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Disruption of Calcium Homeostasis in Alzheimer's Disease: Role of Channel Formation by β Amyloid Protein

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1. Introduction

Alzheimer's disease (AD) is a severe senile type of dementia that affects a large number of elderly people. It is estimated that 5.4 million people in the worldwide are affected by this disease in 2011 and this number continues to grow yearly. AD is characterized by profound memory loss and severe cognitive decline. The pathological hallmarks of AD are the presence of numerous extracellular deposits termed senile plaques and intraneuronal neurofibrillary tangles (NFTs) (Selkoe, 1991). The selective loss of synapses and neurons is observed in the brain of patients, and this decrease in the number of synapses is strongly correlated with memory impairment (Terry et al., 1991). The major component of NFTs is the phosphorylated tau protein, while β-amyloid protein (AβP) is the major component of senile plaques.

Although the pathological cause of AD has not yet strictly been elucidated, numerous biochemical, toxicological, cell biological, and genetic studies have supported the "amyloid cascade hypothesis", which suggests that the neurotoxicity and synaptotoxicity (synaptic degeneration) caused by AβP play a central role in AD (Hardy & selkoe, 2002). Moreover, oligomerization and conformational changes in AβP are important for its neurodegenerative capacity.

There is considerable interest regarding the mechanism by which AβPs cause neurodegeneration. AβPs reportedly cause numerous adverse effects on neuronal survival, e.g., reactive oxygen species (ROS) production, cytokine induction, endoplasmic reticulum (ER) stresses induction, and the abnormal increase in intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) (Small et al., 2001). These adverse effects are complex and may be interwoven. Among them, the disruption of Ca^{2+} homeostasis could be the primary event in the pathogenesis of AD, since Ca^{2+} ions play critical roles in the function and structure of neurons and other cells. Increasing evidence suggests the implications of Ca^{2+} dyshomeostasis in the pathogenesis of AD (Green and LaFerla, 2008; Demuro et al., 2010).

Several possible mechanisms account for AβP-induced Ca^{2+} dyshomeostasis, e.g., its interaction with endogenous Ca^{2+}-permeable ion channels, disruption of membrane integrity, and the formation of Ca^{2+} permeable channels (pores) by the direct incorporation of AβP into membranes. Here, we focus on the 'amyloid channel hypothesis' (Arispe et al., 2007; Lin et al. 2001; Kawahara, 2011b), namely, that the direct incorporation of AβPs and the subsequent imbalances of Ca^{2+} and other ions through amyloid channels may be the primary event in AßP neurotoxicity.

In this chapter, we review the current understanding of the link between Ca^{2+} homeostasis and AD pathogenesis based on the amyloid channel hypothesis the characteristics of AβP-induced neurotoxicity and synaptotoxicity caused by Ca^{2+} dyshomeostasis *via* amyloid channels. We also discuss the possible development of new drugs for the treatment and prevention of AD by attenuating amyloid channels and inhibiting AβP-induced Ca^{2+} dyshomeostasis.

2. Amyloid cascade hypothesis

AβP is a small peptide with 39–43 amino acid residues. It is derived from the proteolytic cleavage of a large precursor protein (amyloid precursor protein; APP) by the cleavage of its N-terminal by β-secretase (BACE), followed by the intra-membrane cleavage of its C-terminal by γ-secretase. There are several species of AβP, such as AβP(1–40), the first 40 amino acid residues, or AβP(1–42), which are generated by the different cleavage processes in its C-terminal domain. Genetic studies of early-onset cases of familial AD indicated that APP mutations and the consequent changes in AβP metabolism are associated with AD (Goate et al., 1991). Moreover, mutations in the presenilin genes also account for the majority of cases of early-onset familial AD (Sherrington et al., 1995). Presenilins have been identified as one of γ -secretase proteins, and their mutations also influence the production of AβP and its neurotoxicity (Selkoe and Wolfe, 2007).

Yankner *et al.* reported that AβP(1–40) caused the death of cultured rat hippocampal neurons and the neurodegeneration in the brains of experimental animals (Yankner et al., 1991). A smaller fragment of AβP (AβP (25–35)) or a longer variant (AβP (1–42)) were also reported to cause neuronal death. AβP is a hydrophobic peptide with an intrinsic tendency to self-assemble to form sodium dodecyl sulfate (SDS)-stable oligomers. In an aqueous solution, freshly prepared and dissolved AβP exists as a monomeric protein with a random coil structure. However, following incubation at 37°C for several days (*aging*), AβPs form aggregates (oligomers) with β-pleated sheet structures, and finally form insoluble aggregates, termed amyloid fibrils (Fig. 1). Pike et al. revealed that *aged* AβP(1–40) peptides were considerably more toxic to cultured neurons than *fresh* (freshly prepared just before the experiment) AβP(1–40) (Pike et al, 1991). The ß-sheet content of AßP solutions, as determined by circular dichroism (CD) spectroscopy, correlates with its neurotoxicity (Simmons et al., 1994). Jarrett and Lansbury demonstrated that the longer peptide variant, AβP(1–42), polymerizes much quicker than AβP(1–40) (Jarrett and Lansbury, 1993). AβP(1–42) enhances the aggregation of AβP(1–40) and functions as a "seed" for amyloid fibrils. AβP (1–42) is more abundant in the brains of AD patients than in age-matched controls. The point mutations of APP are located near the γ-secretase cleavage-site and influences the ratio of AβP(1–40) and AβP(1–42). Mutations of APP and the presenilin genes increase the production of AβP (1–42) in the transfected cell lines.

Recent studies on the identified AβP species further strengthened and refined the amyloid cascade hypothesis. Approaches using size-exclusion chromatography, gel electrophoresis, and atomic force microscopy (AFM) have demonstrated that there are several stable types of oligomers: naturally occurring soluble oligomers (dimers or trimers), AßP-derived diffusible ligands (ADDLs), AβP globulomers, and protofibrils. Walsh et al. found the existence of SDS-stable oligomers in the conditioned medium of cultured cells transfected with the human APP gene. The intracerebral administration of these SDS-stable low-molecular-weight oligomers (dimers, trimers, or tetramers) inhibited long-term potentiation (LTP), which is a form of synaptic information storage that is a well-known paradigm for the mechanisms underlying memory formation (Walsh and Selkoe, 2007). They also demonstrated that LTP was not blocked by AβP monomers or larger aggregates. Natural AβP oligomers derived from the cerebrospinal fluid of AD patients induced the loss of dendritic spines and synapses, and also blocked the oligomers in the conditioned medium. Klein and the colleagues reported that ADDLs obtained by sedimentation with clusterin are highly toxic to cultured neurons. They also reported that ADDLs inhibited LTP and exhibited adverse effects on synaptic plasticity e.g., decreased spine density, abnormal spine morphology, and decreased levels of synaptic proteins (Lacor et al., 2007). Tomiyama et al. found a new AβP variant (AβP E42Δ) that exhibited enhanced oligomerization but no fibrillization (Tomiyama et al., 2008). This unique variant AβP decreased synaptophysin immunostaining and blocked LTP. Since synaptic plasticity is crucial for the process of memory formation and is involved in the early stages of AD, these lines of evidence indicate that the synaptic impairment induced by AβP oligomers is the primary event in the memory impairment observed in AD patients.

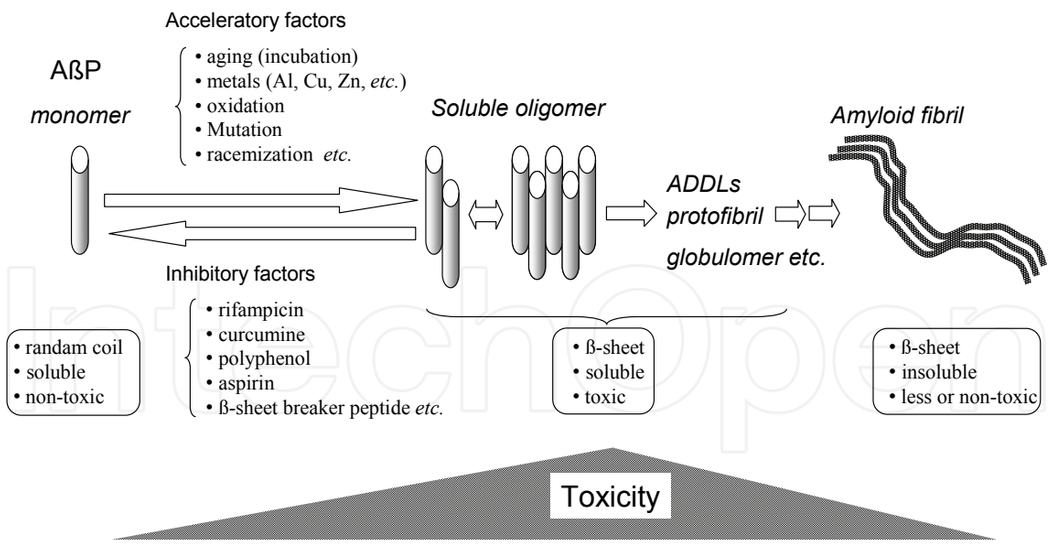


Fig. 1. Oligomerization of AβP

AβP monomers exhibit random-coil structures. However, during aging or in the presence of some acceleratory factors, AβP self-aggregates and forms several types of oligomers (SDS-soluble oligomers, ADDLS, globulomers, protofibrils, *etc.*) and finally forms insoluble aggregates, which are termed amyloid fibrils. Oligomeric soluble AβPs are toxic, although monomers and fibrils are rather nontoxic.

AβP is secreted into the cerebrospinal fluid (CSF) of young individuals as well as in aged or dementia patients (Fukuyama et al., 2000). Therefore, factors that accelerate or inhibit AβP oligomerization may play essential roles in the pathogenesis of AD. Several factors such as peptide concentration, the pH or composition of solvents, and temperature can influence the oligomerization processes. In addition, oxidations, mutations, and racemization of AβP enhance its oligomerization. Substances that can influence the oligomerization processes, e.g., cholesterol or its oxidation products, transthyretin, rifampicin, curcumin, aspirin, docosahexaenoic acid (DHA) and peptides such as the β-sheet breaker peptide, reportedly inhibit AβP oligomerization *in vitro*.

Among these factors, Al is of particular interest considering its epidemiological link with AD (Kawahara and Kato-Negishi, 2011). Al^{3+} has strong positive charges and a relatively small ionic radius in comparison to other metal ions; thus, Al^{3+} firmly binds to metal-binding amino acids (e.g. histidine, tyrosine, arginine *etc.*) or phosphorylated amino acids and acts as a cross-linker. Owing to this property, Al can cause the aggregation of various proteins and induce the conformational changes. Exposure to Al causes the accumulation of AβP in cultured neurons and in the brains of experimental animals and humans (Pratico et al., 2002; Exley and Esiri, 2006).

3. Alzheimer's Disease and calcium homeostasis

Ca^{2+} is required for various normal brain functions and is a component of key enzymes such as kinases, phosphatases, and proteases, it is highly possible that Ca^{2+} dyshomeostasis could be the earliest adverse event among AβP-induced various adverse effects such as ROS production, cytokine induction, endoplasmic reticulum (ER) stresses induction. Once neuronal Ca^{2+} homeostasis is disrupted and $[Ca^{2+}]_i$ is changed, various apoptotic pathways, such as Ca^{2+}-activated neutral protease (calpain) and caspase, are activated, leading to neuronal death. The disruption of Ca^{2+} homeostasis can trigger membrane disruption, the formation of free radicals, and the induction of other adverse effects, which are often observed after exposure to AβP. It is widely recognized that an increase in $[Ca^{2+}]_i$ induces changes in the number and morphology of synapses and spines, and that an imbalances of Ca^{2+} in synapses directly influences neuronal activity and synaptic impairment. Considering that APP localizes in synapses and that AβP is secreted from APP into synaptic clefts by neuronal excitation, the adverse effects caused by AβP-induced Ca^{2+} dyshomeostasis can occur in synaptic compartments and induce synaptotoxicity. Therefore, the influx of Ca^{2+} is tightly controlled and $[Ca^{2+}]_i$ levels are strictly regulated by various mechanisms including voltage-dependent Ca^{2+} channels (VDCC), and neurotransmitter receptors e.g., glutamate (Glu) and acetylcholine (ACh) (Zorumski and Thio, 1992). Moreover, the ER and mitochondoria represent the major intracellular stores of Ca^{2+} (Green and LaFerla, 2008; Leuner et al., 2007).

An increasing amount of data indicates that exposure to AβP causes an abnormal increase in $[Ca^{2+}]_i$ in intoxicated neurons. There is considerable interest regarding the mechanism by which AβPs interact with neurons and disrupts Ca^{2+} homeostasis (Demuro et al., 2010). There are several possible mechanisms that account for AβP-induced Ca^{2+} dyshomeostasis, e.g., interaction with endogenous Ca^{2+}-permeable ion channels, disruption of membrane integrity, and formation of Ca^{2+}-permeable channels (pores) by the direct membrane incorporation of AβP (Fig.6). It is possible that AβP directly binds to membranes and causes their disruption, or that AβP-induced ROS impairs membrane structures.

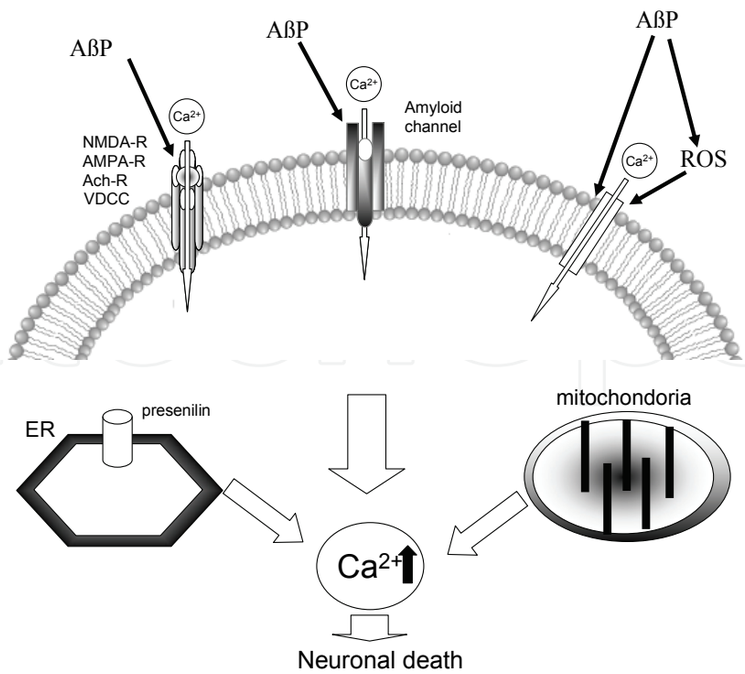


Fig. 2. AβP-induced calcium dyshomeostasis in neurons. It is possible that AßP-induced Ca^{2+} dyshomeostasis by (i) its interactions with endogenous Ca^{2+}-permeable ion channels, (ii) disruption of membrane integrity, and (iii) formation of Ca^{2+} permeable channels (pores) by the direct membrane incorporation of AβP. AβP can directly cause membrane disruption, or AβP-induced ROS can impair membrane structure. Presenilins in the ER or mitochondria can participate in the abnormal increase in $[Ca^{2+}]i$ in neurons

AβP reportedly binds to *N*-methyl D-aspartate (NMDA)-, and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) -type glutamate receptors (Parameshwaran et al., 2008), and nicotinic ACh receptors (Parri and Dineley, 2010). All of these receptors contain Ca^{2+} channels and regulate $[Ca^{2+}]_i$ during membrane depolarization. AβP also influences voltage-gated Ca^{2+} channels and the inositol triphosphate (IP_3) receptor. The influx of Ca^{2+} from the ER is regulated by ryanodine-type Ca^{2+} channels, the sarcoplasmic reticulum Ca-ATPase (SERCA), and presenilins (Green et al., 2008). Presenilins are found in the ER membrane, and are involved in capacitative Ca^{2+} entry, ER Ca^{2+} signaling, Ca^{2+} leakage from the ER, and mitochondrial Ca^{2+} signaling (Querfurth and LaFerla, 2010).

4. Formation of Ca^{2+} permeable pores by AßP

Our and other numerous studies have demonstrated that AβPs are directly incorporated into the surfaces of cellular membrane and create unregulated cytotoxic pore-like channels. In 1993, Arispe et al. first demonstrated that AβP(1–40) directly incorporates into artificial lipid bilayer membranes and forms cation-selective ion channels (Arispe et al., 1993a, 1993b). These channels termed amyloid channels were revealed to be giant multilevel pores that can facilitate the transport of large amounts of Ca^{2+}. Their activity was blocked by Zn^{2+} ions, which are abundant in the brain (Arispe et al., 1996). Electrophysiological studies have revealed that other neurotoxic peptide fragments of AβP including AβP(25–35) and

AβP(1–42) form Ca^{2+}-permeable pores in artificial lipid bilayers. The C-terminal fragment of APP (CT_{105}) including AβP also formed ion channels in Xenopus oocytes (Fraser et al., 1996).

Durell et al. proposed a 3-D structural model of amyloid channels obtained from a computer simulation of the secondary structure of AβP(1–40) in membranes that showed the aggregation of 5- to 8-mers to form pore-like structures on the membranes (Durell et al., 1994). Jang et al. established a model for amyloid channels on membranes and observed the pentameric AβP forms pores (Jang et al, 2009). Strodel et al. proposed a model of AβP(1–42) pores which consist of tetrameric and hexameric β-sheet subunits from the observations in NMR (Strodel et al., 2010). The dimension, shape, and subunit organization of these models were in good agreement with the morphological observations using high resolution AFM that demonstrated that AβPs form pore-like structures on mica plates or on reconstituted membranes (Lal et al., 2007, Jang et al., 2010). Furthermore, the presence of pore-like structures of AßPs *in vivo* was demonstrated in the neuronal cell membrane of the brains of AD patients and of AD-model mice. Using high resolution transmission electron microscopy, Inoue observed in situ AßP pores in the neuronal cell membrane in AD brains (Inoue, 2008). Kayed *et al*. reported that the annular protofibrils (APFs) of AßP exhibit ring-shaped and pore-like structures (Kayerd, et al., 2009). The age-dependent accumulation of APFs was observed on the membranes of AD model mice (APP transgenic mice; APP23) (Kokubo et al., 2009).

It is important to determine whether AβPs form channels in neuronal cell membranes in addition to artificial lipid bilayers. To address this issue, we employed membrane patches from a neuroblastoma cell line (GT1-7 cells), and found that AβP(1–40) formed amyloid channels on GT1-7 cell membranes (Kawahara et al., 1997). GT1-7 cells (immortalized hypothalamic neurons) are derived from murine hypothalamic neurons by site-directed tumorigenesis and exhibit several neuronal characteristics such as the extension of neurites and the expression of neuron-specific proteins and receptors (Mellon et al., 1990). The features of amyloid channels formed on GT1-7 membranes were considerably similar to those observed in artificial lipid bilayers; cation-selective, multilevel, voltage-independent, and long-lasting; channel activity was inhibited by the addition of Zn^{2+}, and recovered by the addition of zinc chelator *o*-phenanthroline. Moreover, Sepulveda et al. revealed that AβP(1-40) formed perforations on membranes excised from hippocampal neurons and induced currents (Sepulveda et al., 2010). The effect of AβP was similar to that of gramicidin and amphotericin which are commonly used to perforate neuron membranes.

Furthermore, we have revealed that AβP directly caused the disruption of liposomal membrane vesicles by observing the release of fluorescent dye (Kawahara et al., 2011b). These results are consistent with the findings that AßP causes membrane disruption, increases membrane permeability, causes hemolysis, and changes membrane fluidity (Eckert et al., 2005).

These results strongly support the hypothetical idea termed amyloid channel hypothesis namely, that the direct incorporation of AβPs and the subsequent imbalances of Ca^{2+} and other ions through amyloid channels may be the primary event in AßP neurotoxicity.

5. Disruption of calcium homeostasis by amyloid channels

In order to test the validity of the amyloid channel hypothesis more precisely, we examined whether AβP alters the $[Ca^{2+}]_i$ levels of GT1-7 cells under the same conditions, using a high-

resolution multi-site video imaging system with Ca^{2+}-sensitive fluorescent dye (fura-2) (Kawahara et al., 2000; Kawahara & Kuroda, 2001). We also observed AβP-induced abnormal increases in $[Ca^{2+}]_i$ in primary cultured rat hippocampal neurons (Kato-Negishi & Kawahara, 2008). Shown in figure 3 are temporal changes of $[Ca^{2+}]_i$ in GT1-7 cells before and after exposure to AβP(1-40) and related peptides. Although a marked increase in $[Ca^{2+}]_i$ was caused by AβP(1–40) (*line (a)*), AβP(1–42) (*line (c)*), and AβP(25–35) (*line (e)*), no remarkable changes were induced by AβP(40–1), control peptide with no toxicity (*line (b)*). There is controversy over whether the AβP-induced $[Ca^{2+}]_i$ changes occur through receptor-mediated pathways or amyloid channels formed by direct incorporation of AβP. To clarify the precise characterization of the AβP-induced $[Ca^{2+}]_i$ changes, we performed detailed and quantitative analysis of the AβP-induced Ca^{2+} influx using a high-resolution multi-site video imaging system. This multisite fluorometry system enables the simultaneous long-term observation of temporal changes in $[Ca^{2+}]_i$ in more than 50 neurons. There are 5 major pieces of evidence supporting the hypothesis that AßP-induced $[Ca^{2+}]_i$ changes occur through amyloid channels. *First*, the AβP-induced $[Ca^{2+}]_i$ rise was highly heterogeneous among genetically identical GT1-7 cells. Even in the same field of view, exposure to the same peptide solution produced differential changes in the $[Ca^{2+}]_i$ levels (Fig. 4A). Considering the heterogeneity, we compared the peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) induced by AβPs, and its latency (the lag between the $[Ca^{2+}]_i$ increase and the time of AβP addition) in each cell to quantitatively analyze Ca^{2+} influx.

Although AβP (1–40) induced an increase in $[Ca^{2+}]_i$ levels either instantly or after some delay, the magnitude and latency differed. In addition, some adjacent cells still did not exhibit any responses. *Second*, the average $\Delta[Ca^{2+}]_i$ was increased and the average latency was shortened in a dose-dependent manner (Fig. 4B and C). These features are considerably similar to those observed in relation to toxic peptide channels formed on membranes. *Third*, the AβP-induced increase in $[Ca^{2+}]_i$ was not influenced by the addition of a Na^+ channel blocker (tetrodotoxin), a Ca^{2+} channel blocker (nifedipine), a glutamate receptor antagonist (D-APV), or a γ-aminobutyric acid (GABA) antagonist (bicuculline). However, antibodies to AβP remarkably inhibited the $[Ca^{2+}]_i$ increase resulting from AβP (Kawahara, 2004). *Fourth*, D-AβP(1–40), AβP(1–40) composed of D-amino acid residues, also caused the elevation of $[Ca^{2+}]_i$ in a manner similar to AβP(1–40) (see Fig. 3, *line (d)*). *Fifth*, the vulnerability of primary cultured rat hippocampal neurons to AβP was changed during the culture period, despite that the expression and the function of neurotransmitter are not changed (Kato-Negishi and Kawahara, 2008). On the basis of these lines of evidence, we conclude that AβP causes the disruption of Ca^{2+} homeostasis *via* the formation of amyloid channels in membranes, ultimately resulting in neuronal death.

Pore formation-induced cytotoxicity is commonly observed in our biological system, particularly in the presence of certain toxins and venoms including the α-toxin of *Staphylococcus aureus*, magainin 2, a 26-residue antimicrobial peptide obtained from *Xenopus laevis*, melitin, a bee venom composed of 28 amino acids, or antibiotics such as amphotericin and gramicidin (Bechinger, 1997). In this respect, AβPs may share the similar toxicity mechanism as various antimicrobial or antifungal peptides that exhibit pore-forming ability and cell toxicity. Indeed, Soscia et al. demonstrated that AβP exerts antimicrobial activity against 8 common and clinically relevant microorganisms (Soscia et al., 2010).

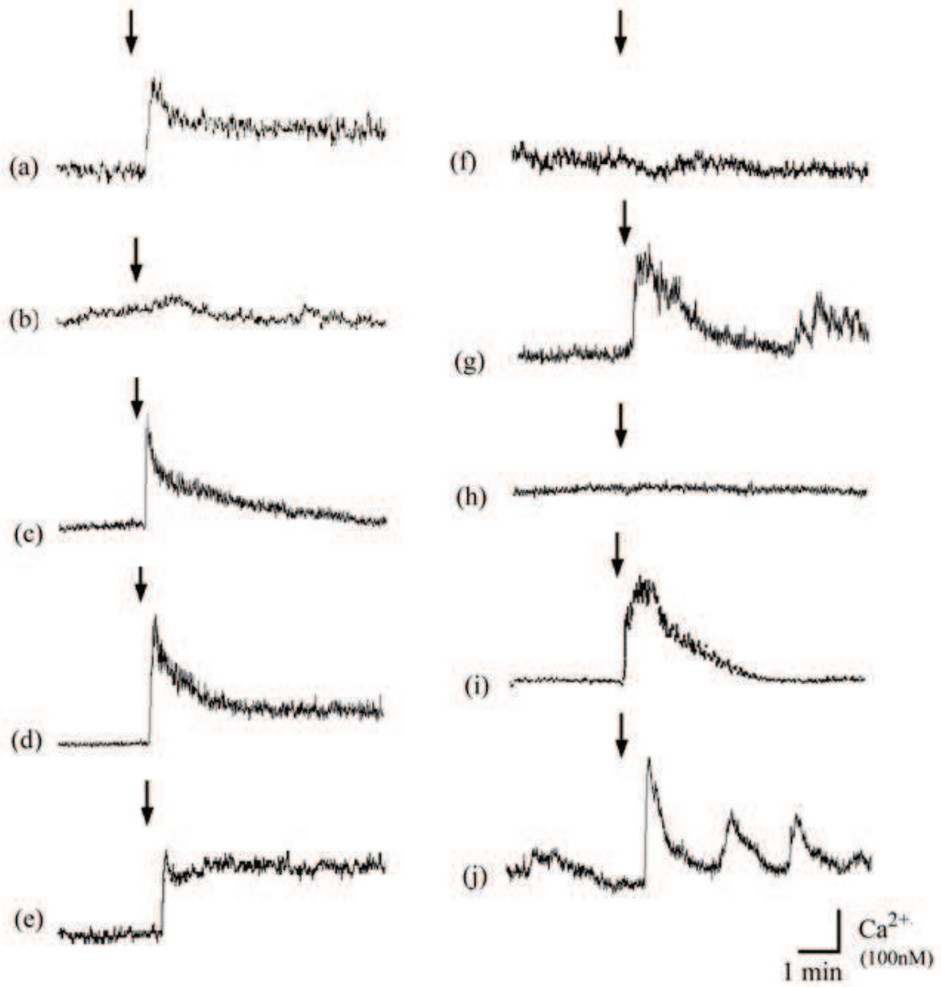


Fig. 3. Characteristics of the elevations in $[Ca^{2+}]_i$ induced by AβP and other amyloidogenic proteins. Typical time course of $[Ca^{2+}]_i$ at 2 min prior to and at 3 min after the application of the peptide is depicted. (a) AßP(1-40); (b) AßP(40-1); (c) AßP(1-42); (d) D-AßP(1-40); (e) PrP106-126; (f) scramble PrP106-126; (g) human amylin; (h) rat amylin; (i) NAC; and (j) magainin 2. All peptides were at 10 μM. The arrow indicates the time of peptide addition

Recently, a new concept of "conformational disease," had been proposed, suggesting that the conformation of disease-related proteins (amyloidogenic proteins) is an important determinant of their toxicity, and consequently, the disease development (Carrell and Lomas, 1997). The conformational diseases includes prion diseases, triplet-repeat diseases, e.g., Huntington's disease, Parkinson's disease and other neurodegenerative diseases that can be categorized under dementia with Lewy bodies (DLB). Increasing evidence indicates that most of these disease-related amyloidogenic proteins or their peptide fragments are directly incorporated into membranes to form ion channels as well as AβP (Lashuel and Lansbury, 2002; Kawahara et al., 2011b). It was also demonstrated that AßP (1–40), α-synuclein, amylin, ABri, or other amyloidogenic peptides morphologically similar common ion channel-like structures and elicit single channel currents using AFM, CD, gel electrophoresis, and electrophysiological recordings (Quist et al., 2005; Lal et al., 2007).

We have also demonstrated that these amyloidogenic peptides including PrP106–126 (a peptide fragment of prion protein), human amylin, NAC, or antimicrobial peptide magainin2 also caused an elevation in the $[Ca^{2+}]_i$ levels similar to that induced by AβPs (Kawahara et al., 2000; Kawahara, 2004). However, rat amylin and a peptide a randomized PrP106–126 sequence (scrambled PrP106–126) did not induce any $[Ca^{2+}]_i$ changes (Fig. 3). We have also demonstrated that PrP106-126 forms β-sheet structures and exhibits neurotoxicity as well as AβP (Kawahara et al., 2011a). In addition, oligomeric α-synuclein causes neuronal death *via* the Ca^{2+} influx (Danzer et al., 2007). Considering these results

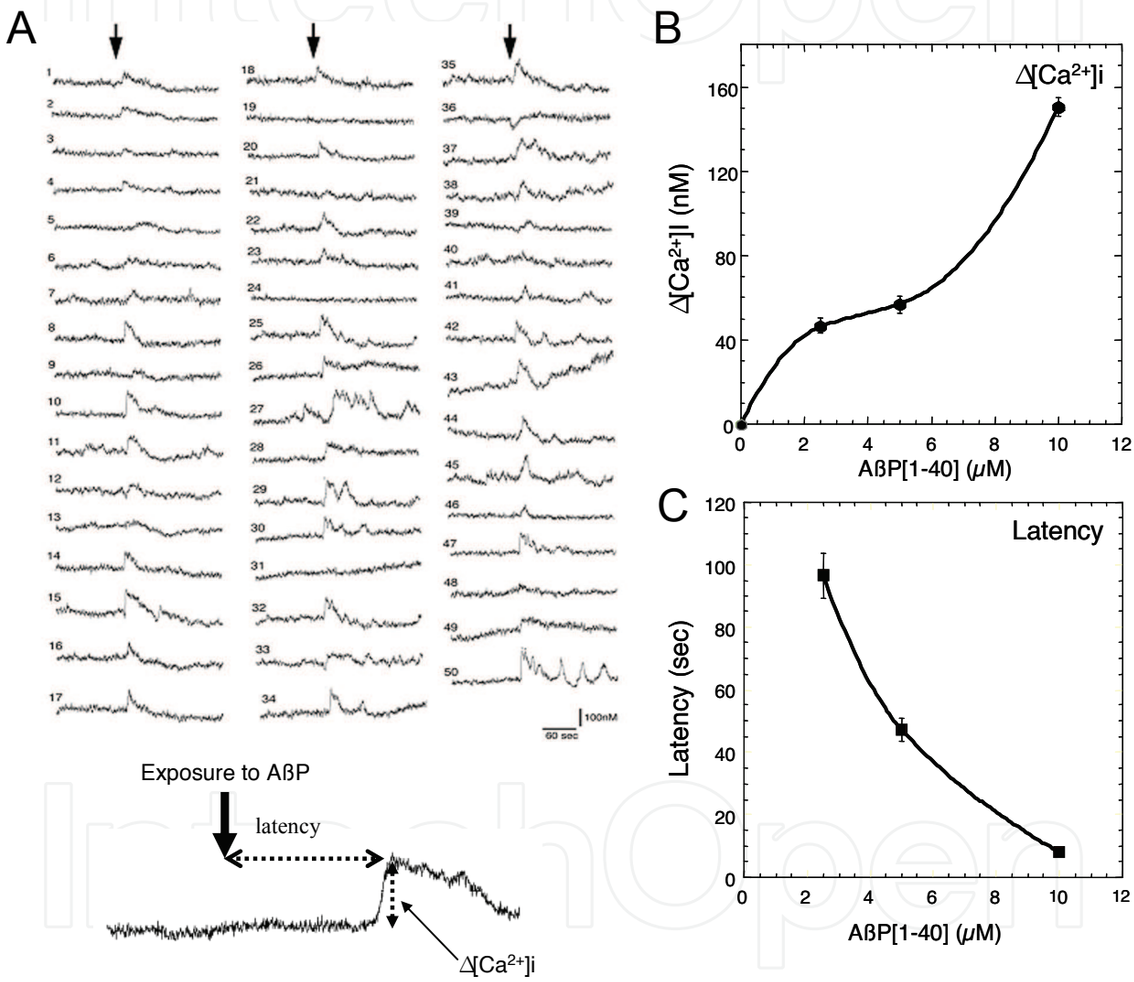


Fig. 4. (A) Heterogeneity of AβP-induced changes in $[Ca^{2+}]_i$ Temporal changes of 50 randomly chosen GT1-7 cells in the same field of view before and after the exposure to AβP(1–40). The arrow indicates the time of peptide addition. B~C: The peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) in each cell and the latency after exposure to AβP (1–40) were analyzed (B) in more than 50 cultured neurons in a single field of view (360×420 μm^3) (mean ± S.E.M., n=300). Typical responses of $[Ca^{2+}]_i$ in cultured neurons following exposure to various concentrations of AβP (1–40) (2.5~10 μM). The peak increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) in each cell (C) and the latency (D) after exposure to AßP (1–40)

together, it is plausible that these disease-related amyloidogenic proteins share similarities in channel formation and the disruption of Ca^{2+} homeostasis as well as β-sheet formation and cytotoxicity. Table 1 summarizes the common properties of these proteins.

Disease	Amyloidogenic protein or its fragment peptide and the primary sequence	ß-sheet formation	Cytotoxicity	Channel formation	Morphological pores	$[Ca^{2+}]_i$ rise
Alzheimer's disease	*AßP(1-40)* DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL MVGGVV	+	+	+	+	+
	AßP(40-1) VVGGVMLGIIAGKNSGVDEAFFVLKQHHVEYGS DHRFEAD	–	–	–	n.d.	–
	AßP(25-35) DVGSNKGAII	+	+	+	+	+
	AßP(1-42) DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGL MVGGVVIA	+	+	+	n.d.	+
Prion disease	*PrP106-126 (prion protein fragment)* KTNMKHMAGAAAAGAVVGGLG	+	+	+	n.d.	+
	Scramble PrP106-126 NGAKALMGGHGATKVMVGAAA	–	–	–		–
Parkinson's disease (DLB; diseases with Lewy bodies)	*α-synuclein* *NAC (α fragment of α-synuclein)* EQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFV	+	+	+	+	+
Triplet-repeat disease	*Polyglutamine* QQQQQQQQ----------	+	+	+	n.d.	n.d.
Familial British dementia	*ABri35* ASNCPAIRHPGNKPAVGTLICSRTVKKNIIGGN	+	+	+	+	n.d.
Diabetes mellitus	*Human amylin* KCNTATCATQRLANFLVHSSNNFGAILSSTNVGS NTY	+	+	+	+	+
	Rat amylin KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGS NTY	–	–	–		–
Medullary carcinoma of the thyroid	Calcitonin CGNLSTCMLGTYTQDFNKFHTFPQTAIGVGAP	+	+	+	+	+

n.d.: not determined

Table 1. Characteristics of amyloidogenic proteins and the related peptides

6. Possible candidates for the treatment of AD

The search for protective agents against AβP-induced neurotoxicity is of great importance. Substances that prevent the oligomerization of AβP such as rifampicin, curcumin, aspirin, DHA can be potential candidates against AβP neurotoxicity (Fig. 2). Reduction of AβP production using BACE or γ-secretase inhibitors is reportedly effective for the treatment of AD. In addition, even though AβP vaccines had been associated with adverse effects, they may be considered as a potential treatment alternative. Conversely, trace metals such as Al, Zn, and Cu enhance AβP oligomerization; thus, chelation therapy with clioquinol, deferoxamine, or silicates has been proposed to be effective in the treatment of AD (Lannfelt et al., 2008; Exley, 2007).

Here, we focused on substances that inhibit the formation of amyloid channels. As discussed, the elevation of $[Ca^{2+}]_i$ by its permeation through amyloid channels is considered to be the primary event of AβP-induced neurotoxicity; therefore, such compounds could serve as the foundation for the new and effective drugs with fewer adverse effects.

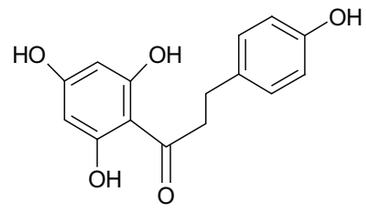
Inorganic cations such as Zn^{2+} inhibit the current induced by amyloid channels. Zn is abundant in presynaptic terminal vesicles and is secreted into synaptic clefts following neuronal excitation. Considering that Zn binds to the His residues of AβP, Arispe et al. found that His-related peptide derivatives, such as His-His, effectively inhibit the current through amyloid channels, attenuate AβP-induced $[Ca^{2+}]_i$ changes, and protect neurons from AβP toxicity (Arispe et al., 2008). They also developed new compounds with pyrimidine structures that inhibit the amyloid channels and investigated its efficacy for treatment (Diaz et al., 2009, Arispe et al., 2010).

We focused substances which modify the membrane properties and inhibit amyloid channels. It is widely accepted that the composition of membrane lipids strongly influences the direct incorporation of peptides into membranes and consequent channel formation (Bechinger and Lohner, 2006; Simakova & Arispe, 2007). In particular, electrostatic interactions between peptides and lipids, namely, the net charges of the membrane surface, and membrane fluidity play crucial roles in the affinity for peptides (Seelig et al., 1994). Several AβP residues (e.g., Arg^5, Lys^{16}, and Lys^{28} residues) have a positive charge at a neutral pH; thus, AβP has an affinity for negatively charged phospholipids, such as phosphatidylserine (PS) or phoshatidylglycerol (PG), but not for neutral phospholipids such as phosphatidylcholine (PC). The formation of ß-sheet structures by AβP (1–40) was increased after the addition of PG. Meanwhile, substances that decrease membrane fluidity are known to enhance membrane stiffness and influence pore formation by toxins (Tomita et al., 1992). Gangliosides also contribute to the net charge of the outer membrane surface and to the binding to AβPs. Micro-circumstances on the membranes, such as rafts, containing gangliosides and cholesterol, may provide suitable locations that facilitate this process (Matsuzaki et al., 2010). Indeed, disruption of raft protected neurons from AβPs-induced neurotoxicity (Malchiodi-Albedi et al., 2010).

Thus, we have developed a screening system for compounds that influence membrane properties by observing the AβP-induced influx of Ca^{2+}. Among tested, we demonstrated that several lipophilic substances such as phloretin, cholesterol, 17β-estradiol, 17α-estradiol, and neurosteroids including dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and pregnenolone, significantly inhibit AβP-induced $[Ca^{2+}]_i$ elevation (Kawahara and Kuroda, 2001, Kato-Negishi and Kawahara, 2008). Figure 5 exhibits the structures of these compounds.

Phloretin, a plant-derived flavonoid, decreases the membrane potential and inhibits the electrostatic interaction between AβP and membrane lipids (Hertel et al., 1997). Cholesterol decreases membrane fluidity and inhibits channel formation by peptides such as α-toxin, gramicidine, amylin, and AβP. The pretreatment with phloretin or cholesterol significantly inhibited the AßP-induced increase in $[Ca^{2+}]_i$ in cultured neurons. Meanwhile, 6-ketocholestanol, which increases the membrane dipole potential, did not influence the AβP-induced increase in $[Ca^{2+}]_i$.

Furthermore, 17β-estradiol, a female hormone, is neuroprotective and affects membrane fluidity (Schwartz et al., 1996). Considering that both of 17β-estradiol and 17α-estradiol inhibit the AβP-induced $[Ca^{2+}]_i$ elevation, this inhibition may depend on their membrane modifying effects (Whiting et al, 2000). All of these compounds inhibit AβP neurotoxicity.



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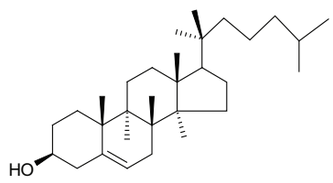
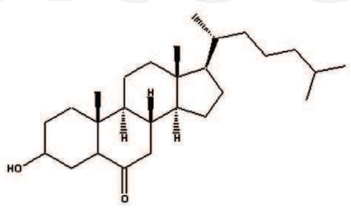
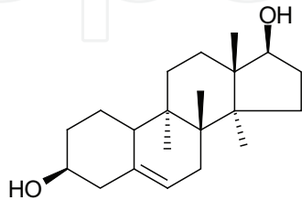


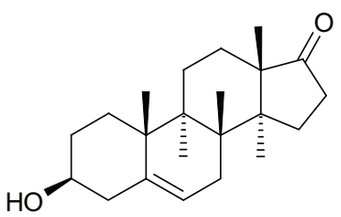
Cholesterol (Chol)



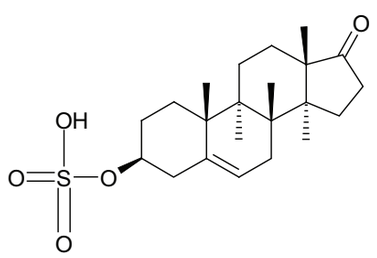
6-ketocholestanol(6KC)



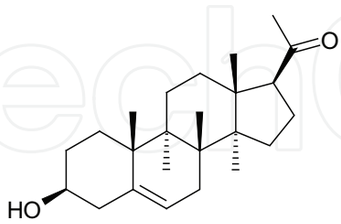
17-ß estradiol



DHEA



DHEA-S



pregnenolone

Fig. 5. Chemical structures of substances which influence AβP-induced $[Ca^{2+}]_i$ elevation

Neurosteroids are steroid hormones synthesized *de novo* in the central nervous system from cholesterol or from peripheral steroid precursors (Tsutuki et al., 2000). Several lines of evidence suggest that neurosteroids modulate various functions of the brain and exhibit neuroprotective activitiy (Mellon, 2007). Thus, neurosteroids have been recognized as anti-

aging hormones, and are widely used as supplements to improve the impaired cognitive functions of the elderly (Huppert et al., 2000). Considering that plasma DHEA-S levels are reduced in healthy individuals in an age-dependent manner and in AD patients (Hillen et al., 2000; Aldred and Mecocci, 2010), neurosteroids may have an important role in the pathogenesis of AD.

7. Amyloid channel hypothesis

Considering the results of our study together with those of the others, we propose the following hypothetical scheme of AβP-induced neurodegeneration (Fig. 6).

AβPs are normally secreted following the cleavage of APP in the synaptic compartment into the cerebrospinal fluid. The secreted AβPs are usually degraded proteolytically within a short period. However, the upregulation of AβP secretion from APP, or an increased ratio of AβP(1–42) to AβP(1–40), which are influenced by APP or presenilin gene mutations, may facilitate the retention of AβP in the brain.

As descried previously, the net charge of the outer membrane surface may be a determinant when secreted AβPs bind to cellular membranes. The distribution of phospholipids on cellular membranes is usually asymmetrical; neutral lipids such as PC usually exist on the outer surface of plasma membranes, whereas negatively charged phospholipids such as PS exist on the inner membrane surface. When this asymmetric distribution is disrupted by apoptotic conditions or aging, AβPs can bind to the membrane surfaces (Fig. 6, step (A)). After incorporation into the membrane, the conformation of AβPs change and the accumulated AβPs aggregate on the membranes. The ratio of cholesterol to phospholipids in the membrane may alter membrane fluidity, thereby affecting the process from step (A) to (B). Considering that apoE, its phenotype is a risk factor of AD, is a cholesterol-binding protein, the relationship between cholesterol and AD is important in the pathogenesis of AD.

Finally, aggregated AβP oligomers form ion channels (Fig. 6 (C)), leading to the various neurodegenerative processes. Once AβP channels are formed in neuronal membranes, the homeostasis of Ca^{2+} and other ions is disrupted. Unlike endogenous Ca^{2+} channels, these AβP channels are not regulated by the usual channel blockers; thus, once formed on the membrane, a continuous flow of Ca^{2+} is initiated. However, Zn^{2+} ions, which are secreted into the synaptic cleft in a neuronal activity-dependent manner, inhibit AβP-induced Ca^{2+} entry, and thus have a protective function in AD. Disruption of Ca^{2+} homeostasis triggers several apoptotic pathways and promotes numerous degenerative processes, including free radical formation and tau phosphorylation, thereby accelerating neuronal death. Presenilins can influence Ca^{2+} homeostasis through the disturbances of the capacitive Ca^{2+} entry or other pathways, and may influence these pathways. Free radicals also induce membrane disruption, by which further amplifies unregulated Ca^{2+} influx. The disruption of Ca^{2+} homeostasis influences the production and processing of APP. Thus, a vicious cycle of neurodegeneration is initiated.

The velocity of channel formation will be regulated by the binding of AßP on membranes and its concentration, considering that *in vitro* aging can enhance the neurotoxicity of AβP and natural oligomers (dimmers or trimers) are more toxic as compared to monomers, it is provable that AßP oligomerization *in vitro* accelerates the velocity from (A) to (B), and enhances the formation of tetrameric or hexameric pores on membranes. These oligomers might easily form tetrameric or hexameric pores and exhibit neurotoxicity. Indeed, O'Nuallain

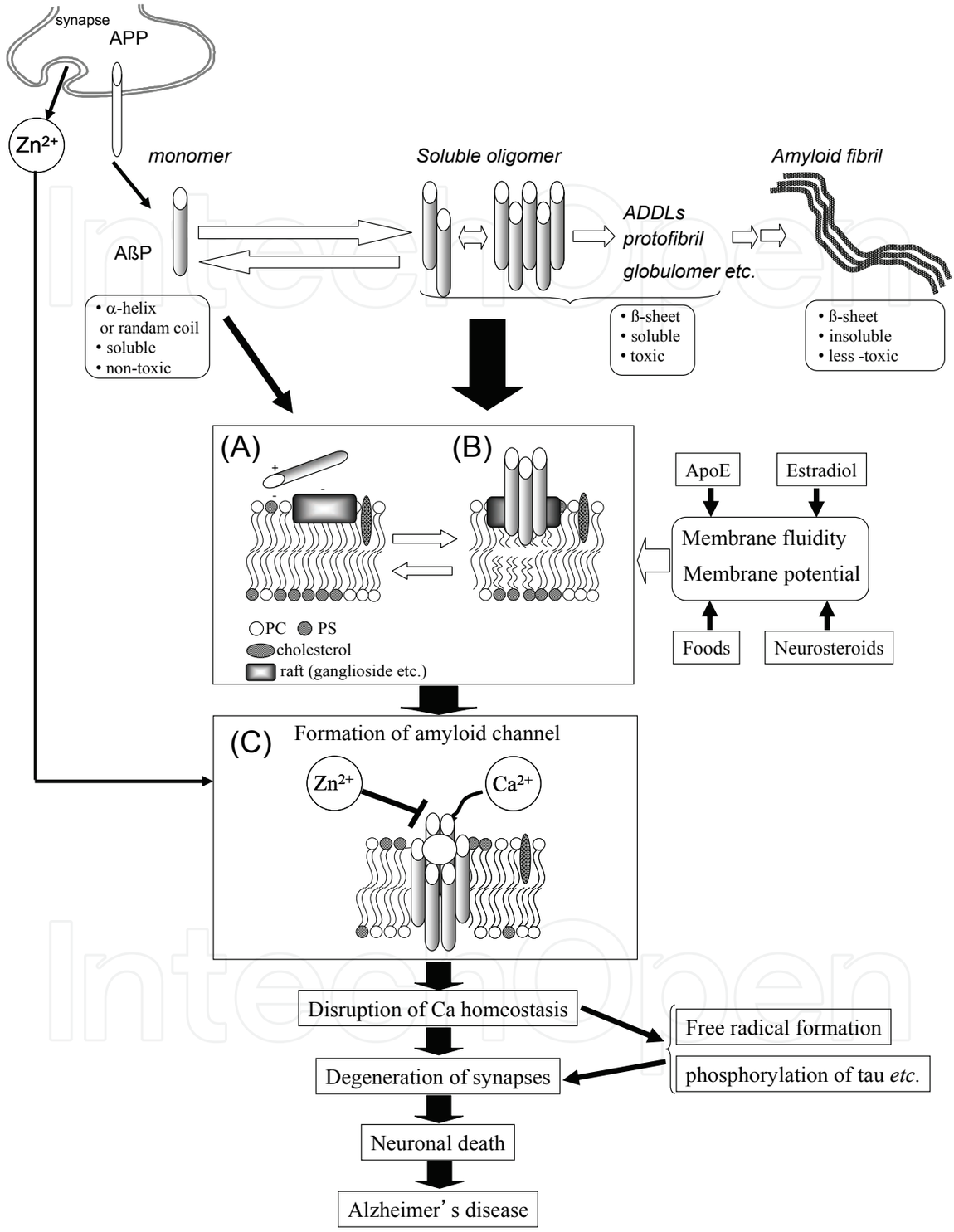


Fig. 6. Hypothesis concerning amyloid channels in the pathogenesis of Alzheimer's disease. AβPs are secreted from into synapses following the cleavage of APP, and then directly incorporated into membranes. The hypothetical scheme for the formation of oligomeric amyloid channels is depicted. Details are shown in the text

et al. demonstrated that AßP dimmers formed toxic protofibrils more rapidly compared to monomer (O'Nuallain et al., 2010). In spite that the exposure to relatively high concentration of AβP *in vitro* causes the acute elevation of $[Ca^{2+}]_i$ and exhibits neurotoxicity, the concentration of the AβP are low and it is difficult to bind to outer membranes. Thus, the processes required for channel formation (from steps (A) to (C)) in our brains may require a long life span and determine the rate of the entire process. This amyloid channel hypothesis explains the long delay in AD development; AD occurs only in aged subjects despite the fact that AβPs are normally secreted in younger or normal subjects. Various environmental factors, such as foods or trace metals, as well as genetic factors will influence these processes and contribute to AD pathogenesis.

8. Conclusion

Despite the immense efforts to develop a therapeutic drug for AD, the results have not been satisfactory. However, we believe that if we can understand the precise pathogenic mechanism more clearly, then potential therapeutic drugs (such as supplements) will be developed in the future. The amyloid channel hypothesis may improve the precise understanding of AD and the development of drugs for AD treatment. Further, it might be worthwhile to consider the application of dietary supplements such as estrogen or neurosteroids for the prevention of AD. Our efforts in developing new drugs will certainly be fruitful for the economy of the nation and the health of the population.

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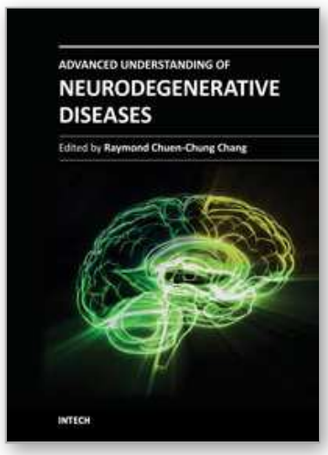

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Advanced Understanding of Neurodegenerative Diseases focuses on different types of diseases, including Alzheimer's disease, frontotemporal dementia, different tauopathies, Parkinson's disease, prion disease, motor neuron diseases such as multiple sclerosis and spinal muscular atrophy. This book provides a clear explanation of different neurodegenerative diseases with new concepts of understand the etiology, pathological mechanisms, drug screening methodology and new therapeutic interventions. Other chapters discuss how hormones and health food supplements affect disease progression of neurodegenerative diseases. From a more technical point of view, some chapters deal with the aggregation of prion proteins in prion diseases. An additional chapter to discuss application of stem cells. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

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