Research Article

Effect of Metal Chelators on γ-Secretase Indicates That Calcium and Magnesium Ions Facilitate Cleavage of Alzheimer Amyloid Precursor Substrate

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Gamma-secretase is involved in the production of Aβ amyloid peptides. It cleaves the transmembrane domain of the amyloid precursor protein (APP) at alternative sites to produce Aβ and the APP intracellular domain (AICD). Metal ions play an important role in Aβ aggregation and metabolism, thus metal chelators and ligands represent potential therapeutic agents for AD treatment. A direct effect of metal chelators on γ-secretase has not yet been investigated. The authors used an *in vitro* γ-secretase assay consisting of cleavage of APP C100-3XFLAG by endogenous γ-secretase from rodent brains and human neuroblastoma SH-SY5Y, and detected AICD production by western blotting. Adding metalloprotease inhibitors to the reaction showed that clioquinol, phosphoramidon, and zinc metalloprotease inhibitors had no significant effect on γ-secretase activity. In contrast, phenanthroline, EDTA, and EGTA markedly decreased γ-secretase activity that could be restored by adding back calcium and magnesium ions. Mg^{2+} stabilized a 1,000 kDa presenilin 1 complex through blue native gel electrophoresis and size-exclusion chromatography. Data suggest that Ca^{2+} and Mg^{2+} stabilize γ-secretase and enhance its activity.

1. Introduction

Gamma-secretase is a key protease activity involved in the production of Alzheimer's disease Aβ amyloid peptides and in regulated intramembrane processing of a subset of membrane receptors, including Notch (reviewed in [1]). Aβ is proteolytically derived from the type I integral amyloid precursor protein (APP) [2] by two sequential cleavages. Shedding of the large APP ectodomain by β-secretase (β-APP cleaving enzyme1, BACE1) [3] produces a 99 amino acid membrane-tethered stub (β-secretase generated APP C-terminal fragment, βCTF, or C99) that is further processed by γ-secretase to liberate Aβ peptides in the extracellu-lar/luminal space. γ-Secretase cleaves the transmembrane domain of APP at multiple sites. Cleavage at the ε-site [4], at position 49-50 according to numbering from Aβ N-terminus mediates the cytosolic release of APP intracellular domain (AICD) together with its binding partners and regulates their nuclear translocation [5]. Further cleavages at the ζ/(46-47) and γ/(40-41) sites generate $Aβ_{40}$ [6]. Pathogenic mutations in APP or in the presenilins result in shifting of ε-cleavage to the (48-49) site and production of $Aβ_{42}$ [7]. APP also undergoes ectodomain shedding by cleavage within the Aβ domain by α-secretase, in a nonamyloidogenic cellular pathway, followed by γ-secretase processing of the corresponding membrane stub (C83) to release AICD and a p3 fragment (reviewed in [8]).

γ-Secretase activity is contained within high molecular complexes formed by assembly of four integral membrane proteins, presenilin, nicastrin, Aph-1, and Pen-2 [9]. Gene knockout experiments [10] and mutation of two con-served aspartates [11] have revealed that the presenilins,

either PS1 or PS2, are membrane proteases and constitute the catalytic subunits of γ-secretase complexes.

The mechanism of γ-secretase and its modulation are yet to be elucidated. We aimed to investigate the effect of metal chelators on γ-secretase activity in vitro. Indeed, biometals and metalloenzymes play an important role in the metabolism of APP and Aβ. APP itself comprises two zinc/copper binding sites, one of them located within the Aβ sequence [12–14]. Although the precise function of APP remains unclear, a wealth of experimental evidence indicates that it plays a role in copper homeostasis [15]. The reduction of Cu^{2+} to Cu^+ by APP is accompanied by the production of hydrogen peroxide resulting in oxidative stress [16]. Also, metal ions, particularly copper, mediate Aβ oligomerization and toxicity [17], therefore metal chelators and ionophores are currently being evaluated as drug candidates for AD treatment (reviewed in [18]). To support the merit of this therapeutic approach, the copper chelator clioquinol (CQ) has been shown to reduce Aβ deposition in the brain of an AD transgenic mouse model [19].

Metals are also implicated in Aβ clearance as the enzymes that metabolize Aβ peptides are zinc-dependent (for a complete review, see [20]), in particular the insulin-degrading enzyme (IDE) [21–24], neprilysin (NEP) [25–27], and the matrix-metalloproteinases MMP2 and MMP9 [28–30]. Secretase processing of APP is also influenced by metal ions since the α-secretase enzymes belong to the family of ADAM proteases, which are zinc-metalloproteases [31], and the β-secretase enzyme; BACE1 comprises a copper-binding site within its cytoplasmic tail, which may regulate its enzymatic activity [32].

In this paper, we have evaluated the direct effects of metal chelators on γ-secretase in an in vitro assay using endogenous enzyme extracted from guinea pig and mouse brains, or from human neuroblastoma SH-SY5Y cells, together with C100-3XFLAG substrate, an analogue of APP β-CTF.

2. Materials and Methods

2.1. Materials and Reagents. Adenosine 5′-triphosphate disodium salt (ATP), ethylene glycol-bis[2-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA), glycerol, 5-chloro7-iodo -8-hydroxyquinoline (clioquinol or CQ), D,L-thiorphan, P-2714 protease inhibitor cocktail, [3-[(3-cholamidopropyl)dimethylammonio-]-2-hydroxy-1-propanesulfonate] (CHAPSO), M2 monoclonal antibody, and anti-FLAG M2-agarose were purchased from Sigma-Aldrich (Sydney, Australia). Glycerol, phosphoramidon and 1,10-phenanthroline were from Merck Biosciences (Victoria, Australia). GM6001 (ilomastat) was obtained from Chemicon (Boronia, Victoria, Australia). L-685,458 inhibitor was obtained from Dr. Mark Shearman (Merck, Sharpe, and Dohme).

2.2. C100-3XFLAG Preparation and Purification. E. Coli transformed with the C100-3XFLAG vector were grown and induced for C100-3XFLAG expression as described before [33]. The cells were harvested, resuspended in 50 mM HEPES, 5 mM MgCl₂, 5 mM CaCl₂, 150 mM KCl, and pH 7.4, supplemented with 1% (w/v) P-2714 (Sigma) protease inhibitor cocktail, and disrupted by sonication. Membranes were isolated by centrifugation for 1 hour at 100,000 g, and were resuspended in homogenisation buffer containing 1% (v/v) CHAPSO, using a Dounce homogenizer, followed by repeated passages through syringe needles of decreasing bore size. The resultant fine suspension was incubated for 1 hour at 4°C, with end-over-end rocking, then centrifuged at 18,000 g for 1 hour at 4°C, and the supernatant containing the solubilized proteins was brought up to 10% glycerol (v/v). C100-3FLAG was affinity-purified on anti-FLAG M2-agarose column and the purified substrate was stored as aliquots at −80°C.

2.3. Preparation of γ-Secretase Activity. All steps were carried out on ice and the centrifugation steps done at 4°C. Guinea pigs and mouse (C57/Bl6) brains were obtained from the Animal Facility of the University of Melbourne Department of Pathology as secondary tissue usage of excess animals. Homogenates from whole brains, minus cerebellum, were prepared as described previously [33]. The crude tissue homogenate prepared in 20 mM HEPES, pH 7.4, containing 250 mM sucrose, and 1% protease inhibitor cocktail (with no pepstatin), was centrifuged at 3,000 g for 30 minutes to remove cellular debris. The supernatant was collected and centrifuged further at 100,000 g for 1 hour in a L8-80M ultracentrifuge equipped with a Ti70.1 rotor (Beckman-Coulter). The pellet was washed with 20 mM HEPES, 150 mM KCl, and pH 7.4, and resuspended in the same buffer plus 1% (v/v) CHAPSO, with mixing on a rotating platform for 1 hour, at 4°C. CHAPSO-extracted membranes were removed by centrifugation at 18,000 g for 20 minutes The supernatant, containing solubilized γ-secretase, was brought up to 10% glycerol (v/v), and stored as aliquots at −80°C.

Alternatively, SH-SY5Y cells were resuspended in 20 mM HEPES, 80 mM KCl, and pH 7.3, supplemented with 1% protease inhibitor cocktail, and homogenized by repeated passages through a 25-gauge needle. The cell homogenate was centrifuged at 1,000 g for 20 min to remove nuclei and cell debris. The resultant supernatant was collected and centrifuged for 1 hour at 100,000 g. The sediment pellet was extracted with 1% CHAPSO.

2.4. Gamma-Secretase Assays. Purified recombinant substrate (~1.5 μM) and CHAPSO-solubilized membranes (5 μg of guinea pig or mouse brain preparation, or 15 μg of SH-SY5Y membrane extract) were incubated for 4 to 18 hours at 37°C in presence of ATP (1.25 mM) and a mixture of phosphotidyl-ethanolamine (PE) and phosphotidylcholine (PC) (50 ng each; Sigma-Aldrich) in a final reaction volume of 20 μL of 20 mM HEPES (pH 7.4) and 0.5% CHAPSO, plus either 2 mM EDTA (buffer E) or 5 mM CaCl₂, 5 mM MgCl₂ and 150 mM KCl (buffer A). Inhibitors were prepared as stocks in DMSO and used at dilutions so that DMSO concentration in incubations was no more than 2.5%. Incubations were terminated by adding Laemmli SDS sample buffer and heating at 95°C for 5 minutes. The samples were electrophoresed for 5 hours on

Tris-tricine gels (two-layer, 10–15% acrylamide discontinuous gradient) using a miniPROTEAN 3 system (Bio-Rad) followed by electrotransfer to nitrocellulose (Trans-blot, Bio-Rad). The blots were boiled in phosphate buffer saline, pH 7.4 (PBS) for 3 min prior to blocking for 1 hour with 0.5% casein in PBS and probing with primary antibody for 2 hours. The blots were developed with the Super Signal West Dura kit (Pierce, Rockford, Il) and exposed to a GeneGnome digital imaging system (Syngene, Cambridge, UK). Data were analysed using GeneTools software (Syngene). γ-Secretase activity was expressed as the ratio of AICD signal to the sum of C100 plus AICD signals after subtracting blank values. Aβ was quantified by DELFIA as described before [34]. In brief, plates were coated with mouse monoclonal antibody G210 (specific for Aβ_{40}) and developed with biotinylated WO2 (anti-Aβ 1–16). Bound antibody was detected with streptavidin-labeled Europium (Perkin Elmer, Inc, Melbourne, Victoria). Results were calculated from a standard curve obtained with Aβ_{40} synthetic peptide.

2.5. BN-PAGE Analysis. SH-SY5Y membrane extract (0.5% CHAPSO), prepared as described above, was mixed with an equal volume of 20 mM HEPES (pH 7.4) containing 0.5% CHAPSO plus one of the following, 2 mM EDTA, 2 mM EGTA, 5 mM MgCl$_2$, or 5 mM CaCl$_2$, and incubated for 18 hours at 19°C. BN-PAGE sample buffer was added and the samples resolved on 3–8% NuPAGE Tris-acetate gels (Invitrogen) as described previously [35].

2.6. Size-Exclusion Chromatography. SH-SY5Y membrane extracts (diluted to 0.5% CHAPSO) prepared as for BN-PAGE were fractionated on a Superose-6 column (GE Healthcare) equilibrated in 20 mM HEPES, 150 mM KCl buffer, and pH 7.3 and eluted at a flow rate of 0.5 mL/min. 0.5 mL fractions were collected and analysed by western blotting with 98/1 antibody [36].

3. Results

3.1. In Vitro γ-Secretase Assay. C100 substrate, expressed in *E. coli,* is based on the human APP C-terminal sequence, which corresponds to the C-terminal fragment produced by β-secretase cleavage of APP (β-CTF, the direct precursor to Aβ) plus N-terminal Met$_{596}$. C100 is expressed with a 3XFLAG C-terminal tag to improve solubility, stability, and detection of AICD [33]. Incubations of C100-3XFLAG with γ-secretase preparations from guinea pig brain membranes produced a 10 kDa C-terminal fragment that was inhibited in a dose-response manner by two specific γ-secretase inhibitors, L-685,458 [37], and DAPT [38] (Figure 1(a)). AICD production was time dependent (Figure 1(b)). Detection of Aβ with WO2 shows an increased production between 2 and 4 hours but the results could not be quantified due to high background and merging of the bands between the lanes.

The effects of phospholipids and ATP were tested to further validate the AICD detection assay and optimize conditions. Adding phospholipids (PC and PE, 2.5 μg/mL

each) enhanced the production of AICD (Figure 1(c)), a result consistent with reports by others [39, 40]. Adding ATP (1.25 mM) to the γ-secretase/APP substrate reaction was also found to cause an average 1.6-fold increase of AICD production (Figures 1(d) and 1(e)), corroborating the report by Fraering et al. [41] who used recombinant γ-secretase solubilized from membranes of cells overexpressing the γ-secretase components. The positive effect of ATP was also obtained using γ-secretase preparations from SH-SY5Y-membranes (data not shown).

3.2. Effect of Zinc and Copper Chelators on γ-Secretase Activity. The effect of metal ion chelators and metalloprotease inhibitors on γ-secretase was evaluated using various compounds (Figures 2(a) and 2(b)). The zinc chelators, thiorphan and phosphoramidon, and the hydroxamate inhibitor ilomastat, which is an inhibitor of α-secretase, were all assayed at 250 nM, a concentration sufficient to completely inhibit their target metalloprotease activities in cells and tissues. There was no significant effect of these compounds in the γ-secretase assay, although thiorphan showed a trend for an increase in AICD signal. Thiorphan is an inhibitor of neprilysin, which might be present in the assay as it is associated with brain membranes. The modest decrease in γ-secretase activity in the presence of the copper chelator clioquinol was not statistically significant. In contrast a 65% decrease in AICD production ($P = .024$; $N = 3$) was observed in the presence of phenanthroline (5 mM), suggesting that some metal ions facilitate γ-secretase activity.

3.3. Metal Chelators of Broad Specificity Decrease γ-Secretase Activity. The reduction of activity in the presence of phenanthroline led us to test the effect of EDTA, another chelator of broad specificity. Indeed, reports in the literature indicate the use of alternative buffers for *in vitro* γ-secretase assays. Notably, these involve the use of buffers that either contain EDTA [41, 42], or that are supplemented with Ca^{2+} and Mg^{2+} [39, 40, 43–45]. Therefore, we compared both conditions in our assay (Figures 3(a) and 3(b)). In the presence of EDTA, AICD production was 28% of that in the presence of buffer with Ca^{2+} and Mg^{2+} ($P = .0002$, $N = 5$). Measurement of Aβ40 production by a sandwich ELISA (Figure 3(c)) showed that 0.035 ± 0.01 ng/mL Aβ40 was produced in the presence of Ca^{2+} and Mg^{2+} whereas 0.011 ± 0.004 ng/mL was produced in the presence of EDTA, further supporting that Ca^{2+} and Mg^{2+} ions facilitate the processing of C100 by γ-secretase. Similar results were obtained when using solubilised SH-SY5Y membranes as the source of γ-secretase, with AICD production in the presence of EDTA being 30% to that in the presence of Ca^{2+} and Mg^{2+} (Figures 3(d) and 3(e)). Comparing incubations with and without Ca^{2+} and Mg^{2+} showed that the addition of these metal ions ameliorates γ-secretase activity by 1.65-fold ($P = .02, N = 3$) (Figures 3(d) and 3(e)). These data are consistent with those obtained with phenanthroline, and suggest that adding Ca^{2+} and Mg^{2+} metal ions facilitate γ-secretase cleavage of APP substrate.
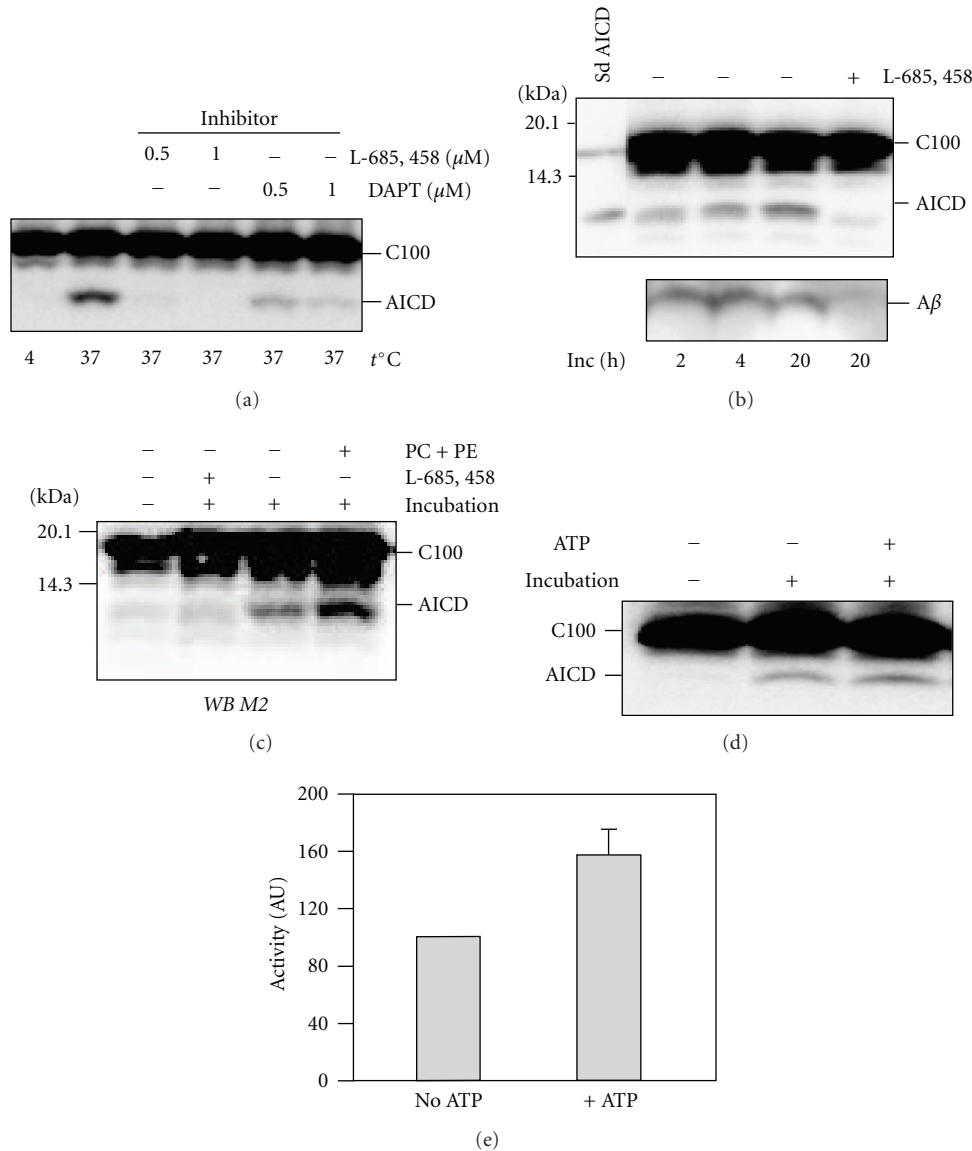
FIGURE 1: Characterization of γ-secretase *in vitro* assay with guinea pig brain enzyme. $\sim$1 μg of purified recombinant C100-3XFLAG was incubated with 2.75 μg of CHAPSO solubilised membranes of guinea pig brain in 20 mM HEPES buffer, pH 7.3, plus 3 mM CaCl$_2$, 3 mM MgCl$_2$ and 150 mM KCl. The reactions were stopped by adding Laemmli buffer, boiled, and separated on Tris-Tricine gels. M2 (anti-FLAG) antibody and WO2 (anti-Aβ 1–16) were used for western blot detection. (a) Production of an AICD fragment in the assay is inhibited by the γ-secretase inhibitors, L-685,458 and DAPT. (b) Both AICD and Aβ are produced in the reaction and inhibited by L-685,458. AICD signal increases in a time dependent manner over 20 h. Aβ signal is increased at 4 h compared to 2 h, but decreased at 20 h, possibly due to degradation. (Inc, incubation at 37°C). (c) Positive effect of phospholipids on AICD production in the γ-secretase assay. PC, phosphatidylcholine; PE, phosphatidylethanolamine. (d) Positive effect of ATP on the γ-secretase assay. ATP was added 1.25 mM concentration. (e) Quantitation of three separate experiments using GeneTools Syngene software shows $\sim$1.6 fold enhancement of γ-secretase activity in the presence of 1.25 mM ATP. The error bars represent SEM.

3.4. Ca^{2+} and Mg^{2+} Facilitate γ-Secretase Activity. To define which of Ca^{2+} and Mg^{2+} ions were important for γ-secretase activity, we tested the effect of EGTA that has much greater affinity for calcium than magnesium. In the reactions carried out with guinea pig or mouse γ-secretase activity, there were similar levels of AICD produced in the presence of EGTA than in the presence of EDTA (Figures 4(a) and 4(b)), suggesting that Ca^{2+} ions are required for the enhancement

of γ-secretase activity. When, reactions were prepared in the presence of 2 mM EDTA and increasing concentrations of CaCl$_2$ or MgCl$_2$ were added, both Ca and Mg ions individually restored AICD production to levels achieved with the Ca and Mg buffer (Figure 4(c)). However, when Mg ions were absent in the reaction, adding 3 mM calcium resulted in precipitation of the C100 substrate. Therefore, both calcium and magnesium are required for optimal assay conditions.
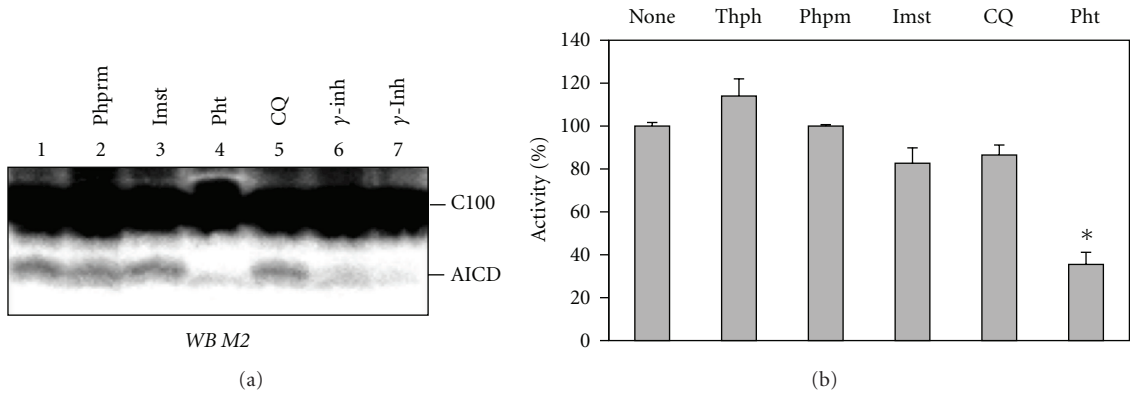
(a)

(b)

FIGURE 2: Effect of metalloprotease inhibitors, and metal chelators on γ-secretase. Chelators and inhibitors were added as DMSO solutions (final DMSO concentration 2.5%) to the guinea pig brain γ-secretase/C100-3XFLAG reactions. (a) Representative western blot of γ-secretase assay in the presence of various inhibitors. Phpm, phosphoramidon; Ilmst, ilomastat; Pht, phenanthroline; CQ, clioquinol; γ-inh, L-685,458, used at 10 nM (lane 6) and 100 nM (lane 7). (b) Effect of metalloprotease inhibitors on the γ-secretase assay. Activity is expressed as the % of substrate converted into AICD, as determined from band density analysis with GeneTools software. The error bars represent SEM. Inhibitor concentrations were selected as follows: thiorphan, 250 nM; phosphoramidon, 25 μM; ilomastat, 250 nM; phenanthroline, 5 mM; clioquinol, 100 μM.
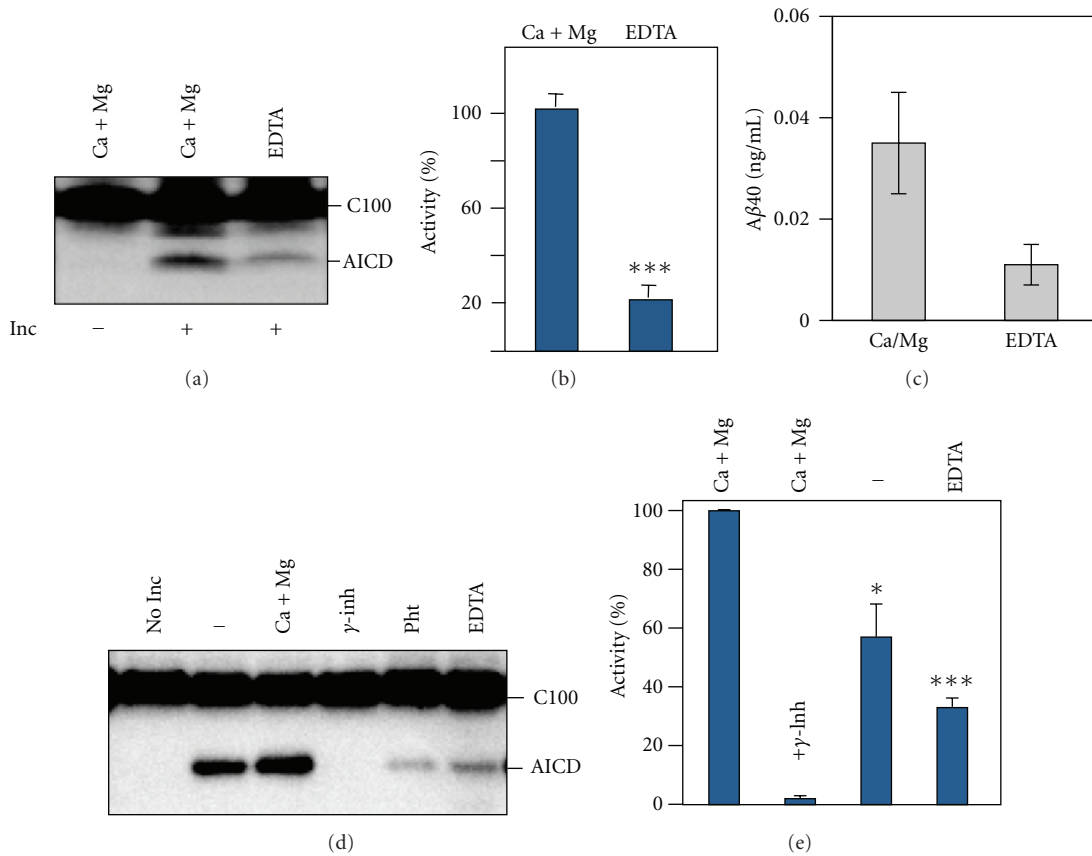

(a)

(b)

(c)

(d)

(e)

FIGURE 3: Effect of EDTA and phenanthroline on γ-secretase activity. (a) Guinea pig γ-secretase activity was assayed on C100-3XFLAG in alternate buffer conditions (2 mM EDTA or 3 mM CaCl$_2$ and 3 mM MgCl$_2$). (b) Quantitative analysis of five separate experiments shows AICD production in the presence of EDTA was 28% of that produced in the presence of CaCl$_2$, and MgCl$_2$. (c) Quantitative analysis of Aβ40 by sandwich DELFIA shows that Aβ production in the presence of EDTA is lower than in the presence of CaCl$_2$, and MgCl$_2$. (d) γ-Secretase activity extracted from SH-SY5Y membranes was assayed in buffer conditions as indicated. Phenanthroline (Pht) concentration was 5 mM. L-685,458 was 0.5 μM. (e) Quantitative analysis of three blots corresponding to assays carried out as in (d) The error bars represent SEM.
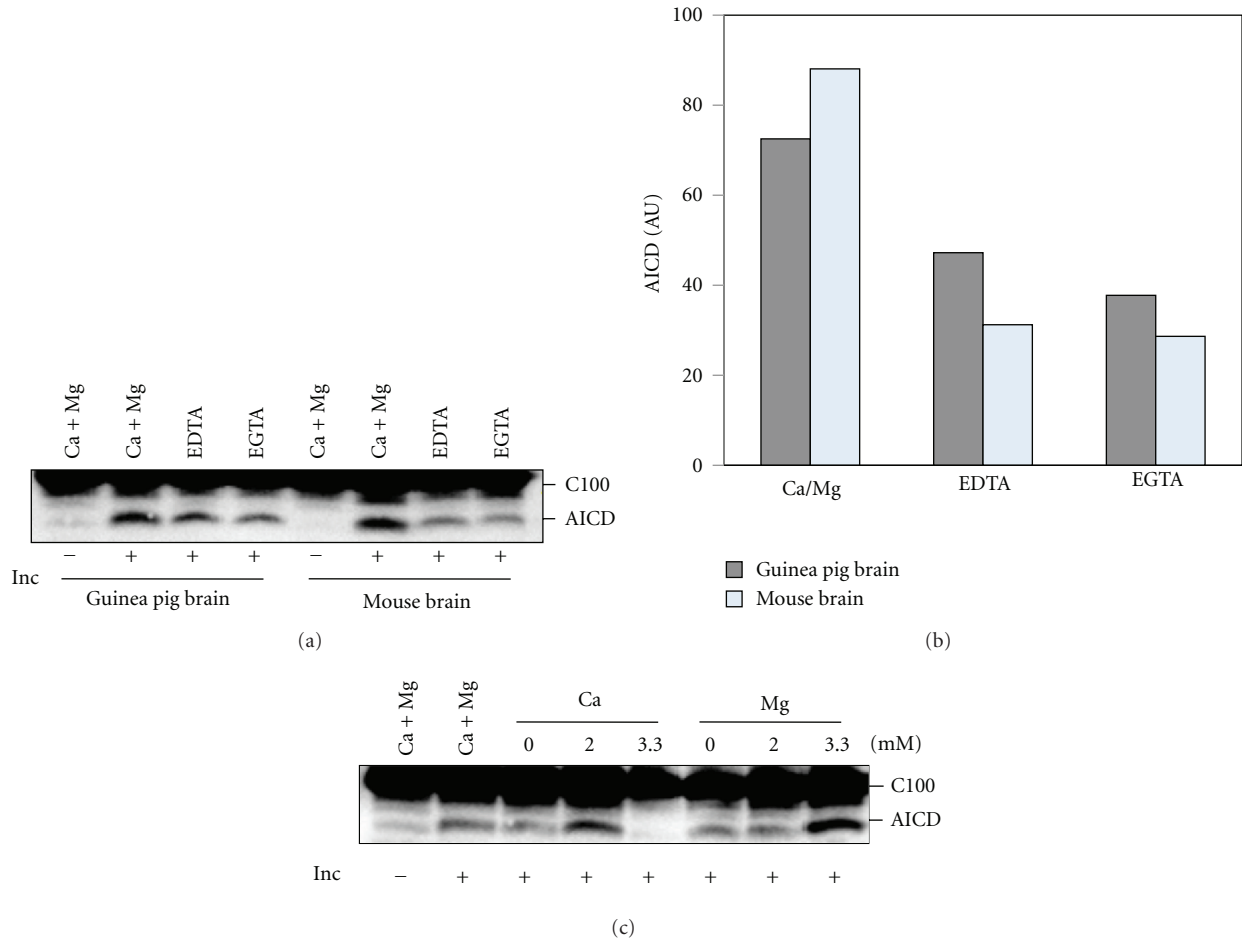
(a)

(b)

(c)

FIGURE 4: Effect of Ca^{2+} and Mg^{2+} on γ-secretase activity. (a) The effects of EDTA (2 mM) and EGTA (2 mM) were compared in assays carried out with γ-secretase preparations from guinea pig or mouse brains. (b) Quantitative analysis of the blot shown in (a). (c) γ-secretase assay with guinea pig brain activity was carried out in HEPES buffer plus 150 mM KCl, in the presence of increasing $CaCl_2$ or $MgCl_2$ concentrations as indicated.

3.5. Calcium and Magnesium Ions Stabilize a High Molecular Weight γ-Secretase Complex.

To determine if calcium and magnesium ions had an effect on the size and stability of γ-secretase complexes, SH-SY5Y membrane-extracts diluted with 20 mM HEPES, and 150 mM KCl (pH 7.3) to a CHAPSO concentration of 0.5% were incubated in the presence of either 2 mM EDTA, 2 mM $MgCl_2$, or 2 mM $CaCl_2$, and analyzed by BN-PAGE and western blotting for PS1 (Figure 5(a)). In HEPES buffer, with no added calcium or magnesium, and in the presence of EDTA or EGTA, PS1 was found mostly associated with a 350 kDa complex, and a minor amount with a 450 kDa complex. In the incubations with Mg^{2+}, with or without ATP, presenilin was found associated with a ~450 kDa complex and also, in a small proportion with a 1,000 kDa complex. In the presence of Ca^{2+}, PS1 was found mostly associated with the ~450 kDa complex. These data suggest that Ca^{2+} and Mg^{2+} ions contribute to stabilization of γ-secretase complexes, possibly in association with binding partners. Further analysis of PS1 γ-secretase complex sizes by gel filtration on a Superose-6 column (Figure 5(b)) showed that, in the presence of

Mg^{2+} ions, most PS1 immunoreactivity is recovered in a symmetrical peak in fractions corresponding to ~1,000 kDa. In the presence of EDTA, the peak of PS1 immunoreactivity was less sharp and broader, suggesting decreased complex stability.

4. Discussion

Biometal ions and metalloenzymes play an important role in the metabolism of APP and Aβ, and the pharmaco-modulation of copper levels in the brain represents a promising therapeutic approach to treat AD [18]. Little is known about the effect of metal ions on γ-secretase activity. We found that the copper-selective chelator, clioquinol did not significantly alter AICD production, suggesting that copper is not directly involved in γ-secretase cleavage of the APP substrate. This finding is consistent with our previous report that CQ does not alter production of Aβ in CHO-APP cells [28]. The zinc chelators, thiorphan and phosphoramidon, and the α-secretase inhibitor, ilomastat, showed no significant effect on the γ-secretase reaction.
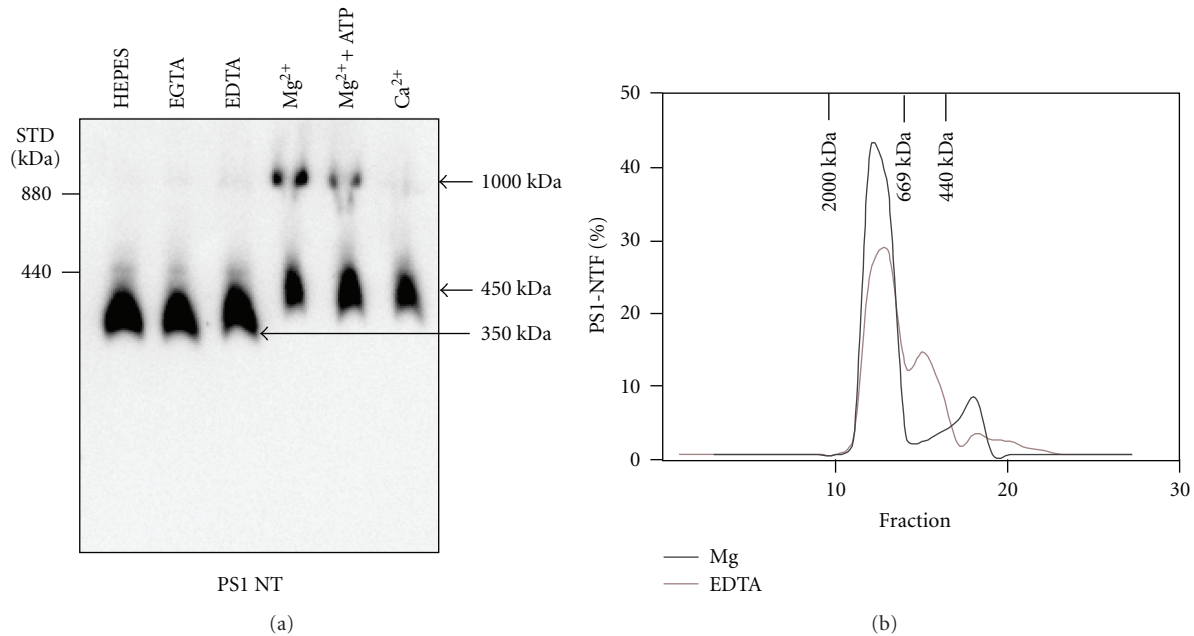
(a)

(b)

FIGURE 5: Effect of Ca^{2+} and Mg^{2+} on the size of γ-secretase complexes analyzed by BNPAGE and size-exclusion chromatography. $10 \mu g$ samples of protein extract from SHSY5Y membranes were incubated with HEPES buffer, pH 7.3, with or without additives, as indicated. EGTA and EDTA concentrations were 2 mM. $CaCl_2$ and $MgCl_2$ concentrations were 3 mM. (a) The incubations were mixed with BN-PAGE sample buffer and electrophoresed on 3–8% Tris-acetate gels. Proteins were transferred to PVDF membrane and the blot probed with anti-PS1 antibody 98/1. (b) SH-SY5Y membranes ($200 \mu g$ per experiment) were extracted with 0.5% CHAPSO in buffer containing either 2 mM EDTA or 3 mM $MgCl_2$. The samples were chromatographed on superose-6 size-exclusion column and eluted fractions were analysed by western blotting with PS1 N-terminal antibody 98/1. Western blot signals were quantified and plotted against fraction numbers.

In contrast, γ-secretase activity was reduced in the presence of EDTA and phenanthroline. EGTA had an effect comparable to that of EDTA, indicating the possible involvement of calcium ions in the assay. Adding back calcium or magnesium to incubations carried out in the presence of EDTA showed that both enhanced AICD production.

In vitro γ-secretase assays described in the literature are carried out in alternative conditions, either in buffers containing EDTA [42, 46], or in buffers supplemented with Ca^{2+} and Mg^{2+} [39, 40, 43–45]. The γ-secretase assays based on $A\beta$ detection are usually carried out in the presence of EDTA, probably to prevent metal-dependent self-aggregation of the peptide that would interfere with antibody capture in immunoassays, whereas the assays based on western blot detection of AICD have been preferably carried out in the presence of calcium and magnesium ions. Parallel incubations in EDTA buffer and in buffer supplemented with $CaCl_2$, and $MgCl_2$, revealed that AICD production was lower by 70–78% in the presence of EDTA than in the presence of Ca^{2+} and Mg^{2+}. $A\beta$ levels, detected by ELISA (Figure 3(c)), were also higher in the presence of Ca^{2+} and Mg^{2+}, suggesting that the same effect of these ions applies to $A\beta$ and AICD production.

To investigate whether calcium and magnesium ions influence the stability of γ-secretase complex, we used both BN-PAGE analysis and gel filtration. These techniques have been previously used to demonstrate that γ-secretase activity associates with high molecular weight complexes [44, 47]. In the present paper, fractionation of γ-secretase preparations by BN-PAGE suggested that Ca^{2+} and Mg^{2+} ions stabilized association of PS1 with high molecular weight complexes of 1,000 and 450 kDa, compared to $\sim$ 350 kDa complexes observed in the presence of EDTA and EGTA. When incubations with EDTA and without Ca^{2+} or Mg^{2+} were treated with a cross-linker prior to BN-PAGE, the $\sim$1,000 complex was also detected in these incubations (data not shown), suggesting that this 1,000 kDa form was normally present but was less stable through electrophoresis in the absence of Ca^{2+} and Mg^{2+} ions, and without cross-linking. Size-exclusion chromatography also suggested that Mg^{2+} ions stabilized HMW γ-secretase complexes. Although only the four subunits, PS, nicastrin, Aph1, and Pen-2 are required for γ-secretase activity and formation of a complex of $\sim$350 kDa, many proteins and other molecules such as phospholipid and ATP have also been shown to associate with γ-secretase complexes and to modulate their activity [48]. For instance, a recent paper reports that PION protein modulates the specificity of γ-secretase towards APP [49]. Three-dimensional reconstruction of the γ-secretase structure from electron micrographs of purified γ-secretase has shown the presence of two weak-density lateral regions [50] and it is tempting to speculate that these could accommodate metal ions. Magnesium ions form complexes with ATP and could mediate the interaction between ATP and presenilin [40]. Alternatively, calcium and magnesium

may play an accessory role by ordering membrane phospholipids. Indeed, it has been reported that substituting EDTA-containing buffer for a buffer supplemented with calcium and magnesium help stabilize and isolate lipid rafts [51]. γ-Secretase components associate with lipid rafts, cholesterol-rich membrane regions where high γ-secretase-specific activity has been detected [52, 53]. Furthermore, experimental evidence indicates that lipids are important for γ-secretase activity [44]. Therefore, calcium and magnesium may facilitate movement of membrane domains and substrate access to the γ-secretase active site. Structural analysis by solid-state NMR and electron microscopy has demonstrated that cations modulate phospholipid bicelle size and that $MgCl_2$ and $CaCl_2$ stabilize large diameter (500 Å) bicelle discs [54]. Thus, the increase in molecular mass of γ-secretase that was observed when adding divalent cations may be consistent with the association of a larger number of detergent and phospholipid molecules with the γ-secretase complex.

Considering that Aβ has been shown to form membrane pores and disrupt neuronal calcium homeostasis by increasing Ca^{2+} influx [55], elevated Aβ could contribute to increased intraneuronal $[Ca^{2+}]$ that might in turn increase γ-secretase activity and Aβ production. Thus, it will be important to clarify how calcium and magnesium tune γ-secretase activity as this may have potential implications in AD pathogenesis.

5. Conclusions

Our study indicates that copper and zinc chelators have no direct effect on γ-secretase activity *in vitro*. It also demonstrates that chelators of broad specificity decrease cleavage of APP C100 substrate. Furthermore, it shows that Ca^{2+} and Mg^{2+} ions facilitate AICD production and contribute to stabilizing HMW γ-secretase complexes. This finding suggests that Ca^{2+} and Mg^{2} mediate molecular associations that modulate γ-secretase activity.

Abbreviations

Aβ: amyloid-β peptide
AICD: APP intracellular domain
APP: amyloid-β precursor protein
ATP: adenosine 5′-triphosphate
BN-PAGE: blue native polyacrylamide gel electrophoresis
C99:   99 amino acid carboxyl terminal fragment of APP
CHAPSO: [3-[(3-cholamidopropyl)dimethylammonio-]-2-hydroxy-1-propanesulfonate]
CTF: C-terminal fragment
CQ: 5-chloro7-iodo-8-hydroxyquinoline, or clioquinol
DMSO: dimethyl sulfoxide
DSP: dithiobis[succinimidylpropionate]
EGTA: ethylene glycol-bis[2-aminoethyl ether]-N,N,N′,N′-tetraacetic acid
HEPES: N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid

MMP: matrix metalloprotease
PC: phosphatidylcholine
PE: phosphotidyl-ethanolamine
PS: presenilin
PVDF: polyvinylidene fluoride
SDS: sodium dodecylsulfate.

References

[1] G. Evin, M. F. Sernee, and C. L. Masters, "Inhibition of γ-secretase as a therapeutic intervention for Alzheimer's disease: prospects, limitations and strategies," *CNS Drugs*, vol. 20, no. 5, pp. 351–372, 2006.

[2] J. Kang, H.-G. Lemaire, A. Unterbeck et al., "The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor," *Nature*, vol. 325, no. 6106, pp. 733–736, 1987.

[3] R. Vassar, "BACE1: the β-secreiase enzyme in Alzheimer's disease," *Journal of Molecular Neuroscience*, vol. 23, no. 1-2, pp. 105–113, 2004.

[4] A. Weidemann, S. Eggert, F. B. M. Reinhard et al., "A novel ε-cleavage within the transmembrane domain of the Alzheimer amyloid precursor protein demonstrates homology with Notch processing," *Biochemistry*, vol. 41, no. 8, pp. 2825–2835, 2002.

[5] X. Cao and T. C. Südhof, "A transcriptivety active complex of APP with Fe65 and histone acetyltransferase Tip60," *Science*, vol. 293, no. 5527, pp. 115–120, 2001.

[6] G. Zhao, M. Z. Cui, G. Mao et al., "γ-cleavage is dependent on ζ-cleavage during the proteolytic processing of amyloid precursor protein within its transmembrane domain," *Journal of Biological Chemistry*, vol. 280, no. 45, pp. 37689–37697, 2005.

[7] Y. Qi-Takahara, M. Morishima-Kawashima, Y. Tanimura et al., "Longer forms of amyloid β protein: implications for the mechanism of intramembrane cleavage by γ-secretase," *Journal of Neuroscience*, vol. 25, no. 2, pp. 436–445, 2005.

[8] G. Evin and A. Weidemann, "Biogenesis and metabolism of Alzheimer's disease Aβ amyloid peptides," *Peptides*, vol. 23, no. 7, pp. 1285–1297, 2002.

[9] T. Iwatsubo, "The γ-secretase complex: machinery for intramembrane proteolysis," *Current Opinion in Neurobiology*, vol. 14, no. 3, pp. 379–383, 2004.

[10] B. De Strooper, P. Saftig, K. Craessaerts et al., "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein," *Nature*, vol. 391, no. 6665, pp. 387–390, 1998.

[11] M. S. Wolfe, W. Xia, B. L. Ostaszewski, T. S. Diehl, W. T. Kimberly, and D. J. Selkoe, "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and γ-secretase activity," *Nature*, vol. 398, no. 6727, pp. 513–517, 1999.

[12] A. I. Bush, G. Multhaup, R. D. Moir et al., "A novel zinc(II) binding site modulates the function of the βA4 amyloid protein precursor of Alzheimer's disease," *Journal of Biological Chemistry*, vol. 268, no. 22, pp. 16109–16112, 1993.

[13] A. I. Bush, W. H. Pettingell, M. D. Paradis, and R. E. Tanzi, "Modulation of Aβ adhesiveness and secretase site cleavage by zinc," *Journal of Biological Chemistry*, vol. 269, no. 16, pp. 12152–12158, 1994.

[14] L. Hesse, D. Beher, C. L. Masters, and G. Multhaup, "The βA4 amyloid precursor protein binding to copper," *FEBS Letters*, vol. 349, no. 1, pp. 109–116, 1994.

[15] G. Multhaup, A. Schlicksupp, L. Hesse et al., "The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I)," *Science*, vol. 271, no. 5254, pp. 1406–1409, 1996.

[16] A. R. White, G. Multhaup, F. Maher et al., "The Alzheimer's disease amyloid precursor protein modulates copper-induced toxicity and oxidative stress in primary neuronal cultures," *Journal of Neuroscience*, vol. 19, no. 21, pp. 9170–9179, 1999.

[17] X. Huang, C. S. Atwood, M. A. Hartshorn et al., "The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction," *Biochemistry*, vol. 38, no. 24, pp. 7609–7616, 1999.

[18] P. J. Crouch, A. R. White, and A. I. Bush, "The modulation of metal bio-availability as a therapeutic strategy for the treatment of Alzheimer's disease," *FEBS Journal*, vol. 274, no. 15, pp. 3775–3783, 2007.

[19] R. A. Cherny, C. S. Atwood, M. E. Xilinas et al., "Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice," *Neuron*, vol. 30, no. 3, pp. 665–676, 2001.

[20] D. J. Selkoe, "Clearing the brain's amyloid cobwebs," *Neuron*, vol. 32, no. 2, pp. 177–180, 2001.

[21] A. Mukherjee, E. S. Song, M. Kihiko-Ehmann et al., "Insulysin hydrolyzes amyloid β peptides to products that are neither neurotoxic nor deposit on amyloid plaques," *Journal of Neuroscience*, vol. 20, no. 23, pp. 8745–8749, 2000.

[22] V. Chesneau, K. Vekrellis, M. R. Rosner, and D. J. Selkoe, "Purified recombinant insulin-degrading enzyme degrades amyloid β-protein but does not promote its oligomerization," *Biochemical Journal*, vol. 351, no. 2, pp. 509–516, 2000.

[23] D. Edbauer, M. Willem, S. Lammich, H. Steiner, and C. Haass, "Insulin-degrading enzyme rapidly removes the β-amyloid precursor protein intracellular domain (AICD)," *Journal of Biological Chemistry*, vol. 277, no. 16, pp. 13389–13393, 2002.

[24] W. Farris, S. Mansourian, Y. Chang et al., "Insulin-degrading enzyme regulates the levels of insulin, amyloid β-protein, and the β-amyloid precursor protein intracellular domain in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 7, pp. 4162–4167, 2003.

[25] N. Iwata, S. Tsubuki, Y. Takaki et al., "Identification of the major Aβ-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition," *Nature Medicine*, vol. 6, no. 2, pp. 143–150, 2000.

[26] R. A. Marr, H. Guan, E. Rockenstein et al., "Neprilysin regulates amyloid β peptide levels," *Journal of Molecular Neuroscience*, vol. 22, no. 1-2, pp. 5–11, 2004.

[27] K. Shirotani, S. Tsubuki, N. Iwata et al., "Neprilysin degrades both amyloid β peptides 1-40 and 1-42 most rapidly and efficiently among thiorphan- and phosphoramidon-sensitive endopeptidases," *Journal of Biological Chemistry*, vol. 276, no. 24, pp. 21895–21901, 2001.

[28] A. R. White, T. Du, K. M. Laughton et al., "Degradation of the Alzheimer disease amyloid β-peptide by metal-dependent up-regulation of metalloprotease activity," *Journal of Biological Chemistry*, vol. 281, no. 26, pp. 17670–17680, 2006.

[29] K. J. Yin, J. R. Cirrito, P. Yan et al., "Matrix metalloproteinases expressed by astrocytes mediate extracellular amyloid-β peptide catabolism," *Journal of Neuroscience*, vol. 26, no. 43, pp. 10939–10948, 2006.

[30] P. Yan, X. Hu, H. Song et al., "Matrix metalloproteinase-9 degrades amyloid-β fibrils in vitro and compact plaques in situ," *Journal of Biological Chemistry*, vol. 281, no. 34, pp. 24566–24574, 2006.

[31] S. Lammich, E. Kojro, R. Postina et al., "Constitutive and regulated α-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3922–3927, 1999.

[32] B. Angeletti, K. J. Waldron, K. B. Freeman et al., "BACE1 cytoplasmic domain interacts with the copper chaperone for superoxide dismutase-1 and binds copper," *Journal of Biological Chemistry*, vol. 280, no. 18, pp. 17930–17937, 2005.

[33] D. E. Hoke, J. L. Tan, N. T. Ilaya et al., "In vitro γ-secretase cleavage of the Alzheimer's amyloid precursor protein correlates to a subset of presenilin complexes and is inhibited by zinc," *FEBS Journal*, vol. 272, no. 21, pp. 5544–5557, 2005.

[34] A. J. George, R. M. D. Holsinger, C. A. McLean et al., "APP intracellular domain is increased and soluble Aβ is reduced with diet-induced hypercholesterolemia in a transgenic mouse model of Alzheimer disease," *Neurobiology of Disease*, vol. 16, no. 1, pp. 124–132, 2004.

[35] G. Evin, L. D. Canterford, D. E. Hoke, R. A. Sharples, J. G. Culvenor, and C. L. Masters, "Transition-state analogue γ-secretase inhibitors stabilize a 900 kDa presenilin/nicastrin complex," *Biochemistry*, vol. 44, no. 11, pp. 4332–4341, 2005.

[36] J. G. Culvenor, G. Evin, M. A. Cooney et al., "Presenilin 2 expression in neuronal cells: induction during differentiation of embryonic carcinoma cells," *Experimental Cell Research*, vol. 255, no. 2, pp. 192–206, 2000.

[37] M. S. Shearman, D. Beher, E. E. Clarke et al., "L-685,458, an aspartyl protease transition state mimic, is a potent inhibitor of amyloid β-protein precursor γ-secretase activity," *Biochemistry*, vol. 39, no. 30, pp. 8698–8704, 2000.

[38] H. F. Dovey, V. John, J. P. Anderson et al., "Functional γ-secretase inhibitors reduce β-amyloid peptide levels in brain," *Journal of Neurochemistry*, vol. 76, no. 1, pp. 173–181, 2001.

[39] P. C. Fraering, W. Ye, J. M. Strub et al., "Purification and characterization of the human γ-secretase complex," *Biochemistry*, vol. 43, no. 30, pp. 9774–9789, 2004.

[40] J. D. J. Wrigley, I. Schurov, E. J. Nunn et al., "Functional overexpression of γ-secretase reveals protease-independent trafficking functions and a critical role of lipids for protease activity," *Journal of Biological Chemistry*, vol. 280, no. 13, pp. 12523–12535, 2005.

[41] P. C. Fraering, W. Ye, M. J. LaVoie, B. L. Ostaszewski, D. J. Selkoe, and M. S. Wolfe, "γ-secretase substrate selectivity can be modulated directly via interaction with a nucleotide-binding site," *Journal of Biological Chemistry*, vol. 280, no. 51, pp. 41987–41996, 2005.

[42] D. Beher, M. Fricker, A. Nadin et al., "In vitro characterization of the presenilin-dependent γ-secretase complex using a novel affinity ligand," *Biochemistry*, vol. 42, no. 27, pp. 8133–8142, 2003.

[43] S. Shah, S. F. Lee, K. Tabuchi et al., "Nicastrin functions as a γ-secretase-substrate receptor," *Cell*, vol. 122, no. 3, pp. 435–447, 2005.

[44] Y. M. Li, M. T. Lai, M. Xu et al., "Presenilin 1 is linked with γ-secretase activity in the detergent solubilized state," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 11, pp. 6138–6143, 2000.

[45] W. T. Kimberly, W. P. Esler, W. Ye et al., "Notch and the amyloid precursor protein are cleaved by similar γ-secretase(s)," *Biochemistry*, vol. 42, no. 1, pp. 137–144, 2003.

[46] G. Tian, S. V. Ghanekar, D. Aharony et al., "The mechanism of γ-secretase: multiple inhibitor binding sites for transition state analogs and small molecule inhibitors," *Journal of Biological Chemistry*, vol. 278, no. 31, pp. 28968–28975, 2003.

[47] T. Ogura, K. Mio, I. Hayashi et al., "Three-dimensional structure of the ^{3}-secretase complex," *Biochemical and Biophysical Research Communications*, vol. 343, no. 2, pp. 525–534, 2006.

[48] T. Wakabayashi, K. Craessaerts, L. Bammens et al., "Analysis of the γ-secretase interactome and validation of its association with tetraspanin-enriched microdomains," *Nature Cell Biology*, vol. 11, no. 11, pp. 1340–1346, 2009.

[49] G. He, W. Luo, P. Li et al., "γ-secretase activating protein is a therapeutic target for Alzheimer's disease," *Nature*, vol. 467, no. 7311, pp. 95–98, 2010.

[50] V. K. Lazarov, P. C. Fraering, W. Ye, M. S. Wolfe, D. J. Selkoe, and H. Li, "Electron microscopic structure of purified, active γ-secretase reveals an aqueous intramembrane chamber and two pores," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 18, pp. 6889–6894, 2006.

[51] J. L. Macdonald and L. J. Pike, "A simplified method for the preparation of detergent-free lipid rafts," *Journal of Lipid Research*, vol. 46, no. 5, pp. 1061–1067, 2005.

[52] S. Wahrle, P. Das, A. C. Nyborg et al., "Cholesterol-dependent γ-secretase activity in buoyant cholesterol-rich membrane microdomains," *Neurobiology of Disease*, vol. 9, no. 1, pp. 11–23, 2002.

[53] Y. Urano, I. Hayashi, N. Isoo et al., "Association of active γ-secretase complex with lipid rafts," *Journal of Lipid Research*, vol. 46, no. 5, pp. 904–912, 2005.

[54] A. Arnold, T. Labrot, R. Oda, and E. J. Dufourc, "Cation modulation of bicelle size and magnetic alignment as revealed by solid-state NMR and electron microscopy," *Biophysical Journal*, vol. 83, no. 5, pp. 2667–2680, 2002.

[55] D. H. Small, R. Gasperini, A. J. Vincent, A. C. Hung, and L. Foa, "The role of Aβ-induced calcium dysregulation in the pathogenesis of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 16, no. 2, pp. 225–233, 2009.