

Review

Metabolism of amyloid β peptide and pathogenesis of Alzheimer's disease

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Abstract: The conversion of what has been interpreted as “normal brain aging” to Alzheimer's disease (AD) via transition states, *i.e.*, preclinical AD and mild cognitive impairment, appears to be a continuous process caused primarily by aging-dependent accumulation of amyloid β peptide ($A\beta$) in the brain. This notion however gives us a hope that, by manipulating the $A\beta$ levels in the brain, we may be able not only to prevent and cure the disease but also to partially control some very significant aspects of brain aging. $A\beta$ is constantly produced from its precursor and immediately catabolized under normal conditions, whereas dysmetabolism of $A\beta$ seems to lead to pathological deposition upon aging. We have focused our attention on elucidation of the unresolved mechanism of $A\beta$ catabolism in the brain. In this review, I describe a new approach to prevent AD development by reducing $A\beta$ burdens in aging brains through up-regulation of the catabolic mechanism involving neprilysin that can degrade both monomeric and oligomeric forms of $A\beta$. The strategy of combining presymptomatic diagnosis with preventive medicine seems to be the most pragmatic in both medical and socioeconomic terms.

Keywords: Alzheimer's disease (AD), mild cognitive impairment (MCI), preclinical AD, amyloid β peptide ($A\beta$), metabolism, neprilysin, secretase

1. Introduction

Aging is a major risk factor for most of the dementing disorders predominantly represented by Alzheimer's disease (AD). The estimated number of AD patients in the world is approximately 50 million and is increasing as the world population ages. More than one out of every two people older than 85 years suffer from either dementia or mild cognitive impairment (MCI), a prodrome of dementia.^{1,2)} This fact was not considered a major threat to the society until a few decades ago when average life expectancy could be well below 70 years, even in the developed countries. Today, however, the average life expectancy in most developed countries is either close to or beyond 80 years. This indicates that dementia is becoming an ultimate form of the manifestation of brain aging. In other words, the chances of the

general population being affected by the AD neuropathology sometime later in life are likely to be greater than 50% unless the disease becomes preventable and curable. Symbolically, China, with a population of more than a billion people, presently exhibits the highest rate of increase in the number of AD patients. A recent epidemiological study in Japan indicates that, of those persons over 65 years of age, 10% suffer from dementia and 7% from MCI.³⁾

Of particular note is that two of the major neuropathological hallmarks of AD, senile plaques and neurofibrillary tangles, take place with the aging of the human brain many years prior to the disease onset.^{4–6)} This suggests that aging is the predominant risk factor for AD, and that a large number of people entering an old age and manifesting these neuropathological structures in their brains are likely to be in a presymptomatic stage of MCI and AD, termed “preclinical AD”.⁷⁾ In this respect, it is most important that a preventive medicine combined with presymptomatic diagnosis allows a substantial portion of aged people to escape from the scourge of dementing syndromes. To achieve this goal, we need

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to clarify the primary cause(s) of AD development and the molecular mechanisms underling the neuro-pathological cascade.

2. Etiology of AD

Pathological and patho-biochemical studies mostly up to 1990 established the chronology of the major pathological events of AD, at least in the neocortex, and identified the molecular components contributing to the pathological structures. Senile plaques, present extracellularly, consist mainly of amyloid β peptide ($A\beta$), while neurofibrillary tangles (NFTs), present intracellularly, contain primarily tau protein. The research community seems to be reaching a consensus that these pathological structures as they appear may not be the direct causes of AD symptoms, but rather, that the processes, not fully identified yet, that is represented by the pathology, such as synaptotoxic $A\beta$ oligomer formation, may be essential in the pathogenesis.⁸⁾

In any case, the temporal distance of decades between the cause and effect has been the most challenging factor in AD research. Even the most aggressive form of autosomal dominant familial AD takes more than 20 years before the disease onset. Besides, the chronology of pathological events alone does not establish any cause-and-effect relationship. AD research thus resembles archaeology in that researchers must collect a large body of consistent circumstantial evidence in order to form a consensus. The observations of pathological structures such as senile plaques and neurofibrillary tangles in AD brains, for instance, have always engendered arguments as to whether these structures represent pathologically essential and significant pathways or just by-products or consequences of something else that is essential in the process.

In this respect, identification of the familial AD-and tauopathy (FTDP-17: frontotemporal dementia with Parkinsonism linked to chromosome 17)-causing gene mutations and analyses of their phenotypes in the 1990s have played a major, indispensable role in resolving the etiology of AD.^{6),9)-12)} Consensus has now been reached in that the decades-long cascade leading to dementia is initiated by the accumulation of $A\beta$ in the brain and that tauopathy is likely to play a major role in the neurodegenerative processes downstream of $A\beta$ deposition although there may possibly exist a tauopathy-independent pathway(s). Thus, the 1990s thus may be called the "decade of familial AD". The next 10 years of the 2000s have witnessed some advancement in the understanding of

the cause-and-effect relationship of sporadic AD, which represents the vast majority of all AD cases,¹³⁾ but the mechanisms still remain largely elusive.

More than 200 mutations that cause familial AD have been identified¹⁴⁾ in the three genes encoding the amyloid precursor protein (APP), presenilin 1 and presenilin 2 proteins, all of which are involved in $A\beta$ generation as indicated by red arrows in Fig. 1. A number of studies in the 1990s described the molecular phenotypes caused by these mutations. Some papers examined the effects of the mutations on $A\beta$ production, while others examined effects on cytoskeletal abnormalities involving tau protein, cell death or apoptosis, endoplasmic reticulum stresses, etc. These studies have established a consensus that the only phenotype commonly shared by essentially all the mutations *in vitro* (in cell culture), *in vivo* (in transgenic and knock-in (KI) mice), and in humans carrying these mutations is the increased production of a specific species of $A\beta$, $A\beta_{42}$, which is much more hydrophobic and thus more apt to oligomerize and polymerize than the other major species, $A\beta_{40}$. These results have strongly suggested that $A\beta_{42}$ is the primary pathogenic agent in the AD cascade.^{10),12)} Typically, most of the presenilin 1 mutations, accounting for more than 70% of all familial AD mutations thus far identified, cause very aggressive presenile $A\beta$ amyloidosis in humans by increasing the steady-state $A\beta_{42}$ level approximately 1.5-fold, as detected in the brains of mutant presenilin 1 transgenic or KI mice.^{15),16)}

Atypical mutations in the intra- $A\beta$ sequences of APP, such as the Dutch, Flemish, Italian and Arctic mutations, have also been identified (indicated by blue arrows in Fig. 1). Most of these mutations result in hemorrhages or strokes caused by unusually severe cerebral amyloid angiopathy (CAA) that also accompanies the pre-senile parenchymal $A\beta$ deposition. These atypical mutations are generally believed to cause $A\beta$ accumulation by augmenting aggregation or protofibril formation.¹⁷⁾⁻¹⁹⁾ Interestingly, the pathological phenotypes of these mutations are not fully identical. Yamamoto *et al.*²⁰⁾ have pointed out that the difference among the closely located mutations may be caused by differential interactions with different ganglioside species. Additionally, we found that these mutations also cause $A\beta$ to be more resistant to the degradation catalyzed by a physiologically relevant peptidase, neprilysin, described in Section 4.²¹⁾ An intra- $A\beta$ mutation, the Tottori mutation that seems to cause typical AD, has recently been identified.²²⁾ This mutation also seems

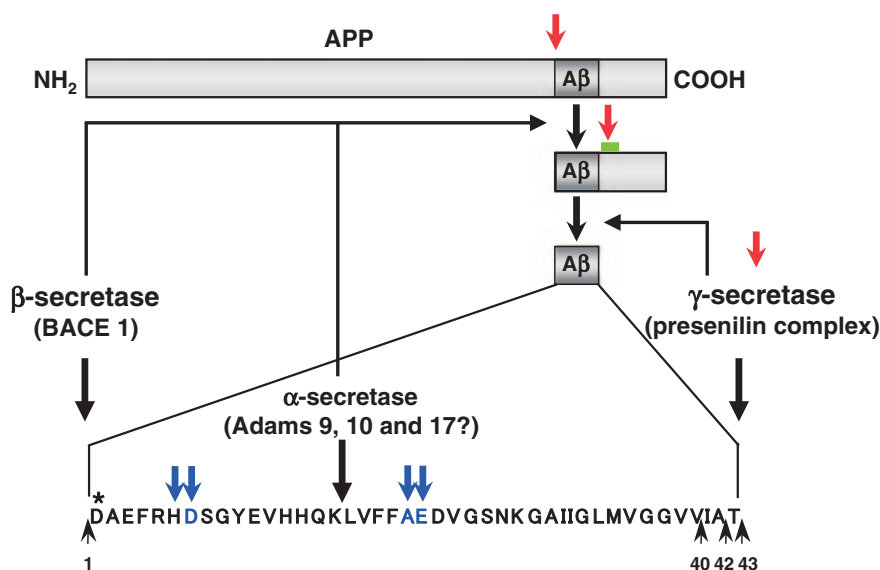


Fig. 1. Generation of A β from its precursor, APP. APP is first cleaved by β - or α -secretase, generating a C-terminal fragment (C99 or C83), which then is cleaved by γ -secretase to generate A β . The major species generated are A β ₄₀ and A β ₄₂. The latter is more hydrophobic and more apt to aggregate and thus is considered to be primarily pathogenic, consistent with the phenotype of the major familial AD-causing mutations. The red arrows indicate where in these processes the major familial AD mutations exert their effects. Atypical mutations (see text) are indicated by blue arrows.

to render A β more resistant to neprilysin-catalyzed proteolysis without altering its aggregation properties (Tsubuki *et al.*, unpublished observations). Therefore, the intra-A β sequence mutations may exert dual pathogenic effects associated not only with aggregation/protofibril formation but also with one major aspect of metabolism, proteolytic degradation.

These autosomal dominant familial AD mutations together account for less than 1% of all AD cases.¹³⁾ Sporadic AD accounts for most cases of AD, and the etiology of sporadic AD remains much more elusive than that of familial AD. However, sporadic AD is essentially indistinguishable from familial AD in both pathological and neurological terms. This suggests that we need to understand why A β accumulates in sporadic AD. From this, inhibition of A β accumulation in the brain is likely to contribute to, at least in part, the prevention of both familial AD and sporadic AD.

Consistent with the extremely high prevalence of sporadic AD in the aged population, most clinically "normal" people start accumulating A β in their brains between the ages 40 and 80 years.^{23)–25)} When A β reaches levels approximately 10,000-times greater than normal, the disease seems to arise; preclinical AD and MCI represent the transition states from "normal aging" to AD development. Carriers of the apolipoprotein E4 genotype, the predominant genetic

risk factor for AD thus far confirmed universally,²⁶⁾ begin accumulating A β at an earlier age than non-carriers.²⁵⁾ These facts further indicate that accumulation of A β is also a primary cause for "Sporadic AD" and that inhibition of A β accumulation would at least provide a preventive measure. Once it becomes possible to manipulate A β levels in the brain, such medical treatment after AD development, before MCI appearance, and even before preclinical AD would relate to "postsymptomatic therapy", "pre-symptomatic prevention" and "aging control (at least in part)" respectively. For presymptomatic prevention to be possible, we need first to establish reliable measures for presymptomatic diagnosis (see Section 6).

3. A β metabolism: three major targets

A β is a physiological peptide secreted from neurons under normal conditions both *in vitro* and *in vivo*.^{27)–29)} A β is generated from APP by sequential limited proteolysis conducted by β - and γ -secretases (Fig. 1). As stated in the previous section, most of the "Familial AD" mutations in APP and presenilin (a γ -secretase catalytic component) genes result in overproduction of A β ₄₂. A β does not appear to play a major physiologically important role; the apparent role of APP processing by the α - and β -secretases is the release of soluble forms of APP, or

APPs, which are known for their neuroprotective and neurotrophic functions.³⁰⁾ Further to this, splice variants containing the insert sequences corresponding to the Kunitz-type protease inhibitor (KPI) domain have been identified as protease Nexin II, an endogenous inhibitor against a group of serine proteases including thrombin.³¹⁾ APP has also been reported to possess a domain that inhibits matrix metalloproteinases.³²⁾ The other possible function of APP processing may be the release of a cytoplasmic fragment (APP intracellular domain: AICD), generated by γ -secretase, which may translocate to the cellular nucleus and play a physiological regulatory role in transcription in a manner similar to the cleavage of Notch by γ -secretase activity.³³⁾ Alternatively, Kim *et al.*³⁴⁾ indicated that AICD could be neurotoxic.

Thus far, several substrates for β -secretase and a number of substrates for γ -secretase other than APP have been identified.^{33),35)–38)} Of all of the known substrates, the fragment that corresponds to $A\beta$ in APP does not seem to possess any biological role. Therefore, at present it is most likely that $A\beta$ is simply an unwanted by-product of APP processing. Discovery of the β - and γ -secretase substrates calls for precaution concerning the anti- $A\beta$ production strategy targeted at the secretases: chronic and over-inhibition of the secretases is likely to cause adverse side-effects. Inhibitors specific to β -secretase appear to be more suitable in terms of clinical applicability because the phenotype of β -secretase deficiency is apparently milder,³⁹⁾ whereas that of γ -secretase deficiency is lethal.³³⁾ De Strooper and colleagues (Dominguez *et al.*⁴⁰⁾) demonstrated that β -secretase deficiency causes partial lethality in neonatal mice using two independent knockout lines, indicating that β -secretase inhibition could also provoke a side effect. There however still remains a hope that we may be able to produce a medication that selectively inhibits the generation of $A\beta_{42}$ without altering metabolism of APP or other γ -secretase substrates.³³⁾

In any case, APP processing takes place constitutively in the brains of both young and old, and, at least in the brains of young and healthy individuals, no $A\beta$ deposition takes place. Taken together, these observations clearly indicate that, under normal conditions, $A\beta$ is constantly anabolized and then rapidly catalyzed before it can be deposited. This catabolism can take place inside the brain or in the circulatory system after transport out of the brain. The kinetic relationships between the three metabolic processes of anabolism, catabolism and

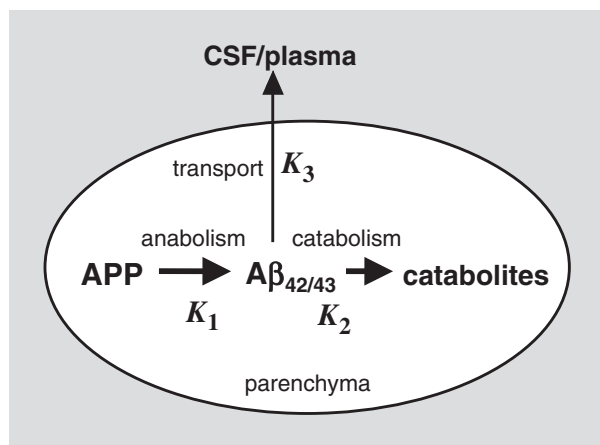


Fig. 2. Kinetic relationships between production, degradation within the brain and transport out of the brain. The steady-state $A\beta$ ($A\beta_{42}$) level in the brain, $[A\beta]$ ($[A\beta_{42}]$), is primarily a function of the APP level, $[APP]$, the rate constants for production, $[K_1]$, in-brain degradation, $[K_2]$ and out-of-brain transport, $[K_3]$. See Section 3 for details.

transport are schematized in Fig. 2. ($A\beta_{40}$ is left out for the sake of simplicity.)

K_1 , K_2 , and, K_3 are the rate constants for production, in-parenchyma degradation, and out-of-brain transport of $A\beta$, respectively. Under the assumptions that the kinetics of the reactions can essentially be analyzed linearly, that these rate constants are independent of each other, and that these processes exist in a dynamic steady-state equilibrium (see previous reviews for details regarding these assumptions^{41),42)}), the relationship between the amounts of $A\beta_{42}$ and APP, represented as $[A\beta_{42}]$ and $[APP]$, respectively, can be expressed by the following equation:

$$[A\beta_{42}] = K_1 / (K_2 + K_3) \times [APP] \quad [1]$$

Formula [1] is consistent with the phenotypes of almost all the familial AD mutations in APP and presenilin 1 genes; K_1 is approximately 1.5-fold greater than that in normal controls, meaning that $[A\beta_{42}]$ also becomes 1.5-fold greater. It is also consistent with one of the phenotypes of Down's syndrome caused by trisomy of chromosome 21 carrying the APP gene; $[APP]$ is 1.5-fold greater than in normal controls and $[A\beta_{42}]$ also becomes 1.5-fold greater.

Therefore, an increase in the rate constant for production (K_1) or decreases in the rate constants for in-parenchyma degradation (K_2) and out-of-brain transport (K_3) can elevate $[A\beta_{42}]$ and thus be causal for pathological $A\beta$ deposition, or oligomer

formation. This logic also indicates that down-regulation of K_1 or up-regulation of K_2 and K_3 can reduce $A\beta$ deposition in the brain. Note that the activation of α -secretase(s) would also contribute to reducing K_1 .^{36),43)} Taken together, these three major targets provide theoretical possibilities for anti- $A\beta$ therapy.

There has been some discussion regarding the relative importance of K_2 (in-parenchyma degradation) versus K_3 (out-of-brain transport) in $A\beta$ clearance.⁴⁴⁾ If K_2 is excessively greater than K_3 , Formula [1] would tend towards $[A\beta_{42}] \approx K_1/K_2 \times [APP]$, whereas, if K_2 is excessively smaller than K_3 , it would be $[A\beta_{42}] \approx K_1/K_3 \times [APP]$. We assume that the rate constants are such that $K_2 > K_3$ and thus $[A\beta_{42}] \approx K_1/K_2 \times [APP]$. The basis of this reasoning is as follows: (i) Brains are rich sources of various peptidases, which can proteolyze $A\beta$ as long as they are accessible to the substrate; (ii) Because the amounts of $A\beta$ in the brain and in the CSF or plasma are poorly correlated with each other in AD patients,^{45),46)} there does not seem to exist an effective transport-dependent mechanism that clears $A\beta$ out of the brain, at least in humans under the pathological condition where $A\beta$ levels in the brain are 1,000–10,000 times greater than in normal brains;²³⁾ (iii) If reduced efficiency of transport out of the brain to the circulatory system was a major cause of the pathological $A\beta$ deposition, one would expect vascular amyloid deposition to arise earlier than parenchymal deposition, but the reality is generally the other way around, particularly in humans; (iv) Human brains are approximately 1,000 times larger than mouse brains in size. Therefore, the advantage of in-parenchyma degradation over transport-dependent clearance is likely to be even greater in humans than in mice, particularly if the major transport pathway is via the interstitial fluid (ISF) and from there to the cerebrospinal fluid (CSF) and plasma. It should be noted that the primary function of proteolysis is the recycling of amino acids; therefore in-parenchyma degradation is more economical from a metabolic (energy consumption) point of view than out-of-brain transport, especially in the case of humans.

The importance of the transport mechanism has however been experimentally highlighted by the $A\beta$ vaccination approach.^{47),48)} Interestingly, DeMattos *et al.*⁴⁹⁾ demonstrated that intravenous injection of anti- $A\beta$ antibody into APP transgenic mice induces rapid elevation of $A\beta$ levels in the plasma in a manner correlating to $A\beta$ burdens in the brain, implying the

presence of active mechanism(s) that transport $A\beta$ from within the brain to the circulatory system. Even so, the molecular and cellular mechanisms underlying $A\beta$ vaccination are still elusive and whether or not the observations made by DeMattos *et al.*⁴⁹⁾ for the mouse model can be extrapolated to humans remains to be determined. Nevertheless, the $A\beta$ vaccination is a strong candidate approach to reduce $A\beta$ burdens since a study using non-human primates⁵⁰⁾ provided data that support the results of Schenk *et al.*⁴⁷⁾ In our view, passive immunization using humanized monoclonal anti- $A\beta$ antibody, rather than active immunization using $A\beta$ or $A\beta$ derivatives, seems to be more promising and safer because the former is likely to generate consistent effects while the latter may result in unexpected side-effects⁵¹⁾ if used for several years. Passive immunization would however be a costly medication requiring a large amount of purified monoclonal antibody without any antigenicity. An antibody to an amino-terminal truncated form of $A\beta$ starting from pyroglutamate at position 3 may provide an interesting medical treatment because this form of $A\beta$ is present in human brains only under pathological conditions.^{52),53)}

4. Identification and characterization of $A\beta$ -degrading mechanisms

More than a decade ago, we hypothesized that down-regulation of $A\beta$ degradation may be a primary relevant cause for $A\beta$ accumulation in sporadic AD.^{41),54),55)} This hypothesis was made on the basis that there is little evidence supporting the up-regulation of $A\beta$ generation upon aging prior to $A\beta$ deposition in the brain, that sporadic AD patients seem to accumulate less $A\beta_{42}$ compared to $A\beta_{40}$ than familial AD patients⁵⁶⁾ and because aging generally accompanies down-regulation rather than up-regulation of enzyme activities (except for those associated with inflammation and other pathological processes).

In order to test this working hypothesis, we first set out to identify the major *in vivo* $A\beta$ -degrading enzyme in the brain. Whereas the mechanism of $A\beta$ generation had been examined in depth using molecular and cellular biological approaches,^{36),43),57),58)} the mechanism of $A\beta$ degradation remained elusive. This was because not only the cellular topology but also the complex structural organization of the brain tissue composed of various types of cells needed to be taken into consideration in analyzing $A\beta$ degradation taking place in the brain. Therefore, we started our series of degradation studies by establishing a novel

in vivo experimental paradigm in which we injected synthetic internally multi-radio-labeled $A\beta_{1-42}$ into the hippocampus of anesthetized live rats and analyzed the degradation process using high-pressure liquid chromatography directly connected to a flow-type scintillation counter.⁵⁵⁾ Experiments using a panel of more than 20 peptidase inhibitors highlighted that a neutral endopeptidase family member, similar or identical to neprilysin, appears to play a major role in the $A\beta_{1-42}$ catabolism because thiorphan, a well-characterized neutral endopeptidase inhibitor,⁵⁹⁾ was the most potent inhibitor. In accordance with this, short-term and long-term infusions of thiorphan into the rat hippocampus resulted in biochemical and pathological accumulation of endogenous $A\beta$, respectively. Dolev and Michaelson⁶⁰⁾ recently demonstrated that thiorphan infusion induces $A\beta$ accumulation in an apolipoprotein E genotype-dependent manner, consistent with the human pathology.^{25),26)}

The next task was to identify the major responsible $A\beta$ -degrading peptidase among members of the neutral endopeptidase family. We made efforts to determine the molecular identity by using biochemical and molecular biological approaches and, consequently, predicted that neprilysin is likely to be the primary candidate.^{61),62)} We subsequently confirmed our prediction by examining the degradation of radio-labeled $A\beta$ in neprilysin-knockout (KO) mouse brains using their wild-type littermates as positive controls.⁶³⁾ Neprilysin is a type II membrane-associated peptidase whose active site faces the lumen or extracellular side of membranes^{59),64),65)} (Fig. 3). This topology is suited for the degradation of extracytoplasmic peptides such as $A\beta$. Using immunofluorescence microscopy, we confirmed that neprilysin is essentially exclusively expressed in neurons, not in glia, and that the peptidase, after synthesis in the soma, is axonally transported to presynaptic terminals,⁶⁶⁾ presumably in a manner similar to the way APP is transported. Therefore, presynaptic terminals and nearby intracellular (lumen-side) locations are likely to be the sites of $A\beta$ degradation by neprilysin.⁶⁷⁾

Based on the above observations, we examined the ability of neprilysin-KO mouse brains to degrade $A\beta$ *in vivo* and quantified the endogenous $A\beta$ levels in the brains.⁶³⁾ Due presumably to redundancy in the neutral endopeptidase family,^{59),65),68)} the KO mice show normal characteristics in relation to reproduction, development, and adult anatomy to the best of our knowledge.⁶⁹⁾ The ability to degrade

the radiolabeled $A\beta$ was significantly reduced in the KO mouse brains. Consistently, both the endogenous $A\beta_{40}$ and $A\beta_{42}$ levels were elevated approximately 2-fold, in a manner comparable to or even greater than what has been described in familial AD-causing mutant presenilin transgenic or knock-in mice.^{15),16)} More importantly, the elevation of $A\beta$ levels was inversely correlated with the gene dose of neprilysin and thus with the enzyme activity.^{63),70)} These observations suggest that even partial loss of neprilysin expression/activity causes the elevation of $A\beta_{1-42}$ levels and thus could induce $A\beta$ amyloidosis on a long-term basis in a manner similar to that of familial AD-causing gene mutations. These results also indicate that the rate constant for the in-parenchyma degradation of $A\beta_{42}$ by neprilysin accounts for about 50% of total clearance activity, or $K_2 + K_3$ in Formula [1]. Therefore, as it is improbable that K_2 is negligibly smaller than K_3 , K_2 is consequently likely to be even greater in human brains for the reasons stated in the previous section.

In a similar manner, some of the other $A\beta$ -degrading enzyme candidates were examined by a reverse genetic approach, *i.e.*, by measuring the $A\beta$ levels in the brains of KO-mice (Table 1). To our knowledge, neprilysin seems to be the dominant peptidase regulating the steady-state level of the primarily pathogenic $A\beta$ species, $A\beta_{42}$, as do the pathogenic familial AD presenilin mutations. The 2-fold increase in the $A\beta$ levels in neprilysin-KO mouse brains indicates that neprilysin accounts for approximately 50% of total clearance mechanism ($K_2 + K_3$ in Formula [1]). Endothelin-converting enzymes (ECEs) are interesting because they resemble neprilysin in structure, belonging to the same family of proteases (M13 family),⁷⁰⁾ and because they degrade $A\beta$ in acidic intracellular compartments.⁷¹⁾ Notably, not only neprilysin but also a number of other $A\beta$ -degrading enzyme candidates, including ECEs and insulin-degrading enzyme (IDE) (Table 1), are zinc-requiring enzymes. Further to this, all the α -secretase candidates, a disintegrin and metalloproteinase (ADAMs) 9, 10 and 17,^{36),43)} which could contribute to reduction of $A\beta$ synthesis, also require zinc for their proteolytic activities. Therefore, although too much zinc would be obviously harmful,⁷²⁾ a heightened zinc deficiency is likely a negative risk factor for AD.

McGeer and colleagues reported that neprilysin mRNA levels are significantly reduced in the brain areas vulnerable to $A\beta$ pathology in preclinical AD patients at a relatively early stage (Braak stage II)

Table 1. $A\beta$ -degrading enzyme candidates: Effect of genetic deficiency on $A\beta$ levels in the brain

Mice	$A\beta_{42}$	$A\beta_{40}$	References
Neprilysin-KO(-/-)	2-fold	2-fold	Iwata <i>et al.</i> ⁶³⁾
Neprilysin-KO(+/-)	1.5-fold	1.5-fold	Iwata <i>et al.</i> ⁶³⁾
ECE 2-KO (-/-)	1.3-fold	1.3-fold	Eckman <i>et al.</i> ⁷¹⁾
ECE 2-KO (+/-)	1.2-fold	1.2-fold	Eckman <i>et al.</i> ⁷¹⁾
ECE 1-KO (+/-)	1.3-fold	1.3-fold	Eckman <i>et al.</i> ⁷¹⁾
IDE-KO (-/-)	Not significant	1.1- to 1.2-fold	Farris <i>et al.</i> ⁹⁴⁾
IDE-KO (-/-) ¹	1.4-fold	1.6-fold	Miller <i>et al.</i> ¹³³⁾
tPA-KO (-/-)	Not significant	Not significant	unpublished data by Iwata <i>et al.</i>
uPA-KO (-/-)	Not significant	Not significant	Ertekin-Taner <i>et al.</i> ¹³⁴⁾
ACE-KO (-/-)	Not significant	Not significant	unpublished data by Takaki <i>et al.</i>
Plasmin (-/-) ²	Not significant	Not significant	Tucker <i>et al.</i> ¹³⁵⁾
Presenilin 1-KI ³ (positive control)	1.5-fold increase	Not significant	Nakano <i>et al.</i> ¹⁶⁾

KO: (gene) knock-out; KI: (gene) knock-in; tPA: tissue-type plasminogen activator; uPA: urokinase-type plasminogen activator; ACE: angiotensin-converting enzyme. ¹Only water-soluble $A\beta$, which accounts for less than 30% of total $A\beta$ was quantified. ²Plasminogen-KO mice were examined as plasmin-KO mice. ³The data from mutant presenilin 1-KI mice, which show typical pathogenic alterations in $A\beta$ levels, leading to accelerated $A\beta$ accumulation in the brain, are presented as a positive control. Quantification was performed using an identical enzyme-linked immunosorbent assay.¹³⁶⁾ The mice were from 8 to 10 weeks of age.

as compared to age-matched normal controls.^{73),74)} These observations are consistent with our hypothesis. Nevertheless, as it was still unclear whether reduction of neprilysin expression or activity precedes $A\beta$ pathology during the course of aging, we thus examined the effect of aging on the expression/activity of brain neprilysin using two methods: (i) A biochemical assay to measure neutral endopeptidase activity in the brain (The advantage of this is that it is more quantitative than the latter, whereas the disadvantage is that the method fails to provide information regarding the local or spatial activity/expression of neprilysin).; (ii) An immunofluorescence detection method using an anti-neprilysin antibody.⁶⁶⁾ Representative immunofluorescence results are shown in Fig. 4. The selective reduction of neprilysin expression upon aging is apparent in the polymorphic cellular layer, inner molecular layer, and outer molecular layer of the dentate gyrus and also in the stratum lucidum of the CA3 sector of the hippocampus.⁷⁵⁾ The differences were also proven to be statistically significant by quantitative image analyses. The results of the enzymatic quantification were also consistent with these observations; we observed a statistically significant reduction of approximately 10% of the enzyme activity per year in the entire hippocampus for two years and a 10% reduction in the neocortex in two years. If the 1.5-fold increase of $A\beta_{42}$, caused by the pathogenic mutations in the presenilin gene, is truly sufficient for the

development of early-onset familial AD, then a 1% reduction of neprilysin activity per year, leading to 50% reduction of neprilysin activity at the age of 50 and thus about a 1.5-fold elevation of the $A\beta$ levels in the brain, would be sufficient to be causative of late-onset AD in humans.

Since then there have been an overwhelming number of reports indicating that neprilysin expression/activity declines with aging and in AD.^{76)–81)} An exception is a report by Miners *et al.*,⁷⁹⁾ showing that neprilysin enzyme activity increases with aging. This paper however suffers from two major weaknesses. First, the authors used tissues from Brodmann area 6 for their analysis. This area, composed of the premotor cortex and the supplementary motor area, is much less affected by $A\beta$ pathology than such regions as temporal cortex and hippocampus and thus is inappropriate for observing representative aging-dependent changes of neprilysin expression/activity in brain. Second, the immunocapture-enzyme assay used by the authors lacks appropriate controls. We thus analyzed WT, heterozygous and homozygous neprilysin-KO, and aged mice using Western blotting, immunocapture assay and immunocapture-activity assay. The immunocapture-activity assay exhibited very poor linearity (Saito and Saido, unpublished observation) and therefore is only qualitative. This probably accounts for the discrepancy between the neprilysin protein contents and enzyme activities in the Miners' paper.

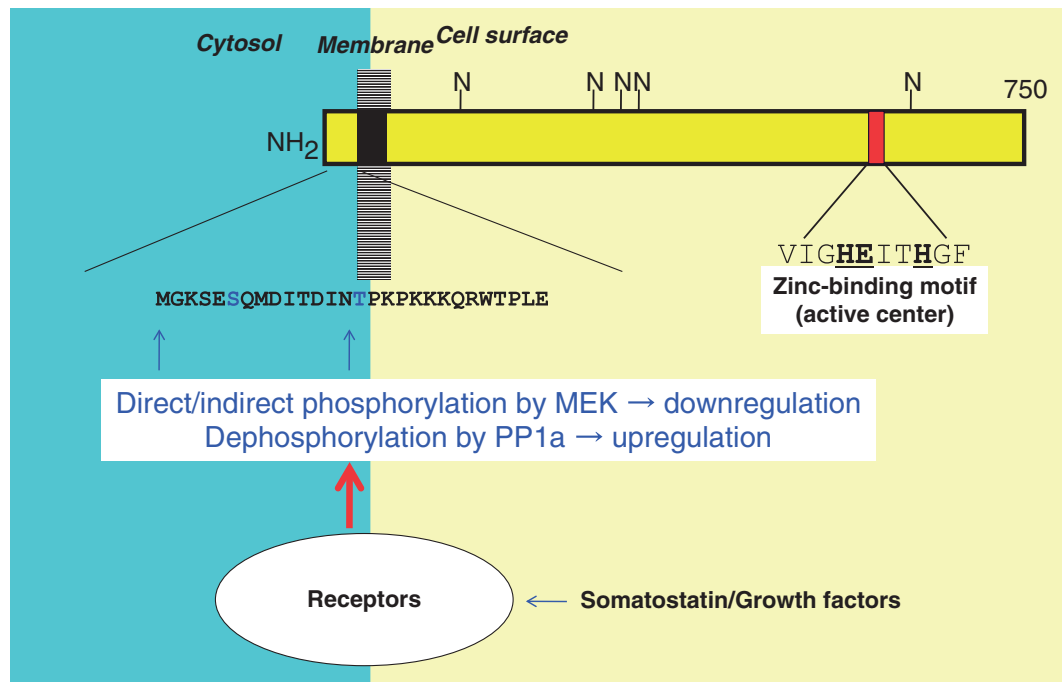


Fig. 3. Schematic structure and cellular topology of neprilysin. Neprilysin is a type II membrane-associated peptidase whose active site faces the lumen or extracellular side of membranes. The enzyme requires the presence of zinc for proper functioning. N: N-glycosylation sites. The glycosylation is reported also to be required for the enzymatic activity to be fully expressed.¹³⁷⁾

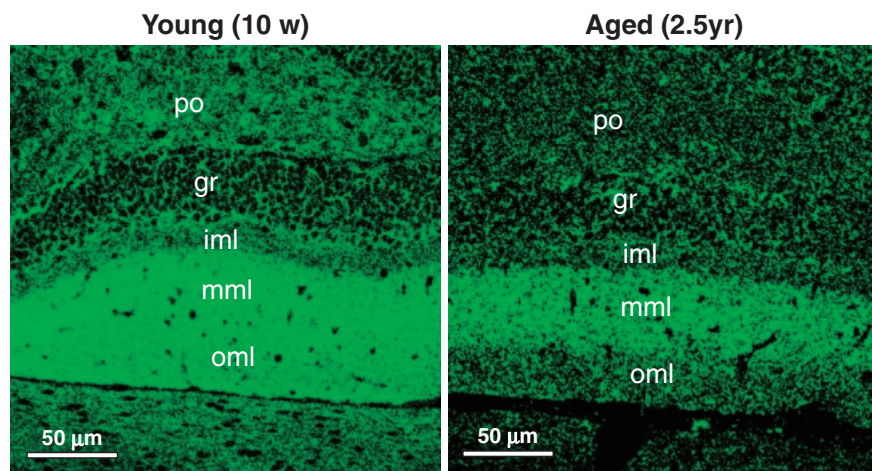


Fig. 4. Aging-dependent reduction of neprilysin in polymorphic cell layer, inner molecular layer and outer molecular layer of the dentate gyrus. The selective reduction of neprilysin expression is apparent in the polymorphic cellular, inner molecular layer and outer molecular layer of the dentate gyrus. See Iwata *et al.*⁷⁵⁾ for further details. Reproduced with permission from John Wiley & Sons, Inc.

The immunofluorescence observations (Fig. 4) indicate that the areas where we see selective aging-induced reduction of neprilysin expression correspond to the terminal zones of mossy fibers and perforant path, which suggest that local A β concentrations are particularly elevated at the presynaptic locations

originally projecting from the entorhinal cortex. It is notable that the entorhinal cortex is the region where the initial neurodegeneration takes place in AD brains.⁸²⁾ Indeed, M lanie *et al.*⁸³⁾ reported that not only parenchymal A β amyloidosis but also amyloid angiopathy correlates inversely with neprilysin levels

in control and AD patients. Several reports also describe the possible role of neprilysin in inhibiting $A\beta$ accumulation in the brain.^{66),84)–87)} Other reports have associated neprilysin gene polymorphisms with the incidence of AD as summarized elsewhere,⁶⁷⁾ although the aging dependent decline of neprilysin activity seems to be a natural process.^{75),88)}

Our findings also indicate that regulation of neprilysin activity in a manner specific to brain regions that are vulnerable to $A\beta$ deposition could provide an effective strategy to reduce $A\beta$ burdens in the brain. The advantages of utilizing neprilysin activity for the purpose of regulating brain $A\beta$ levels were discussed previously.^{70),89)} A relatively straightforward approach in experimental terms, but not necessarily in clinical terms, is the application of gene therapy. Indeed, we demonstrated using an *in vitro* paradigm that overexpression of neprilysin, but not of an inactivated mutant form, in primary cultured neurons caused by the Sindbis virus leads to clearance of both the extracellular and cell-associated $A\beta_{40}$ and $A\beta_{42}$ forms.⁹⁰⁾ We also succeeded in regulating $A\beta$ levels *in vivo* and in reducing $A\beta$ burdens in APP transgenic mice by expressing neprilysin using adeno-associated virus.⁸⁷⁾ Similar results were also reported by the groups of Hersh⁸⁵⁾ and of Masliah.^{91),92)} These approaches however require neurosurgery and thus are quite invasive. In this respect, the success in global brain delivery of neprilysin gene by intravascular administration of AAV vector in mice⁹³⁾ stands as a major breakthrough in the field of gene therapy.

Selkoe and colleagues demonstrated that transgenic overexpression of neprilysin and IDE results in a reduction of the $A\beta$ pathology in APP transgenic mice.⁸⁶⁾ For a number of reasons, the effect of IDE is likely to have been mediated by alteration of APP processing by increased insulin levels rather than by the direct proteolysis of $A\beta$. First, insulin is a better substrate than $A\beta$ for IDE,⁹⁴⁾ and insulin signaling regulates neuronal APP processing and trafficking.^{95),96)} Also, IDE expression failed to reduce $A\beta$ levels in a *Drosophila* model overexpressing $A\beta_{42}$ (not APP), whereas neprilysin expression significantly decreased $A\beta$ levels and inhibited $A\beta_{42}$ -induced neurodegeneration.⁹⁷⁾ The advantages of neprilysin utilization over IDE utilization for the purpose of reducing $A\beta$ levels in the brain are the following: (i) IDE is primarily a cytosolic protein capable of degrading the APP intracellular domain *in vivo* far better than any known substrates⁹⁴⁾ and thus is unlikely to have direct access to $A\beta$ unless

the cells are permeabilized; (ii) Neprilysin degrades extracytoplasmic peptides particularly at synapses^{66),87),98),99)} where $A\beta$ may cause neuronal dysfunction; (iii) The *in vivo* effect of neprilysin on brain $A\beta$ seems to be unexpectedly selective because neprilysin deficiency does not seem to alter the levels of "neprilysin substrate" neuropeptides such as enkephalin, cholecystokinin, neuropeptide Y, substance P and somatostatin in the brain (Iwata & Saido, unpublished data); (iv) Neprilysin can degrade $A\beta$ oligomers, which can impair neuronal plasticity,¹⁰⁰⁾ both in tubes¹⁰¹⁾ and *in vivo*¹⁰²⁾ whereas IDE can degrade only $A\beta$ monomers.¹⁰³⁾

The ability of neprilysin to degrade $A\beta$ oligomer provides an important implication about the spatial relationship between the cause and effect in AD etiology. Even under normal conditions, hippocampus exhibits the highest levels of $A\beta$ in the presence or absence of neprilysin.⁶³⁾ Because neprilysin deficiency increases the quantity of synaptotoxic $A\beta$ oligomers,¹⁰²⁾ oligomer formation is likely primary in AD pathogenesis while plaque formation secondary.

Since the gene therapy approach, which requires surgical procedures, is not yet a realistic approach for clinical application to humans, we have sought a pharmacological means to selectively up-regulate brain neprilysin activity as a new therapeutic candidate.¹⁰⁴⁾ A rationale for this strategy was based on the fact that at least two cell type-specific ligands capable of up-regulating neprilysin activity have been identified: opioids for monocytes¹⁰⁵⁾ and substance P for bone marrow cells¹⁰⁶⁾ as part of negative feedback mechanism. It is notable that receptors for these ligands are G protein-coupled receptors (GPCRs). We found that somatostatin (SST) regulates the metabolism of $A\beta$ peptide in the brain via the modulation of proteolytic degradation catalyzed by neprilysin: Among various effector candidates, only SST up-regulated neprilysin activity in primary cortical neurons; A genetic deficiency of SST altered hippocampal neprilysin activity/localization and increased the quantity of a hydrophobic 42mer form of $A\beta$, $A\beta_{42}$, in a manner similar to presenilin gene mutations that cause familial AD.⁹⁹⁾ These results indicated that SST receptor(s) now emerge as pharmacological target candidates for the prevention and treatment of AD (Fig. 3).⁶⁷⁾

Thus far, five SST receptor subtypes have been identified, all of which are GPCRs,¹⁰⁷⁾ the most suitable pharmacological target category of proteins in the history of pharmaceutical science. Among the five subtypes, types two and four may serve as

primary candidate targets because they are relatively potently expressed in the neocortex and hippocampus.^{107),108)} Synthesis of blood brain barrier-permeable agonists that can distinguish between different receptor subtypes, which should not be an impossible task in modern medicinal chemistry,¹⁰⁷⁾ would make it possible to achieve a medical application to our findings. Alternatively, the use of an “anti-dementia” compound such as FK960 that elevates hippocampal SST levels¹⁰⁹⁾ in brain may provide another effective approach. One potential benefit of harnessing neprilysin activity by agonizing SST receptor(s) among other A β -reducing strategies is that, if used conservatively, it is unlikely to be accompanied by major adverse side effects.

The expression of SST in the brain is known to decline with age in various mammals, including rodents, apes and humans.^{110),111)} In human brains, SST mRNA is one of approximately 50 transcripts, the expression of which significantly declines after the age of 40, among approximately 11,000 transcripts examined.¹¹¹⁾ This indicates that the aging-dependent reduction of SST expression in the brain is a biologically specific and universal process. A prominent decrease in SST also represents a pathological characteristic of AD.¹¹²⁾ These facts, combined with our observations that SST regulates neuronal neprilysin activity, led us to propose the following scenario for the etiology of sporadic AD development.¹¹³⁾ First, the aging-dependent reduction of SST causes a decrease of neprilysin activity, which then causes the steady-state A β levels in brain to increase. Chronic elevation of the A β levels may result in further downregulation of SST levels,¹¹²⁾ oxidative inactivation of neprilysin,⁷⁶⁾ increased expression of APP and β -secretase because APP is a stress-responsive protein¹¹⁴⁾ and because expression both APP and β -secretase have been reported to increase in the relatively downstream cascade of AD development.^{74),115)} These events form a vicious circle leading to a catastrophic accumulation of A β in the brain.^{23)–25)} Expression of cortistatin, a recently discovered SST-like neuropeptide that can activate SST receptors,¹⁰⁷⁾ may also decline upon aging. If this hypothesis turns out to be true, we will not only be able to understand the etiology of sporadic AD but will also have identified a primary strategic target for the prevention and treatment of AD.

5. Proposal for cocktail therapy strategy

Currently, four major, distinct strategies to reduce A β burdens in brain have been proposed:

β -secretase inhibition,⁵⁷⁾ γ -secretase inhibition,⁵⁸⁾ A β vaccination,^{51),116)} and enhanced degradation of A β .^{42),85)–87),104)} Each strategy, if practiced too extensively, is likely to cause chronic adverse effects. For instance, inhibition of β - and γ -secretases will interfere with proteolytic processing of other secretase substrates such as sialyltransferase³⁵⁾ and Notch,^{33),36)} respectively, possibly resulting in perturbation of the immune system. The side effect of A β vaccination has already been reviewed.⁵¹⁾

However, if medications based on these strategies are used at relatively low doses or in a mild fashion so that A β is downregulated by just 30% in each case, then the side effects will probably become negligibly small. Still, if such medications are combined as a cocktail (putting vaccination aside), a very selective and potent effect on the A β level can be expected; a combination of β -secretase inhibitor, γ -secretase inhibitor and neprilysin activator, for instance, at doses that would lower the A β levels by 30% each would bring down A β to 34% ($= 70\% \times 70\% \times 70\%$) or down by 66%, while other substrates down only to 70% (Fig. 5). Indeed, Asai *et al.*¹¹⁷⁾ have shown that a cocktail of secretase inhibitors affects A β levels in an additive manner. Because any medications used to prevent or treat AD would be administered to patients for a long period of time, *i.e.*, a few years or more, we propose that seeking both safety and effectiveness in this cocktail therapy approach would be given high priority as a potential near-future strategy to combat AD. The successful history of the cocktail therapy in the conquest of human immunodeficiency virus¹¹⁸⁾ supports this proposal. This strategy may be extended to the targeting of other downstream pathological processes, such as inflammation,¹¹⁹⁾ tauopathy¹²⁰⁾ and calpain activation^{121)–124)} to achieve maximum effects.

6. Future perspectives

One currently unresolved issue in AD research is the elucidation of the precise mechanism(s) by which A β deposition causes subsequent pathological effects, *i.e.*, tauopathy, dysfunction and degeneration of neurons. Unraveling this mystery will open more opportunities for therapeutic interventions at various time points between A β deposition and neurodegeneration. The accelerating effect of excessive amounts of A β on tauopathy demonstrated in mouse models,^{125),126)} although potentially problematic as stated above, may indicate the presence of an unknown phenomenon that relays pathological signals from the

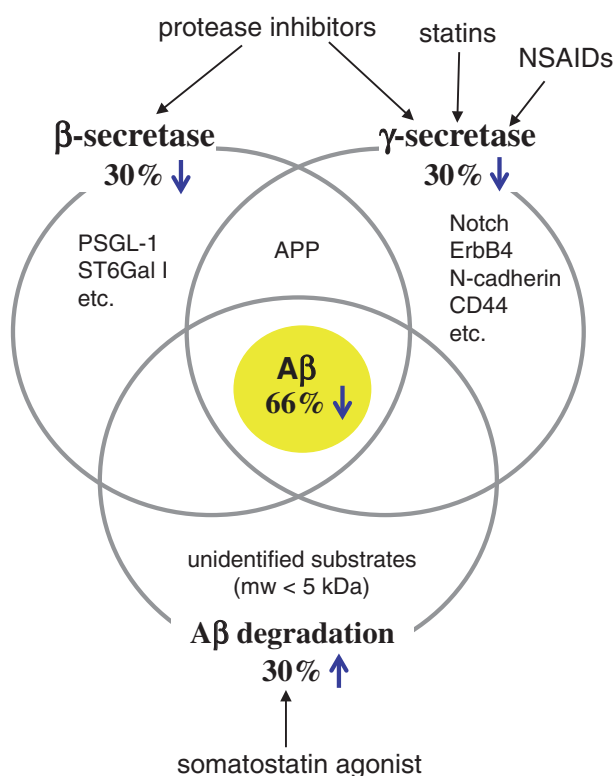


Fig. 5. Proposal for a cocktail therapy strategy targeted at three distinct aspects of A β metabolism. The inhibition of secretases or upregulation of A β degradation to the extent that they independently lower A β levels by 30% is unlikely to generate adverse side effects by influencing the metabolism of substrates other than APP and A β . When used in combined fashion, however, they would achieve a selective 62% decrease of the A β level. Substrates subjected to processing by the target molecule are presented in the circles. NSAIDs: non-steroidal anti-inflammatory drugs; PSGL-1: P-selectin glycoprotein ligand 1; ST-6Gal: sialyltransferase that produces a sialyl α 2,6galactose residue.

former to the latter. However, perhaps the most important and fundamental question yet to be answered is why A β is deposited in sporadic AD which accounts for 99% or more of all AD cases. It should be noted that the number of sporadic AD patients will grow as the average life expectancy increases, whereas the number of familial AD patients will simply remain proportional to the total population. Although our hypothesis described in Section 4 is one of the relevant possibilities, other possibilities also need to be pursued.

Recent studies indicate that virtually all humans start to accumulate A β in the brain upon aging.^{5),23)–25)} This suggests that A β deposition is an important factor that could determine the life span of our brain. It provides us with the hope that a large

portion of AD cases can be prevented if we can make the brain life span longer than the body life span by reducing A β deposition (Fig. 6). The other way of looking at brain aging and Alzheimer's disease is that aging causes abnormal proteolysis, resulting in abnormal deposition of proteins followed by neuronal dysfunction and degeneration as schematized in Fig. 7. The importance of proteolysis in AD pathogenesis is obvious because both generation (Fig. 1) and degradation (Table 1) of A β are performed by proteases and because proteases such as calpain¹²⁷⁾ and caspase^{128),129)} may be involved in the downstream pathological cascade. The importance of proteolysis has been highlighted in other neurodegenerative disorders including Parkinson's disease as well; loss-of-function mutations associated with juvenile Parkinsonism have been identified in the gene encoding Parkin,¹³⁰⁾ which functions as an E3 ubiquitin ligase involved in the proteasome-dependent proteolysis.¹³¹⁾ Although Parkin mutation carriers generally do not display the neuropathology of Lewy bodies composed of α -synuclein,¹³²⁾ it is possible that impairment of proteolytic α -synuclein metabolism due to as yet unidentified mechanism(s) could be a cause of Lewy body formation in most cases of sporadic Parkinson's disease. It is also true that the mechanism that accounts for normal tau metabolism has not yet been identified; tau metabolism is difficult to analyze because tau exists in two forms, microtubule-bound and unbound, in neurons. Identification of protease(s) involved in normal tau metabolism may lead to elucidation of the mechanism of tauopathy formation and contribute to the development of a more relevant AD mouse model than is provided presently by transgenic mice.

Figure 7 also indicates that there are a number of potential therapeutic targets for the prevention and treatment of dementing disorders. If it becomes possible to pre-diagnose the preclinical AD, a pre-symptomatic intervention can be initiated that includes the A β -reducing strategies described in Fig. 5. Because the conversion of "normal aging" to AD via preclinical AD and MCI appears to be a continuous process caused primarily by the gradual acceleration of A β accumulation, we may even be able to partially control some very significant aspects of brain aging by maintaining low A β levels throughout our lives (Figs. 2 and 6). Recently, Mawuenyega and colleagues observed decreased clearance rather than increased production of A β in sporadic AD patients,¹³⁸⁾ suggesting up-regulation of A β degradation may indeed be an effective

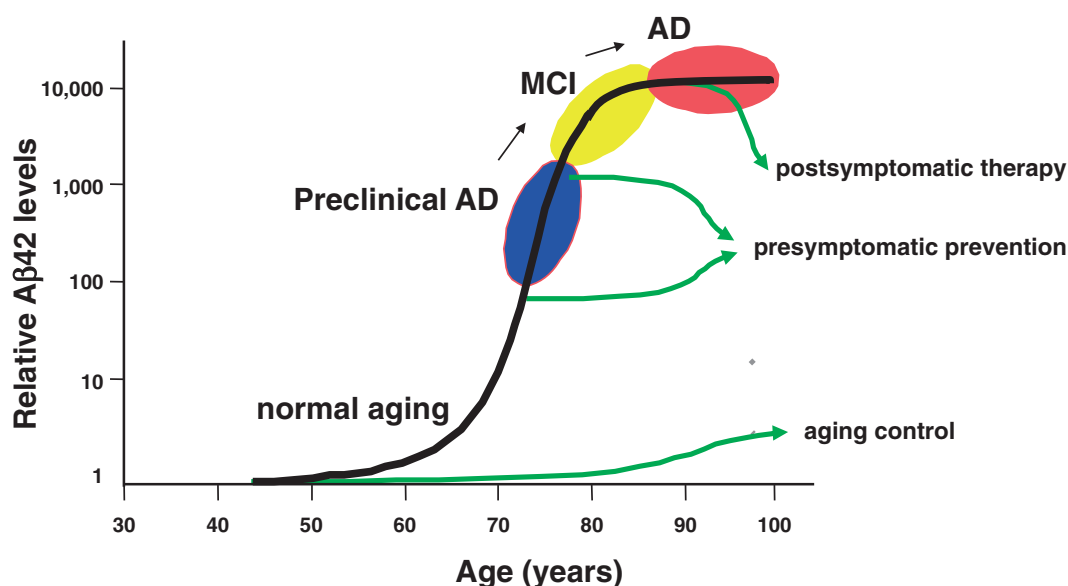


Fig. 6. Relationship between $A\beta$ deposition and normal aging, preclinical AD, MCI and AD. Treatment after the onset, during preclinical AD and normal aging would be postsymptomatic therapy, presymptomatic prevention and aging control.

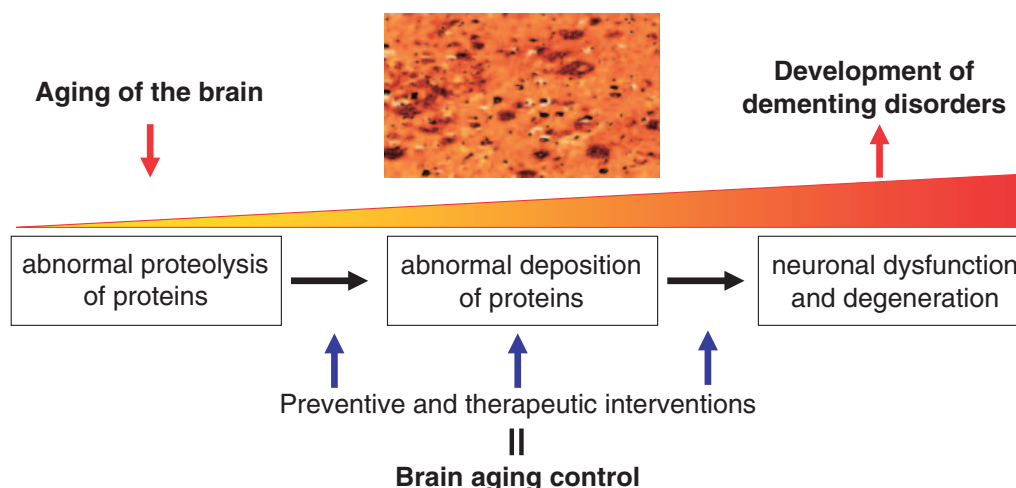


Fig. 7. Brain aging, proteolysis, protein deposition, neuronal dysfunction/degeneration and dementia. See Section 6.

preventive measure. We also need to learn a lesson from what has happened to the $A\beta$ vaccination.⁵¹⁾ We need to be patient and cautious in bringing the knowledge obtained from experimental science to clinical applications; we should move from mice to humans only after confirming effectiveness and safety of a given approach in non-human primates.

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Profile

Takaomi C. Saido was born in 1959 and started his research career in 1988 with studies on the biological and pathological roles of calpain, calcium-activated neutral protease, at Tokyo Metropolitan Institute of Medical Science. One of the pioneering works has been to devise antibodies that distinguish proteolytic products from their intact precursors. In particular, antibodies specific to calpain-cleaved spectrin have been used by a number of researches all over the world. He further applied this technique to processing of amyloid precursor protein (APP) and amyloid β peptide (A β) that are closely associated with pathogenesis of Alzheimer's disease (AD). He discovered that ischemic conditions accelerates amyloidogenic processing of APP and that the major A β species that accumulates in aged and AD brains starts with pyroglutamate (pE) at position 3. A β _{3(pE)-42} is now considered to be one of the most toxic A β species. He then moved to RIKEN Brain Science Institute to start a new laboratory as a laboratory head in 1977 to further develop the research on AD and calpain. As a milestone work in the AD research field, he and colleagues have discovered that a neutral endopeptidase, neprilysin, is a major A β -degrading enzyme in brain. This finding has led to successful gene therapy of AD using mouse models. Preclinical and clinical trials are expected to bring this gene therapy to reality for prevention and treatment of AD in the near future. Other findings at RIKEN include the following: (1) *In vivo* detection of A β plaques by MRI; (2) Regulation of neprilysin activity by somatostatin; (3) Deterioration of A β pathology by calpain hyperactivation; (4) Generation of single **APP** locus knockin mouse models that overproduce A β without overexpressing APP. He was awarded Young Investigator's Award from the Japanese Biochemical Society in 1995 and Tokizane Award from the Japan Neuroscience Society in 2007. He serves as a board member for Japan Society for Dementia Research.

