

## Forum Minireview

# Molecular Approaches to the Treatment, Prophylaxis, and Diagnosis of Alzheimer's Disease: Tangle Formation, Amyloid- $\beta$ , and Microglia in Alzheimer's Disease

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**Abstract.** Pathological hallmarks of Alzheimer's disease (AD) include senile plaques, neurofibrillary tangles (NFTs), synaptic loss, and neurodegeneration. Senile plaques are composed of amyloid- $\beta$  ( $A\beta$ ) and are surrounded by microglia, a primary immune effector cell in the central nervous system. NFTs are formed by the intraneuronal accumulation of hyperphosphorylated tau, and progressive synaptic and neuronal losses closely correlate with cognitive deficits in AD. Studies on responsible genes of familial AD and temporal patterns of pathological changes in brains of patients with Down's syndrome (Trisomy 21), who invariably develop neuropathology of AD, have suggested that  $A\beta$  accumulation is a primary event that influences other AD pathologies. Although details of the interaction between AD pathologies remain unclear, experimental evidences to discuss this issue have been accumulated. In this paper, we review and discuss recent findings that link the AD pathologies to each other. Further studies on the interaction between pathologies induced in AD brain may contribute to provide deep insight into the pathogenesis of AD and to develop novel therapeutic, prophylactic, and early diagnostic strategies for AD.

**Keywords:** amyloid- $\beta$ , tau, actin, microglia, interaction, Alzheimer's disease

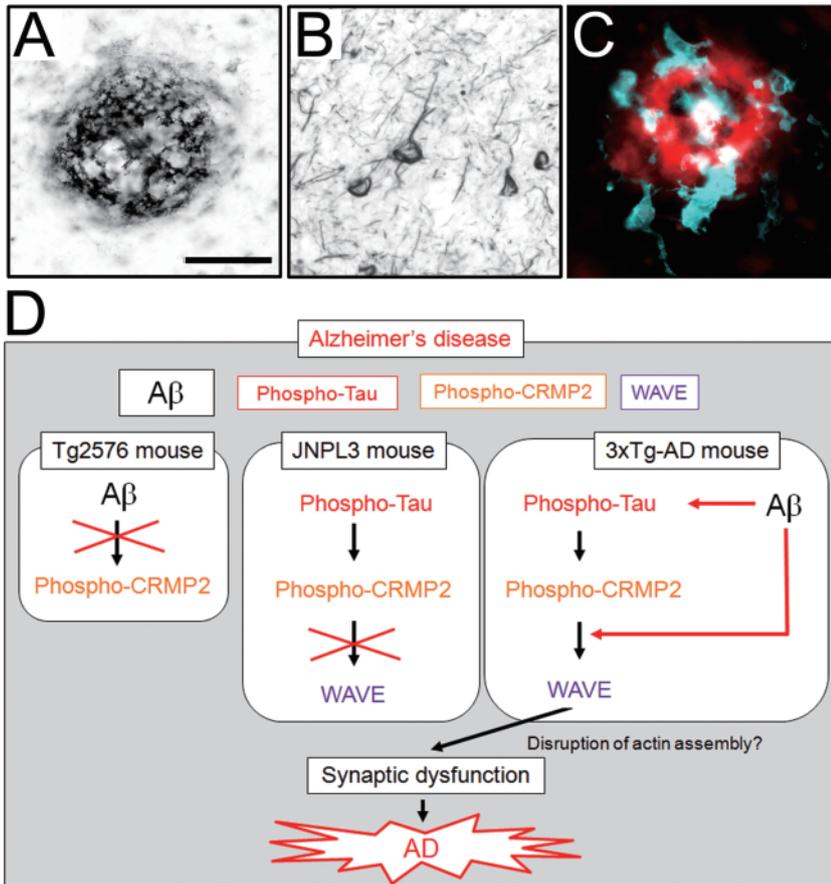
## 1. Introduction

Alzheimer's disease (AD) is characterized by progressive cognitive impairment that is a consequence of extensive neuronal loss (1). Principal pathological features of AD are extracellular deposition of fibrillar amyloid- $\beta$  ( $A\beta$ ) (Fig. 1A) and formation of intraneuronal neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau (Fig. 1B). It is also well known that microglia markedly accumulate on the  $A\beta$  depositions (senile plaques) in AD brain (Fig. 1C) (2). Microglia are primary immune effector cells distributed ubiquitously throughout the central nervous system (CNS) and have an ability as phagocytes. Furthermore, progressive synaptic dysfunction is found in AD brain and closely correlates with cognitive deficits (3, 4).

$A\beta$  peptides are produced from amyloid precursor protein (APP) by combination cleavages with  $\beta$ -secretase

and  $\gamma$ -secretase.  $A\beta$  is composed of 37 – 43 amino acid residues because  $\gamma$ -secretase, which is a protein complex containing presenilin (PS), generates the C-terminal of  $A\beta$  with different length (5). In genetic studies on AD (6), mutations in genes of *APP*, *PS1*, and *PS2* have been found, and transgenic mice models carrying these familial AD-linked mutations enhance the  $A\beta$  production in their brains (7 – 9). Furthermore, in patients with Down's syndrome (Trisomy 21), who invariably develop neuropathology of AD, the mRNA expression of the *APP* gene on chromosome 21 is 1.5-fold, and the formation of  $A\beta$  plaques is preceded about 10 years before the onset of NFTs formation (10, 11). These findings strongly suggest that the accumulation of  $A\beta$  in brain is a primary event driving other AD pathogenesis, such as NFTs formations, synaptic dysfunction, and neuronal loss (amyloid cascade hypothesis) (12). However, details in the relationships between  $A\beta$  accumulation and other AD pathologies remain poorly understood. Therefore, findings on the interaction between AD pathologies may provide important clues to the pathogenesis of AD and contribute to develop novel therapies, prophylaxis, and

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**Fig. 1.** Pathological changes in human AD brain and a possible mechanism of interaction between  $A\beta$ , tau, and synaptic dysfunction. A:  $A\beta$  plaque (senile plaque) was stained with anti- $A\beta$  antibody (clone 6E10). B: Neurofibrillary tangles were stained with anti-phosphorylated tau antibody (clone AT8). C: AD brain tissue was stained with antibodies against ionized calcium-binding adaptor molecule 1 (Iba1; cyan) and  $A\beta$  (clone 6E10; red) antibody. Microglia (cyan) was accumulated on the senile plaque in AD. Scale bar = 50  $\mu\text{m}$  for all panels. D: In AD brain, WAVE is co-aggregated with hyperphosphorylated tau and phosphorylated CRMP2 in NFTs and abnormal neurites. Although neither CRMP2 nor WAVE accumulation was observed in Tg2576 mice, accumulation of phosphorylated CRMP2 but not WAVE accumulation was detected in JNPL3 mice. Both accumulations of phosphorylated CRMP2 and WAVE were recapitulated in 3xTg-AD. Thus, accumulation of phosphorylated CRMP2 was induced by hyperphosphorylated tau, while combined  $A\beta$  and tau pathologies were required for WAVE accumulation. Results imply that WAVE-mediated disturbance of actin assembly may be closely associated with synaptic deficits in AD brain and that WAVE pathology could be a cross-linker between  $A\beta$ , tau, and synaptic pathologies manifesting in AD brains through the abnormality of CRMP2.

early diagnosis of AD. In this paper, we review and discuss current research on the interaction between AD pathologies, especially focusing on the  $A\beta$  interaction. We further discuss the role of microglial accumulation on the senile plaques as an  $A\beta$ -induced pathological change in AD brain.

## 2. Interaction of $A\beta$ in the formation of NFTs

In AD brain, NFTs consist of paired helical filaments (PHFs). PHFs are also seen in dendrites as neuropil threads (curly fibers) and in some axonal terminals as dystrophic neurites surrounding senile plaques. The main component of PHFs has been identified as abnormally dislocated and hyperphosphorylated tau protein (13). Tau is a microtubule-associated protein and physiologically promotes tubulin assembly into microtubules and regulates motor-driven transport in axons (14), whereas the abnormally hyperphosphorylated tau self-aggregates into PHFs and impairs the promotional activity for tubulin assembly (15). Thus, an aspect of the neurodegenerative event in AD may include a disturbance of microtubule assembly and its destabilization induced by the hyper-

phosphorylation of tau (16). Regarding the tau phosphorylation, sequential phosphorylation by cyclin-dependent kinase (Cdk5) and glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) is strongly suggested to be involved in the pathogenic hyperphosphorylation of tau (17).

In the experimental study crossing transgenic mice expressing mutant human APP and mice expressing mutant human tau, the formation of hyperphosphorylated tau-positive NFTs-like structure was increased as compared with mice expressing tau alone, whereas the structure and number of their  $A\beta$  plaques were essentially unaltered (18). Similarly, injection of synthetic  $A\beta$  into mutant tau transgenic mice exacerbated the NFTs-like pathology (19). Furthermore,  $A\beta$  immunization in 3xTg-AD mice, which express human mutant proteins of APP, tau, and PS1 and develop  $A\beta$  plaques and NFTs-like pathologies, resulted in reduced levels of not only  $A\beta$  but also hyperphosphorylated tau (20). These results above further support the amyloid cascade hypothesis. Therefore, regarding the interaction between  $A\beta$  and tau, a crucial question could be how  $A\beta$  effects the hyperphosphorylation of tau and NFTs formation. Recent studies have provided suggestive evidences for this question

indicating that processes leading to excessive  $A\beta$  generation increase GSK-3 $\beta$  activity (21, 22). Thus,  $A\beta$  may interact in the tau pathologies through the up-regulation of tau kinases.

A novel interaction between  $A\beta$  and tau, which is related to mitochondrial dysfunction, has been suggested. At the protein and activity levels, mitochondrial dysfunction was found in a mouse model of AD, and it is suggested that  $A\beta$  and tau independently induce mitochondrial dysfunction by inhibiting complex IV and complex I, respectively (23). Furthermore, another group reported that tau mediates  $A\beta$  neurotoxicity via disturbance of axonal transport of mitochondria (24). Thus,  $A\beta$  and tau may amplify their neurotoxic effects through the inhibition of mitochondrial function and its axonal transport. It is also suggested that the  $A\beta$ -induced endoplasmic reticulum (ER) stress (25, 26) is enhanced under the mitochondrial dysfunction conditions (27).

### 3. Interaction of $A\beta$ in the synaptic dysfunction

Integrity of the synaptic function is maintained by the precise formation of dendritic spines that are major sites of synaptic contacts, and the formation is structurally regulated by the actin cytoskeleton. Progressive synaptic dysfunction and loss are found in AD brain and closely correlate with cognitive deficits (3, 4). Thus, the disruptions of the actin cytoskeleton as well as microtubule assembly are suggested to be involved in the developmental cascade in AD. However, the relationship between disruptions of microtubule and actin cytoskeletons and the involvement of  $A\beta$  and tau pathologies remain poorly understood.

The Wiskott-Aldrich syndrome protein family verprolin-homologous protein (WAVE) has been identified as a key molecule for actin assembly (28), and it forms a complex with collapsin response mediator protein 2 (CRMP2), a regulator for microtubule assembly as well as tau (29). Previously, association of highly phosphorylated CRMP2 with NFTs is reported in AD brain (30, 31). In this context, abnormal CRMP2 possibly influences the properties of WAVE and furthermore actin assembly. In other words, there is a possibility that CRMP2 and WAVE could be factors that link the NFTs and synaptic dysfunction in AD brain. Therefore, we investigated the expression pattern of WAVE in AD brain and found that WAVE is co-aggregated with hyperphosphorylated tau and phosphorylated CRMP2 in NFTs and abnormal neurites, such as neuropil threads and dystrophic neurites (32, 33). We also found the association between hyperphosphorylated tau, CRMP2, and WAVE in the cytosol fraction of AD brain by immunoprecipitation (33). To examine the mechanism of

WAVE accumulation, we further investigated the brains of three transgenic mouse models of AD, that is, Tg2576 mice developing  $A\beta$  plaques, JNPL3 mice developing NFTs-like hyperphosphorylated tau tangles, and 3xTg-AD mice developing combined pathologies of  $A\beta$  plaques and hyperphosphorylated tau tangles (33). Although neither CRMP2 nor WAVE accumulation was observed in Tg2576 mice, accumulation of phosphorylated CRMP2 but not WAVE accumulation was detected in JNPL3 mice. Interestingly, both accumulations of phosphorylated CRMP2 and WAVE were recapitulated in 3xTg-AD. Thus, the accumulation of phosphorylated CRMP2 was induced by hyperphosphorylated tau, while combined  $A\beta$  and tau pathologies were required for WAVE accumulation. Recent studies have suggested that  $A\beta$  induces hyperphosphorylation of CRMP2 through the activation of GSK-3 $\beta$  (21), and the hyperphosphorylation of CRMP2 is detected in 3xTg-AD mice but not in JNPL3 mice (34). Therefore, it is suggested that hyperphosphorylation of CRMP2 induced by  $A\beta$  pathology is important for the WAVE accumulation. These results imply that WAVE-mediated disturbance of actin assembly may be closely associated with synaptic deficits in AD brain and that WAVE pathology could be a cross-linker between  $A\beta$ , tau, and synaptic pathologies manifesting in AD brains through the abnormality of CRMP2 (Fig. 1D).

Another mechanism of  $A\beta$  interaction in synaptic dysfunction has been reported (35, 36). It is suggested that hyperphosphorylation of tau induced by  $A\beta$  pathology makes tau detach from microtubules and accumulate into the somatodendritic compartment (including dendritic spine) of neurons. The abnormally dislocated and hyperphosphorylated tau recruits the tyrosine protein kinase Fyn to the dendritic spine, and then Fyn phosphorylates *N*-methyl-D-aspartate receptors (NMDARs), thereby mediating complex formation of NMDARs with the post synaptic density protein 95 (PSD95). The NMDAR-PSD95 interaction is required for excitotoxic downstream signaling, and this pathway renders neurons more susceptible to  $A\beta$  toxicity in dendrites. Although the interaction between  $A\beta$  and synaptic dysfunction has been gradually elucidated, further studies are needed to establish or confirm the exact mechanisms.

### 4. Microglial accumulation on $A\beta$ depositions in AD brains

Studies on the  $A\beta$  interaction described above as well as the amyloid cascade hypothesis strongly suggest that the accumulation of  $A\beta$  may play a key role in the pathogenesis of AD, and the reduction of brain  $A\beta$  is a primary therapeutic target for AD. It is conceivable that up-regu-

lations of  $A\beta$  production and/or inhibitions of  $A\beta$  clearance are involved in the  $A\beta$  accumulation in AD brain. Recently, Kaneko et al. have suggested that the dysfunction of endoplasmic reticulum-associated degradation system in neurons is possibly involved in the mechanism that induces the up-regulation of  $A\beta$  production in AD brain (26).

A possible mechanism in an aspect of the inhibition of  $A\beta$  clearance in AD brain may involve dysfunction of microglia. It is well known that microglia transform their morphology into the activated phenotype and markedly accumulate on senile plaques in AD brain (2). Although the microglial accumulation was initially postulated to be involved in the formation of  $A\beta$  plaques in the brain, later experimental studies demonstrated the ability of microglia to uptake  $A\beta$  peptide. We have also investigated the microglial function using primary cultured rat microglia and have found that microglia markedly phagocytose  $A\beta$  as a compensatory reaction against the  $A\beta$  accumulation (37, 38). Despite the presence of abundant plaque-associated microglia in brains of AD, microglia seems to fail to efficiently phagocytose  $A\beta$  deposits. Streit's group reported age-related microglial dystrophy and suggested that microglial senescence may occur to a greater extent in neurodegenerative conditions such as AD (39). On the other hand, we have found that high mobility group box protein-1 (HMGB1), an abundant non-histone chromosomal protein, is deposited into the senile plaques with  $A\beta$  peptides in the AD brain and have demonstrated that the extracellular HMGB1 inhibited microglial  $A\beta$  phagocytosis in primary cultured rat microglia and delayed the  $A\beta$  clearance in the brain of  $A\beta$ -injected rats (40). Furthermore, Koenigsnecht-Talboo and Landreth reported that microglial  $A\beta$  phagocytosis is inhibited in the presence of proinflammatory cytokines including interleukin- $1\beta$ , tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , monocyte chemotactic protein-1, and CD40L (41). Thus, in AD brain, microglial dysfunction may be induced by senescence itself and/or other factors, and it is speculated that the progression of AD pathogenesis may result from the decreased ability of microglia to clear  $A\beta$ . However, Akiyama and McGeer reported that in the cortical area affected by incomplete ischemia in a typical case of AD, intense accumulation of reactive microglia and a reduction of senile plaques were observed (42). Therefore, microglial activation seems to be an effective tool for the clearance of  $A\beta$  in AD.

A number of studies have demonstrated that the up-regulation of microglial  $A\beta$  phagocytosis could be mediated by various factors, such as nitric oxide (43) and estrogen (44), and have suggested the promotion of the microglial  $A\beta$  phagocytosis as a reasonable therapeutic

strategy for AD. In transgenic mouse models of AD, immunization with synthetic  $A\beta$  peptides or injection of anti- $A\beta$  antibodies significantly reduces the formation and deposition of  $A\beta$  and restored cognitive function. One proposed mechanism of  $A\beta$  vaccines (with synthetic  $A\beta$  peptides or anti- $A\beta$  antibodies) on the  $A\beta$  clearance is the enhancement of microglial  $A\beta$  phagocytosis: antibodies enter the brain, accumulate surrounding the  $A\beta$  plaques and enhance microglial  $A\beta$  phagocytosis via Fc receptors (45). Furthermore, in a case report of the human clinical trial, phagocytic microglia were found in the brain area devoid of  $A\beta$  plaques, and microglial contribution in the  $A\beta$  clearance in the AD brain was also suggested (46). Therefore, we verified this mechanism using deglycosylated antibodies against  $A\beta$  in primary cultured rat microglia (47). Glycosylation of immunoglobulin is critically involved in binding to Fc receptors. While deglycosylated antibodies maintain binding affinity to their antigen, they have reduced interaction with Fc receptors. We investigated the effect of intact and deglycosylated antibodies on the microglial  $A\beta$  phagocytosis and found that the deglycosylated antibodies fail to increase the microglial  $A\beta$  phagocytosis. This result distinctly demonstrated that anti- $A\beta$  antibodies up-regulate microglial  $A\beta$  phagocytosis via Fc receptors expressed on the microglia (47). We further found that extracellular heat shock proteins (HSPs), such as HSP90, enhance rat microglial  $A\beta$  phagocytosis through Toll-like receptor (TLR) 4 in both an in vitro study (37) and an in vivo study (48). Other groups also suggested that activating TLRs, such as TLR2, TLR4, or TLR9, and their coactivator CD14 with their specific ligands were shown to stimulate the microglial  $A\beta$  phagocytosis (49, 50).

We have recently provided new evidence that modulation of microglial  $\alpha 7$  nicotinic acetylcholine receptors (nAChRs) by galantamine or stimulation of  $\alpha 7$  nAChRs by nicotine enhances  $A\beta$  phagocytosis in primary cultured rat microglia (51). Galantamine sensitizes microglial  $\alpha 7$  nAChRs to choline and nicotine by binding to the allosterically potentiating ligand-binding site on  $\alpha 7$  nAChRs. Thus, galantamine requires extracellular choline or other acetylcholine-like agonists to enhance microglial  $A\beta$  phagocytosis, whereas nicotine directly induces the promotion of microglial  $A\beta$  phagocytosis. Furthermore, we also demonstrated that the  $Ca^{2+}$  signaling cascade followed by the calmodulin (CaM) – CaM-dependent protein kinase II (CaMKII) pathway and the CaM–Rac1 (a small G-protein) pathway for the regulation of the microglial actin cytoskeleton may be involved in the phagocytic enhancement in microglia (Fig. 2). Subsequently, we verified that galantamine enhanced  $A\beta$  clearance in brains of  $A\beta$ -injected rats and a transgenic mouse model of AD. Results suggest a further advantage

of galantamine as a therapeutic drug for AD and the significant therapeutic potential of microglial  $\alpha 7$  nAChRs in novel drug development for the treatment of AD (51).

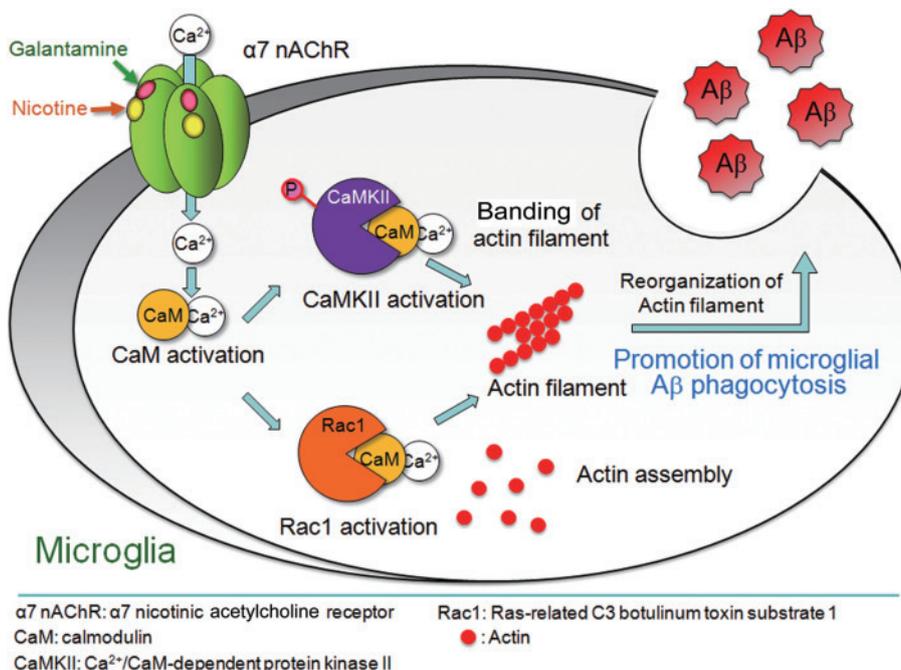
Bard et al. indicated the possibility that exogenously administered microglia also may have an effect on the clearance of  $A\beta$  in an experimental ex vivo study (52). These findings suggest the potential of exogenous microglia for the cell therapeutic approach in AD. Therefore, we transplanted rat primary cultured microglia into the lateral ventricle of the  $A\beta$ -injected rat and found that exogenous microglia accumulated on the  $A\beta$  deposits and increased  $A\beta$  clearance in the brain of  $A\beta$ -injected rat (53). In the study, we transplanted microglia into the lateral ventricle, while Sawada's groups reported that intra-arterially injected microglia but not macrophages can migrate through the intact or injured blood–brain barrier (BBB) to the brain parenchyma (54, 55). Therefore, we speculate that the transplantation of exogenous microglia into the systemic circulation may be effective for the  $A\beta$  clearance in brain parenchyma. Taken together, the transplantation of freshly prepared exogenous microglia may contribute to the clearance of  $A\beta$  in the in vivo brain; suggesting the possibility of cell therapeutic strategies for AD.

## 5. Conclusions

In the brain of Alzheimer's disease, various pathological changes are induced. Extracellular deposition of  $A\beta$ , intraneuronal accumulation of hyperphosphorylated tau,

and synaptic and neuronal losses are the typical pathologies. So far, most studies on the pathogenic mechanism in each change had been individually investigated. Recently, evidences which show a close interaction between the pathologies have been gradually accumulated. In this review, we summarized the recent findings focusing on the  $A\beta$  interaction. In the interaction,  $A\beta$  seems to play a key role in the initiations and/or exacerbations of other pathological changes. Therefore,  $A\beta$  clearance from the brain as early as possible could be a primary target for therapeutic strategies in AD. For this purpose, the development of a method for early and precise diagnosis would be the most important step (56). In contrast, by the elucidation of the interaction between pathologies, novel targets for the diagnosis may be found, and the developmental opportunities and possibilities could be increased. Furthermore, stage-dependent diagnosis may be established. The same possibilities may be provided to the therapeutic and prophylactic strategies for AD.

The role of microglia in AD attracted considerable attention, especially for therapeutic use. In this review, we also focused on the microglial  $A\beta$  phagocytosis and summarized microglial beneficial effects on the  $A\beta$  pathology in AD brain. It is very important to regulate and/or activate microglia to effectively function in phagocytosis. For this purpose, several target receptors and factors have been elucidated, possibly suggesting that the transplantation of microglia [and/or bone marrow stem cell-, embryonic stem (ES) cell-, or induced pluripotent stem (iPS) cell-derived microglia] could be a powerful strategy for the therapeutic approach in AD. Indeed, in



**Fig. 2.** A possible mechanism of galantamine-enhanced microglial  $A\beta$  phagocytosis. Galantamine sensitizes microglial  $\alpha 7$  nicotinic acetylcholine receptors (nAChRs) to acetylcholine agonists such as choline and nicotine by binding to the allosterically potentiating ligand-binding site on  $\alpha 7$  nAChRs. Subsequently,  $Ca^{2+}$  influx through the  $\alpha 7$  nAChRs activates the CaM–CaMKII pathway and the CaM–Rac1 pathway for the promotion of actin filament reorganization, which is regulated by the banding of actin filament and actin assembly, respectively. The reorganization of actin filaments may promote the microglial  $A\beta$  phagocytosis.

vivo studies by Rivest's group demonstrated that bone marrow stem cell-derived microglia have the ability to populate entire brain parenchyma (57) and play a critical role in the restriction of A $\beta$  plaques (58). Furthermore, in our in vitro study, we recently indicated that bone marrow stem cells can be differentiated into the microglia-like A $\beta$  phagocytic cells (59). Thus, further studies on the biology and regulation of microglia function may provide a novel insight into the therapies for AD.

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