; E) Cell lysates analyzed by 10-16% SDS-PAGE and probed with 6E10; F) Prolonged exposure of (D); G) Cell lysates analyzed by 10% SDS-PAGE and probed by anti-actin as loading control. The antibody specificity: Monoclonal antibody 6E10 recognizes residues 1-17 of the A $\beta$  sequence. Monoclonal antibody 22C11 recognizes the N-termini of APP. Polyclonal antibody C15 is raised against the C-terminal 15 residues of human APP

high concentration of MDL28170, the accumulation of CTF $\epsilon$  declined.

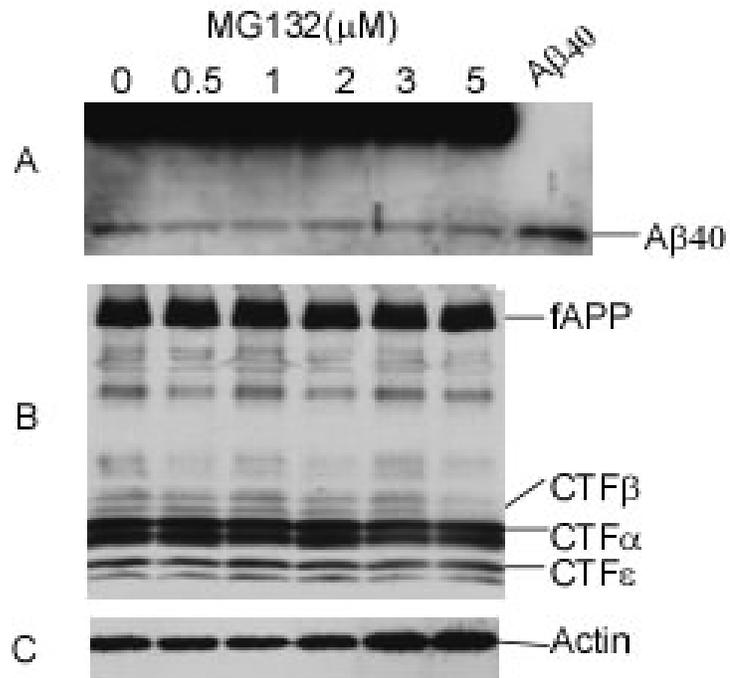
MDL28170 hardly had any affect on the production of soluble APP $\alpha$  and soluble APP $\beta$ .

A small portion of conditioned media were analyzed by 10% SDS-PAGE directly. (fig.4\_B, C). Here the amount of soluble APP $\alpha$  and soluble APP $\beta$  were unchanged across different dosages of calpain inhibitors.

Proteasome inhibitor MG132 has no effect on the accumulation of A $\beta$  and other APP derivatives.

To determine whether the MDL28170 caused accumulation of A $\beta$  is due to non-specific inhibition of proteasomal degradation, cells were treated with proteasome inhibitor MG132. Conditioned media, processed by immunoprecipitation first, were analyzed by 11% urea-SDS-PAGE. As shown in Fig. 5A, there is no increase in A $\beta$  level was observed in MG132-treated cells

When cell lysates were analyzed by regular 10-16% SDS-PAGE (fig.5\_B), it was observed that the amount of intracellular accumulation of C-terminal fragments was approximately constant across different dosages of MG132.



**Figure 5: The effect of MG132 on APP derivatives**

Western blot results: A) Secreted A $\beta$ 40 immunoprecipitated from conditioned media analyzed by 11% urea-SDS-PAGE and probed by 6E10; B) Cell lysates analyzed by 10-16% SDS-PAGE and probed with C15; C) Cell lysates analyzed by 10% SDS-PAGE and probed by anti-actin as loading control. The antibody specificity: Monoclonal antibody 6E10 recognizes residues 1-17 of the A $\beta$  sequence. Polyclonal antibody C15 is raised against the C-terminal 15 residues of human APP.

## **Part II: Treatment with siRNA**

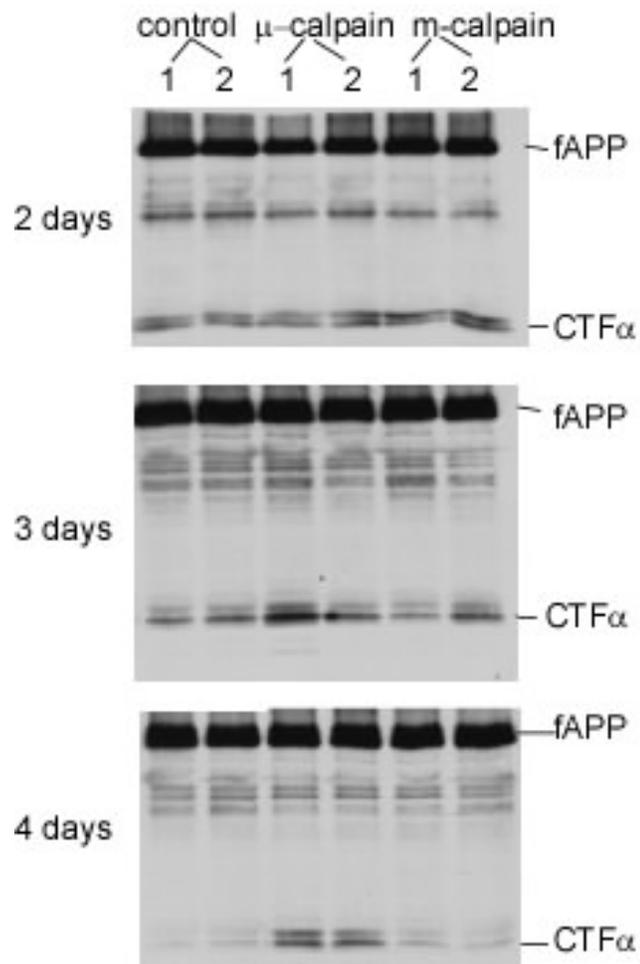
N2a cells stably expressing both wild-type PS1(PS1wt) and myc-tagged Swedish mutant APP (APP<sup>sw</sup>), which have been used in previous studies, were treated with calpain-specific siRNA complexes. Cells were cultured in DMEM containing 10% FBS for the time periods indicated. After that, conditioned media and/or cell lysates were analyzed by SDS-PAGE. Control 1 was treated with non-silence siRNA complexes while control 2 was treated with only HiPerfect delivering reagent. 1 and 2 for each kind of calpain specific siRNA represents different targeted domains.[Fig.6]

Cells treated by  $\mu$ -calpain-specific siRNA show significant increase in CTF $\alpha$  after 3 or 4 days' treatment.

Cell lysates were analyzed by regular 10-16% SDS-PAGE. (fig.6)The levels of CTF $\alpha$  were almost the same among different groups after 2 days' treatment. After 3 or 4 days, the CTF $\alpha$  levels for  $\mu$ -calpain group demonstrated significant increase.

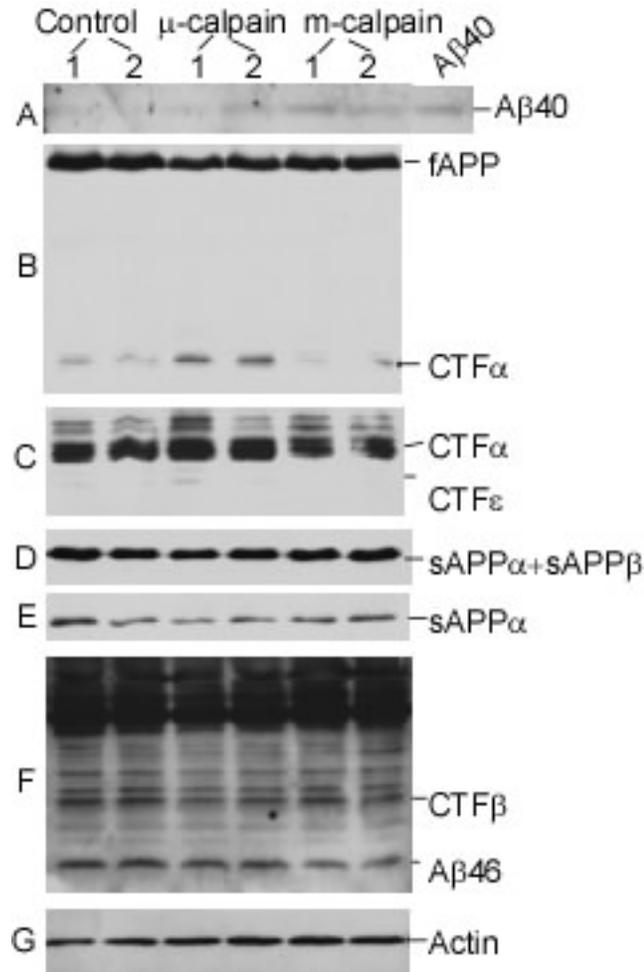
Knocking down m-calpain levels tends to cause an increase in secreted amyloid- $\beta$  peptides (3 days' treatment)

The majority of conditioned media was first processed by immunoprecipitation, and then was analyzed by 11% urea SDS-PAGE. (fig.7\_A), m-calpain siRNA group showed an increase in A $\beta$ 40 at 3 days.



**Figure 6: Preliminary experiments indicated calpain-specific siRNA effects the accumulation of CTF $\alpha$  after 3 or 4 days' treatment**

Western blot results: Cell lysates analyzed by 10-16% SDS-PAGE and probed with C15. The time listed beside the figures indicated how long the cells were treated with siRNA complexes. The antibody specificity: Polyclonal antibody C15 is raised against the C-terminal 15 residues of human APP



**Figure 7: The effects of different calpain-specific siRNA on APP derivatives**

Western blot results: A) Secreted A $\beta$ 40 immunoprecipitated from conditioned media analyzed by 11% urea-SDS-PAGE and probed by 6E10; B) Cell lysates analyzed by 10-16% SDS-PAGE and probed with C15; C) Prolonged exposure of (B); D) Conditioned media analyzed by 10% SDS-PAGE and probed by 6E10; E) Conditioned media analyzed by 10% SDS-PAGE and probed by 22C11; F) Cell lysates analyzed by 10-16% SDS-PAGE and probed with 6E10; G) Cell lysates analyzed by 10% SDS-PAGE and probed by anti-actin as loading control. The antibody specificity: Monoclonal antibody 6E10 recognizes residues 1-17 of the A $\beta$  sequence. Monoclonal antibody 22C11 recognizes the N-termini of APP. Polyclonal antibody C15 is raised against the C-terminal 15 residues of human APP

Knocking down  $\mu$ -calpain levels causes intracellular accumulations of CTF $\alpha$  (3 days' treatment)

Cell lysates were analyzed by regular 10-16% SDS-PAGE (fig.7\_B,C) and probed with C15. CTF $\alpha$  levels for  $\mu$ -calpain siRNA group demonstrated significant increase compared with the control.

siRNA hardly has any effect on the production of soluble APP $\alpha$  and soluble APP $\beta$ . (3 days' treatment)

A small portion of conditioned media were analyzed by 10% SDS-PAGE directly. (fig.7\_D,E) Here the amount of soluble APP $\alpha$  and soluble APP $\beta$  were constant across different groups.

Knocking down calpain levels hardly has any effect on CTF $\beta$  and A $\beta$ 46 (3 days' treatment)

Cell lysates were analyzed by regular 10-16% SDS-PAGE (fig.7\_F) and probed with 6E10. Intracellular CTF $\beta$  and A $\beta$ 46 accumulations were constant across different groups.

## Discussion

In cells either treated by calpain inhibitors or calpain-specific siRNA complexes, neither of the  $\alpha$ - and  $\beta$ - secretase mediated APP processing steps are affected.

In non-amyloidogenic pathway, APP would be cleaved by  $\alpha$ - secretase, producing sAPP $\alpha$  and CTF $\alpha$ . CTF $\alpha$  undergoes  $\gamma$ - secretase-mediated processing, while sAPP $\alpha$  is hardly subjected to any further proteolysis. Therefore, the level of sAPP $\alpha$  could be considered as a good indicator of the activity of  $\alpha$ - secretase-mediated APP processing (non-amyloidogenic pathway). Because sAPP $\alpha$  levels were approximately the same among different treatment and control groups in calpain inhibitors experiments and the siRNA experiments, the  $\alpha$ - secretase mediated APP processing steps seemed to be unaffected by calpain enzymes.

Similarly, in amyloidogenic pathway the  $\beta$ - secretase mediated APP processing steps appeared unaffected by calpain enzymes, too.

Calpain enzymes are involved in the metabolism of APP and the formation and accumulation of A $\beta$

At low concentration, calpain inhibitor MDL28170 caused a dose-dependent increase in A $\beta$ 40 in the conditioned media of cultured cells. This increase could either caused by increase in A $\beta$  production or decrease in A $\beta$  degradation. As what we applied is an enzyme inhibitor, it is very likely that the increase in the A $\beta$ 40 is the result of inhibition on the degradation of A $\beta$ 40. In the same

experiment, we also observed that at high concentration of MDL28170, the increased A $\beta$  level declined and eventually back to the basal level. It is very possible that at high concentration, MDL28170 may not only slow down the degradation of A $\beta$ , but also non-specifically inhibit the  $\gamma$ -secretase activity, resulting in a decrease in A $\beta$  production. As a result of the inhibition of degradation and the inhibition of the production of A $\beta$ 40, at high concentration (40  $\mu$ M), MDL28170 showed no effect on the final level of A $\beta$ 40.

At low concentration, MDL28170 also protected CTF $\epsilon$  from degradation.

However, in the presence of high concentration of MDL28170, the CTF $\epsilon$  was not increased, but rather decreased. This result strongly suggests that at high concentration, MDL28170 non-specifically inhibit  $\gamma$ -secretase activity resulting in reduced production of CTF $\epsilon$ . This result is consistent with the effects of MDL28170 on the accumulation of A $\beta$ .

CTF $\alpha$  and CTF $\beta$ , which are substrate of  $\gamma$ -secretase mainly undergo  $\gamma$ -secretase processing, while a portion of them are also subjected to degradation. As shown in the panel D and E in figure 4, at low concentration, MDL28170 caused a slight accumulation of CTF $\alpha$  and CTF $\beta$ , suggesting MDL28170 inhibited the degradation of these CTFs. However, at higher concentrations, MDL28170 caused dramatic increase in the accumulation of CTF $\alpha$  and CTF $\beta$ . This is

apparently a result of the additive effects of the inhibition of both random degradation and  $\gamma$ -secretase processing of these CTFs.

It is possible that in our experiment calpain inhibitors block the function of proteasomes, which mainly degrade proteins by proteolysis in cells and thus leads to the buildup of APP derivatives during proteolytic processing. Here, proteasome inhibitor MG132 was applied the same way as MDL28170 was done. No increment in the accumulation of intracellular C-terminal fragments was found. Neither did the accumulation of A $\beta$ 40. Therefore, we can confidently rule out the possibility that MDL28170 affects APP metabolism through blocking proteasomes.

So, at this step, we can say that calpain inhibitor sensitive enzymes are involved in the degradation of APP derivatives produced during proteolytic processing of APP.

Our observation that the effect of MDL28170 on the accumulation of A $\beta$  is completely dose dependent may provide an answer to the controversial results reported by previous studies. Some reports that MDL28170 inhibited the formation of A $\beta$ 40, while a further study showed that at low concentrations, MDL28170 increased A $\beta$ 40. Our data demonstrate that the effect, either an enhancing or an inhibiting effect, of MDL28170 on the formation of secreted A $\beta$ 40, is totally dependent on the dose of the inhibitor used. Thus, the conflicting

results reported by previous studies may be due to differences in the dose of inhibitors used.

### Different isoforms of calpain enzymes function differently in A $\beta$ production and accumulation

Various subcellular compartments including the ER, the IC, the Golgi/TGN, endosomes/lysosomes, and the cell surface have been demonstrated to be sites for A $\beta$  generation. It is known that different A $\beta$  species are generated in distinct subcellular compartments, and the sites for generation of intracellular cellular and secreted A $\beta$  are also different. This may explain our observation that different isoforms of calpain have different effects on the metabolism of different APP derivatives.

In the RNAi experiment, the activities of  $\alpha$ - and  $\beta$ - secretase mediated APP processing are not affected by the treatment of calpain-specific siRNA. Thus, the finding that the knock down of the level of  $\mu$ -calpain enzyme leads to the accumulation of CTF $\alpha$  and CTF $\epsilon$ , without affecting A $\beta$  or CTF $\beta$  levels, may reasonably indicate that degradation of CTF $\alpha$  and CTF $\epsilon$  in the non-amyloidogenic pathway is closely related with  $\mu$ -calpain enzyme.

On the other hand, the knock down of the level of  $m$ -calpain enzyme resulted in the A $\beta$ 40 accumulation without affecting C-terminal fragments or intracellular

A $\beta$ 46, it suggests that m-calpain enzyme is involved in the degradation of secreted A $\beta$ 40.

## Conclusions

Calpain plays an active role in APP processing and metabolism. As there are no specific inhibitors for calpains, we first used a potent inhibitor MDL28170 to investigate the relationships and speculated its inhibitory effect on APP derivatives. To further identify the enzyme(s) that is responsible for calpain inhibitor-regulated A $\beta$  formation and accumulation, we applied the siRNA approach to investigate the effect of knockdown of calpains on the formation and accumulation of A $\beta$ . Our results suggest that m-calpain enzyme may be involved in the degradation of secreted A $\beta$ 40, while  $\mu$ -calpain may be involved in the degradation of intracellular C-terminal fragments produced in the non-amyloidogenic pathway. This information may lead to a better understanding of the mechanism underlying the abnormal accumulation of A $\beta$  peptide in the Alzheimer's disease brain and provide new insight into the pathogenesis of this disease.

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