Synergistic effect of N-terminal pyroglutamyl amyloid β protein in Alzheimer's disease and in normal aging

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Abstract

Amyloid β protein (Aβ) has been considered as the main pathogenetic basis of Alzheimer's disease (AD). Substantial evidence indicates that the soluble Aβ aggregates containing N-terminally truncated Aβ starting with pyroglutamate at position 3 ($A\beta_{PE3}$) and position 11($A\beta_{PE11}$) account for the major neuronal toxicity of AD. In addition to the heterogeneity in soluble Aβ aggregate, the composition ratio of Aβ variants in the brain from AD and in normal aging possess a significant role for the development of AD. For this reason, we postulate that Aβ variants with different composition ratio may cause aggregation behavior entirely different. In this study, two mixtures, AD and NA, composed of three Aβ variants ($A\beta_{1-40}$, $A\beta_{PE3-40}$, $A\beta_{PE11-40}$) with different composition ratio were investigated. Thioflavine T fluorimetric assay revealed that AD mixture with a high $A\beta_{PE3-40}$/$A\beta_{PE11-40}$ composition ratio has highly increased β-sheet structure compared with the three individual Aβ variants. By contrast, NA mixture with a low $A\beta_{PE3-40}$/$A\beta_{PE11-40}$ composition ratio leads to an unobvious increase. This suggests that $A\beta_{PE3-40}$ may have synergistic effect to regulate the aggregation propensities of the Aβ mixtures. Surface plasmon resonance kinetics assay demonstrated that the aggregation rates of the three soluble Aβ variants interacting both AD and NA mixtures have a consistent of order as follows, $A\beta_{PE3-40}$> $A\beta_{PE11-40}$> $A\beta_{1-40}$. Both $A\beta_{PE3-40}$ and $A\beta_{PE11-40}$ have a higher aggregation rate than $A\beta_{1-40}$ to form aggregates. Therefore, the investigated N-terminal pyroglutamyl Aβ variants and their composition ratio in mixtures may play an important role to regulate aggregation behaviors and to influence the development of AD. [Life Science Journal. 2009; 6(3): 80– 85] (ISSN: 1097 – 8135).

Key words: Alzheimer's disease, amyloid, surface plasma resonance, synergistic effect, amyloid β protein (Aβ), amyloid β precursor protein (AβPP), Alzheimer's disease (AD), normal aging (NA), surface plasmon resonance (SPR), thioflavine T (ThT)

1 Introduction

lzheimer's disease (AD), a neurodegenerative disease, is the most common cause of dementia in the elderly population. This widespread progressive neurodegeneration characterized by the presence of proteinaceous deposits in the brain is described as amyloid. The extracellular deposition of amyloid β protein (Aβ) and the intracellular generation of neurofbrillary tangles are the main histopathological features of AD (1,2).

Aβ is a 39- to 43-amino acid polypeptide, and is a normal metabolic product which can be found in cerebrospinal fluid and plasma (3). Aβ is derived from the proteolytic product of amyloid β precursor protein (AβPP) through the cleavage of β-secretase and γ-secretase (4,5). Authentic evidence indicates that several factors can lead to the formation of amyloid plaques in AD (2) including (i) genetic mutations of APP resulting in early-onset familial AD (FAD), and the over expression of APP resulting from elevated gene dosage in trisomy 21 (Down's syndrome), (ii) FAD-causing mutations on chromosome 14 and 1 in genes encoding the homologous presenilin proteins PS1 and PS2, which affect APP processing, (iii) apolipoprotein E4 allele which lower the average age of AD. These factors can result in two predominant aggregates of Aβ including $A\beta_{1-40}$ and $A\beta_{1-42}$ which are the primary component in senile plaques (6,7).

Although previous studies demonstrate fibrillar form of Aβ is inferred as a key role leading to the pathogenesis of AD. Recent data show that the more neurotoxic forms of Aβ are small, still water-soluble oligomers, amyloid-derived diffusible lignds (8) and protofibrils (9) which correspond better than fibrils with neurodegeneration. In addition to A $_{1-40}$ and A $_{1-42}$, N-terminal truncated forms of water soluble Aβ were also seen in A plaques of the brain of AD and Dome syndrome patients. The most common forms of N-terminally truncated A is post-translationally modified N-terminal pyroglutamyl Aβ variants, termed A $_{PE3-40/42}$, A $_{PE11-40/42}$ and p3 (A $_{17-40/42}$) (10,11). The C-terminal heterogeneity of Aβ and its role in the pathogenesis of AD have been well characterized (2,12). Several studies demonstrated that N-terminal pyroglutamyl A variants, A $_{P\ 3-40/42}$ and A $_{P\ 11-40/42}$, can stabilize the peptides against degradation and they appear very early in the disease progress to show an enhanced cytotoxicity (13,14).

Most recent investigation show that the molecular composition ratio of water-soluble Aβ variants in the

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soluble Aβ aggregates between AD patients and normal aging (NA) individuals is unlike; the major differentiation is the molecular composition ratio of N-terminal pyroglutamyl Aβ variants in aggregates which can make different depositability and cytotoxicity for the development of AD (15). In this study, the mixtures of three Aβ variants, including two pyroglutamyl Aβ variants (Aβ$_{PE3-40}$ and Aβ$_{PE11-40}$) and a full-length Aβ$_{1-40}$ at different molecular composition ratios, were investigated to study the variations of aggregation propensies induced by composition change

2. Materials and Methods

All solvents and chemical used were either of analytical grade or chemically pure. Aβ peptides, including Aβ$_{1-40}$, Aβ$_{PE3-40}$ and Aβ$_{PE11-40}$, were purchased from AnaSpec (San Jose, CA). Thioflavine T (ThT), dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) were obtained from Sigma Chemical (St. Louis, MO). All of the surface plasmon resonance (SPR) experiments used in kinetics assay of Aβ variants aggregation were performed on a Biacore X apparatus, at 25 °C. The instrument, sensor chips (type CM5), and coupling reagents, including (N-ethyl-N'- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and ethanolamine HCl), were from Biacore AB (Uppsala, Sweden).

Preparation of soluble A*β* Solutions.

Prior to analysis, the lyophilized amyloid peptides were subjected to a disaggregation procedure described by Dahlgren et al. (16). Afterward, stock solutions of A$_{1-40}$, A$_{PE3-40}$ and A$_{PE11-40}$ in a concentration of 1 mM were prepared in pure DMSO. Aβ solutions treated in this way have been described to be free of oligomeric species (17,18).

Two soluble Aβ variants mixtures at the molecular composition ratios referring to the investigation on AD and NA individuals described by Piccini et al. (15) with little modifications, AD (A$_{1-40}$, 36%; A$_{PE3-40}$, 48%; A$_{PE11-40}$, 16%) and NA (A$_{1-40}$, 40%; A$_{PE3-40}$, 29%; A$_{PE11-40}$, 31%), and three soluble Aβ variants were suspended in PBS and kept for 24h at room temperature, at a final concentration of 1μM, PH 7.2, for subsequent analyses.

SPR Kinetics Binding Assay

SPR biosensing technology has been chosen as analytical tool to study ligand-ligate binding kinetics, which is capable of the ability to detect specific binding events between target biomolecules in liquid phase (ligate) and a specific binding partner immobilized on chip surface (ligand) without the use of labeling molecules on the target molecules and tedious processing procedures keeping peptides in native state.

In this study, SPR biosensor was adopted to investigate the real-time aggregation kinetics of the two Aβ mixtures and the three Aβ variants in detail. The three Aβ variants kept for 24h at room temperature were separately immobilized onto chip surface as ligand by using standard amine coupling method (19). Sensor chips were first activated with an injection of a 1:1 ratio of 0.4M EDC and 0.1M NHS at a flow rate of 20μL/min for

7min. The three Aβ variants, at 10μM in 10 mM sodium acetate, pH 4.0, were injected over the activated surface for 7 min. The remaining activated surface groups were blocked with a 7-min injection of 1M ethanolamine, pH 8.0. The SPR signals from each of the Aβ variants result in 500-800 Biacore response units (RU).

These three immobilized Aβ variants were then used to interact with the incubated soluble Aβ variants and the two incubated soluble Aβ mixtures. The binding data were analyzed using the BIA evaluation program.

Thioflavine T Binding Assay

The three Aβ peptides and two mixtures, AD and NA, were aggregated in 100 l of RPMI buffers, at a concentration of 100nM, for 24h at room temperature. Ten l of each reaction mixture were mixed with 990 l of ThT (3 M in 50 mM sodium phosphate, pH 6.0), and the fluorescence was subsequently measured at Ex/Em of 450/482 nm by a fluorescence spectrophotometer (Hitachi F-4500). The relative fluorescence intensity was defined by taking fluorescence of 100 nM Aβ$_{1-40}$ aggregated for 24 h as 100 %.

3. Results

Thioflavine T binding to amyloid is a specific interaction for anti-parallel β-pleated sheet secondary structure which produces a change in the emission spectrum of ThT (20). Thereby, the emission intensity of ThT is proportional to the total quantity of β-pleated sheet amyloid. Fig. 1 shows that after a 24h of incubation time, Aβ$_{PE11-40}$ revealed a highest amount of β-pleated sheet amyloid among the three tested Aβ variants and AD mixture displayed a much higher amount of β-pleated sheet amyloid than does NA mixture. In our experiments, both two mixtures have a close composition ratio of Aβ$_{1-40}$, but AD mixture having a high Aβ$_{PE3-40}$/Aβ$_{PE11-40}$ (48:16) composition ratio revealed a much higher increase in the amount of β-pleated sheet amyloid than the three tested A variants under the same test condition of peptide concentration. By contrast, the NA mixture, which has a low A$_{PE3-40}$/A$_{PE11-40}$ (29:31) composition ratio, leads to a less amount of β-pleated sheet amyloid than does AD mixture. The amount of β-pleated sheet amyloid of NA mixture is only a little higher than does A$_{1-40}$.

To measure the aggregation propensies of the three individual Aβ variants, SPR biosensing technique was used to directly detect specific biomolecular interactions in real time through a molecular recognition mechanism (21) which is a noninvasive optical method better than the traditional approaches for measuring aggregation kinetics (22). In Fig. 2, the sensogram, showing real-time aggregation kinetics of the three individual soluble Aβ variants, revealed that the order of aggregation rates was as follows, Aβ$_{PE11-40}$ > Aβ$_{PE3-40}$ > Aβ$_{1-40}$. The time response of the two pyroglutamyl Aβ variants showed that Aβ$_{PE3-40}$ and Aβ$_{PE11-40}$ are capable of much higher aggregation rate than does Aβ$_{1-40}$.

Meanwhile, the aggregation propensies of both AD and NA mixtures with the three individual Aβ variants were measured. In Fig. 3a, the three immobilized Aβ variants interacting with AD mixture shows that Aβ$_{PE3-40}$ has a highest aggregation rate and Aβ$_{PE11-40}$ has a

relatively lower aggregation rate. In Fig. 3b, the three immobilized Aβ variants interacting with NA mixture show that $Aβ_{PE3-40}$ has a highest aggregation rate, but this time response just a little higher than does $Aβ_{PE11-40}$. Both two mixtures, AD and NA, revealed a lowest aggregation rate with $Aβ_{1-40}$.

4. Discussion

In ThT binding assay, the three studied Aβ variants show that the more charges the N-terminal pyroglutamyl-containing Aβ peptides lose in the N terminus, the peptides have a higher amount of β-pleated sheet secondary structure. Thereby, $Aβ_{PE11-40}$ has a highest quantity of β-pleated sheet structure and $Aβ_{1-40}$ has a least quantity of this specified structure. Since the lose of three charges for $Aβ_{PE3}$ and six charge for $Aβ_{PE11}$ could alter their conformational properties and make them more hydrophobic to forward amyloid formation. In addition, The N-terminal glutamic acid residues of Aβ peptides develop pyroglutamyl species after post-translational modification making these peptides less susceptible to further proteolysis (23). The resistance to proteolysis of pyroglutamyl Aβ peptides, $Aβ_{PE3}$ and $Aβ_{PE11}$, probably results in a varying degree of accumulation relative to other N-terminally truncated pyroglutamyl Aβ showing in neuritic plaques and in diffuse plaques. However, AD mixture in ThT binding assay containing a high $A_{PE3-40}/A_{PE11-40}$ composition ratio revealed a much higher quantity of β-pleated sheet structure. This is even higher than does $Aβ_{PE11-40}$ alone. The enhanced aggregation mechanism is not clear; one possible interpretation is that A_{PE3-40} in AD mixture may be capable of a positive synergistic effect in promoting turnover of conformational change. By contrast, NA mixture containing a low $A_{PE3-40}/A_{PE11-40}$ composition ratio shows a low quantity of β-pleated sheet secondary structure by comparing with the three tested Aβ variants. This is even less than does $Aβ_{PE3-40}$ alone. In contrast to AD mixture, the role of $Aβ_{PE3-40}$ in NA mixture could be a negative synergistic effect to prohibit the formation of amyloid. The aggregation propensity of pyroglutamyl-containing Aβ peptides is mainly due to a stabilized formation of β-pleated sheet secondary structure (13), however, the composition ratio should be taken into account. In this study, the ThT fluorescence binding assay demonstrated that the two pyroglutamyl-containing Aβ variants have relatively higher amount of β-pleated sheet amyloid than $Aβ_{1-40}$. In addition, by varying the composition ratio of pyroglutamyl Aβ variants in the tested mixtures can produce different synergistic effects to change the depositability of Aβ mixtures.

Previous ThT binding assay is used to differentiate the quantity of β-pleated sheet secondary structure of the three Aβ variants. It can be used to interpret the enhancement in conformational transition by the composition ratio of the composed three Aβ variants in the tested mixtures. In order to provide the binding kinetics of AD and NA mixtures with the three studied Aβ variants, SPR kinetics assay was analyzed which can illustrate the differentiation in aggregation behaviors of the three Aβ variants with AD and NA mixtures.

SPR kinetics assay displayed that the order of aggregation rates of the three Aβ variants is correspond to the quantity of β-pleated sheet structure of the Aβ variants. This suggests that intra- and intermolecular interactions between hydrophobic parts of the Aβ sequence leads to the formation of Aβ aggregates. The peptide by lose of charge repulsion and stabilized β sheet structure can obviously enhance aggregation rate (24). However, this aggregation behavior cannot be applied directly to the tested mixtures. Among the three tested Aβ variants, both AD and NA mixtures have a highest aggregation rate with $Aβ_{PE3-40}$, not the more hydrophobic $Aβ_{PE11-40}$. In addition, AD mixture has a much higher aggregation rate with $Aβ_{PE3-40}$ than with $Aβ_{PE11-40}$. This may explain that AD mixture has a high $Aβ_{PE3-40}/Aβ_{PE11-40}$ composition ratio. By contrast, NA mixture shows a similar aggregation rate with both $Aβ_{PE3-40}$ and $Aβ_{PE11-40}$. This may explain that NA mixture has a low $Aβ_{PE3-40}/Aβ_{PE11-40}$ composition ratio.

In this study, we found that the elevated $A_{PE3-40}/A_{PE11-40}$ composition ratio can provide positive synergistic effect for the formation of β–pleated sheet secondary structure and both two mixtures have highest aggregation rate with A_{PE3-40}. These results suggest that higher composition of A_{PE3-40} can form more amyloidogenic structure and higher affinity to aggregate with pathogenic Aβ mixture. $Aβ_{1-40}$ has less two hydrophobic C-terminal alanine and isoleucine residues than full-length $Aβ_{1-42}$ resulting in a lower aggregation propensity. A pronounced elevation of only $Aβ_{1-40}$ does not lead to plaque formation but can actually really retard the deposition of $Aβ_{1-42}$ in the brain (25). If $Aβ_{1-40}$ is mixed with a high $A_{PE3-40}/A_{PE11-40}$ composition ratio, that can result in larger pathogenic plaques. Therefore, a adequate control on the pyroglutamyl-containing Aβ variants and the composition ratio can be used to define therapeutic strategy of AD.
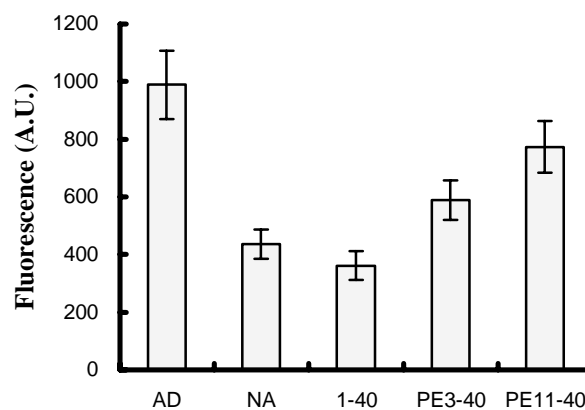

Fig. 1. Thioflavine T binding assay of A_{1-40}, A_{PE3-40}, $A_{PE11-40}$, and two mixtures, MD and MA. Data are expressed as fluorescence intensity in arbitrary unit as mean values ± S.D. measured from three experiments.
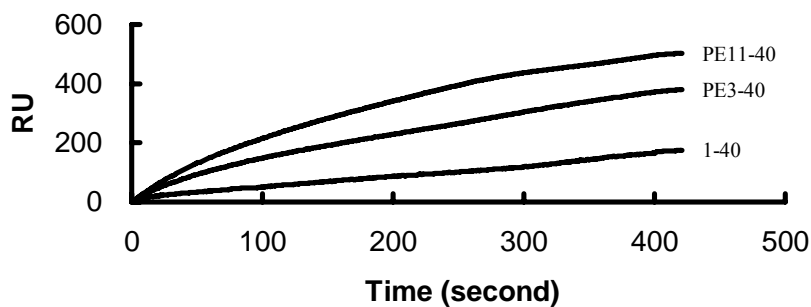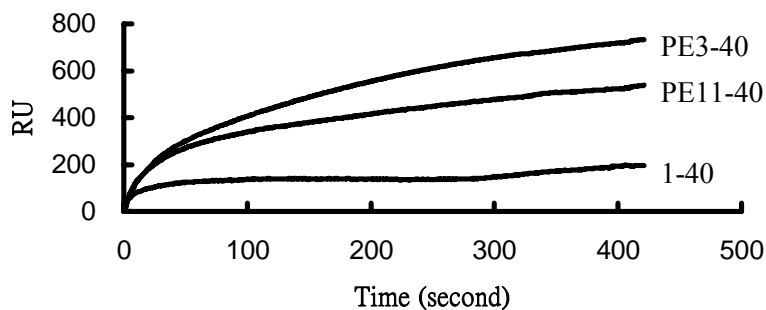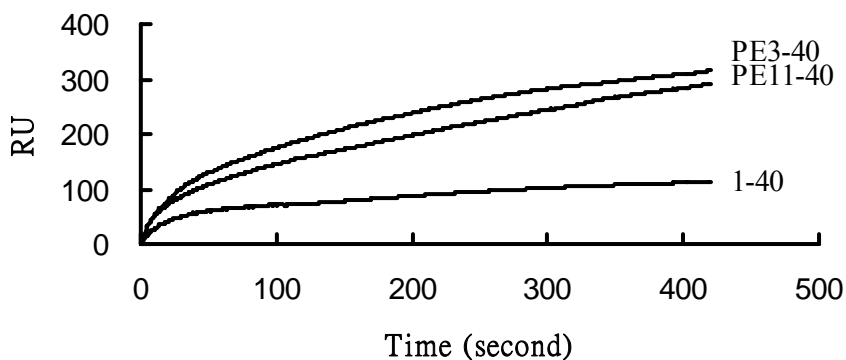
Fig.2. SPR analysis of the aggregational kinetics of $A\beta_{1-40}$, $A\beta_{PE3-40}$, and $A\beta_{PE11-40}$.After 7mins of polymerization, $A\beta_{PE11-40}$ revealed the highest aggregation rate, $A\beta_{PE3-40}$ is next, and $A\beta_{1-40}$ is lowest.

(a)

(b)

Fig.3 SPR analyses of the aggregational kinetics of mixtures (a) AD, (b) NA interact with $A\beta_{1-40}$, $A\beta_{PE3-40}$, and $A\beta_{PE11-40}$, respectively. $A\beta_{PE3-40}$ displayed a highest aggregation rate with both AD and NA mixtures. To compare with $A\beta_{PE3-40}$, $A\beta_{PE11-40}$ displayed a similar aggregation rate with NA mixture.

References

1. Selkoe, D. J. (1996) Amyloid β-protein and the genetics of Alzheimer's disease, *J. Biol. Chem.* 271, 18295–18298.

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

3. Wisniewski, T., Ghiso, J., Rogers, J. (1994) Alzheimer's disease and soluble Aβ, *Neurobiol. Aging* 15, 143-152.

4. Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., Selkoe, D. J. (1995) The Swedish mutation causes early-onset Alzheimer's disease by -secretase cleavage within the secretory pathway, *Nature Med* 1, 1291-1296.

5. Esler, W. P., Wolfe, M.S. (2001) A portrait of Alzheimer secreases- new features and familiar faces, *Science* 293, 1449-1454.

6. Rogers, J., Cooper, N. R., Websters, N. R., Schultz, J., McGeer, P. L., Styren, S. D., Civin, W. H., Brachova, L., Bradt, B., Ward, P. (1992) Complement activation by beta-amyloid in Alzheimer disease, *Proc. Natl. Acad. Sci. U.S.A.* 89, 10016-10020.

7. Turner, R. S., N. Suzuki, A. S. C. Chyung, S. G. Younkin, Lee, V. M.-Y. (1996) Amyloids beta(40) and beta(42) are generated intracellularly in cultured human neurons and their secretion increases with maturation, *J. Biol. Chem.* 271, 8966-8970.

8. Walsh, D. M., Klyubin, I.,, Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., Selkoe, D. J. (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal longterm potentiation in vivo, *Nature* 416(6880), 535-539.

9. Walsh, D. M., Hartley, D. M., Kusumoto, Y., Fezoui, Y., Condron, M. M., Lomakin, A., Benedek, G. B., Selkoe, D. J., Teplow, D. B. (1999) Amyloid β-Protein Fibrillogenesis, *J. Biol. Chem.* 274, 25945-24952.

10. Saido, T. C., Yamao-Harigaya, W., Iwatsubo, T., Kawashima, S. (1996) Amino- and carboxyl-terminal heterogeneity of beta-amyloid peptides deposited in human brain, *Neurosci Lett* 215, 173-176.

11. Saido, T. C., Iwatsubo, T., Mann, D. M., Shimada, H., Ihara, Y., Kawashima, S. (1995) Dominant and differential deposition of distinct beta-amyloid peptide species, A beta N3(pE), in senile plaques. *Neuron* 14, 457-466.

12. Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N., and Ihara, Y. (1994) Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43). *Neuron* 13, 45–53.

13. He, W., Barrow, C. J. (1999) The Aβ 3-pyroglutamyl and 11-pyroglutamyl peptides found in senile plaque have greater beta-sheet forming and aggregation propensities in vitro than full-length Aβ. Biochemistry 38, 10871-10877.

14. Russo, C., Violani, E., Salis, S., Venezia, V., Dolcini, V., Damonte, G., Benatti, U., D'Arrigo, C., Patrone, E., Carlo, P., Schettini, G. (2002) Pyroglutamate -modified amyloid β-peptides- AβN3(pE)-strongly affect cultured neuron and astrocyte survival. *J. Neurochem.* 82, 1480-1489.

15. Piccini, A., Russo, C., Gliozzi, A., Relini, A., Vitali, A., Borghi, R., Giliberto, L., Armirotti,

A., D'Arrigo, C., Bachi, A., Cattaneo, A., Canale, C., Torrassa, S., Saido, T. C., Markesbery, W., Gambetti, P., Tabaton, M. (2005) β-Amyloid is different in normal aging and in Alzheimer disease. *J. Biol. Chem.* 280, 34186–34192.

16. Dahlgren, K. N., Manelli, A. M., Stine, W. B., Jr., Baker, L. K., Krafft, G. A., LaDu, M. J. (2002) Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J. Biol. Chem.* 277, 32046-32053.

17. Hardy, J., Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297, 353–356.

18. Stine, W. B., Jr., Dahlgren, K. N., Krafft, G. A., and LaDu, M. J. (2003) In vitro characterization of conditions for amyloid-β, peptide oligomerization and fibrillogenesis. *J. Biol. Chem. 278*, 11612-11622.

19. Johnsson, B., Lofas, S., Lindquist, G. (1991) Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* 198, 268–277.

20. LeVine, H. (1993) Thioflavine T interaction with synthetic Alzheimer's disease β-amyloid peptides: Detection of β amyloid aggregation in solution. *Protein Sci* 2, 404-410.

21. McDonnell, J. M. (2001) Surface plasmon resonance: towards an understanding of the mechanisms of biological molecular recognition. *Curr. Opin. Chem. Biol.* 5,572-577.

22. Harper, J., Lansbury, P. J. (1997) Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins. *Annu Rev Biochem.* 66, 385-407.

23. Sahasrabudhe, S. R., Brown, A. M., Hulmes, J. D., Jacobsen, J. S, Vitek, M. P., Blume, A. J., Sonnenberg, J. L. (1993) Enzymatic generation of the amino terminus of the β-amyloid peptide. *J. Biol. Chem.* 269, 16699–16705.

24. Schlenzig, D., Manhart, S., Cinar Y., Kleinschmidt, M., Hause, G., Willbold, D., Funke, S. A., Schilling, S., Demuth, H.-U. (2009) Pyroglutamate formation influences solubility and amyloidogenicity of amyloid peptides. Biochemistry 48 , 7072–7078.

25. McGowan, E., Pickford, F., Kim, J., Onstead, L., Eriksen, J., Yu, C., Skipper, L., Murphy, M. P., Beard, J., Das, P., Jansen, K., DeLucia, M., Lin, W.-L., Dolios, G., Wang, R., Eckman, C. B., Dickson, D. W., Hutton, M., Hardy, J., Golde, T. (2005) Aβ42 Is Essential for Parenchymal and Vascular Amyloid Deposition in Mice. *Neuron* 47, 191-199.