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博 士 論 文 概 要
Doctoral Thesis Synopsis

Arctic mutant amyloid β protein interferes the functions of CHRNA7 through specific binding

Arctic変異型アミロイド β 蛋白によるCHRNA7機能の抑制とその分子メカニズムの解析

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Alzheimer's disease (AD) constitutes the most common neurodegenerative disorder. AD describes a set of symptoms, which include memory loss, mood changes, communicational problems and reasoning problems. The neuropathological and neurochemical hallmarks of AD include synaptic loss and selective neuronal cell-death, decrease of the markers for certain neurotransmitters, and abnormalities in neurons and their processes as well as in the extracellular space. There are several hypotheses about AD pathology causes, among which the 'amyloid hypothesis' is the primary basic hypothesis for most of the researches. The traditional formulation of amyloid hypothesis suggests that the cytotoxicity of mature aggregated amyloid fibrils are believed to be the toxic form of the protein, responsible for disrupting the cellular calcium ion homeostasis and thereby inducing apoptosis. That means, the accumulation of amyloid beta protein (Aβ) fragments in the brain trigger the disruption and destruction of nerve cells that cause AD. This hypothesis is supported by the observation that a variety of Aβ form the senile plaque. Aβ protein is thus considered to play a significant role in the pathogenesis of AD because of their ability to aggregate into β-sheets, which constitute the amyloid plaques in AD brains.

Aβ is formed after sequential cleavage of the amyloid precursor protein (APP) by β- and γ- secretase. The γ-secretase, which produces the C-terminal end of the Aβ peptide, cleaves within the transmembrane region of APP and generates a number of isoforms of 36-43 amino acid residues in length. Among them, Aβ40 is the predominant one and the main compose of senile plaque. Several autosomal dominant mutations causing early-onset familial AD (FAD) have been identified in the APP gene, suggesting that this protein affects either the metabolism of Aβ or its properties of aggregation. Those mutations cause a rapidly and progressive and severe form of the disease. Clinical features of FAD are indistinguishable from those of sporadic cases and disease onset occurs at a much younger age.

Point mutations in the Aβ sequence associated with FAD are clustered around the central hydrophobic core of Aβ. The 'Dutch' mutation in the Aβ sequence, Aβ[E22Q] gives rise to a highly distinct phenotype of severe amyloid angiopathy, leading to recurrent cerebral hemorrhage. The 'Iowa' mutation, Aβ[D23N], is associated with severe amyloid angiopathy as well. The Aβ[E22G] mutation, found to cause AD in Swedish families, was first reported in 2001 by Nilsberth et al. It is named the Arctic mutation. This mutation has a purely cognitive phenotype, typical of AD, without showing the presence of marked amyloid angiopathy. Carriers of the Arctic mutation have decreased amounts of plasma Aβ(1-42) and Aβ(1-40), and demonstrated that Aβ(1-40)E22G forms protofibrils much faster and more abundantly than the wild-type Aβ, whereas the rate of fibrillization remains the same. Oligomerization pattern of Arctic mutation was reported different from wild type Aβ with a tendency to form larger oligomers. Fibril structures were also observed and studied previously. Therefore, abnormal aggregation pattern of Arctic Aβ has been suggested to be a primary result of 'Arctic' mutation. However, none of these studies targeted any molecular signaling pathway including membrane receptors.

The nicotinic acetylcholine receptors, key players in neuronal communication, convert neurotransmitter binding into membrane electrical depolarization. One of the receptors, alpha-7 nicotinic acetylcholine receptor, is composed of homologous subunits known as neuronal acetylcholine receptor subunit alpha-7 (CHRNA7). This receptor is mainly located in the brain where activation yields pre- and postsynaptic excitation, mainly by

increased Ca^{2+} permeability. CHRNA7 regulates numerous Ca^{2+}-dependent events in the nervous system, and its activation can mediate long-term potentiation (LTP) at glutamatergic synapses. CHRNA7 has been reported to show the neuroprotective function by its activation, both *in vitro* and *in vivo* as well. Previous studies have found that Aβ42 binds to CHRNA7 with high affinity. Furthermore, the decline of senile plaques has been detected in Aβ-over-expressed transgene mice by blocking of CHRNA7. Moreover, Aβ has been found to interact with CHRNA7 resulting in impaired receptor functions. Meanwhile, Aβ has been reported to enhance its aggregation in the lipid raft with CHRNA7 exiting in it. Combine together, these results have led to the hypothesis that the CHRNA7 subunit plays a role in AD.

Because the molecular mechanism of Arctic mutation-mediated FAD remains unknown, this study investigates the relationship between Arctic Aβ and CHRNA7 in order to search whether CHRNA7 is participating in the molecular mechanism of Arctic mutation-mediated FAD.

In Chapter 3-1, *in vitro* binding assay was performed to search the direct interaction between CHRNA7 and Arctic Aβ. 3 different mutant Aβ40s were utilized and only Arctic Aβ40 bound to CHRNA7 with high affinity. In Chapter 3-2, Arctic Aβ42 bound to CHRNA7 with much higher affinity than wild-type, similar to the result showed in Chapter 3-1. However, the bands corresponded to aggregation forms were also detectable. Therefore, to exclude the effect of self-aggregation of Arctic Aβ in order to focus on the effect of the mutation on CHRNA7, Arctic Aβ1-40 peptides was utilized to do the further research. Moreover, extracellular N-terminal domain of CHRNA7 was determined to play a critical role in the direct interaction between wild-type Aβ as well as Arctic Aβ with CHRNA7 (Chapter 3-9-1).

Since Arctic mutation was reported to have an abnormal aggregation pattern, whether aggregation of Arctic Aβ could be affected by CHRNA7 was investigated (Chapter 3-3). 2 kinds of methods were used: Thioflavin T (ThT) assay and Transmission electron microscopy (TEM). The ThT assay showed that Arctic Aβ started to aggregate at 6h when co-incubated with CHRNA7, while Arctic Aβ alone itself did not aggregate at this time point. Furthermore, aggregation of Arctic Aβ was enhanced at 9h to 24h when co-incubated with CHRNA7, significantly greater than Arctic Aβ alone itself. Wild-type Aβ40 did not aggregate with or without the co-incubation of CHRNA7. When co-incubation with CHRNA7 N-terminal, Arctic Aβ enhanced its aggregation, showing the similar phenomenon when co-incubation with CHRNA7-full-length protein (Chapter 3-9-2). GST showed lack of the influence on the aggregation of Arctic Aβ (Chapter 3-9-2). By using TEM, the same phenomenon was observed, that accumulation of Arctic Aβ significantly enhanced when co-incubated with CHRNA7 for 24h compared with Arctic Aβ alone.

In order to search whether Arctic Aβ had any influence on the function of CHRNA7, Ca^{2+} response to nicotine and the downstream signaling protein ERK1/2 activation were measured (Chapter 3-4, 3-5). In Chapter 3-4, Ca^{2+} response was measured immediately after Aβ addition into CHRNA7-over-expressed CHO-K1 cells. Wild-type Aβ42 induced Ca^{2+} flux rapidly. However, Arctic Aβ40 did not modify (i.e. activate or block) the calcium response mediated through CHRNA7. The activation of Ca^{2+} downstream signaling ERK1/2 was further tested, that Arctic Aβ40 did not modify the function.

Since Arctic Aβ40 enhanced its aggregation when CHRNA7 exists (Chapter 3-3), I searched whether the incubation of Arctic Aβ40 could affect the function of CHRNA7 in Chapter 3-5. Nicotine was used to activate the calcium flux mediated through CHRNA7. Ca^{2+} rapidly increased with the addition of nicotine in CHRNA7-over-expressed-CHO-K1 cells. This response was significantly diminished within the cells incubated with Arctic Aβ40. Therefore, Arctic Aβ tended to attenuate Ca^{2+} response in addition to enhanced its aggregation when co-incubated with CHRNA7. The incubation of Arctic mutant Aβ inhibited the activation of ERK1/2 as well (Chapter 3-5). The results indicated that Arctic Aβ suppressed the functions of CHRNA7 via the inhibition of Ca^{2+} flux and activation of ERK1/2 after incubation.

The pathology of AD is associated with increased oxidative stress on a molecular level. Arctic mutation-mediated FAD has also been reported to have the increased oxidative stress on a molecular level. CHRNA7 tends to have neuroprotection both *in vivo* and *in vitro*. SH-SY5Y human neuroblastoma cell lines are widely used for the study of neuronal cell death. In order to search the neuroprotective effect of CHRNA7, H_2O_2 was applied as an oxidative stress inducer and nicotine as to activate CHRNA7. In Chapter 3-6, we used cells without shRNA transfected as the control. Scramble shRNA vector which did not target any endogenous transcript was used as the negative control for RNAi, shRNA1 and shRNA2 vectors were to knock down the expression of CHRNA7. By knocking down the expression of CHRNA7, both of these cell models failed to represent the neuroprotective function of CHRNA7. These results confirmed the neuroprotection of CHRNA7 in SH-SY5Y cells activated by nicotine against oxidative stress induced by H_2O_2.

The result in Chapter 3-7 showed that Arctic mutant Aβ inhibited the neuroprotective function of CHRNA7 within the cell viability using neuronal CHRNA7-over-expressed SH-SY5Y cells, measured by MTS assay and fluorescence microscopy. This phenomenon was not observed within the mock-transfected cells, indicating that this interference effect of Arctic Aβ on the neuroprotective function was actually mediated through CHRNA7 but not endogenous CHRNA7 or other unrelated channels in SH-SY5Y cells. Combine together, these results served a brief interpretation that the interference of Arctic Aβ on CHRNA7 may be one of the key molecular mechanisms within the Arctic mutation-mediated FAD.

Several different signaling cascades are reported to be participating in cell survival within nicotine anti-apoptotic action, including the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK1/2) pathway, the JNK pathway, as well as v-akt murin thymoma viral oncogene homolog (AKT) pathway. In Chapter 3-8, the activation of these signal proteins was investigated to see which signaling proteins were participating in the nicotine-activated functions of CHRNA7. Our results illustrated that only ERK1/2 activation but no other survival signaling proteins was involved in the nicotine-mediated functions through CHRNA7. Furthermore, the activation of ERK1/2 was found to mediate the nicotine effect in neuroprotection (Chapter 3-8). By inhibiting the activation of ERK with PD98059, nicotine failed to rescue the cells from oxidative stress. This effect was proven to be specific to Arctic mutation, but no other familial AD Aβ (Chapter 3-9-4).

In short, this study indicates that Arctic Aβ protein interferes the functions of CHRNA7 through specific binding, suggesting a novel understanding of the molecular mechanism of Arctic mutation-mediated FAD.

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講演	・　国際学会（ポスター発表） **Ju Y**., Asahi T., Sawamura N. Arctic Amyloid beta inhibits CHRNA7's function by specifically binding to and aggregating on this membrane receptor. 10th International symposium on electrochemical micro & nanosystem technologies (EMNT2014)、沖縄、2014 年 11 月 **Ju Y**., Asahi T., Sawamura N. Arctic mutant Aβ modifies CHRNA7's functions through specific binding. Neuroscience 2013, 43rd Annual meeting of Society for Neuroscience, San Diego, USA,　2013 年 11 月 ・　国内学会（ポスター発表） キョウ　ヨウ,朝日　透,澤村　直哉. Arctic Aβ による CHRNA7 の神経保護作用の抑制とその分子メカニズムの解析.　第 39 回日本分子生物学会年会、横浜、2016 年 12 月 ・　国内学会（口頭発表） **Ju Y**., Asahi T., Sawamura N. Determination of the key domains of CHRNA7 in the interacting actions of Arctic mutant Aβ. 第 58 回日本神経化学大会、埼玉、2015 年 9 月 **Ju Y**., Asahi T., Sawamura N. Arctic mutant Aβ affects CHRNA7's functions. 第 56 回日本神経化学大会 (Neuro2013)、京都、2013 年 6 月 **Ju Y**., Asahi T., Sawamura N. Arctic mutant Aβ modifies CHRNA7's functions. 第 85 回日本生化学会大会、福岡、2012 年 12 月

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その他 （講演）	・ 国内学会（ポスター発表） キョウ　ヨウ,朝日　透,澤村　直哉. ニコチン性アセチルコリン受容体とアミロイド β タンパクの結合部位の検討.早稲田大学ナノテクノロジーフォーラム、東京、2016 年 3 月 **Ju Y**., Asahi T., Sawamura N. Aβ aggregation requires CHRNA7 as scaffold molecule.　日本薬学会第 132 回年会、札幌、2012 年 2 月 **Ju Y**., Asahi T., Sawamura N. CHRNA7 serves as scaffold molecule for Aβ aggregation.　2nd workshop for Diamond Researchers. 東京、2012 年 2 月 **Ju Y**., Asahi T., Sawamura N. CHRNA7 as scaffold molecule for Aβ aggregation.　第 54 回日本神経化学会大会、金沢、2011 年 9 月