Synaptecx-227 in the treatment of Alzheimer's disease

Dr. Asha Amritraj. Ph.D.

Alzheimer's disease (AD) is the most common cause of dementia, responsible for 100,000 deaths annually in North America alone, making it the seventh leading cause of death. AD is a progressive neurodegenerative disease that manifests as mild cognitive, language and behavioral deficits which gradually worsens in severity and eventually leads to dementia. The progression of AD has no remission, nor are there any disease-stabilizing drugs currently available. The disease onset is inevitably followed by increasing mental and physical incapacitation, loss of independent living, institutionalization and death. In addition to the enormous emotional burden suffered by people with AD and their families, there exists an estimated financial burden of $172 billion per year in the U.S. alone (Alzheimer's Disease Facts and Figures 2010, www.alz.org).

AD is characterized by two hallmarks first identified by Alois Alzheimer in 1907: extracellular protein deposits known as 'amyloid' plaques and intracellular protein aggregates known as neurofibrillary tangles (NFTs) (Glenner and Wong, 1984; Wisniewski et al., 1989). These accumulated protein deposits results in neuronal loss leading to cognitive deficits observed in AD. The amyloid plaques are primarily composed of a protein called amyloid-β, or Aβ, while the tangles are composed of hyperphosphorylated form of tau protein. AD plaques are spherical structures of Aβ, 15 to 20 µM in diameter, surrounded by a peripheral rim of dystrophic neuritis and glial cells (Glenner and Wong, 1984; Masters et al., 1985).

Aβ is proteolytically derived from a larger integral membrane protein called the amyloid precursor protein (APP). APP is a type I membrane glycoprotein containing the Aβ region. APP has a 28 amino acid ectodomain and 12 to 14 amino acid transmembrane domain (Selkoe, 2001; Marks and Berg, 2003). APP can be processed by at least three secretases: α-, β-, and γ-secretases. The site of cleavage of each of these enzymes is shown in (Fig. 1). In the nonamyloidogenic pathway, α-secretase cleaves the APP within the Aβ domain. The cleavage within the Aβ domain prevents deposition of the intact amyloidogenic peptide. α-secretase activity generates a soluble N-terminal fragment of APP known as sAPP α, and its C-terminal counterpart remains embedded in the membrane. The 10- to 11-kd C-terminal product may undergo an additional cleavage by γ-secretase. This process leads to the formation of p3 and its complementary product AICD (Fig. 1). In the amyloidogenic pathway, the protease termed β-secretase initiates Aβ generation by cleaving APP, resulting in an approximately 12-kd membrane retained C-terminal fragment with the intact Aβ residues at its N-terminus. This results in the secretion of a truncated soluble APP molecule, called sAPPβ. The 12-kd fragment may then undergo γ-secretase cleavage within the hydrophobic transmembrane domain to release the 38 to 43 residue Aβ peptides. The varying C-terminal of Aβ may be a feature of crucial importance because Aβ peptides display distinct physical properties and, in particular, exhibit aggregation behavior that can vary according to

their length (Sisodia et al., 1999; Vassar, 2004; Cole and Vasser, 2007).

β-secretase is an aspartyl proteases (BACE1, also named Asp2 or memapsin2) (Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Hussain et al., 1999; Lin et al., 2000) whereas γ-secretase is a high-molecular-weight complex that cleaves its substrates within the lipophilic environment of the transmembrane domain (Wolfe et al., 1999; Haass and Steiner, 2002). Being the key enzymes in the production of Aβ, the therapeutic inhibition of either β-secretase or γ-secretases would essentially decrease the production of all forms of Aβ, including the pathogenic $A\beta_{42}$. But this strategy has not been very successful so far as these enzymes play important role in the brain.

β-secretase participates in the proteolytic processing of neurolegulin 1 and, also acts as a ligand for members of the ErbB family of receptor-tyrosine kinases (Hu et al., 2006; Willem et al., 2006). This signaling pathway has numerous roles in the central nervous system (CNS), including synapse formation, plasticity, neuronal migration, myelination of central and peripheral axons, and regulation of neurotransmitter expression and function (Falls, 2003; Michailov et al., 2004). Similarly the protease, γ-secretases has several substrates like APLP 1 and 2, Notch 1-4, Notch ligands, p75 (NTR), ErbB4, E- and N-cadherins which are involved in numerous functions in the CNS like neurogenesis, neuritic growth (Sestan et al., 1999) and neural stem cell maintenance (Hitoshi et al., 2002), as well as synaptic plasticity and long-term memory, which have been related to cognitive function (Poirazi and Mel., 2001; Shors et al., 2001). Therefore, complete inhibition of β-secretase and γ-secretase as a therapeutic approach to treat AD might be risky owing to its vast number of substrates; although partial inhibition can prove to be very useful.
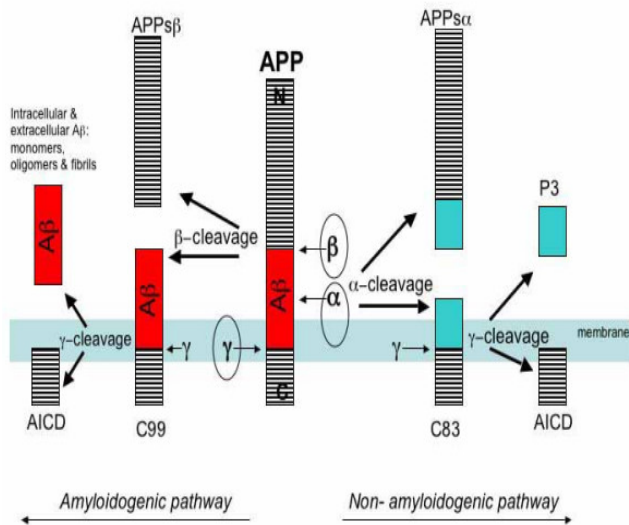
Adapted from Cole and Vasser, 2007

Fig. 1. APP metabolism by the secretase enzymes.

APP is sequentially cleaved by BACE1, the β-secretase, and γ-secretase, a complex comprised of presenilin, nicastin, Aph1 and Pen2, to generate Aβ. BACE1 cleavage of APP is a prerequisite for Aβ formation and is putatively the rate-limiting step in Aβ genesis. BACE1 cleavage of APP forms the N-terminus of the peptide, and two cleavage fragments are liberated: APPsβ, a secreted ectodomain, and C99, a membrane bound fragment. C99 is the substrate for γ-secretase, and C99 cleavage generates the AICD together with the C-terminus of Aβ. Aβ formation is precluded by the activities of α-secretase, which has been identified as TACE, ADAM9 and ADAM10. α-secretase cleaves APP to generate the secreted ectodomain, APPsα and membrane bound fragment, C83. C83 is subsequently cleaved by the γ-secretase complex to yield the 3 KDa fragment, P3 and the AICD.
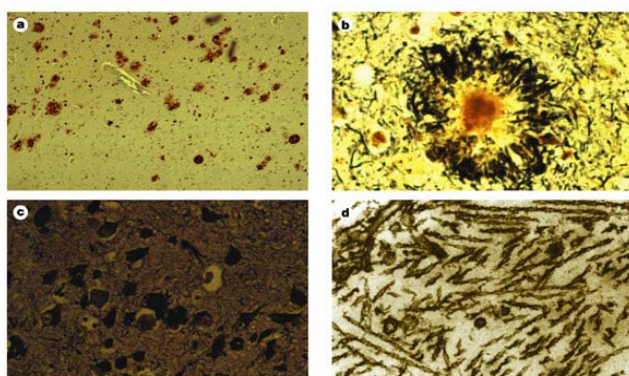
Adapted from Sisodia and St. George-Hyslop, 2002

Fig. 2. Neuropathology of Alzheimer's disease.

(A) Low power amyloid plaques, (B) high power amyloid plaque, (C) neurofibrillary tangles, silver stained, and (D) electron micrograph of neurofibrillary tangles composed of hyperphosphorylated tau (Courtesy of Sisodia and St. George-Hyslop, 2002).

The chief peptide component of the AD plaque, Aβ, has been isolated as a peptide of 40 to 43 amino acids in length (Younkin, 1998; Selkoe, 2001). Brain Aβ has both soluble and insoluble species with aggregation states from monomer to higher molecular-weight oligomers. Soluble brain Aβ is predominately a random coil and an α -helical folded peptide. Insoluble Aβ is β-sheeted and forms either fibrillar or amorphous deposits. These Aβ fibrillar aggregates are thought to act as a nidus for subsequent deposits of other proteins, including α-antichymotrypsin, components of the complement cascade, and Apo E, and Apo J (Cataldo et al., 2004). It is believed that β-sheet formation is the general mechanism of aberrant protein aggregation leading to AD (Walsh et al., 1999). In addition, C-terminal fragments are considered more harmful than N-terminal fragments of Aβ, which may induce the development of dystrophic neurites by a toxic effect rather than by physical injury (Lin et al., 2001; Kasa et al., 2003). Several reports have showed evidence that there could be two distinct pools of Aβ: an extracellular pool which is thought to cause dystrophic neuritis and neuroinflamation and the intracellular pool causing neurotoxicity and cell death especially the cholinergic neurons (Christensen et al., 2008). Recent studies also suggest that soluble Aβ oligomers extracted directly from AD brain impairs synaptic structure and function, and that the Aβ N-terminus is the key sequence causing the cognitive impairment (Cleary et al., 2005; Shankar et al., 2008). Aβ can also stimulate inflammatory responses from microglia that can inhibit neuritic outgrowth, and can activate protein phosphorylation, which is neurotoxic (Selkoe, 2001). In addition, oxidation of Aβ can promote aggregation by peptide cross-linking (Dikalov et al., 2003), a potentially important factor in light of increasing evidence of the involvement of oxidative stress in AD. Exposure of neurons, cell lines, or endothelial cells to high concentration of aggregated Aβ causes cell death, although it has been suggested that low concentrations of Aβ are neurotrophic (Bush et al., 2000). Altogether, it is thought that the deleterious effect of Aβ can cause the pathogenic features that lead to AD. Moreover, Aβ accumulation in the brain during AD could be dependent on the misbalance of production and turnover of this peptide, with neprilysin and insulin degrading enzyme (IDE) known to be important Aβ degrading enzymes in the brain (Eckman and Eckman, 2005).

$Aβ_{42}$ has a greater propensity to aggregate owing to two additional hydrophobic residues (Ile41 and Ala42) at the C-terminus (Jarrett et al., 1993) and its assembly into

fibrils does not occur in a linear fashion; rather, distinct aggregation intermediates or oligomers are formed, which either give rise to fibrils or do not (Wetzel, 2006). The structural form of Aβ is influenced by a variety of intrinsic, as well as extrinsic factors that cause conformational transition of Aβ from a random-coil to the predominantly β-sheet structure. These factors include peptide concentration (Barrow et al., 1992), low pH (Matsunaga et al., 2002; Petkova et al., 2004), metal ions (Drago et al., 2007), high cholesterol (Kakio et al., 2001; Yanagisawa and Matsuzaki, 2002) and pressure (Lin et al., 2002). Moreover, temperature-dependent transition of Aβ40 plays an important role in the structural transformation from α-helix and random-coil to β-sheet form in aqueous solution by heating above 37°C (Gursky and Aleshkov, 2000) or at 45°C (Lin et al., 2003).

Hence, in terms of therapeutic development, drugs targeted towards Aβ aggregation and fibrillization may have the most favorable outcome. Inhibitors aimed at halting aggregation or disrupting preformed aggregates will be very useful in terms of therapeutics. Neurochem's tramiprostate - a small molecule reported to bind to Aβ monomers and maintain it in a non-fibrillar form progressed into Phase III clinical trials, but did not demonstrate sufficient efficacy. Hence, in addition to being highly efficacious, the therapeutic compound must be non-toxic and have 'drug-like' characteristics, i.e. be readily absorbed, able to cross into the brain, and remain in the body for an extended period of time.
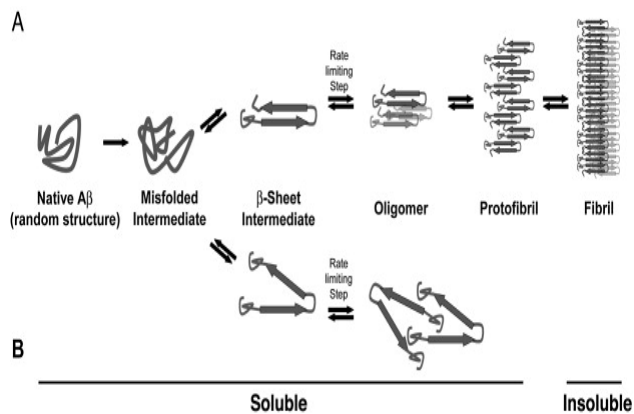
Adapted from DaSilva et al., 2010

Fig. 3. Amyloid-β aggregation

Native Aβ adopts a random-coil structure. Fibrillogenesis is initiated when Aβ undergoes a conformational change to a misfolded intermediate. This intermediate undergoes a conformational change to a β-sheet rich intermediate. (A) This intermediate is unstable, and

aggregates in a rate-limiting step into higher-order oligomers composed of multiple monomer units. Recruitment of additional monomers results in the formation of protofibrils, which assemble further into insoluble fibrils. (B) An alternative pathway of aggregation includes the formation of "off-pathway" oligomers, which are assembly-incompetent, and do not form fibrils.

Although AD had already been described about 100 years ago and despite enormous research efforts, at present only few symptomatic treatment options exist for the more than 25 million patients worldwide. The available drugs used in the treatment of AD mainly aim at increasing the cholinergic activity or decreased *N*-methyl-*D*-aspartate (NMDA) function (using Memantine) of the remaining healthy neurons, but none of them address the underlying cause of the disease. One of the therapeutic approaches developed was vaccination against the N- and C-terminals of Aβ. Passive immunization against the C-terminal increased brain-soluble $Aβ_{42/43}$, decreased insoluble $Aβ_{40}$ and $Aβ_{42/43}$ and reduced plaque formation (Asami-Odaka et al., 2005). However, the appearance of severe side effects during clinical trials has warranted the need for improved safety and efficacy. Moreover, certain anti-inflamatory agents had modest effect in the clinical trials with side effects. While all these factors may be of little help for those where the disease has already struck, they may offer some incentives for those at risk. Nevertheless, it is evident that effective therapeutics is urgently needed, and it is hoped that anti-amyloid strategies will offer a significant step towards a causal therapy.

Results and Discussion

Aβ induced toxicity in mice hippocampal cultured neurons

Mouse hippocampal neurons were treated with Aβ and neuronal viability was assessed using a colorimetric assay that converts MTT [3-(4,5-dimethylthiozolyl)-2,5-diphenyl-tetrazolium bromide] from yellow to a blue formazan crystal by dehydrogenase enzymes in metabolically active cells. Mouse primary hippocampal cultured neurons are vulnerable to $Aβ_{25–35}$ or $Aβ_{1–42}$-mediated toxicity, as evident from a reduction in MTT values. (Fig. 4). As reported earlier a concentration-dependent (0.01–20 μm) effect of $Aβ_{25–35}$ and $Aβ_{1–42}$ over a 24-hr treatment revealed a significant decrease in MTT values from 1 μm onwards, reaching a plateau at 10 μm concentration of $Aβ_{1–42}$ or 20 μm concentration of $Aβ_{25–35}$ (data not shown). Exposure of 10 μm $Aβ_{1–42}$ or 20 μm

Aβ$_{25-35}$ to cultured neurons decreased MTT values in a time-dependent (1–72 hrs) manner, with a significant reduction in cell viability observed at 12 hrs and reaching maximal effect (50% decrease) at 2 days post-treatment (Fig. 4).

20μM Aβ(25- 35)

10μM Aβ(1- 42)
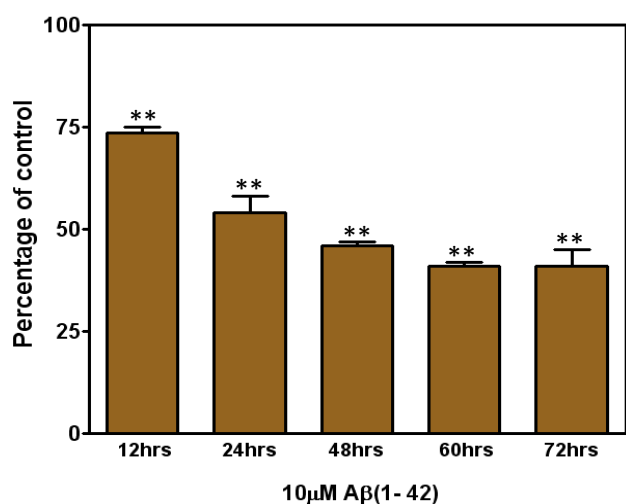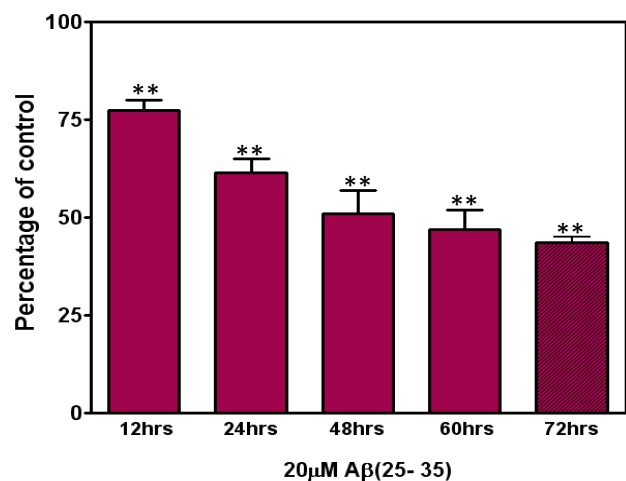
Fig. 4. Aβ-induced toxicity in mouse primary hippocampal cultured neurons.

Histograms show the time course effect of Aβ$_{25-35}$ and Aβ$_{1-42}$ on viability of hippocampal cultured neurons as revealed by (A and B) MTT reduction. Both the forms of Aβ causes significant toxicity to the hippocampal cultured neurons. Data are presented as % of control (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001.
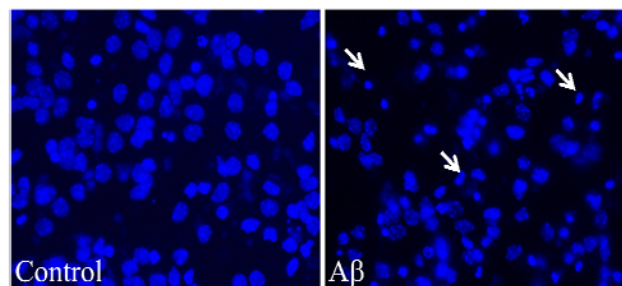
Fig. 5. Aβ-induced neuronal apoptosis in mouse primary hippocampal cultured neurons.

Effects of Aβ treatment on neuronal apoptosis in mouse primary hippocampal cultured neurons as evident by Hoechst 33258 labeling. Representative pictomicrographs show a relative increase in Hoechst 33258 labeled apoptotic nuclei following treatment with 10 μm Aβ$_{1-42}$ for 48 hrs. Each experiment was performed in triplicates and these pictures are representative images of the results obtained.

A range of in vitro studies have shown that Aβ peptides can induce toxicity by triggering intracellular apoptotic cascade in a variety of cell lines and primary mouse and human cultured neurons. The toxic potency of the peptide is related to its ability to form specific oligomeric forms and/or insoluble aggregates which depend to some extent on the concentration of Aβ peptide used (Ribe et al., 2004; Smith et al., 2006). In a parallel series of experiments, neuronal apoptosis was assessed by using the nuclear marker Hoechst 33258 as described earlier (Song et al., 2008). The Aβ toxicity on hippocampal cultured neurons was supported by an increase in the number of Hoechst 33258-positive apoptotic condensed nuclei (Fig. 5). Our results show that Aβ$_{25-35}$ and Aβ$_{1-42}$ can induced significant toxicity in mice hippocampal cultured neurons in a dose and time dependent manner.

Synaptecx-227 and Aβ$_{25-35}$-induced toxicity

To determine the potential neuroprotective role of Synaptecx-227, hippocampal cultured neurons were treated with various concentrations of Synaptecx-227 (1 : 2500, 1:5000) with 20 μm Aβ$_{25-35}$, and then cell viability was assessed using MTT or live/dead assay. Our MTT results, show that Synaptecx-227 (1:2500) can significantly protect cultured neurons against Aβ-induced toxicity. When compared to the untreated control, the viability of cells treated with Synaptecx-227 was increased by 25% and 26% for 24 and 48 hrs respectively (Fig. 6). Neuronal viability was also assessed using Live/Dead assay kit containing calcein AM and ethidium homodimer (EthD-1) as the fluorescent probes. Calcein AM is a cell-permeant dye that fluoresces in live cells with a functional intracellular esterase, whereas EthD-1 is a membrane-

impermeable DNA-binding dye that is excluded from live cells with an intact plasma membrane. Our experimental data shows increased EthD-1 positive dead cells in the Aβ treated samples (red). While the samples that were treated with Syneptecx-227 show relatively lesser number of EthD-1 positive dead cells (Fig. 7). As the effect of Synaptecx-227 is more pronounced at (1:2500) dilution, this concentrations was selected for the subsequent experiments. The results obtained from the above experiment indicates that Synaptecx-227 (1:2500) can considerably improve survival of neurons treated with Aβ.
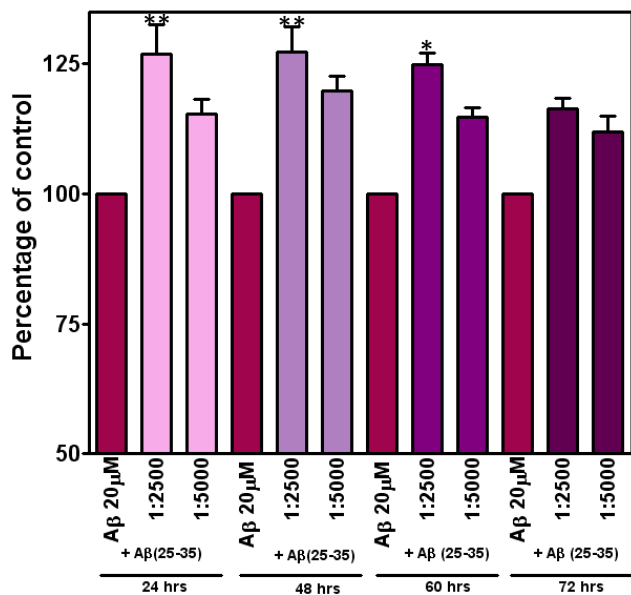

Fig. 6. Effects of Synaptecx-227 on Aβ$_{25-35}$ -induced toxicity.

Mice hippocampal cultured neurons were treated with 20 μm Aβ$_{25-35}$ in the presence or absence of different concentrations of Synaptecx-227 and cellular viability was assessed by MTT reduction. Synaptecx-227 offers significant protection to the hippocampal cultured neurons treated with Aβ from 24 hrs. Data are presented as % of control (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001.

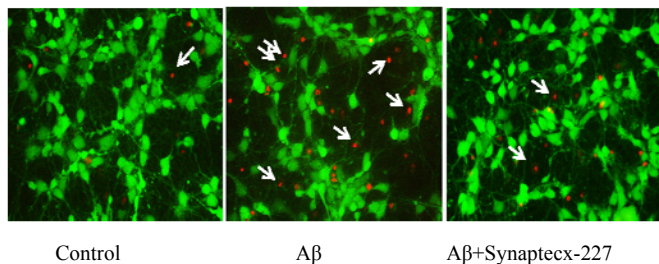**Fig. 7. Effects of Synaptecx-227 on Aβ$_{25-35}$ -induced toxicity.**

Mice hippocampal cultured neurons were treated with 20 μm Aβ$_{25-35}$ in the presence or absence of Synaptecx-227 (1:2500) and cellular viability was assessed by Live/dead assay. Note that treatment of hippocampal cultures with Synaptecx-227 (1:2500) can significantly protect the neurons against Aβ-mediated toxicity as evident by decreased number of EthD-1 positive neurons. Each experiment was performed in triplicates and these pictures are representative images of the results.

Synaptecx-227 post-treatment and Aβ$_{25-35}$-induced toxicity

There are no effective treatment available in the market for a pre-existing diagnosed case of Alzheimer's disease and most of the available drugs are targeted towards alleviating the symptoms. To evaluate if Synaptecx-227 is effective for the treatment of pre-diagnosed AD conditions, Synaptecx-227 was treated to the cells pre-insulted with Aβ for 24 hrs. Synaptecx-227 was added to cultured hippocampal neurons after 24 hrs of exposure to 20μm Aβ$_{25-35}$ and the viability of neurons were measured using MTT. Synaptecx-227 mediated minimal cell survival at 60 hrs but at 72 hrs, it was able to significantly protect the cultured hippocampal neurons pretreated with Aβ (Fig. 7). As evident by our results, Synaptecx-227 (1:2500) rescued the cultured hippocampal neurons even after 24hrs of Aβ pre-treatment.

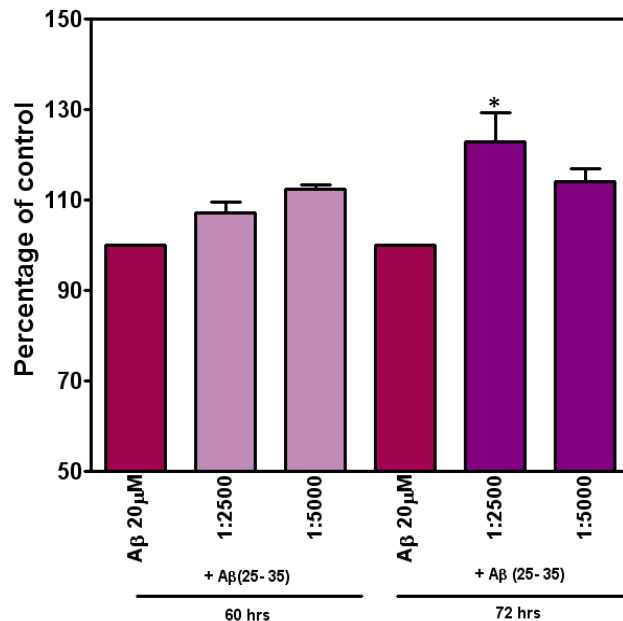**Fig. 8. Effects of post treatment of Synaptecx-227 on Aβ$_{25-35}$ -induced toxicity.**

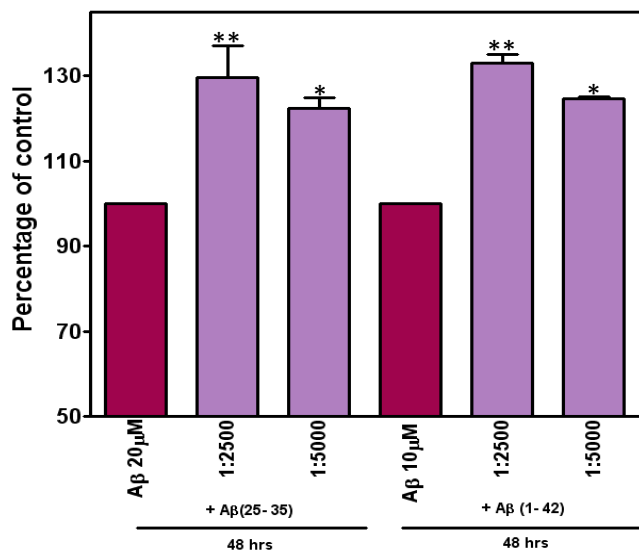Mice hippocampal cultured neurons were treated with different concentrations of Synaptecx-227 after 24hrs of pre-treatment with 20 μm Aβ$_{25-35}$ and cellular viability was assessed by MTT reduction. Synaptecx-227 offers significant protection to the Aβ pretreated hippocampal

cultured neurons. Data are presented as % of control (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001.

Synaptecx-227 and Aβ$_{25-35}$/ Aβ$_{1-42}$ induced toxicity

As most of our experiments were performed using Aβ$_{25-35}$, we validated the potency of Synaptecx-227 using Aβ$_{1-42}$ which is the most toxic Aβ isoform. Hippocampal cultured neurons were treated with the most potent concentrations of Synaptecx-227 (1 : 2500, 1:5000) with 20 μm Aβ$_{25-35}$, or 10 μm Aβ$_{1-42}$ and then cell viability was assessed using MTT. Our data reveal that even though the Aβ$_{1-42}$ is more toxic when compared to Aβ$_{25-35}$, the protective effect offered by Synaptecx-227 was comparable (Fig. 9). This could be due to the usage of twice the concentration of Aβ$_{25-35}$ in this study to match the toxicity offered by 10 μm Aβ$_{1-42}$.

Fig. 9. Effects of Synaptecx-227 on Aβ$_{25-35}$ and Aβ$_{1-42}$ - induced toxicity.

Mice hippocampal cultured neurons were treated with 20 μm Aβ$_{25-35}$ and 10 μm Aβ$_{1-42}$ in the presence or absence of Synaptecx-227 and cellular viability was assessed by MTT reduction. Synaptecx-227 offers significant protection to both Aβ$_{25-35}$ and Aβ$_{1-42}$ treated hippocampal cultured neurons. Data are presented as % of control (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001.

Synaptecx-227 and Aβ$_{1-42}$ fibrils

Aβ peptides are prone to self-aggregation, in vivo and in vitro (Wolfe et al., 1999; Herreman et al., 2000). Mature amyloid fibrils are filamentous with a cross β-sheet structure. Aggregation of physiologically produced soluble

Aβ to insoluble, neurotoxic fibrils is a crucial step in the pathogenesis of Alzheimer's disease. As Aβ aggregates impair neuronal viability and function (De Strooper et al., 1998), drugs that inhibit the aggregation process may serve as effective therapeutics.

We evaluated the effect of Synaptecx-227 on Aβ oligomerization by the well-known thioflavin T assay. The fluorescence of thioflavin T (ThT) at 490 nm is enhanced dramatically in the presence of Aβ aggregates. We aggregated the 10 μM concentration of Aβ$_{1-42}$ at 37^{0}C with or without Synaptecx-227 (1:2500). The samples were incubated for different time intervals (0, 12, 24, 48, 60 or 72 hrs) and the Aβ aggregation was measured using the ThT assay. Our results indicate that the aggregation of Aβ$_{1-42}$ increased exponentially with time. Whereas in the samples that were incubated with Synaptecx-227 (65-70% decrease) showed a drastic decrease in the ThT florescence and their florescence did not increase with increasing incubation time unlike the Aβ samples (Fig. 10). Samples treated with Synaptecx-227 had lower ThT florescence indicating higher potency of this compound to inhibit or decrease the Aβ$_{1-42}$ fibril formation.
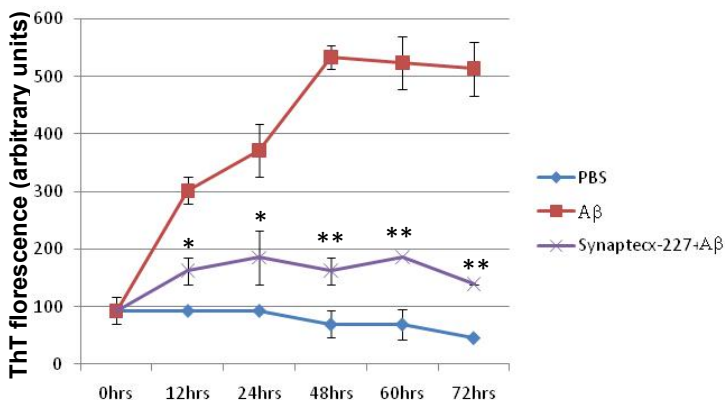

Fig. 10. Effects of Synaptecx-227 and both together on Aβ$_{1-42}$ – aggregation.

Aβ$_{1-42}$ (10 μm) was incubated with or without Synaptecx-227 at 37^{0}C for various time periods and the Aβ aggregation was evaluated as an increase in thioflavin T fluorescence. ThT fluorescence was dramatically decreased in the Aβ$_{1-42}$ samples treated with Synaptecx-227. Data are presented as arbitrary units (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001

In order to further understand if Synaptecx-227 was capable of preventing the progression of fibril formation or efficient in breaking the preformed fibrils, Synaptecx-227 was incubated with Aβ$_{1-42}$ fibrils that was pre-aggregated for 46.5 hrs at 37^{0}C. The potent concentrations of

Synaptecx-227 (1:2500) was added to the preformed fibrils and the amount of ThT florescence was measured. Our results indicate that after 1.5 hr of incubation with Synaptecx-227, the amount of ThT fluorescence dramatically decreased in the $A\beta_{1-42}$ pre-aggregated samples (Fig. 11), which would suggest that this compound is efficient in breaking the preformed $A\beta_{1-42}$ fibrils seen during the later stages of Alzheimer's disease. In addition, Synaptecx-227 prevented the further fibril formation. In all our aggregation experiments, the Synaptecx-227 reduced 65-70% of preformed fibrils as evidenced by the reduction in the ThT florescence.
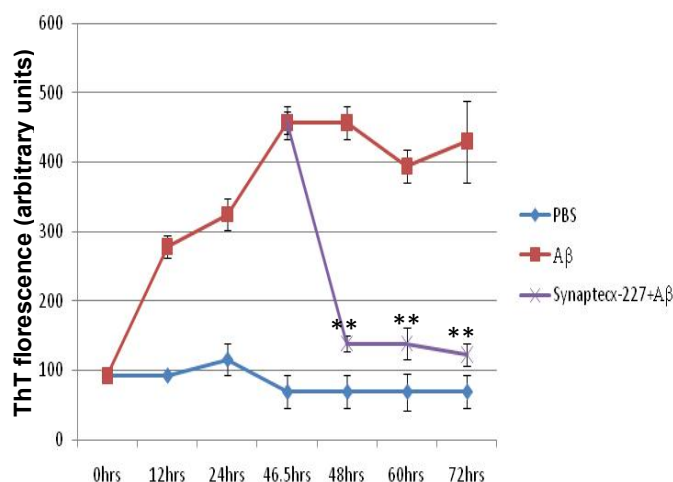

Fig. 11. Effects of Synaptecx-227 on pre-aggregated $A\beta_{1-42}$.

$A\beta_{1-42}$ (10 μm) was pre-aggregated for 24 hrs and were then treated with or without Synaptecx-227 for various time periods and $A\beta$ aggregation was measured as an increase in thioflavin T fluorescence. ThT fluorescence was dramatically decreased in the pre-aggregated $A\beta_{1-42}$ samples treated with Synaptecx-227. Data are presented as arbitrary units (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001

Electron microscopy: Synaptecx-227 and $A\beta_{1-42}$ fibrils

In order to validate the ThT assay data, we performed Electron Microscopy (EM) on the *in vitro* $A\beta_{1-42}$ samples. $A\beta_{1-42}$ samples were incubated alone or with Synaptecx-227 (1:2500) and samples were visualized using electron microscopy. The EM data shows that $A\beta_{1-42}$ forms strong fibrils after 48 hrs of incubation at 37^0C (Fig. 12: A). Whereas the $A\beta_{1-42}$ samples that were incubated with Synaptecx-227, showed a significant decrease in $A\beta_{1-42}$ fibril like structures (Fig. 12: B). In addition, the $A\beta_{1-42}$ samples that were incubated for 5 days showed very dense $A\beta_{1-42}$ fibrils. And when the $A\beta_{1-42}$ samples were co-incubated with Synaptecx-227 for 5 days, there was a significant decrease in $A\beta_{1-42}$ fibril like structures (Fig. 12: C, D). Similar results were also obtained for pre-aggregated $A\beta_{1-42}$ samples that were incubated with synaptecx-227 (data not shown). Our results noticeably indicate that Synaptecx-227 is very efficient in clearing the $A\beta_{1-42}$ fibrils, further confirming the ThT assay data.
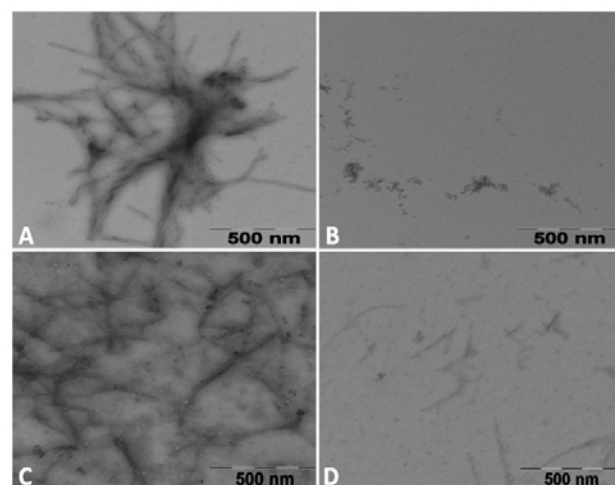
**Fig. 12. Effects of Synaptecx-227 on $A\beta_{1-42}$-aggregation.**

Electron microscopy images of 10 μm $A\beta_{1-42}$ treated with or without Synaptecx-227 at 37^0C for various time periods. Representative pictomicrographs indicate that Synaptecx-227 is very efficient in reducing the $A\beta$ fibril formation. Each experiment was performed in triplicates and these pictures are representative images of the results.

Synaptecx-227 and γ-secretase activity

Presenilin proteins and APP are frequently mutated in familial Alzheimer's disease (AD), and presenilin controls the γ-secretase-mediated processing of APP. The level of γ-secretase activity controls the amount of $A\beta$ formation from the APP intermediate fragment C99 (see Fig.1). Familial AD-associated mutations in presenilin lead to a specific increase in production of more fibril-prone Aβ42 variant (Hardy, 1997), the peptide species linked to the early pathological changes seen in AD (Iwatsubo et al., 1994; Näslund et al., 2000). Hence, it is of interest to develop pharmaceutical approaches that modulate the level of γ-secretase activity acting on APP. In this study we measured the γ-secretase activity using the Yeast-based β-galactosidases reporter assay for monitoring γ-secretase cleavage on APP.

Our Yeast-based reporter system has been validated using the known Nonsteroidal anti-inflammatory drugs (NSAID's) that are known to alter the γ-secretase activity.

As reported earlier, NSAID, Sulindac Sulphide does inhibit the γ-secretase activity whereas Naproxen does not affect the γ-secretase activity (Takahashi et al., 2003) (Fig. 13). Moreover, the effective concentration of Synaptecx-227 (1:2500) in the cultured hippocampal neurons caused a decrease in the γ-secretase activity as measured by β-galactosidases activity, but these values did not reach significance (Fig. 14). To optimize the concentration of Synaptecx-227 in yeast system we used lower as well as higher concentrations of the compound. Interestingly, four times lower concentration of Synaptecx-227 was effective in lowering the γ-secretase activity significantly (Fig. 15).
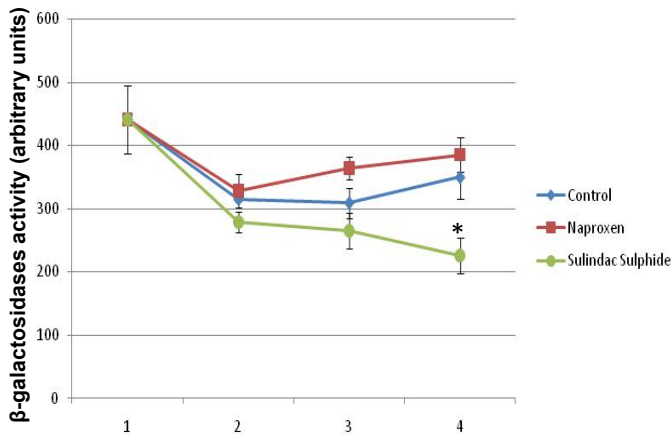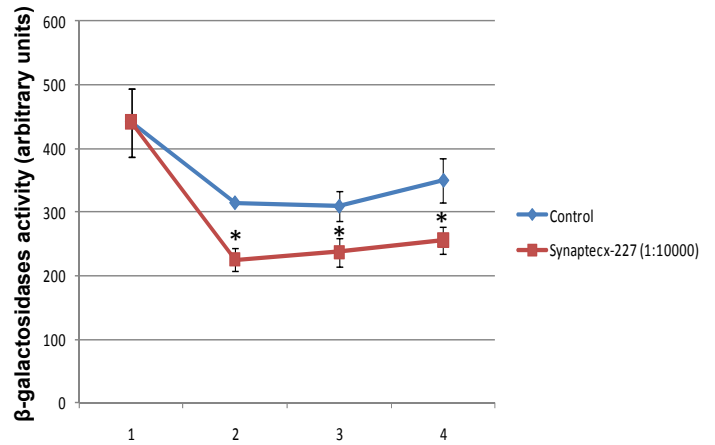

Fig. 13. Effects of known NSAID's on γ-secretase activity.

Yeast cells overexpressing the intact γ-secretase components and APP were treated with known NSAID's that do (sulindac sulphide) or don't (Naproxen) inhibit γ-secretase activity. These data indicate that this Yeast expression system could be used to assess the affect of Synaptecx-227 on γ-secretase activity. Data are presented as arbitrary units (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001
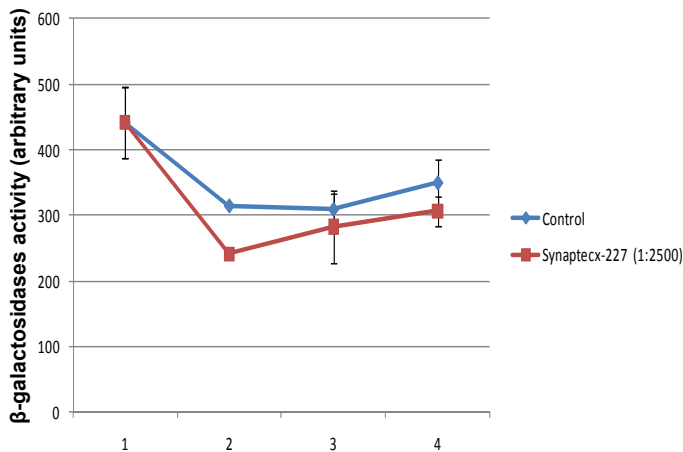
Fig. 14. Effects of Synaptecx-227 on γ-secretase activity.

Yeast cells overexpressing the intact γ-secretase components and APP were treated with Synaptecx-227 and the γ-secretase activity was measured with the increase in β-galactosidases activity. The results indicate that Synaptecx-227 can reduce the γ-secretase activity. Data are presented as arbitrary units (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05

Fig. 15. Effects of lower concentrations of Synaptecx-227 on γ-secretase activity.

Yeast cells overexpressing the intact γ-secretase components and APP were treated with Synaptecx-227 and the γ-secretase activity was measured as the increase in β-galactosidases activity. The results indicate that Synaptecx-227 can significantly reduce the γ-secretase activity. Data are presented as arbitrary units (means ± SEM) and were obtained from three to five separate experiments, each performed in triplicate. *P < 0.05 and **P < 0.001

The results indicate that, Synaptecx-227 exhibits a 25-35% inhibitory effect on the β-galactosidases reporter output. It is known that depression of Aβ output by only 12% reduces amyloid plaque burden by 50% and delays the synaptic deficits in transgenic mice (McConlogue et al., 2007).

Conclusion

Given the increased incidence of AD with age, and the aging of our population, estimates suggest that by the year 2050, there will be 11-16 million cases of AD in the United States, unless medical breakthroughs identify ways to prevent or more effectively treat the disease (Alzheimer's Disease Facts and Figures 2010, www.alz.org).

Data obtained so far indicate that Synaptecx-227

- is not toxic to cultured hippocampal neurons

- can protect 26 to 30% of cultured hippocampal neurons against Aβ induced toxicity
- is also able to protect 20% of the neurons pre-insulted with Aβ
- can inhibit 70-75% of Aβ aggregation
- can also induce the disaggregation of the preformed Aβ fibrils by 65-70%
- can reduce 25-35% of γ-secretase activity

Synaptecx-227 could be a complete package for the treatment of AD encompassing Aβ disaggregation, decreased Aβ production, increased neuronal survival and is exceptionally promising as it can not only limit progression of existing pathology but can also be used in the treatment of pre-existing conditions.

Reference

A. Asami-Odaka, Y. Obayashi-Adachi, Y. Matsumoto, H. Takahashi, H. Fukumoto, T. Horiguchi T. (2005). Passive immunization of the Aβ42(43) C-terminal-specific antibody BC05 in a mouse model of Alzheimer's disease. Neurodegener Dis 2: 36–43.

A. Herreman, L. Serneels, W. Annaert, D. Collen, L. Schoonjans, De Strooper B.(2000) Total inactivation of gamma-secretase activity in presenilin-deficient embryonic stem cells. Nat Cell Biol. 2: 461-462.

A. M. Cataldo, S. Petanceska, N. B. Terio, C. M. Peterhoff, R. Durham, M. Mercken, P. D. Mehta, J. Buxbaum, V. Haroutunian and R.A. Nixon. (2004) Abeta localization in abnormal endosomes: association with earliest Abeta elevations in AD and Down syndrome, Neurobiol. Aging 1263–1272.

A. Kakio, S. L. Nishimoto, K. Yanagisawa, Y. Kozutsumi, K. Matsuzaki (2001). Cholesterol-dependent formation of GM1 ganglioside-bound amyloid beta-protein, an endogenous seed for Alzheimer amyloid. J Biol Chem 276: 24985–24990.

A. I. Bush, C.S. Atwood, L.E. Goldstein, X. Huang, J. Rogers. Could Abeta and AbetaPP be Antioxidants? J Alzheimers Dis.2:83-84.

A. T. Petkova, G. Buntkowsky, F. Dyda, R. D. Leapman, W. M. Yau , R. Tycko (2004). Solid state NMR reveals a pH-dependent antiparallel beta-sheet registry in fibrils formed by a beta-amyloid peptide. J Mol Biol 335: 247–260.

B. De Strooper, P. Saftig, K. Craessaerts, H. Vanderstichele, G. Guhde, W. Annaert, K. Von Figura and F. Van Leuven. (1998) Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein, Nature 391: 387–390.

C. Haass, H. Steiner. (2002) Alzheimer disease gamma-secretase: a complex story of GxGD-type presenilin proteases. Trends Cell Biol. 12:556-62.

C. J. Barrow, A. Yasuda, P. T. Kenny, M. G. Zagorski (1992). Solution conformations and aggregational properties of synthetic amyloid beta peptides of Alzheimer's disease: analysis of circular dichroism spectra. J Mol Biol 225: 1075–1093.

D. Drago, M. Folin, S. Baiguera, G. Tognon, F. Ricchelli, P. Zatta (2007). Comparative effects of Abeta(1–42)-Al complex from rat and human amyloid on rat endothelial cell cultures. J Alzheimers Dis 11: 33–44.

D. J. Falls. (2003) Neuregulins: functions, forms, and signaling strategies. Exp Cell Res. 284:14-30.

D. J. Selkoe. (1986) Altered structural proteins in plaques and tangles: what do they tell us about the biology of Alzheimer's disease? Neurobiol. Aging 7:425–432.

D. J. Selkoe. (2001) Alzheimer's disease: genes, proteins, and therapy. Physiol. Rev. 81:741–766.

D .Z. Christensen, T. A. Bayer, O. Wirths. (2010) Intracellular Abeta triggers neuron loss in the cholinergic system of the APP/PS1KI mouse model of Alzheimer's disease. Neurobiol Aging.31: 1153-1163.

D. M. Walsh, D. M. Hartley, Y. Kusumoto, Y. Fezoui, M. M. Condron, A. Lomakin, G. B.Benedek, D. J. Selkoe, D. B. Teplow. (1999) Amyloid β-peptide fibrillogenesis. Structure and biological activity of protofibrillar intermediates. J Biol Chem 274: 25945–25952.

E. A. Eckman, C. B. Eckman.(2005) Abeta-degrading enzymes: modulators of Alzheimer's disease pathogenesis and targets for therapeutic intervention. Biochem Soc Trans.33: 1101-1105

E. M. Ribe, M. Perez, B. Puig, I. Gich, F. Lim, M. Cuadrado, T. Sesma, S. Catena, B. Sanchez, M. Nieto, P. Gomez-Ramos, M. A. Moran, F. Cabodevilla, L. Samaranch, L. Ortiz, A. Perez, R. A. Fuentealba, G. Farias, J. Scheu, M. Bronfman, M. P. Marzolo and N. C. Inestrosa, (2004) Signal transduction during amyloid-beta-peptide neurotoxicity: role in Alzheimer disease, Brain Res. Rev. 47: 275–289.

G. G. Glenner. (1989) The pathobiology of Alzheimer's disease. Annu. Rev. Med. 40: 45–51.

G. G. Glenner and C. W. Wong, (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein, Biochem. Biophys. Res. Commun. 120: 885–890.

G. V. Michailov, M. W. Sereda, B. G. Brinkmann, T. M. Fischer, B. Haug, C. Birchmeier, L. Role, C. Lai, M. H. Schwab, K. A. Nave. (2004) Axonal neuregulin-1 regulates myelin sheath thickness. Science. 304:700-703.

G. M. Shankar, S. Li, T. H. Mehta, A. Garcia, N. E. Hepardson, I. Smith F.M Brett, M.A. Farrell, M.J. Rowan, C.A. Lemere, C.M. Regan, D.M. Walsh, B.L. Sabatini, D.J. Selkoe.. (2008). Amyloid-β protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. Nat Med 14: 837–842.

H. Lin, R. Bhatia, R. Lal (2001). Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology. FASEB J 15: 2433–2444.

I. Hussain, D. Powell, D. R. Howlett, D. G. Tew, T. D. Meek, C. Chapman, I. S. Gloger, K. E. Murphy, C. D. Southan, D. M. Ryan, T. S. Smith, D. L. Simmons, F. S. Walsh, C. Dingwall, G. Christie. (1999) Localization of β-Secretase Memapsin 2 in the Brain of Alzheimer's Patients and Normal Aged Controls Mol. Cell. Neurosci.14: 419.

J. T. Jarrett, E. P. Berger and P. T. Lansbury. (1993) The carboxy terminus of the beta amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease, Biochemistry 32; 4693–4697.

J. Hardy. (1997) Amyloid, the presenilins and Alzheimer's disease. Trends Neurosci. 20:154-159.

K. Yanagisawa, K. Matsuzaki (2002). Cholesterol-dependent aggregation of amyloid beta-protein. Ann N Y Acad Sci 977: 384–986.

K. A. DaSilvaa, J. E. Shawa and J. McLaurin. (2010) Amyloid-β fibrillogenesis: Structural insight and therapeutic intervention.Exp Neurol. 223: 311-21.

K. M. Wisniewski., J.Wegiel, K.C. Wang, M.Kujawa, B. Lach. (1989) Ultrastructural studies of the cells forming amyloid fibers in classical plaques. Can. J. Neurol. Sci. 16: 535–542.

L. Masters, G. Multhaup, G. Simms, J. Pottigiesser, R.N. Martins and K. Beyreuther. (1985) Neuronal origin of a cerebral amyloid: neurofibrillary tangles of Alzheimer's disease contain the same protein as the amyloid of plaque cores and blood vessels, EMBO J. 4: 2757–2763.

L. McConlogue, M. Buttini, J.P. Anderson, E.F. Brigham, K.S. Chen, S.B. Freedman, D. Games, K. Johnson-Wood, M. Lee, M. Zeller, W. Liu, R. Motter, S. Sinha. (2007) Partial reduction of BACE1 has dramatic effects on Alzheimer plaque and synaptic pathology in APP Transgenic Mice. J Biol Chem. 282: 26326-2634.

M. S. Wolfe, W. Xia, B. L. Ostaszewski, T. S. Diehl, W. T. Kimberly, D. J. Selkoe. (1999) Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature. 398: 513-517.

M. Willem, A. N. Garratt, B. Novak, M. Citron, S. Kaufmann, A. Rittger, B. DeStrooper, P. Saftig, C. Birchmeier, C. Haass. (2006) Control of peripheral nerve myelination by the beta-secretase BACE1. Science. 314:664-666.

M. S. Song, G. Rauw, G. B. Baker, S. Kar. (2008) Memantine protects rat cortical cultured neurons against beta-amyloid-induced toxicity by attenuating tau phosphorylation.Eur J Neurosci.28:1989-2002.

N. Marks and M. J. Berg, (2003) APP processing enzymes (secretases) as therapeutic targets: insights from the use of transgenics (Tgs) and transfected cells. Neurochem. Res. 28: 1049–1062.

N. Sestan, S. Artavanis-Tsakonas and P. Rakic. (1999) Contact-dependent inhibition of cortical neurite growth mediated by notch signaling, Science 286: 741–746.

O. Gursky, S. Aleshkov (2000). Temperature-dependent beta-sheet formation in beta-amyloid abeta(1–40) peptide in water: uncoupling beta-structure folding from aggregation. Biochim Biophys Acta 1476: 93–102.

P. Poirazi and B. W. Mel. (2001) Impact of active dendrites and structural plasticity on the memory capacity of neural tissue. Neuron 29: 779–796.

P. Kasa, H. Papp, J. Zombori, P. Mayer, F. Checler (2003). C-terminal fragments of amyloid-β peptide cause cholinergic axonal degeneration by a toxic effect rather than by physical injury in the nondemented human brain. Neurochem Res 28: 493–498.

R. Vassar. (2004) BACE1: the beta-secretase enzyme in Alzheimer's disease, J. Mol. Neurosci. 23: 105–114.

R. Vassar, B.D. Bennett, S. Babu-Khan, S. Kahn, E.A. Mendiaz, P. Denis, D.B. Teplow, S. Ross, P. Amarante, R. Loeloff, Y. Luo, S. Fisher, J. Fuller, S. Edenson, J. Lile, M.A. Jarosinski, A.L. Biere, E. Curran, T. Burgess, J. C. Louis, F. Collins, J. Treanor, G. Rogers and M. Citron. (1999) Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE, Science 286: 735–741.

R. Yan, M. J. Bienkowski, M. E. Shuck, H. Miao, M. C. Tory, A. M. Pauley, J. R. Brashier, N. C. Stratman, W. R. Mathews, A. E. Buhl, D. B. Carter, A. G. Tomaselli, L. A. Parodi, R. L. Heinrikson, M. E. Gurney. (1999) Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature, 402: 533-537.

R. Wetzel. (2006) Kinetics and thermodynamics of amyloid fibril assembly, Acc. Chem. Res. 39: 671–679.

S. L. Cole and R. Vassar. (2007) The Alzheimer's disease β-secretase enzyme, BACE1, Molecular Neurodegeneration. 2: 22

S. Hitoshi, T. Alexson, V. Tropepe, D. Donoviel, A. J. Elia, J. S. Nye, R. A. Conlon, T. W. Mak, A. Bernstein and D. van der Kooy. (2002) Notch pathway molecules are essential for the maintenance, but not the generation of mammalian neural stem cells, Genes Dev. 16: 846–858

S. S. Sisodia, P. H. St George-Hyslop. (2002) gamma-Secretase, Notch, Abeta and Alzheimer's disease: where do the presenilins fit in? Nat Rev Neurosci.3: 281-90.

S. Sinha, J. P. Anderson, R. Barbour, G. S. Basi, R. Caccavello, D. Davis, M. Doan, H. F. Dovey, N. Frigon, J. Hong, K. Jacobson-Croak, N. Jewett, P. Keim, J. Knops, I. Lieberburg, M. Power, H. Tan, G. Tatsuno, J. Tung, D. Schenk, P. Seubert, S. M. Suomensaari, S. Wang, D. Walker, J. Zhao, L. McConlogue, V. John. (1999) Purification and cloning of amyloid precursor protein beta-secretase from human brain.Nature 402: 537-540.

S. G. Younkin. (1998) The role of Abeta 42 in Alzheimer's disease. J Physiol 92:289-292.

S. I. Dikalov, M. P. Vitek, R. P. Mason. (2004) Cupric-amyloid beta peptide complex stimulates oxidation of ascorbate and generation of hydroxyl radical. Free Radic Biol Med.36: 340-347.

S. S. Sisodia, S. H. Kim and G. Thinakaran. (1999) Function and dysfunction of the presenilins, Am. J. Hum. Genet. 65: 7–12.

S. Y. Lin, H. L. Chu, Y. S. Wie. (2002) Pressure-induced transformation of alpha-helix to beta-sheet in the secondary structures of amyloid beta (1–40) peptide exacerbated by temperature. J Biomol Struct Dyn 9: 619–625.

S. Y. Lin, H. L. Chu, Y. S. Wie (2003) Secondary conformations and temperature effect on structural transformation of amyloid beta(1–28), (1–40) and (1–42) peptides. J Biomol Struct Dyn 20: 595–601.

J. P. Cleary, D. M. Walsh, J. J. Hofmeister, G. M. Shankar, M. A. Kuskowski, D. Selkoe and K. H. Ashe. (2005). Natural oligomers of the amyloid-β protein specifically disrupt cognitive function. Nat Neurosci 8: 79–84.

J. Näslund, V. Haroutunian, R. Mohs, K.L. Davis, P. Davies, P. Greengard, J.D. Buxbaum. (2000) Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline.JAMA.283: 1571-1577.

T. J. Shors, G. Miesegaes, A. Beylin, M. Zhao, T. Rydel and E. Gould. (2001) Neurogenesis in the adult is involved in the formation of trace memories, Nature 410:372–376.

T. Iwatsubo, A. Odaka, N. Suzuki, H. Mizusawa, N. Nukina, Y. Ihara. (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.

W. E. Klunk , H. Engler , A. Nordberg , B. J. Bacskai , Y. Wang , J. C. Price, M. Bergström , B.T. Hyman , B. Långström , C. A. Mathis. (2003) Imaging the pathology of Alzheimer's disease: amyloid-imaging with positron emission tomography. Neuroimaging Clin N Am. 13:781-789.

W. W. Smith, M. Gorospe and J. W. Kusiak. (2006) Signaling mechanisms underlying Abeta toxicity: potential therapeutic targets for Alzheimer's disease. CNS Neurol. Disord, Drug Targets 5: 355–361.

X. Lin, G. Koelsch, S. Wu, D. Downs, A. Dashti, J. Tang, (2000) Human aspartic protease memapsin 2 cleaves the b-secretase site of b-amyloid precursor protein. Proc. Natl. Acad. Sci. USA 97: 1456-1460.

X. Hu, C. W. Hicks, W. He, P. Wong, W. B. Macklin, B. D. Trapp, R. Yan. (2006) Bace1 modulates myelination in the central and peripheral nervous system. Nat Neurosci. 9:1520-1525.

Y. Matsunaga, N. Saito, A. Fuji, J. Yokotani, T. Takakura, T. Nishimura, H. Esaki, T. Yamada (2002). A pH-dependent conformational transition of Aβ peptide and physicochemical properties of the conformers in the glial cell. Biochem J 361: 547–556.

Y. Takahashi, I. Hayashi, Y. Tominari, K. Rikimaru, Y. Morohashi, T. Kan, H. Natsugari, T. Fukuyama, T. Tomita, T. IwatsuboT. (2003) Sulindac sulfide is a noncompetitive gamma-secretase inhibitor that preferentially reduces Abeta 42 generation. J Biol Chem. 278: 18664-18670.