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Drosophila model of Alzheimer's Disease

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Hsueh-Cheng Chiang

We, the dissertation committee for the above candidate for the Doctor of Philosophy degree, hereby recommend acceptance of this dissertation.

Yi Zhong – Dissertation Advisor
Professor, Department of Neuroscience

Maurice Kernan – Chairperson of Defense
Associate Professor, Department of Neuroscience

Josh Huang – Committee member
Professor, Department of Neuroscience

Wen-Biao Gan – Committee member
Associate Professor, Department of Physiology and Neuroscience, Skirball Institute

This Dissertation is accepted by the Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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AD is a neurodegenerative disease affecting 20 % of people over 75 years of age resulting in a reduction of intellectual abilities, memory loss and dementia. Genetic studies of early-onset familial AD (EOFAD) provide a strong causative link between Aβ42 and AD. The neurotoxicity of Aβ42 has been well established in various experimental models and the toxicity of Aβ42 often correlated with its aggregation propensity. In order to investigate the role of Aβ42 on memory loss we generated Aβ42 transgenic flies. This Aβ42 transgenic fly recapitulated key markers of AD such as: age-dependent memory loss and neuronal degeneration. Further analysis of the aggregation-prone, EOFAD-related Arctic mutation (Aβ42Arc) and an artificial mutation (Aβ42art) that is known to suppress aggregation of Aβ42 *in vitro* showed that Aβ42 with different aggregation properties can induce distinct pathological phenotypes. Aβ42Arc caused greater neuron loss and memory damage than did Aβ42. Aβ42art induced a more severe memory defect than did Aβ42. Using targeted expression of Aβ42 to presynaptic or postsynaptic cells, we found that different types of cells may secrete distinct forms of Aβ42, leading to different modulation of synaptic functions. Aβ42 oligomers secreted from neurons inhibit neurotransmitter release and exert no effect on long-term depression (LTD). Larger-sized aggregates, possibly Aβ42 fibrils secreted from muscle cells, enhance synaptic transmission and LTD. Importantly, we found that the synaptic dysfunction produced by Aβ42 can be corrected by inhibition of Phosphatidylinositol-3 kinase (PI3K). Consistently, reducing PI3K activity can prevent both memory loss and the Aβ42 aggregation. Altogether, our data show that the different aggregation propensities of Aβ42 variants can result in qualitative shifts in the pathology induced *in vivo*. Furthermore, PI3K modulates the Aβ42 aggregates and the observed behavior change resulting from expression of Aβ42.

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ABBREVIATIONS

Aβ40: amyloid-β-40

Aβ42: amyloid-β-42

Aβ42Arc: amyloid-β-42 Arctic mutation

Aβ42Art: amyloid-β-42 Artificial mutation

AD: Alzheimer's disease

ADAM: a disintegrin and metalloprotease

AFM: atomic force microscope

AICD: amino terminal intracellular domain

ALS: Amyotrophic lateral sclerosis

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

APP: beta-amyloid precursor protein

APPL: amyloid precursor proteins like

CHO: Chinese hamster ovary

CREB: cAMP-response-element- binding protein

DAE: days-after eclosion

DS: Down syndrome

EGG: electroencephalography

EJCs: Excitatory Junction Currents

EM: electromicroscope

EOFAD: early-onset familial AD

ER: endoplasmic reticulum

FTD: fronto-temporal dementia

HD: Huntington disease

LOFAD: Late-onset familial AD

LTD: long-term depression

LTM: long-term memory

LTP: long-term potentiation

MCI: mild-cognitive impairment

mEJCs: miniature EJCs

NFTs: neurofibrillary tangles

NMJ: Neuromuscular Junction

NMDA: N-methyl-D-aspartic acid

NMR: Nuclear magnetic resonance

PBS: phosphate buffered saline

PD: Parkinson disease

PI: performance index

PI3K: Phosphatidylinositol-3 kinase

Pi3,4P: phosphatidylinositol-3,4-bisphosphate

Pi3,4,5P: phosphatidylinositol-3,4,5-trisphosphate

PS: presenillin

PTEN: phosphatase and tensin homolog

SOD1: Copper zinc-superoxide dismutase 1

TTX: tetrodotoxin

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Chapter 1

Introduction

Ever since Dr. Alois Alzheimer, a German psychiatrist, first defined Alzheimer's disease (AD) in Tübingen, Germany in 1906, AD has remained a mystery (Alzheimer, 1907).

This research study attempts to reveal one aspect of AD, the role of Aβ42 in the AD pathogenesis. For a better understanding of this study, I will divide this topic into two parts: 1) a general introduction of AD, and 2) an examination of using Drosophila as a model system to study AD. Finally, I will discuss the logical experimental design for this thesis work.

PART I:

1. What is Alzheimer's disease?

AD, a neurodegenerative disease and the most common form of dementia, is a progressive neuronal disorder associated with aging. Due to a population with increasing longevity and the current lack of a cure or preventive treatment, AD is reaching epidemic proportions. In fact, it affects about 2% of the population in the industrialized countries (Mattson, 2004), and it now affects over five million Americans (http://www.alz.org).

AD starts with difficulty in acquiring new knowledge and memory, which gradually leads to the loss of both declarative and non-declarative memory. AD worsens over time, leading to difficulty in performing normal activities and, ultimately, death (Selkoe, 2002).

Autopsies have shown that people who died with AD had atrophied neurons and shrinkage in the size of the brain, especially in the area responsible for learning and memory, including the temporal cortex and frontal lobe (Mattson, 2004).

Because there are a variety of reasons that may cause memory loss and atrophied neurons, a postmortem examination is often required for a certain diagnosis of AD. At the pathological level, AD is characterized by two hallmarks: senile plaque and neurofibrillary tangles (NFTs). Senile plaques are extra-cellular deposits and buildup of aggregated insoluble beta-amyloid (Aβ) peptides. Neurofibrillary tangles are intracellular aggregates that are composed by hyperphosphorylate tau. Tau is a microtubule binding protein responsible for axonal transportation and cellular communication (Price and Sisodia, 1998). Plaques and NFTs are present mainly in the area of the brain that involves learning and memory, as well as emotions, such as the hippocampus, basal forebrain and amygdala (Welsh-Bohmer and White, 2009).

In general, there are two forms of AD based on the age that AD is diagnosed. Late onset AD (LOAD), also called sporadic AD, is diagnosed after the age of 65 and comprises approximately 90-95% of all AD sufferers. Early onset familial AD (EOFAD) is diagnosed before the age of 65, usually in the 40s or 50s, and makes up about 5-10% of all AD sufferers (Tanzi and Bertram, 2005).

1.1 Discovery of the mutation of APP gene in EOFAD

Glenner and Wong first identified amyloid beta protein in samples of cerebrovascular amyloid in Down syndrome (DS) patients (Glenner and Wong, 1984). The reason they researched DS patients is that it has been long known that DS patients also have amyloid

plaque and NFTs that are typical in AD. Later in 1985, Masters et al. isolated Aβ from senile plaques (Masters et al., 1985). These two papers first suggested the presence of Aβ in samples of brain tissue from AD patients. Shortly thereafter, the beta-amyloid precursor protein (APP) gene, which encodes Aβ on chromosome 21, was identified.

APP is a type one transmembrane protein and a receptor-like protein that is wildly expressed in neural and non-neural cells (Haass and Selkoe, 2007). The physiological function of APP is not well understood. However, there are reports that suggest APP may have trophic properties (Saitoh et al., 1989; Meziane et al., 1998), cell adhesion properties (Young-Pearse et al., 2007) and gene transcription activity (Pardossi-Piquard et al., 2005; Zhang et al., 2007). Animals with an APP deletion have less body mass and less synaptic marker (Thinakaran and Koo, 2008), suggesting a role of APP during their development.

The APP can be cut and processed in two ways, and the APP cutting enzymes have been identified. The process that does not generate Aβ is called the non-amyloidogenic pathway, while the process that generates Aβ is called the amyloidogenic pathway (LaFerla et al., 2007) (see figure 1). The reason why APP needs to be processed in different ways, as well as the relevant physiological functions of APP cleavage products, is not clear. However, the non-amyloidgenic pathway is more prevalent than the amyloidogenic pathway in a healthy brain.

With the discovery of the APP gene, 25 genetic linkages between APP and AD, especially in early onset familial Alzheimer's disease, were reported (Tanzi and Bertram, 2005). Although the age of developing AD differs between EOFAD and LOAD, clinically and histologically, phenotypes are similar. More importantly, the mutations of

the APP gene in the region close to the Aβ domain that result in an increase in Aβ production are detected in EOFAD. This finding provides a causative link between Aβ and AD.

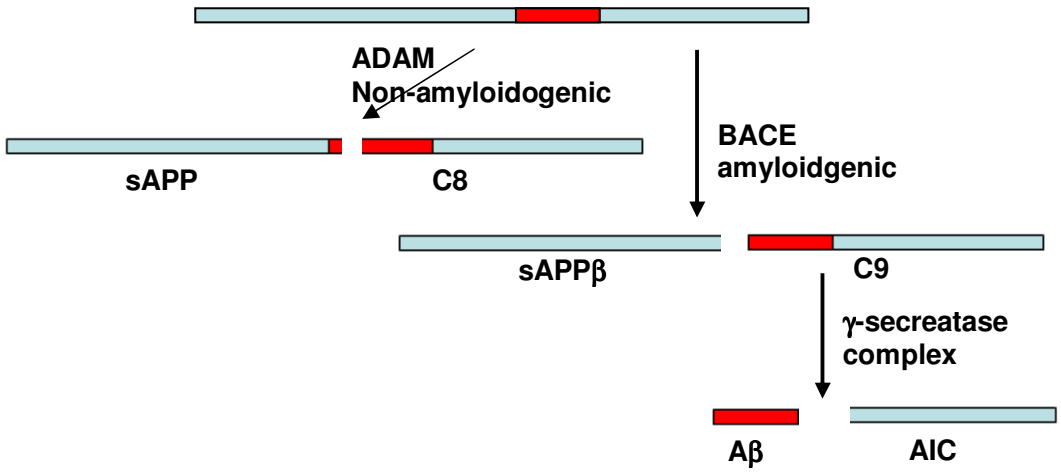


Figure 1. APP proteolysis. Left: the Non-amyloidogenic pathway, the first cutting enzyme called α-secretase, a disintegrin and metalloprotease (ADAM) family. After cutting by α-secretase, sAPPα will be released and the rest of the domain, C83, will be further cut by γ-secretase, a complex including PS1, Nicastrin, PEN2 and APH-1, to generate P3. Right: the Amyloidgenic pathway, after the APP is cut by β-secretase, also called BACE, sAPPβ will be released. The remaining part, C99, will be processed by γ-secretase to generate Aβ peptides and amino terminal intracellular domain (AICD).

1.2 The Aβ Cascade Hypothesis

Although EOFAD makes up only 5-10% of all AD cases and the onset of the disease is much earlier than LOAD, EOFAD phenocopies LOAD. Thus, EOFAD provides a genetic model to study the progression of cognitive impairment in AD.

Studies from EOFAD and related hereditary cerebral amyloid angiopathy have identified more than 25 mutations in APP. The mutation of APP leads to two possible consequences: an increase in the production of Aβ peptides and/or a change in the

propensity of Aβ aggregation (Tanzi and Bertram, 2005) (I will discuss Aβ aggregation later). Either one is believed to eventually lead to the senile plaques' formation in the case of EOFAD.

As mentioned above, Aβ peptides are cleavage products of APP. There are many different Aβ peptides that, depending upon the length, which could be anywhere from 39 to 43 amino acids long, could be produced after APP is processed in the amyloidogenic pathway. In the case of EOFAD, it has been observed that, in most circumstances, there is a change in the ratio of Aβ ending at position 42 (Aβ42) to Aβ ending at position 40 (Aβ40) (Haass and Selkoe, 2007).

These findings led to a hypothesis that the accumulation of Aβ can be pathogenic in AD. The Amyloid Cascade Hypothesis was first proposed by John Hardy and David Allsop in 1991 (Hardy and Allsop, 1991). This hypothesis was based on the fact that an early event of EOFAD is a mis-metabolism of APP, which leads to an increase of Aβ production and aggregation, especially for Aβ42. The aggregation of Aβ ultimately forms senile plaques and triggers subsequent pathological events, such as the formation of NFTs and synaptic dysfunction, eventually causing neuronal death and dementia.

With an increasing number of experiments and research being performed, this hypothesis has been gradually modified, but Aβ peptides are still highly accepted as a causative of AD.

2. What are the features of Aβ ?

In order to understand how Aβ is involved in AD, it is important to first characterize the properties of Aβ. In the following section, I will present various issues ranging from the structure of Aβ to its physiological function and its toxicity.

2.1 Aβ assembly

Aβ hypothesis suggests that accumulation of Aβ aggregates will trigger a neurotoxic cascade that leads to clinical manifestation. As a result, it is important to understand how Aβ accumulates and aggregates, resulting in pathological features.

Since it is quite difficult to directly study the progress of Aβ aggregate *in vivo*, most research on Aβ aggregation is done *in vitro*. Although most protocols used to generate Aβ aggregation employ non-physiological solvents to dissolve Aβ peptides and usually contain a high concentration of Aβ peptides (uM), they still provide a useful, detailed analysis of the structure of Aβ aggregates.

In vitro studies have shown that Aβ aggregates can be roughly categorized—from the smallest to the biggest size—as monomers, oligomers, protofibrils and fibrils, under examination by electromicroscope (EM) and atomic force microscope (AFM).

- *Monomers*: According to a nuclear magnetic resonance (NMR) study, monomeric Aβ is most likely an extension of a region of β strand (Hou et al., 2004) with a molecular weight of about 4.5 kDa.
- *Oligomers*: These are globular aggregates with a molecular mass usually within 50 kDa and have a spherical structure with a diameter around 5 nm (Lambert et al., 1998).
- *Protofibrils*: These are linear with a length within 200 nm (Harper et al., 1999).

- *Fibrils*: These are β-sheet structures, 1 um long and 7-12 nm in diameter (Makin and Serpell, 2005; Murphy, 2007).

The physical characteristic of most of the secondary structures of Aβ aggregates is the β-sheet structure. With the increase of the Aβ peptides' concentration, the amount of β-sheets contained dramatically increases. In general, there are three factors that affect Aβ aggregation: peptide concentration, pH, and salt concentration (Bharadwaj et al., 2008; Selkoe, 2008).

In contrast to the artificial synthesis of Aβ aggregates *in vitro*, naturally formed Aβ aggregates generated *in vivo* are often in lower concentrations (nanomolar) (Gravina et al., 1995; Näslund et al., 2000). It has been documented that a variety of oligomer aggregates like dimer, timer and tetramer have been detected by western blot methods in hAPP transgenic mice (Klyubin et al., 2005; Shankar et al., 2008) (an AD mouse model, expressing a mutant human APP gene) and in AD patients. In addition, protofibrils have also been observed *in vivo* (Lue et al., 1999; McLean et al., 1999; Lesné et al., 2006). These aggregates observed *in vivo* have been suggested as a brick to buildups of fibril and senile plaques. The toxicity of aggregates will be further discussed later in this chapter.

2.2 Physiological function of Aβ

Understanding the physiological function of Aβ may shed light on how Aβ plays a role in AD pathogenesis.

A study conducted by the Roberto Malinow group proposes a negative feedback model, which suggests that increased neuronal activity enhances Aβ secretion and the

enhanced Aβ production subsequently leads to a decrease of neuronal activity by suppressing the synaptic functions (Kamenetz et al., 2003). as shown in fig. 2.

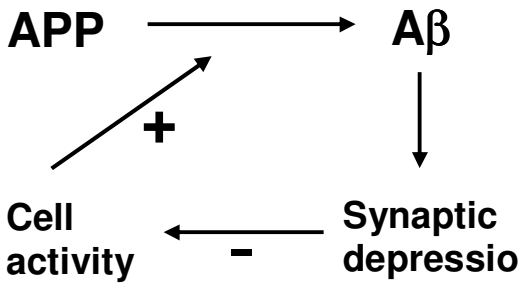


Figure 2. A negative feedback model. Cell activity enhances Aβ production through increasing the activity of β-secreatase. However, the produced Aβ will consequently decrease synaptic functioning and suppress neuronal activity.

In this study, they showed, first of all, that with the increase of cell activity in the hippocampus slice culture with an hAPPwt expression, Aβ production is further promoted. Second, they found that by using different hAPP constructs (some with the Aβ domain deleted and some with only the Aβ domain remaining), they could confirm that the synaptic suppression by over-expressing hAPP is due to Aβ production. Finally, with tetrodotoxin (TTX) treatment, a sodium channel blocker, they showed that neuron activity is required for APP to produce Aβ and, thus, suppress synaptic activity.

According to this model, the authors suggest that the loss of sensitivity of Aβ in synapse results in continuous Aβ production, causing neuronal toxicity (Kamenetz et al., 2003).

However, this model is inconsistent with observations in AD patients and mice models of AD. It is well known that AD patients suffer epileptic seizures, and the hAPP

transgenic mice have shown abnormally increased neuronal activity (Romanelli et al., 1990; Lozsadi and Larner, 2006; Palop et al., 2007). These studies are all from a population subject to Aβ over-production. These findings conflict with the negative feedback model, which proposes that an over-production of Aβ results in reduced neuronal activity.

Obviously, more studies are required to further understand the physiological/pathological function of Aβ. This question will be one of the principal issues addressed later in this work.

2.3 Aβ toxicity

Ever since APP was discovered and the Aβ cascade was proposed, much work has been conducted to understand the potential mechanisms of how the toxicity of Aβ is involved in AD pathogenesis.

In this section, I will discuss several perspectives of this topic, from *in vitro* to *in vivo* and from Aβ fibrils to Aβ oligomers.

Is senile plaque the source of Aβ toxicity in AD?

Based on an observation that postmortem autopsies of the brains of AD patients showed severe plaque deposits in the brain, it was originally thought that senile plaques or mature fibrils were toxic. Indeed, there are some studies suggesting that fibrils are toxic to neurons (Lorenzo and Yankner, 1994, 1996; Puzzo and Arancio, 2006; Yoshiike et al., 2007).

However, in postmortem examinations on elderly patients, scientists sometimes found those who died without being diagnosed with AD also showed senile plaque buildups in

the brain. And other elder patients, who had severe dementia, did not show an increase in senile plaque buildups (Schmitt et al., 2000; Goldman et al., 2001; Bennett et al., 2006; Erten-Lyons et al., 2009). This can be quite confounding because if senile plaques are toxic, then why is it that patients who had severe buildup of senile plaques in the brain had normal cognitive function? This finding urges scientists to revisit the question of where Aβ toxicity comes from. One of the important features of Aβ peptides is that this peptide tends to aggregate into different aggregate forms. As a result, people began looking at different Aβ aggregates in hopes of discovering other possible explanation(s).

Study of cell cultures

In order to address this question first, biochemists have developed many different protocols that make it possible to artificially synthesize different aggregate forms of Aβ (Dahlgren et al., 2002; Klein, 2002). Experimental data has shown that different Aβ aggregates have different levels of toxicity, which is reflected in cell viability in cell culture systems (Dahlgren et al., 2002). Aβ oligomers are more toxic forms than Aβ fibrils and Aβ monomers. *In vitro* experiments further provide explanations for how Aβ causes cell death. Application of Aβ to the cells results in increasing intracellular oxidative stress by promoting lipid oxidation (Chen, 2008; Liu, 2008), intracellular calcium concentration (Canevari et al., 2004) or endoplasmic reticulum (ER) stress (Ghribi, 2006) and eventually leads to cell death (Praticò, 2008).

In vitro experiments also make it possible to directly compare the toxicity effects from different lengths of Aβ peptides (Dahlgren et al., 2002). Amongst all the Aβ peptides, Aβ42 is the most toxic. This result is consistent with the observation from

EOFAD, since one of the common features of EOFAD is an increase in the ratio of Aβ42 to Aβ40.

Study of the electrophysiology

Synaptic plasticity has long been suggested as a cellular level of memory. Since AD is characterized by memory damage, much work has been done on looking at how Aβ affects synaptic functioning. The application of synthetic Aβ42 oligomers to hippocampus slice cultures shows synaptic transmission is affected and the long-term potentiation (LTP) is disrupted in comparison to the control group, which only provided a vehicle solution (Lacor et al., 2004; Takahashi et al., 2004; Knobloch et al., 2007; Rowan et al., 2007).

Natural secretion Aβ aggregates have also been used to illustrate the effects of Aβ on synaptic functions. In one study, Chinese hamster ovary (CHO) cells expressing V717F hAPP mutation in APP751 constantly secreted substantial amounts of Aβ. When the conditioned medium taken from this cell culture was applied to a hippocampus slice culture, LTP was disrupted. This experiment not only demonstrates that the natural secretion of Aβ aggregates can be pathogenic but also shows that the effects on LTP suppression are likely to be mediated by Aβ dimers and/or trimers (Walsh et al., 2002).

In order to address how Aβ peptides affect synaptic function *in vivo*, Szegedi et al. (2005) injected Aβ peptides directly into the hippocampus region of a rat through iontophoretic Pontamine Sky Blue injection and did extracellular recording in the CA1 region. NMDA, AMPA or kainic acid was injected prior to Aβ injection. After injecting Aβ42 aggregates into the brain (most of the aggregates they injected were fibrils or

protofibrils), they found an increased response in fEPP evoked by NMDA, but a reduction in the response to AMPA and kainic acid (Szegedi et al., 2005).

All *in vitro* studies demonstrate that Aβ affects the synaptic function and is toxic to cells. These findings suggest that Aβ might play a role in disrupting memory and degeneration.

***In vivo* studies**

In order to investigate *in vivo* potential mechanisms of AD, several hAPP transgenic mice were developed. In general, the strategy used to develop these mice introduced a mutated human APP gene into the mice genome. Since there are various AD-associated mutations of APP, different hAPP transgenic mice have been developed. The reason that scientists do not use mouse APP is because rodent APP yields significantly less Aβ peptides, as a result of changes in relevant amino acids, and the Aβ peptides produced from rodent APP lack the propensity to form β-sheets and, therefore, are unable to aggregate similarly to those discovered using human Aβ fragments (Johnstone et al., 1991; De Strooper et al., 1995).

In studies, hAPP transgenic mice showed an increase in Aβ production; in particular, there was an increase of the Aβ42 to Aβ40 ratio. Senile plaques were also found in the brain of hAPP mice (Game et al., 1995). These results are consistent with observations in EOFAD.

A hippocampus slice culture from hAPP transgenic mice showed that synaptic transmission and synaptic plasticity, LTP, was affected (Fitzjohn et al., 2001). Moreover, a decrease of Aβ production by reducing β- or γ-secreatase was able to recover the

synaptic deficit, indicating that the synaptic damage in hAPP transgenic mice was due to Aβ overproduction (Kamenetz et al., 2003).

In addition, in the hAPP transgenic mice studies, animals showed significant impairment in learning and memory. These cognitive impairments in the hAPP transgenic mice could be corrected by immunizing the hAPP transgenic mice with an Aβ antibody injection (Klyubin et al., 2005) (the application of immunization in AD will be further discussed later).

In vitro studies have suggested that Aβ dimers or trimers can initiate synaptic dysfunction. What about *in vivo*? Recent studies that attempted to isolate specific Aβ aggregates from APP transgenic mice responsible for memory decay in transgenic animals showed that there is correlation between memory damage and Aβ oligomers' formation, especially an extracellular dodecameric form of 56 kDa. These authors demonstrated that injections of the Aβ 56 kDa in mice resulted in memory deficiency (Lesne´ et al., 2006).

In short, *in vitro* and *in vivo* studies have shown that Aβ peptides are toxic to cells, cause synaptic dysfunction, and induce behavioral deficiencies.

3. The involvement of Aβ in AD pathogenesis

I will first discuss the reasons to focus on Aβ, the main component of plaques, but not tau, a component of the tangles, during AD pathogenesis.

1) EOFAD provides a causative link between Aβ and AD.

2) Tau transgenic mice produce fronto-temporal dementia (FTD), which is different from AD (Hutton et al., 1998).

3) Elevations of Aβ lead to tau phosphrylation (Small and Duff, 2008). A recent data shows that memory damage in hAPP transgenic mice is suppressed with tau reduction, indicating that Aβ is the prime pathogenic driver (Roberson et al., 2007).

Studies of EOFAD demonstrate that there is a causative link between Aβ and AD. Biochemical and physiological studies also demonstrate that Aβ is toxic. This data only suggests that Aβ may possibly be involved in AD pathogenesis. In this section, I will list some findings that provide *in vivo* suggesting Aβ involvement in AD pathogenesis.

First, the expression of Aβ peptides causes animals to have learning and memory deficiencies and the presence of plaques.

Second, recent work showed that Aβ dimers accumulate in the brain of AD sufferers. The application of isolated Aβ dimers from the AD patients' brains to the rat hippocampus showed marked synaptic dysfunction (Shankar et al., 2008). More importantly, rats that received purified Aβ dimers showed memory damage.

Third, the immunization of hAPP transgenic animals, either through passive methods, the injection Aβ antibodies into animals, or active methods, injecting Aβ homologues peptides into animals, results in the recovery of damaged memory or reduces the burden of senile plaques in the brain (Schenk, 2002; Klyubin et al., 2005).

The reasons why immunization works are not clear, but there are several possibilities: 1) Aβ antibodies bind to the Aβ aggregates and destroy the aggregation (Bacskai et al., 2001); 2) The targets of Aβ antibodies are the Aβ monomers. The structure of Aβ monomers may be stabilized after binding with antibodies, preventing further Aβ aggregates (Solomon et al., 1996); 3) These antibodies may also activate microglia to clear Aβ in the brain through phagocytosis (Bard et al., 2000); and 4) A peripheral-sink

effect reduces the soluble Aβ in the brain by reducing excessive circulation of soluble Aβ (DeMattos et al., 2001).

Although it is not clear which mechanism(s) is responsible for the recovery of behavior, reducing Aβ accumulation is the main purpose. Even though clinical trials were stopped due to an acute meningoencephalitis response (Wisniewski and Konietzko, 2008), most of the AD patients that received immunization showed a decrease of plaques in the brain, and some of them showed improvement in some cognitive testing (Wisniewski and Konietzko, 2008).

Altogether, these *in vivo* data demonstrate that the expression of Aβ reproduces the AD phenotype and reduces Aβ toxicity, enabling recovery of the behavioral deficiencies in hAPP transgenic mice and AD patients.

4. Treating AD: Current hypothesis and its caveat

Although AD is currently incurable, there are many hypotheses proposing various treatments for AD. Next, I will discuss the current treatment for AD, a potential treatment for AD, the most promising treatment as of yet, and, finally, recent evidence that has proposed treating AD as a diabetic form. I will briefly mention the rationale and its caveats.

4.1 Current treatment for AD: Acetylcholinesterase inhibitors

At the moment, acetylcholinesterase inhibitors are the only drugs that the Food and Drug Administration (FDA) has approved for AD treatment. The purpose of employing acetylcholinesterase inhibitors is to inhibit acetylcholine from degrading and boosting the

cholinergic system (Francis et al., 1999). The cholinergic hypothesis proposes that the loss of cholinergic neurons in the basal forebrain and their neurotransmission in the cerebral cortex with other areas contribute to the cognitive dysfunction observed in AD patients (Francis et al., 1999). This hypothesis is based on some findings that reduction of choline acetyltransferase and acetylcholine synthesis correlates with the degree of cognitive impairment in AD (Bartus et al., 1982).

In addition, *in vitro* data shows that Aβ reduces the choline uptake and acetylcholine release (Auld et al., 1998). Furthermore, tau phosphorylation is reduced after culture cells have obtained an over-expression of M1 muscarinic receptors treated with cholinergic agonist (Sadot et al., 1996).

The caveats are as follows: 1) Not all AD patients showed a reduction of acetylcholine (Barten and Albright, 2008); 2) It is still questionable whether cholinergic impairment directly or indirectly contributes to the disease process (Francis et al., 1999); and 3) All the acetylcholinesterase inhibitors only "delay" the progression of the disease (www.alz.org).

4.2 Potential treatment for AD: Immunotherapy

Since the first paper on using immunotherapy for AD treatment, published in 1999, demonstrated that immunotherapy is able to reduce the AD-like pathology in the PDAPP mouse, hAPP with valine at residue 717 substituted by phenylalanine (APP V717F) (Schenk et al., 1999), immunotherapy has been drawing a lot of attention for treating AD.

The idea to use Aβ specific antibodies for therapy was based on a finding that antibodies can recognize and disrupt some Aβ aggregates, especially oligomers. *In vitro*

experiments showed that the disruption of hippocampus LTP by intracerebroventricular injections of Aβ oligomers from CHO cells' condition medium could be completely reversed by the Aβ antibody (Kllubin et al., 2005).

Later, further evidence illustrated that through either passive or active immunization, behavioral deficiencies in hAPP transgenic mice were rescued (Wisniewski and Konietzko, 2008). Right now, many pharmacological companies are trying to develop a promising vaccine for application to AD therapy. The advantage of this treatment is that it is specific to the toxicity of Aβ. Unlike other treatments that can also affect other cells' functions through nonspecific side effects, this method specifically targets Aβ peptides.

The caveats of this treatment are as follows: 1) A strong immune response is induced by this therapy. The first clinical trials failed during phase II due to the acute meningoencephalitis in some patients (Wisniewski and Konietzko, 2008); and 2) In the six-year follow-up study, the first group of the clinical trial phase 1 showed no evidence in delaying the progression of the disease. One of the possible reasons could be that the treatment was started too late in the disease's progression. Early detection before clinical testing is required for the treatment (Holtzman, 2008).

4.3 Treating AD as diabetes type 3

Diabetes, diabetic mellitus, is characterized by hyper-insulinemia, hyper-glycemia, and a lessened response to insulin stimulus (Zhao and Townsend, 2008). It has been shown that there is a close link between diabetes and cognitive dysfunction, such as AD. Clinical studies show that about 80% of AD patients have diabetes or show abnormal

blood glucose levels (Janson et al., 2004). Also, people who suffer from diabetes often have a two- to three-fold increased risk for AD (Ott et al., 1999; Grant, 1999).

One clinical study suggested that there is insulin resistance in AD patients. Craft et al. (1998) compared the insulin level in 25 AD patients and 14 age-matched control groups. There was a higher plasma insulin and lower cerebrospinal fluid insulin in the AD patients. This result suggests that there is an association between insulin resistance and AD (Craft et al., 1998). This notion is also supported by data from AD mice models. In hAPP transgenic mice, the glucose metabolism or tolerance is affected. In addition, a diet high in fat, which leads to insulin resistance, also accelerates Aβ deposition (Ho et al., 2004; Pedersen et al., 2006). Based on these findings, AD appears to share several features with diabetes. Many investigators suggest that AD is type 3 diabetes (Zhao and Townsend, 2008).

The similarity between AD and diabetes encourages scientists to use similar treatments for diabetic patients and AD patients; however, one should use caution in treating AD as type 3 diabetes. Just because there are similarities in the phenotype does not mean that the mechanism is also the same. In fact, it is still controversial to use insulin to treat AD.

5. Insulin-Phosphoinositide-3 kinase (PI3K) signaling pathway in AD pathogenesis

The insulin-PI3K pathway has attracted a lot of attention for its pivotal role in cellular functioning, such as cell survival and proliferation and regulation of the synaptic function (Duronio, 2008). The simplify Insulin-PI3K pathway is illustrated in figure 3.

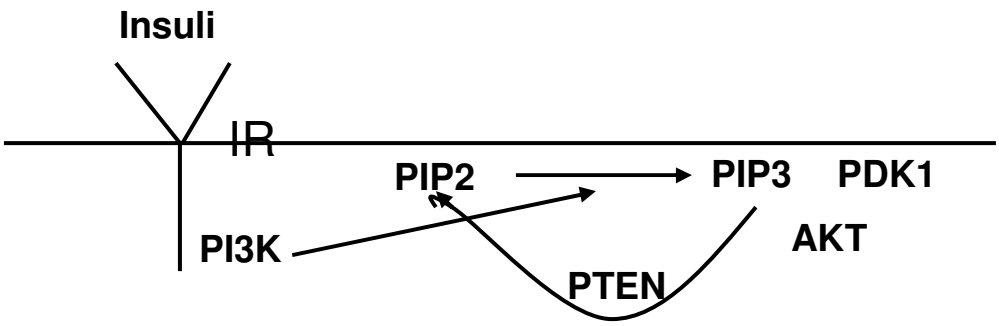


Figure 3. Insulin-PI3K signaling pathway.

The role of this signaling pathway in the Aβ pathogenesis is still not well defined and remains controversial. In studies, postmortem analysis of the brains of AD sufferers showed an increase of PKB activity and a reduction of phosphatase and tensin homolog (PTEN) in the temporal cortex (Rickle et al., 2006). Furthermore, the activity of insulin receptors in the brains of sporadic AD patients increased (Frölich et al., 1998). Acute insulin infusion increased cerebrospinal fluid Aβ42 in patients over the age of 70 (Watson et al., 2003). In addition, the oral application of wortmannin, a specific PI3K inhibitor, to Tg2576, transgenic mice expressing the human mutant APP (Swedish mutation), resulted in the reduction of Aβ accumulation (Haugabook et al., 2001). These results suggest that this signaling pathway is increased in AD pathogenesis.

In contrast, studies also showed that PI3K activity in AD patients was reduced from the intracellular soluble pool of the frontal cortex as compared to the control (Rickle et al., 2004). For rats that received the insulin growth factor 1, the infusion increased the clearance of Aβ in the brain (Carro and Torres-Aleman, 2004). Also, *in vitro* experiments showed that increased PI3K signaling activity was able to reduce the toxicities of Aβ and improved the cells' viability (Nakagami, 2004; Townsend et al., 2007; Lee et al., 2008).

A recent review tried to explain these contradictions. The authors proposed that there are optima insulin concentrations for different tissues. A lower concentration for one tissue probably would be too high for another, and different cases may require different concentrations (Cohen and Dillin, 2008).

In summary, a variety of treatment hypotheses have been proposed, ranging from hormone therapy and growth factor treatments to anti-inflammatory agents, but most of them have not shown any signs of being effective (Grundman and Thal, 2000). The need to treat AD is urgently required.

PART II:

The *Drosophila* model

In this section, I will elucidate the rationale behind using *Drosophila* as a model to study AD. First, I will cover the background of using *Drosophila* to study genes that relate to learning and memory, and then I will present current research employing *Drosophila* to study human diseases. Finally, I will discuss *Drosophila* as a model in researching AD.

II-1 Using *Drosophila* to study learning and memory

The cellular and molecular mechanisms in memory formation are preserved in the brains of life forms from lower invertebrates to higher mammalian systems. For memory formation, for both mammals and fruitflies, the same components of memory—short- , middle- and long-term memory—emerge at different times after acquiring new knowledge, according to the duration and time of onset. Also, on the molecular level, there are preserved functions among various species. In *Drosophila*, for example, studies on cAMP-response-element-binding protein (CREB) transcription factors in the fruitflies showed that this protein is involved in long-term memory (LTM) formation (Yin and Tully, 1996). Mutation of this gene causes mental retardation in humans and an LTM deficit in mouse models (Alarcón et al., 2004; Wood et al., 2005).

Furthermore, the ability to easily and rapidly manipulate the genes of *Drosophila* makes it a great model to study learning and memory. *Drosophila* has been used for studying the impact of genes on behavior for more than 30 years (McGuire et al., 2005). It was Seymour Benzer who, about 30 years ago, first proposed that *Drosophila* could be used to identify phenotypic mutants by single gene defects introduced through chemical mutagenesis (Benzer, 1971).

Several methods have been used with *Drosophila* to study genes associated with learning and memory, such as courtship, taste, and visual and olfactory cues. Among them, olfactory associative learning is the most intensively studied. In 1974, Quinn and colleagues first developed a reliable assay that was used to measure olfactory learning and memory in fruitflies (Quinn et al., 1974). The first assay was successful but had low performance due to the effects of poor training. Later in 1985, Tully and Quinn

developed a classical conditioning paradigm that strengthened the training effects, resulting in higher performance (Tully and Quinn, 1985).

Since then, using *Drosophila* as a tool to study learning and memory through the olfactory paradigm has been intensively employed.

II-1.1 The study of synaptic function in *Drosophila* Neuromuscular Junction (NMJ)

Synaptic plasticity, the process by which connections between a neuron and its target are modified, is believed to be the mechanism at the cellular level of learning and memory. Studies in fruitflies and rodents have shown that the synaptic molecules as well as the mechanism that regulates synaptic function are common. The glutaminergic synapse in *Drosophila* larval NMJ is particularly attractive for studying synaptic plasticity because of its ease of gene manipulation, anatomical accessibility, and suitability to quantitative studies of synaptic transmission and plasticity at identifiable synapses (Koh et al., 2000). Therefore, the use of fruitflies as a model provides a great opportunity to study and discover the molecular mechanisms of synaptic function.

Drosophila larval NMJ has been used to study genes that are involved in learning and memory on the synaptic transmission and activity-dependent plasticity. For example, in *Drosophila*, two mutation genes, *rutabaga* and *dunce*, identified through chemical mutageneis analysis, have been found to diminish the capacity of learning and memory associated with an altered cAMP level. Studies of these two mutation genes in NMJ further demonstrate their role change in synaptic plasticity and synapse morphology, which provides a cellular mechanism in explaining the learning and memory deficiency (Zhong and Wu, 1991; Zhong et al., 1992).

In addition, this preparation has been used for studying mutant genes that propagate human disease. For example, changes in *Drosophila's* fragile X mental retardation protein (dFMRP) level alter the synaptic morphology (Tessier and Broadie K, 2008). Mutations of presenillin (PS), part of subunits of gamma-secreatase that are involved in EOFAD, have shown a learning deficit among adult flies as well as altered synaptic functioning in *Drosophila* larva NMJ (Lu et al., 2007).

II-2 *Drosophila* as a model to study human disease

Genes that control basic development have been highly preserved among species, giving an opportunity to use different animal models to study developmental human disease. Genomic analysis has indicated that over **70%** of identified human disease genes have orthologs in *Drosophila*. In addition, more than one-third of human disease genes have sufficiently well-preserved homologues in *Drosophila* (Fortini et al., 2000; Iijima and Iijima-Ando, 2008). Also, in many cases, genes found in vertebrates can be used to functionally replace genes in *Drosophila*. Furthermore, in some cases, newly identified genes in *Drosophila* can also be helpful in studying human disease. For example, the *Delta* gene identified in *Drosophila* codes a ligand for Notch. In previous studies, it was shown that mutations to this gene caused a thickened wing-vein phenotype. Later, in mice, it was shown that mutations of the Delta-like 3 gene induced spinal malfunction (Fiúza and Arias, 2007).

Drosophila has been used to study various different aspects of human diseases, such as cancer, neurological disorders, cardiac disease, and many more. The advantages of using *Drosophila* as a model system to study human diseases are the subjects' short life

span and large number of progeny, as well as the ease of doing gene manipulation along with the absence of gene redundancy. The most common way to analyze human disease in *Drosophila* is to create a mutant line or insert an extra copy of genes that could be used to model human disease.

One can reasonably expect that there are similar molecular bases affected by inserting a gene to model human disease into *Drosophila*. This assumption is based on the consideration and observation that there are many highly preserved functional genes in both vertebrates and invertebrates, as well as similarities in the developmental growth between species (Bier, 2005).

There are several current methodological applications using *Drosophila* as a model to study human disease, especially those related to neurodegenerative disease. The following examples are using *Drosophila* to study neurodegenerative diseases.

II-2.1 Parkinson's disease (PD)

PD is characterized by a locomotion disability and a loss of dopaminergic neurons in the substantia nigra forming cytoplasmic aggregates known as Lewy bodies. The α-synuclein gene is linked to the family of Parkinson diseases and causes the formation of Lewy bodies and locomotion deficit. The first α-synuclein model to study Parkinson's disease was established in the year 2000 (Feany and Bender, 2000).

The recapitulation of the PD phenotype in α-synuclein flies resulted in the following observations: 1) age-dependent loss of dorsomedial dopaminergic neurons, 2) cytoplasmic inclusion, Lewy body-like, in the head of α-synuclein flies, and 3) locomotive deficiency (Feany and Bender, 2000).

II-2.2 Huntington disease (HD)

HD is one of several trinucleotide repeated disorders that causes the expansion of the unstable CAG tracts associated with the degeneration of brain-specific regions. Huntington is the responsible gene and encodes a cytoplasmic protein of ~350 KDa. The instability of CAG repeated tracts makes this protein vary in sizes and aggregate easily. The first Huntington transgenic fly models were established in 1998 (Jackson et al., 1998; Warrick et al., 1998).

The recapitulation of the HD phenotype in Huntington flies resulted in the following observations: 1) polyglutamine-dependent degeneration, and 2) accumulation of Huntingtin in the nucleus is age- and polyglutamine-length dependent.

II-2.3 Amyotrophic lateral sclerosis (ALS)

ALS is a progressive neurodegenerative disease that leads to loss of neuronal motor functions and to early death. Copper zinc-superoxide dismutase (SOD1) is the most intensively studied gene associated with ALS. In studies, the loss of functionality of this gene in mice did not induce the ALS phenotype; only the expression of a mutated human gene produced the phenotype similar to that found in the human disease. The first SOD1 model of *Drosophila* was not established until 2008 (Watson et al., 2008).

The recapitulation of the ALS phenotype in SOD1 flies resulted in the following observations: 1) progressive motor dysfunction, and 2) insoluble species with higher molecular aggregation (Watson et al., 2008).

II-3 *Drosophila* model in Alzheimer's disease

II-3.1 Physiological relevance of using *Drosophila* to study AD

A conserved function of hAPP in the *Drosophila*

APPL is a homologue of hAPP in *Drosophila*. The functionality between hAPP and amyloid precursor proteins like (APPL) is preserved in *Drosophila*. It has been shown that phototoxic damage in APPL-deleted flies is rescued by transgenic expressions of wild-types of APPL and also hAPP (Luo et al., 1992; Torroja et al., 1999).

APPL has similar processing pathways to hAPP; a recent finding has demonstrated that besides γ-secreatase, there is a β-secreatase-like enzyme in *Drosophila* (dBACE). After expression of APPL and dBACE in *Drosophila,* a cleavage product, a c-terminal fragment of APPL, is detected by western blot indicating that APPPL has been processed.

dAβ has a similar toxicity effect as Aβ in mammals

Carmine-Simmen and colleagues discovered that *Drosophila* Aβ (dAβ) is presented in the *Drosophila*, and this peptide is able to deposit and cause cell degeneration in the brain.

Overall these results suggest that there is physiological relevance for employing fruitflies to research AD.

Develop *Drosophila* AD model

In 2004 three papers demonstrated the possibility of using *Drosophila* as a model system to study AD. Although they all aimed to establish a model system, their experimental designs were all quite different.

Greeve et al. co-expressed hAPP with β- or γ- secreatase in the eyes of *Drosophila*. The rationale for using hAPP and secreatase was to mimic APP processing and to produce more hAβ peptides in the *Drosophila*. (Greeve et al., 2004).

First, they showed that the co-expression of hAPP and secreatase produces Aβ peptides. In this system, the authors did not specify which Aβ peptides played a major role in the formation of the resulting phenotypes. Second, they showed that those Aβ peptides are aggregated and can be detected by thioflavin-S staining, a dye used to stain β-sheet structures and commonly used as an indicator of aggregate proteins. They found that accumulation of Aβ peptides leads to cell degeneration in the eyes and is rescued by feeding a β-secreatase inhibitor, indicating that producing Aβ peptides in the fly causes a degenerative phenotype (Greeve et al., 2004).

The Konsolaki group used a very different strategy. Instead of expressing hAPP and β-tasecreatse in the fly, they directly expressed human Aβ42 peptides in the *Drosophila* eyes. The rationale for using hAβ42 was that Aβ42 is the most toxic peptide and has been implicated as playing a major role in AD pathogenesis. Furthermore, in doing so they can avoid other cleavage products from APP processing that may interfere with experimental results.

They observed rough eyes as an indication for cell degeneration, resulting from the expression and accumulation of hAβ42 in the eyes. Wild-expression of Aβ42 in all neuronal cells reduced the flies' life span. All the observed phenotypes were rescued by expressing the Aβ peptides degradating enzyme, neprilysin (Finelli et al., 2004).

Besides focusing solely on Aβ42, Iijima and colleagues also expressed Aβ40 separately. Although it has been suggested that Aβ42 is the most toxic of peptides, Aβ40 is far more abundant in the brain. Why Aβ42 is more toxic than Aβ40 is not clear, but there is evidence suggesting that Aβ42 tends to aggregate more than Aβ40, and the extra two amino acids added onto Aβ42 allow Aβ42 to easily interact with membrane drafts

that affect the properties of the membrane as well as proteins on it. However, how these two different peptides affect behavior differently is not clear. So this paper focused not only on pathological observations, but also on behavior outcomes in Aβ42 and Aβ40 transgenic flies. As expected, Aβ40 showed less deposits and less neuronal degeneration than Aβ42. Notably, both of them showed the same age-dependant learning damage. These results suggest that neuronal dysfunction and degeneration may be mediated by different mechanisms (Iijima et al., 2004).

Altogether, these three early papers showed the evidence that the *Drosophila* model of AD can recapitulate most AD phenotypes, such as age-dependant, cell degenerative, damage learning, shorten lifespan, and accumulate and deposit Aβ peptides.

II-3.2 The significance of using the *Drosophila* model to study AD

Many models have been established for studying AD, such as *C. elegans*, rats, mice, non-human primates and *Drosophila* (Götz and Ittner, 2008). Among them, the rodent model has been the best characterized and the most intensively studied. Next, I will compare the fly model to the mouse model and list significant findings from the fly model.

One of the important features of postmortem examinations of AD brains is global cell loss; however, all the hAPP transgenic mice models used so far have no such phenotype. The reason for the lack of mass cell degeneration in the mice models has yet to be determined (Iijima and Iijima-Ando, 2008). Consequently, this calls into question utilizing the rodent model to study neurodegeneration in AD. In contrast, there is great age-dependent cell loss in the AD flies (Iijima and Iijima-Ando, 2008). Two important

features, memory damage and cell degeneration, present in AD flies make the fly model a useful model to study AD pathogenesis.

The use of the *Drosophila* model of AD has been suggested for different applications. The genetic screening of *Drosophila*, one of the most powerful advantages of this model, gives us the ability to perform this operation easily on a large scale. To find a novel gene that may modify the disease's progression, the Konsolaki group tested 1,963 different mutants and identified 23 genes that may either improve or exacerbate the Aβ42 toxicity. The discovery of these genes may provide not only the mechanisms of Aβ42 toxicity but also potential therapeutic targets for future treatment of AD (Cao et al., 2008).

A large quantity of isogenic progeny and the ease of maintenance make *Drosophila* attractive to perform large-scale drug screening. Although so far there is no report regarding drug screening performed with the AD *Drosophila* model, the invaluable finding from this screening will yield significant findings for the future drug treatments of AD.

Summary

AD is a devastating, currently incurable neurodegenerative disease, and the need to develop an effective treatment is urgent. A body of evidence suggests that Aβ42 plays a

crucial role in the onset of AD. Recent studies suggest that accumulations of Aβ42 aggregates will initiate a neurotoxic cascade that leads to the AD pathogenesis. However, the underlying mechanism by which Aβ42 causes AD remains undetermined. The proposed research aims to study the mechanisms underlying the toxicity of Aβ42. In this research, the *Drosophila* as a model system to study AD is employed.

By examining studies that validate our *Drosophila* AD model, we demonstrate that the expression of Aβ42 in the *Drosophila* can recapitulate most AD phenotypes, such as deposition of Aβ42 in the fly brain, age-dependant memory loss, a shorter lifespan, and damage of locomotive capabilities in the Aβ42 flies. In addition, our data further elaborates on the relationship between Aβ42 toxicity and its aggregation. Strikingly, we show that different propensities of Aβ42 aggregation result in a distinct pathology *in vivo.* The Aβ42art mutant (which will be further discussed in Chapter 3) suppresses the aggregation of Aβ42. Deposits, mostly in the neuropil region, show memory damage but prolong lifespan compared to the Aβ42wt. On the other hand, the Aβ42arc mutant (to be discussed in Chapter 3) experience an enhanced aggregation of Aβ42, depositing mostly in the cell body and causing both memory damage and a shortened lifespan.

Confident that this model is applicable to studying the pathogenesis of Aβ42, we can now ask how Aβ42 affects memory. We look at how synaptic functions are affected by Aβ42. Synaptic plasticity is considered to be the cellular level of memory. In the fourth chapter, we demonstrate that different endogenous secretions of Aβ42 aggregates affect synaptic function differently. Depending on the different cell types, neuron cells' secret Aβ42 oligomers have a different effect on synaptic functioning than Aβ42 fibrils secreted

from muscle cells. The observation of how Aβ42 affect synaptic plasticity offers an opportunity to find the mechanism that involves the effect of Aβ42 on synaptic functioning and the possibility of employing this mechanism to discover genes that are involved in the pathogenesis of Aβ42.

In the fifth chapter, we seek to find out which genes can be utilized to prevent the toxicity of Aβ42. Here we demonstrate that PI3K is involved in the pathogenesis of Aβ42. Our results show that a change in the activity of PI3K is able to improve or enhance the toxicity of Aβ42 and moderate the aggregation of Aβ42. Our data shows, for the first time, that the activity of PI3K is required for Aβ42 aggregation and toxicity *in vivo*.

Chapter 2

Materal and Methods

Drosophila genetics and stocks

cDNA fragments encoding the human Aβ42, Aβ42Arc, and Aβ42art peptides were amplified by PCR from human APP cDNA, fused to the rat pre-proenkephalin signal peptide, cloned into the *pUAST Drosophila* transformation vector and microinjected into fly embryos of the w1118 (isoCJ1) genotype. Several transgenic lines for each Aβ construct were established. The flies were raised and maintained at 25^{o}C, under conditions of 70% humidity and a 12 h: 12 h light: dark cycle. The transgenic *UASCD8:: GFP; OK107* line was a kind gift from Dr. L. Luo (Watts, et al., 2003). *G7-Gal4* and *C57-Gal4* are muscle expression Gal4 drivers (Budnik et al., 1996; Renden and Broadie, 2003). *UAS-dPTEN* flies *(*Britton, et al., 2002), *UAS-PTEN c124s* flies (Huang et al., 1999), *UAS-p60 RNAi* flies was from the Vienna Drosophila RNAi Center. *UASnlsGFP* and *elav-GAL4c155* flies were obtained from the Bloomington Drosophila Stock Center. For Pavlovian olfactory conditioning, the *elav-GAL4c155* line was outcrossed with w1118 (isoCJ1) flies, an isogenic line, for 5 generations.

Western blot analysis

For sequential extractions, fly heads were homogenized in RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.5% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl) containing 1% SDS. Lysates were centrifuged at 100,000 g for 1h, and supernatants were collected (SDS-soluble fraction). SDS-insoluble pellets were further homogenized in 70% formic acid (Sigma) followed by centrifugation at13,000 rpm for 20 min and the supernatants

were collected (FA fraction). Formic acid was evaporated by Speed Vac (Savant, SC100) and protein was resuspended in dimethyl sulfoxiside (Sigma). Protein extracts were immunoprecipitated with the anti-Aβ antibody 6E10 (Signet), separated on 10–20% Tris-Tricine gels (Invitrogen), and transferred to nitrocellulose membranes (Invitrogen). The membranes were boiled in phosphate buffered saline (PBS) for 3 min, blocked with 5% non-fat dry milk (Nestle´) and blotted with the 6E10 antibody or anti-tubulin antibody (Sigma). To quantify levels of expression of the Aβ peptide, flies heads were homogenized in Tris-Tricine sample buffer (Invitrogen), centrifuged at 13,000 rpm for 20 min and the supernatants were subjected to Western blotting, as described above. The signal intensity was quantified using ImageJ (NIH).

Climbing assay

Approximately 25 flies were placed in an empty plastic vial. The vial was gently tapped to knock the flies to the bottom and the number of flies at the top, middle, or bottom of the vial was scored after 10 seconds under red light (Kodak, GBX-2, Safelight Filter). Experiments were repeated more than three times, and a representative result was shown.

Survival assay

Food vials containing 25 flies were placed on their sides at 25uC, under conditions of 70% humidity and a 12 h:12 h light:dark cycle. Food vials were changed every 2–3 days, and the number of dead flies was counted each time. At least four vials for each genotype

were prepared. Experiments were repeated more than three times, and a representative result was shown.

Pavlovian olfactory associative learning

Approximately 100 flies were trained by exposure to electroshock paired with one odour [octanol (OCT, 1023(v/v)) or methylcyclohexanol (MCH, 1023(v/v))] for 60 s and subsequent exposure to the other odour without electroshock for 60 s (Tully and Quinn, 1985). Immediately after training, learning was measured by allowing flies to choose between the two odours for 120 s. For one hour memory, trained flies were transferred to food vials, which were placed on their side in the dark at 25^{o}C and 70% humidity, and tested after one hour. The performance index (PI) was calculated by subtracting the number of flies making the incorrect choice from those making the correct one, dividing by the total number of flies, and multiplying by 100. Absolute odour avoidance was quantified by a T-maze with one of the two odours (octanol [103 (vol/vol)] or methylcyclohexanol [103 (vol/vol)]) coming from one side and air from the other side. Naı¨ve flies avoid odours, and the performance index was calculated by subtracting the number of flies that chose the odour side of the T-maze from those in the air side, dividing by the total number of flies and multiplying by 100.

Electric shock reactivity was tested by putting approximately 100 flies in a T-maze having one arm with electric shock and one arm without electric shock. The performance index was calculated by subtracting the number of flies that chose the electric shock arm of the T-maze from those in the arm without shock, dividing by the total number of flies and multiplying by 100.

Quantification of neurodegeneration

Heads were fixed in 4% paraformaldehyde (Electron Microscopy Sciences), processed to embed in paraffin blocks, and sectioned at a thickness of 6 mm. Sections were placed on slides, stained with haematoxylin and eosin (Vector), and examined by bright-field microscopy. To quantify neurodegeneration in the cell body and neuropil of the mushroom body structures, images of the sections which included the Kenyon cell body and/or Calyx were captured, and the area of the vacuoles in the Kenyon cell body or Calyx region was measured in each image. The ratio was calculated by dividing the sum of the vacuole areas by the total area of the Kenyon cell body or Calyx region. Seven to nine hemispheres from five flies were analyzed for each genotype. To quantify the atrophy of dendritic and axonal structures of the mushroom body neurons, the GFP signal in whole fly brains carrying *UAS-CD8::GFP;;OK107* was analyzed using confocal microscopy (Carl Zeiss LSM510). The area of Calyxes (dendritic structures of the Kenyon cells), Lobes (axon bundles of the Kenyon cells) and the size of the brains was measured using LSM Image software. Six hemispheres from three flies were quantified for each genotype.

Whole-mount immunostaining and Thioflavin S staining

Fly brains were dissected in cold PBS and fixed in PBS containing 4% paraformaldehyde (EMS), and then placed under vacuum in PBS containing 4% paraformaldehyde and 0.25% Triton X-100. After permeabilization with PBS containing 2% Triton X-100, the brains were treated with 70% formic acid (Sigma), and stained with a mouse monoclonal anti-Aβ antibody (Chemicon) followed by detection with biotin-XX

goat anti-mouse IgG and streptavidin-Oregon Green 488 conjugate (Molecular Probes). Nuclei were counterstained with propidium iodide (Molecular Probes). To detect nuclei of glial cells, fly heads were stained with an anti-Repo antibody (DSHB) followed by detection with Texas Red goat anti-mouse IgG (Molecular Probes). The brains were analyzed using a confocal microscope (Carl Zeiss LSM 510). For Thioflavin S staining, the brains were permeabilized and incubated in 50% EtOH containing 0.1% Thioflavin-S (Sigma) overnight. After washing in 50% EtOH and PBS, the brains were analyzed using a confocal microscope. ThioflavinS-positive deposits and neurites were quantified from six hemispheres from three flies per genotype.

Immuno-gold labelling and electron microscopy

Probosces were removed from decapitated heads, which were then immersion-fixed overnight in 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS. Samples were post-fixed 1 h in ferrocyanide-reduced osmium tetroxide (1% osmium tetroxide and 1.5% potassium ferrocyanide). Fixation was followed by dehydration in a graded alcohol series and infiltration with LR White resin (2 h in 50% LR White in ethanol and 24 h in 100% LR White) using constant rotation. After transferring the samples to gelatin capsules with fresh LR White resin, the samples were polymerized overnight at 60^{o}C. Thin sections (100 nm) of Kenyon cells and neuropil regions of the mushroom body were collected on nickel grids (100 mesh, Veco-EMS). For immunogold labelling of Aβ42 transgenic and control fly heads, thin sections were first incubated for 2 minutes in 10% hydrogen peroxide for antigen retrieval, jetrinsed in distilled water, and then placed on drops of 1% deacetylated BSA in PBS for 5 min. The grids were then transferred to drops of a rabbit

antibody specific for human Aβ42 (Chemicon-Millipore) diluted 1:10 in PBS and incubated for 2 h at room temperature. Unbound primary antibody was removed by rinsing the grids through 5 drops of PBS. Antibody was detected by incubating grids for 1h in 10 nm colloidal gold conjugated goat anti-rabbit H&L (GE Healthcare) diluted 1:10 in PBS. Grids were then rinsed in 10 drops of distilled water and air-dried. Thin sections were counterstained for 5 minutes in 3% uranyl acetate dissolved in 30% ethanol and then rinsed in distilled water.

Drosophila S2 cell culture

Drosophila Schneider's cells (S2 cells) were maintained in Schneider's *Drosophila* Medium (Gibco) supplemented with 10% FBS (GEMINI) and an Antibiotic-Antimycotic mixture (Gibco). The cells were transiently transfected with *Actin-Gal4* and *UAS-Aβ* plasmid constructs using a calcium phosphate transfection kit (Invitrogen). Culture medium was replaced at 12 h post-transfection, and cells were cultured for an additional 24 h. The cells and culture medium were then harvested and subjected to immunoprecipitation followed by Western blot analysis as described above.

Electrophysiology

Electrophysiological recordings of two-electrode voltage clamp were performed as described previously (Guo and Zhong, 2006). In brief, wall-climbing third-instar larvae from large fresh bottles were chosen for dissection. Larvae were dissected at room temperature and in Ca^{2+}-free hemolymph-like (HL-3) solution containing the following (mM): 70 NaCl, 5 KCl, 4 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose, 5 HEPES, and 115 sucrose.

All recordings were made at the longitudinal muscles of segments A4–A5, muscle fiber 12 with $CaCl_2$ (concentrations are indicated in the text and the figure legends). The segmental nerve was stimulated at 1.5 times the stimulus voltage required for a threshold response for Excitatory Junction Currents (EJCs). For recordings of LTD, the nerve was stimulated at baseline frequency of 0.05 Hz for 5mins and 30Hz for induction of LTD. Current signals were amplified with an Axoclamp 2B amplifier (Molecular Devices, Palo Alto, CA). The signals were filtered at 0.1 kHz on-line and converted to a digital signal using a Digidata 1320A interface (Molecular Devices) and acquired by pClamp 9.0 software (Molecular Devices).

FM1-43 dye imaging

This method is widely used for analyzing vesicle trafficking (Kilic, 2002; Kuromi and Kidokoro, 1999). The preparation was performed as described previously (Renden and Broadie, 2003) with some modification. Wall-climbing third-instar larvae were dissected in HL-3 solution as described in the preceding text. For loading, the preparation was incubated for 5 mins in HL-3 solution with 90mM K^+ and Ca^{2+} (1.8mM for *G7-Gal4* group and 0.9mM for *Elav-Gal4* group) containing 10 μM FM1-43 dye (Molecular Probes, Eugene, OR). The preparation was washed with HL-3 solution without Ca^{2+} for 10 mins. NMJs were imaged using two-photon imaging; a custom-built two-photon laser scanning microscope was used as described previously (Lendvai et al., 2000). For unloading, larva were stimulated by incubating in 90 mM K^+ with 0.4 mM Ca^{2+} for 20-30 seconds, followed by 10 mins wash. Boutons of muscle fiber 12 on a section were circled

and after subtracting background fluorescence, mean intensity from loaded and unloaded conditions were compared.

Aβ Preparations

Aβ1-42 peptides were purchased from Sigma. Aggregated Aβ1-42 was prepared based on protocols developed in previous studies (Dahlgren, et al., 2002). Aβ powder was initially dissolved to 1 mM in hexafluoroisopropanol (Sigma) then vacuumed in a Speed Vac to remove hexafluoroisopropanol. The film was first resuspended in dry dimethyl sulfoxide (Me_2SO, Sigma) to a concentration of 5 mM. For fibrillar conditions, 10 mM HCl was added to reach a final peptide concentration of 100 μM and incubated for 24 hr at 37°C. For oligomeric conditions, Ham's F-12 (phenol red-free, BioSource, Camarillo, CA) was added to bring the peptide to a final concentration of 100 μM and incubated at 4 °C for 24 h.

Lipid analysis

Wall-climbing third-instar larvae from large fresh bottles were chosen for dissection. Larvae were dissected at room temperature and in Ca^{2+}-free hemolymph-like (HL-3) solution containing the following (mM): 70 NaCl, 5 KCl, 4 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose, 5 HEPES, and 115 sucrose. After incubated with indicated drugs for 30 mins, animal is fixed with 4% paraformaldehyde for 20 mins then change to 0.5% spatonin for 20 mins. After blocking with normal goal serum overnight the animal is incubated with indicated antibody. Imagine is acquired by Confocal.

Five different cells from each animal is picked for quantify the lipid content. A line is draw cross an individual cell. A ratio peripheral region to central region fluorescence is used to compare the level of lipid between different genotypes.

Data analyses and statistics.

To minimize variation, each experimental group was only compared to a dedicated control group that had a similar genetic background and was recorded in the same batch of experiments. To be compatible for different Ca^{2+} concentrations, all EJC amplitudes were normalized to the respective controls. Evoked and spontaneous responses were analyzed using the Mini Analysis Program (Synaptosoft, Decatur, GA). All between-group comparisons were performed using t-tests.

Chapter 3

Aβ42 Mutants with Different Aggregation Profiles Induce Distinct Pathologies in *Drosophila*

ABSTRACT:

Aggregation of the amyloid-β-42 (Aβ42) peptide in the brain parenchyma is a pathological hallmark of Alzheimer's disease (AD), and the prevention of Aβ aggregation has been proposed as a therapeutic intervention in AD. However, recent reports indicate that Aβ can form several different prefibrillar and fibrillar aggregates and that each aggregate may confer different pathogenic effects, suggesting that manipulation of Aβ42 aggregation may not only quantitatively but also qualitatively modify brain pathology. Here, we compare the pathogenicity of human Aβ42 mutants with differing tendencies to aggregate. We examined the aggregation-prone, EOFAD-related Arctic mutation (Aβ42Arc) and an artificial mutation (Aβ42art) that is known to suppress aggregation and toxicity of Aβ42 *in vitro*. In the *Drosophila* brain, Aβ42Arc formed more oligomers and deposits than did wild type Aβ42, while Aβ42art formed fewer oligomers and deposits. The severity of locomotor dysfunction and premature death positively correlated with the aggregation tendencies of Aβ peptides. Surprisingly, however, Aβ42art caused earlier onset of memory defects than Aβ42. More remarkably, each Aβ induced qualitatively different pathologies. Aβ42Arc caused greater neuron loss than did Aβ42, while Aβ42art flies showed the strongest neurite degeneration. This pattern of degeneration coincides with the distribution of Thioflavin S-stained Aβ

aggregates: Aβ42Arc formed large deposits in the cell body, Aβ42art accumulated preferentially in the neurites, while Aβ42 accumulated in both locations. Our results demonstrate that manipulation of the aggregation propensity of Aβ42 does not simply change the level of toxicity, but can also result in qualitative shifts in the pathology induced *in vivo*.

Introduction:

The amyloid-β-42 (Aβ42) peptide has been suggested to play a central role in the pathogenesis of Alzheimer's disease (AD), a devastating, and currently incurable, neurodegenerative disorder (Selkoe, 2001). Aggregation of Aβ42 peptide in the brain parenchyma is a pathological hallmark of AD (Thal, et al., 2001). Genetic studies of early-onset familial AD (EOFAD) provide a strong causative link between Aβ42 and AD (Tanzi and Bertram, 2005), and some mutations in the Aβ peptide promote amyloid fibril formation (Nilsberth, et al., 2001; Johansson, et al., 2006). These data suggest that Aβ42 aggregation might be involved in AD pathogenesis (Lansbury and Lashuel, 2006), and Aβ42 aggregation is therefore an attractive target for therapeutic intervention in AD (Gestwicki, et al., 2004).

In vitro, the neurotoxicity of Aβ42 has been often correlated with the tendency of Aβ42 to aggregate (Yankner, et al., 1989; Murakami, et al., 2003). However, recent evidence indicates that Aβ42 can form a variety of misfolded structures, including multiple monomer conformers, different types of prefibrillar assemblies, and structurally distinct amyloid fibrils, and that such structural polymorphisms may mediate the diverse toxic effects of Aβ42 (Caughey and Lansbury, 2003; Klein, et al., 2004; Petkova, et al., 2005; Slow, et al., 2006). These results suggest that manipulation of Aβ42 aggregation *in vivo* may not simply change the magnitude of toxicity, but also qualitatively modify its pathogenic effects.

We have previously shown that expression of the human Aβ42 peptide in *Drosophila* brains induces age-dependent memory defects,

locomotor dysfunction, and neurodegeneration accompanied by Aβ42 deposits (Iijima, et al., 2004). Using this model system, we investigated the correlation between the aggregation tendencies of Aβ42 and memory defects, as well as neurodegeneration, through genetic manipulation of Aβ42 aggregation. We demonstrated that manipulation of the aggregation propensity of Aβ42 qualitatively as well as quantitatively modified the pathogenicity of Aβ42 *in vivo*.

Results

The human Aβ42 with the Arctic mutation (E22G substitution, Aβ42Arc) (Figure 1A), which causes early onset familial AD (EOFAD) (Nilsberth, et al., 2001) is more aggregation-prone and toxic *in vitro* (Johansson, 2006; Whalen, et al., 2005) and accelerates the formation of amyloid deposits in the brains of AD model mice (Cheng, et al., 2004; Lord, et al., 2006). In contrast, an artificial mutation, (L17P substitution, Aβ42art) (Figure 4A), suppresses amyloid fibril formation and toxicity *in vitro* (Murakami, et al., 2003; Morimoto, et al., 2004) and prevents the formation of amyloid deposits in C. elegans muscle (Fay, et al., 1998).

A signal sequence was fused to the N-terminus of each Aβ (Iijima, et al., 2004), to target the peptide to the secretory pathway. Multiple transgenic lines carrying a *UAS-Aβ42*, *UAS-Aβ42Arc*, or *UAS-Aβ42art* transgene were established. Expression of each Ab in the brain was driven by the pan-neuronal *elav-Gal4c155* driver (Brand and Perrimon, 1993). Since *elav-Gal4* is on the X chromosome, male progeny expressed more Aβ peptide and developed stronger phenotypes than female progeny due to dosage compensation (data not shown). The results presented in this study are from male flies, unless otherwise indicated.

Western blot analysis detected monomeric forms of Aβ42, Aβ42Arc, and Aβ42art as 4 kDa signals (Figure 4B). Monomeric and oligomeric forms of Aβ42art migrated slower than those of Aβ42 due to an amino acid substitution (Figure 4B and D).

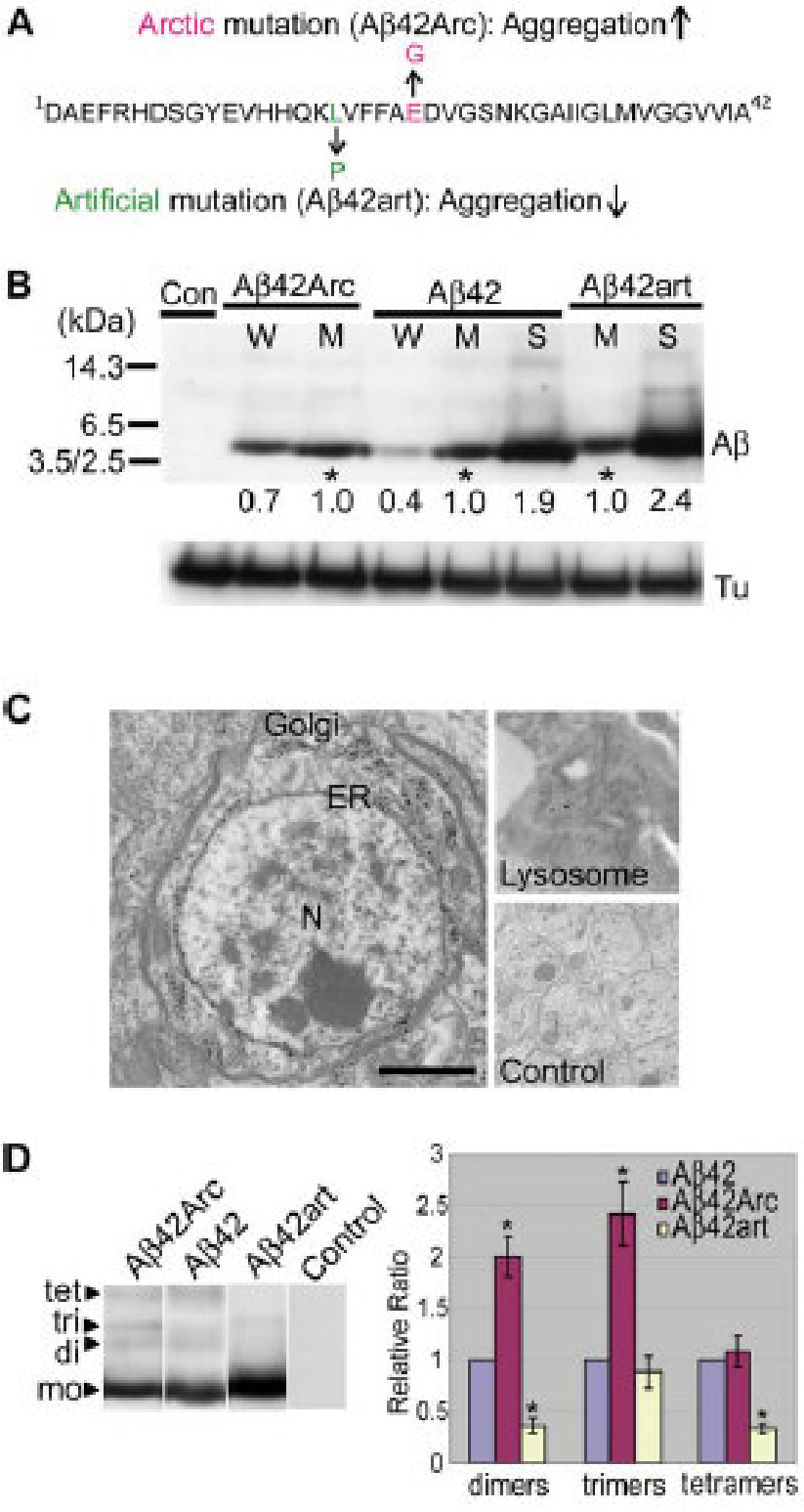

A
Arctic mutation (Aβ42Arc): Aggregation↑
G
1DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA42
P
Artificial mutation (Aβ42art): Aggregation↓
B
(kDa)
Con
Aβ42Arc
Aβ42
Aβ42art
W M W M S M S
14.3
6.5
3.5/2.5
Aβ
0.7 1.0 0.4 1.0 1.9 1.0 2.4
Tu
C
Golgi
ER
N
Lysosome
Control
D
Aβ42Arc
Aβ42
Aβ42art
Control
tet
tri
di
mo
Relative Ratio
Aβ42
Aβ42Arc
Aβ42art
dimers
trimers
tetramers

Figure 4.

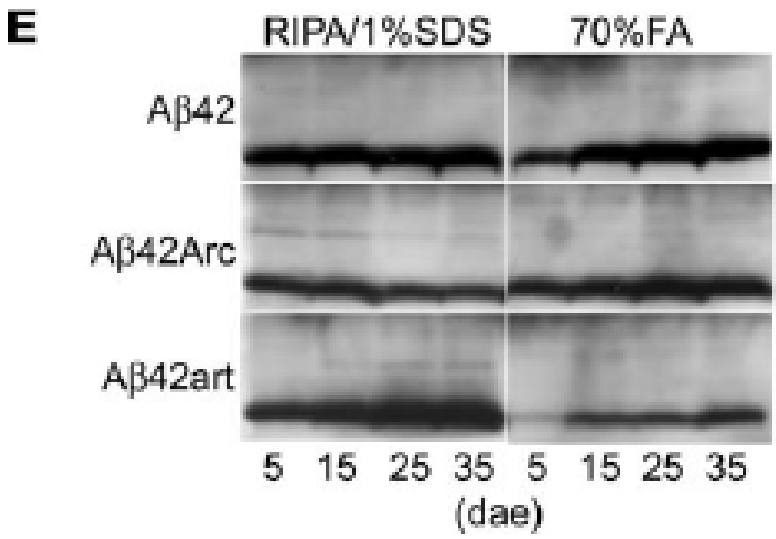


Figure 4, continued. Expression, distribution, aggregation, and accumulation profiles of mutant Aβ42 peptides in fly brains. (A), Sequences of Aβ42, Aβ42Arc, and Aβ42art. (B), Expression levels of Aβ in independent transgenic lines (W; weak, M; moderate, S: strong expression) at 1-2dae (top panel, Aβ42), when accumulation of each Aβ in the insoluble fraction was minimum, were compared, and relative ratios were shown below each lane and in Table S1 (n = 3). Asterisks indicate the fly lines primarily used in this study. *elav-Gal4c155* flies were used as control. Tubulin was used as a loading control (bottom panel: Tu). (C), ImmunoEM detection of Aβ42 in the endoplasmic reticulum (ER) and Golgi, as well as a lysosome. Gold particles are absent in the control (Control). N: nucleus, Scale bar: 1 mm. Neurons in Kenyon cell region of Aβ42 fly brains at 25dae were analyzed. (D), Detection of dimers (di), trimers (tri) and tetramers (tet) in fly brains. The level of each oligomer was shown as a ratio relative to that of Aβ42. Asterisks indicate significant differences from Aβ42 (n = 3, P,0.05, Student's t-test). (E), Age-dependent accumulation of Aβ peptides in detergent-soluble and insoluble fractions. The ages of the flies are indicated at the bottom. Data was done by Iijima.

Immunoprecipitation followed by mass spectrometry analysis confirmed that the fused signal peptide was correctly cleaved, and intact Aβ42, Aβ42Arc, and Aβ42art peptides were produced (Figure 5).

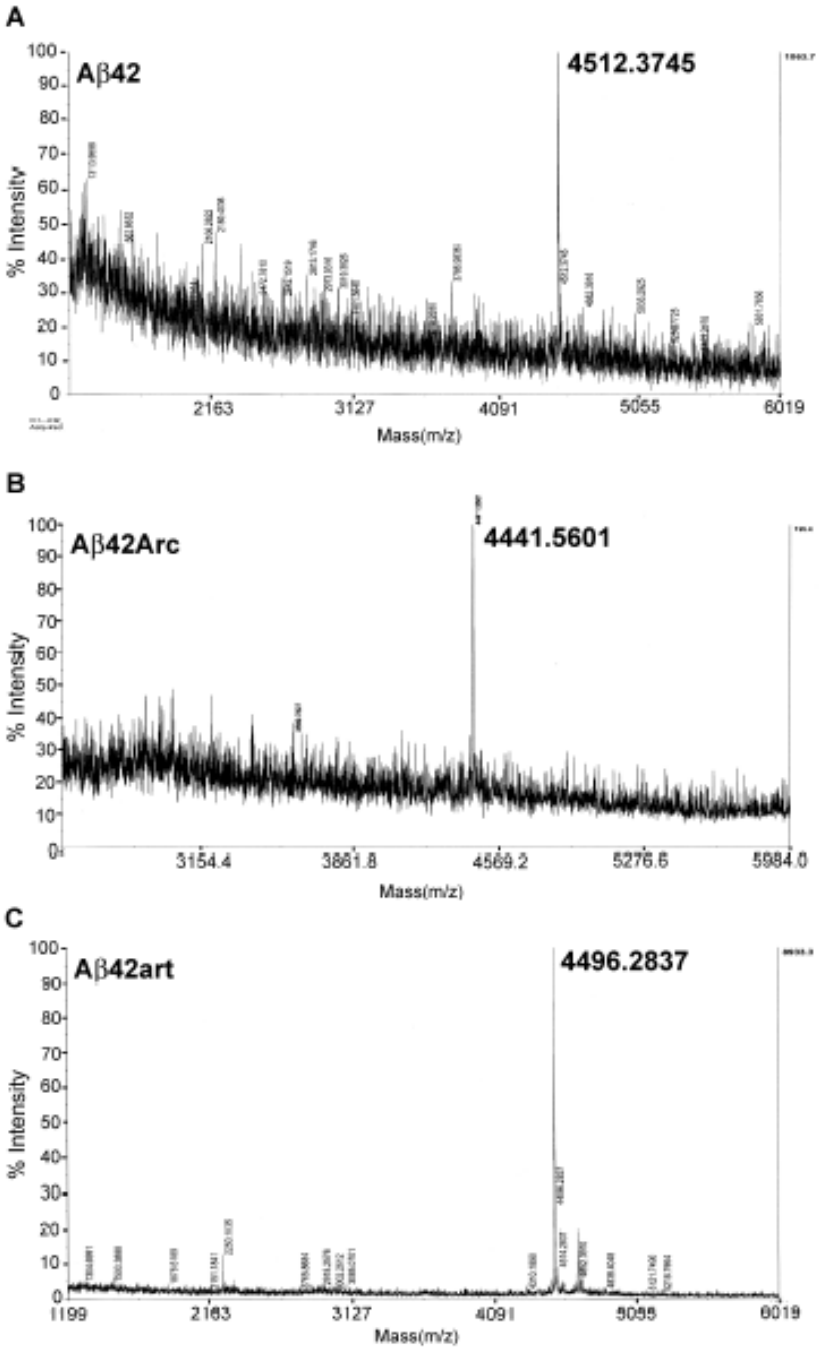


Figure 5 MS/IP analysis of Aβ peptides expressed in fly brains. Each Aβ peptide was immunoprecipitated using the anti-Aβ antibody and subjected to MALDI-TOF mass spectrometry. Aβ42 (A), Aβ42Arc (B), and Aβ42art (C) were each detected at their predicted mass. Data from Iijima

Immuno-electron microscopy (Immuno-EM) detected Aβ42 signals in the secretory pathway, including ER, Golgi, and lysosomes (Figure 4C), with minimal signals in the mitochondria and cytoplasm of neurons in the Kenyon cell region of Aβ42 fly brains. Aβ42Arc and Aβ42art peptides were also detected in the secretory pathway (data not shown). Secretion of Aβ peptides occurred in *Drosophila* cultured cells (Figure 6), and, in *Drosophila* brains, immuno-EM analysis occasionally detected Aβ42 accumulation in glial cells suggesting that Aβ42 peptides were secreted from neurons and then taken up by glial cells (Figure 7).

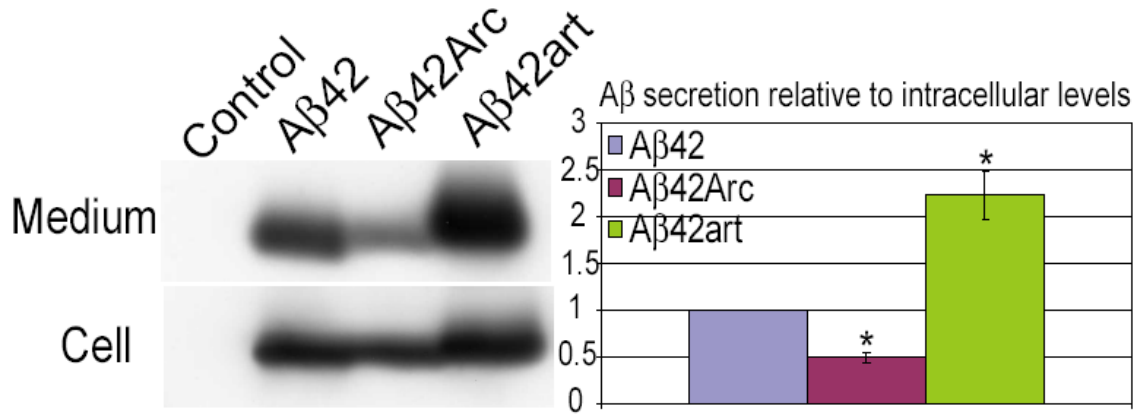


Figure 6. Secretion of Aβ peptides expressed in *Drosophila* S2 cells. The levels of Aβ42 (blue), Aβ42Arc (magenta), and Aβ42art (green) in the culture medium were detected by Western blotting, normalized to intracellular Aβ levels, and shown as a ratio relative to that of Aβ42. Each Aβ peptide was secreted at different levels. Asterisks indicate a significant difference from Aβ42 ($n = 3$, $P<0.05$, Student's t-test). Data from Iijima

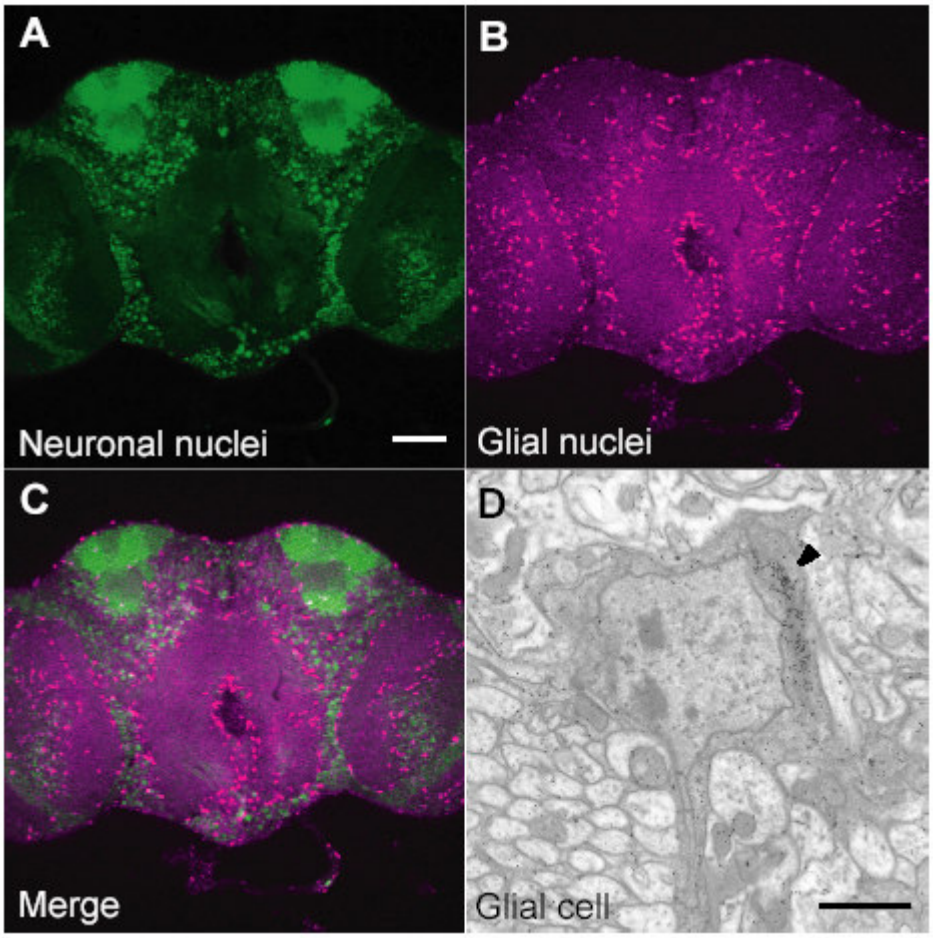
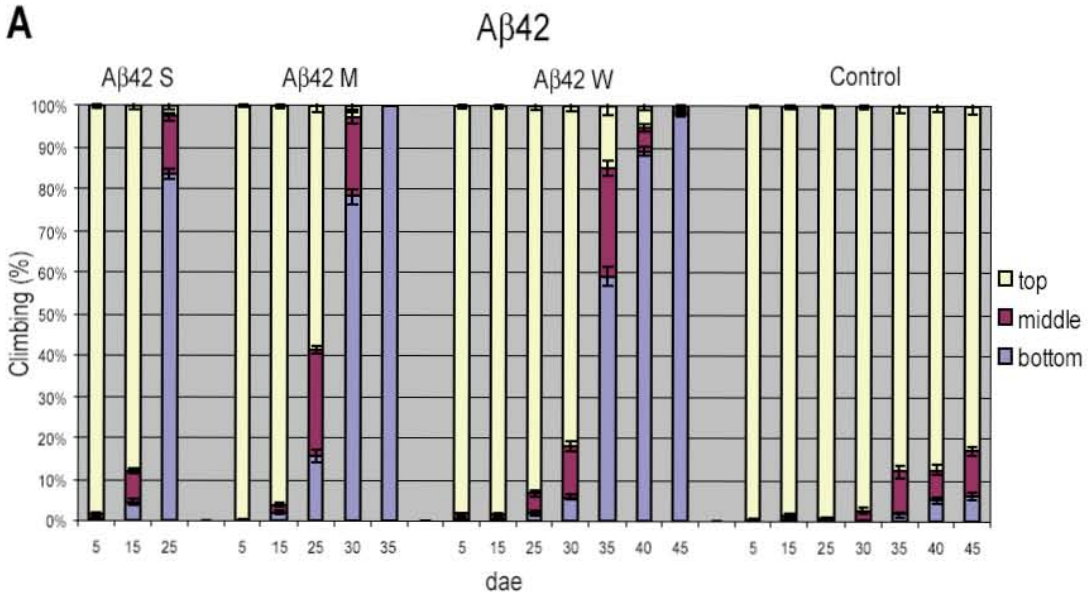


Figure 7. Glial cells accumulate Aβ42 peptide produced in neurons in the *Drosophila* brain. ImmunoEM analysis detected Aβ42 (gold particles, arrowhead) in glial cells in brains of 25 dae flies with Aβ42 expression driven by *elav-Gal4c155* (D). Scale bar in D: 1 µm. Confocal analysis revealed that *elav-Gal4c155* does not drive the expression of transgene in glial cells. All nuclei of neurons in the fly brain were labeled by GFP fused to a nuclear localization signal driven by *elav-Gal4c155* (A, green). The brain was counterstained with anti-Repo, a marker for *Drosophila* glial cells (B, magenta). The overlay image showed no significant overlap between the two signals (C). Scale bar in A, 50 µm. Data from Chiang and Iijima for EM

All Aβ peptides caused late-onset locomotor defects and premature death when expressed in neurons. Since the severity of these phenotypes positively correlated with the expression level of the peptides (Figure 8), we selected transgenic lines with similar

expression levels (Figure 4B, asterisks) to characterize the accumulation profiles and the pathogenic effects of each Aβ peptide in the *Drosophila* brain.



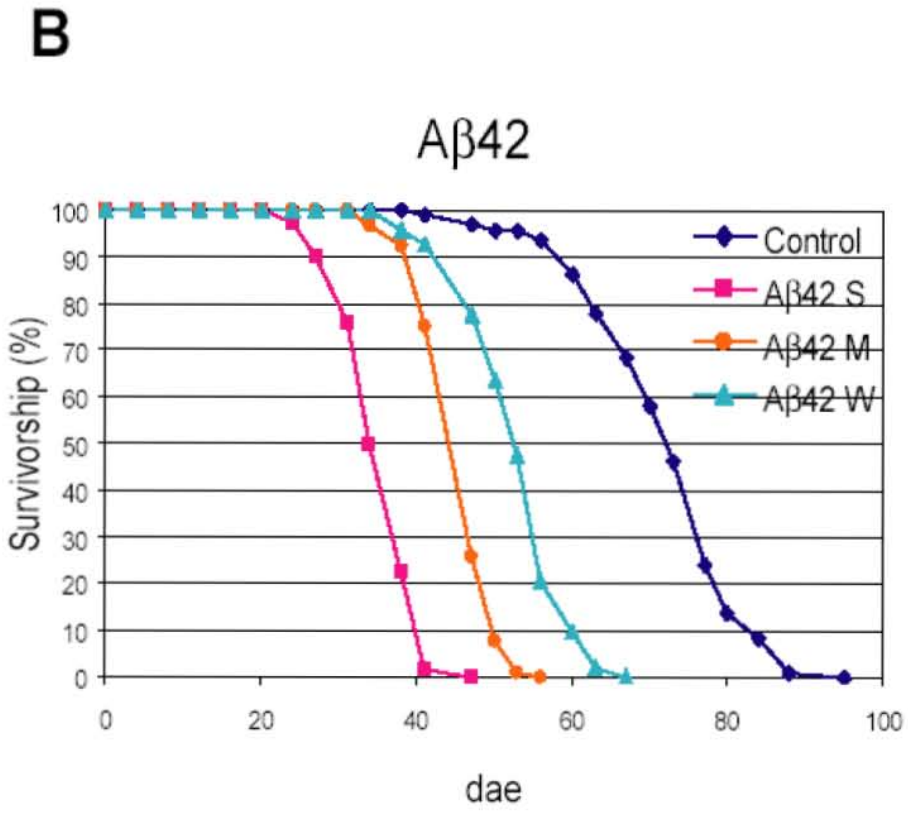
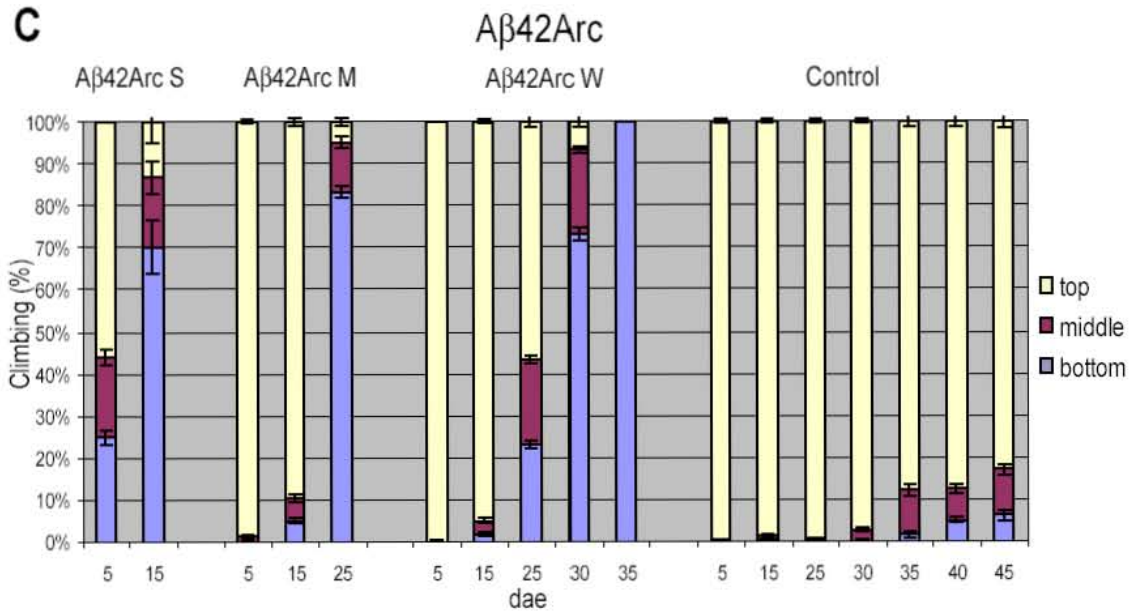


Figure 8.



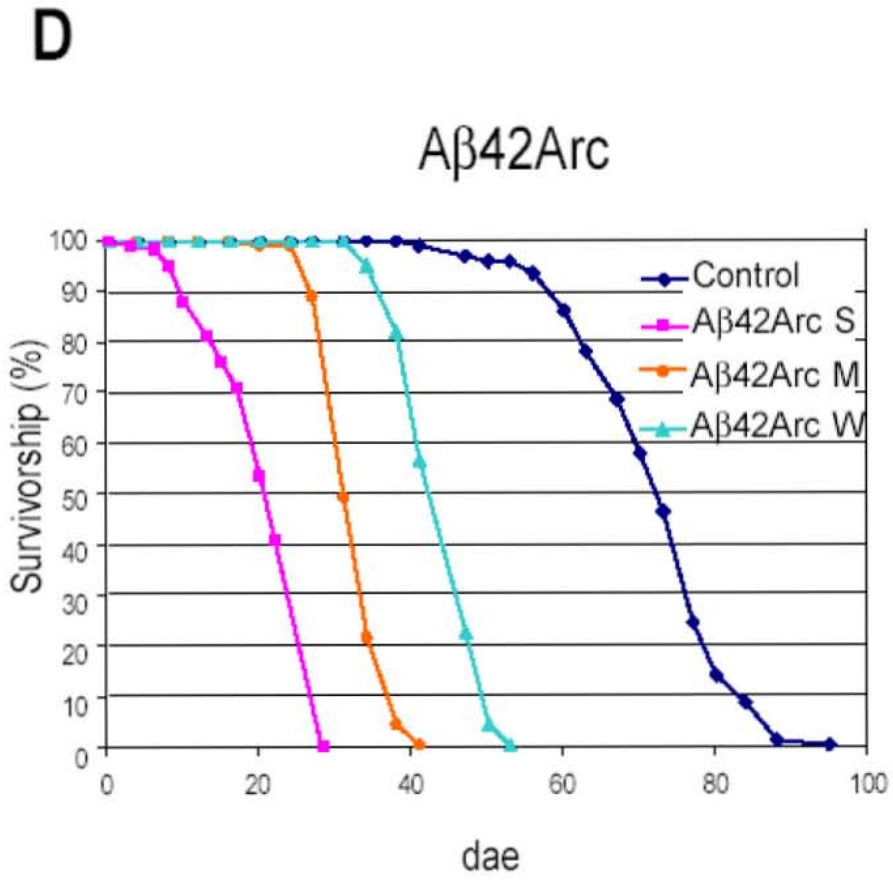
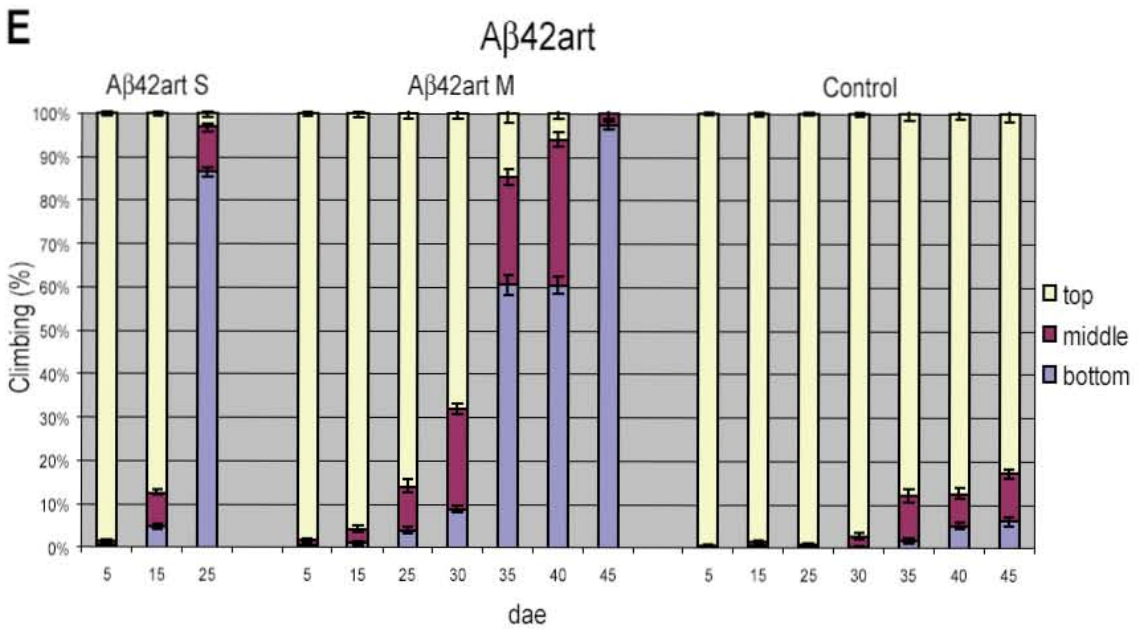


Figure 8, continued



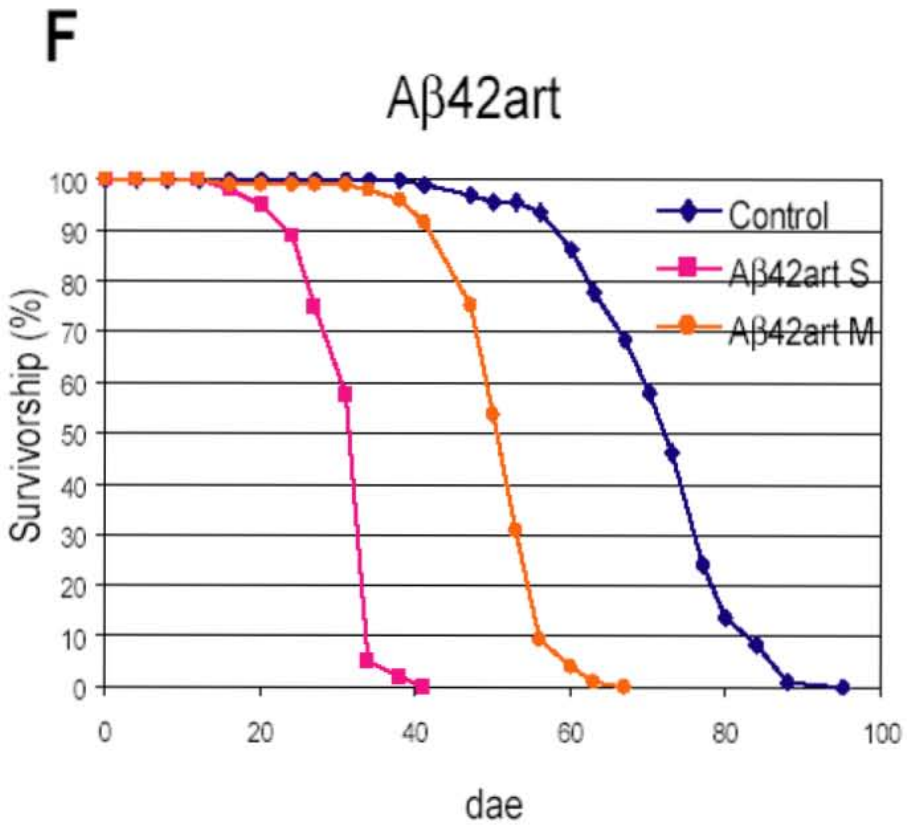


Figure 8, continued, Behavioral defects induced by the expression of Aβ42, Aβ42Arc, and Aβ42art peptides were dose dependent. (A), (C) and (E), Locomotor dysfunction in independent transgenic lines

Figure 8, continued, (W; weak, M; moderate, S; strong expression) of Aβ42 (A), Aβ42Arc (C), and Aβ42art (E). The percent of flies at the top (yellow), middle (magenta), or bottom (blue) of the vial at 10 seconds after knocking flies to the bottom are shown (average±SEM (n = 10)). B, D and F, The percent survivorship of independent transgenic lines (W, M, and S) of Aβ42 (B), Aβ42Arc (D), and Aβ42art (F) was plotted against the age (dae). The expression levels of Aβ peptides in all transgenic lines are shown in Figure 1B, and indicated as S (strong), M (moderate) or W (weak). The results are summarized in table 1. In the main text, the data from Aβ42 M, Aβ42Arc M and Aβ42art M (asterisks in Figure 1B) are presented. Data from Chiang and Iijima.

To compare the ability of each Aβ peptide to form small oligomers, we quantified the levels of dimers (8 kDa), trimers (12 kDa), and tetramers (16 kDa), as detected by Western blotting (Figure 4D). This analysis revealed that Aβ42Arc formed 2-fold more dimers and trimers than did Aβ42, while Aβ42art formed 50% fewer dimers and tetramers.

During aging, Aβ42 and Aβ42Arc accumulated in the insoluble fraction of brain lysates (Figure 4E; extracted by 70% formic acid), with no significant accumulation in the soluble fraction (Figure 4E; extracted by RIPA/1%SDS). Accumulation of Aβ42Arc in the insoluble fraction was more aggressive than that of Aβ42 (Figure 4E, compare Aβ42 and Aβ42Arc in 5 days-after eclosion (dae) flies). In contrast, Aβ42art strongly accumulated in the soluble fraction with greatly reduced accumulation in the insoluble fraction (Figure 4E).

It should be noted that although age-dependent accumulation of Aβ42Arc in FA fraction from 5 to 25dae was clearly observed, the level of Aβ42Arc at 35dae was less than that at 25dae, presumably due to a progressive cell loss in Aβ42Arc fly brains (See

below). These results demonstrate that Aβ42Arc is consistently more, and Aβ42art is significantly less, prone to aggregate *in vivo*.

The severity of locomotor dysfunction and premature death phenotypes of the transgenic flies correlated well with the aggregation proneness of the Aβ peptides. Climbing ability was used to quantify locomotor activity (Ganetzky and Flanagan, 1978). Climbing disability in 80% of the flies occurred by 25, 35, and 45 dae in Aβ42Arc, Aβ42, and Aβ42art flies, respectively (Figure 9A). A similar tendency was observed for the premature death phenotype. The average lifespan of Aβ42Arc flies (32.9 dae) was shorter than that of Aβ42 flies (46.2 dae), while Aβ42art flies (51.4 dae) lived longer than Aβ42 flies (Figure 9B).

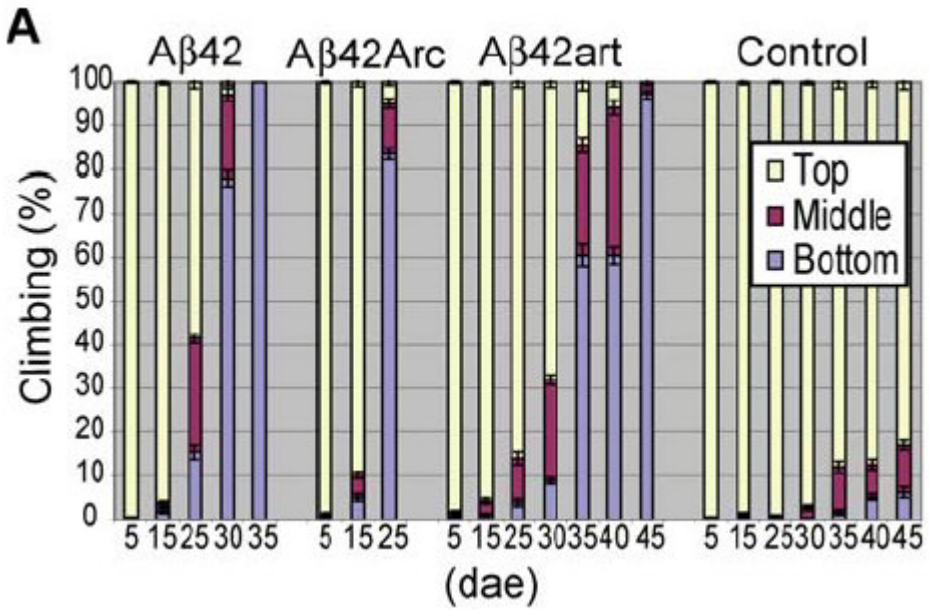


Figure 9.

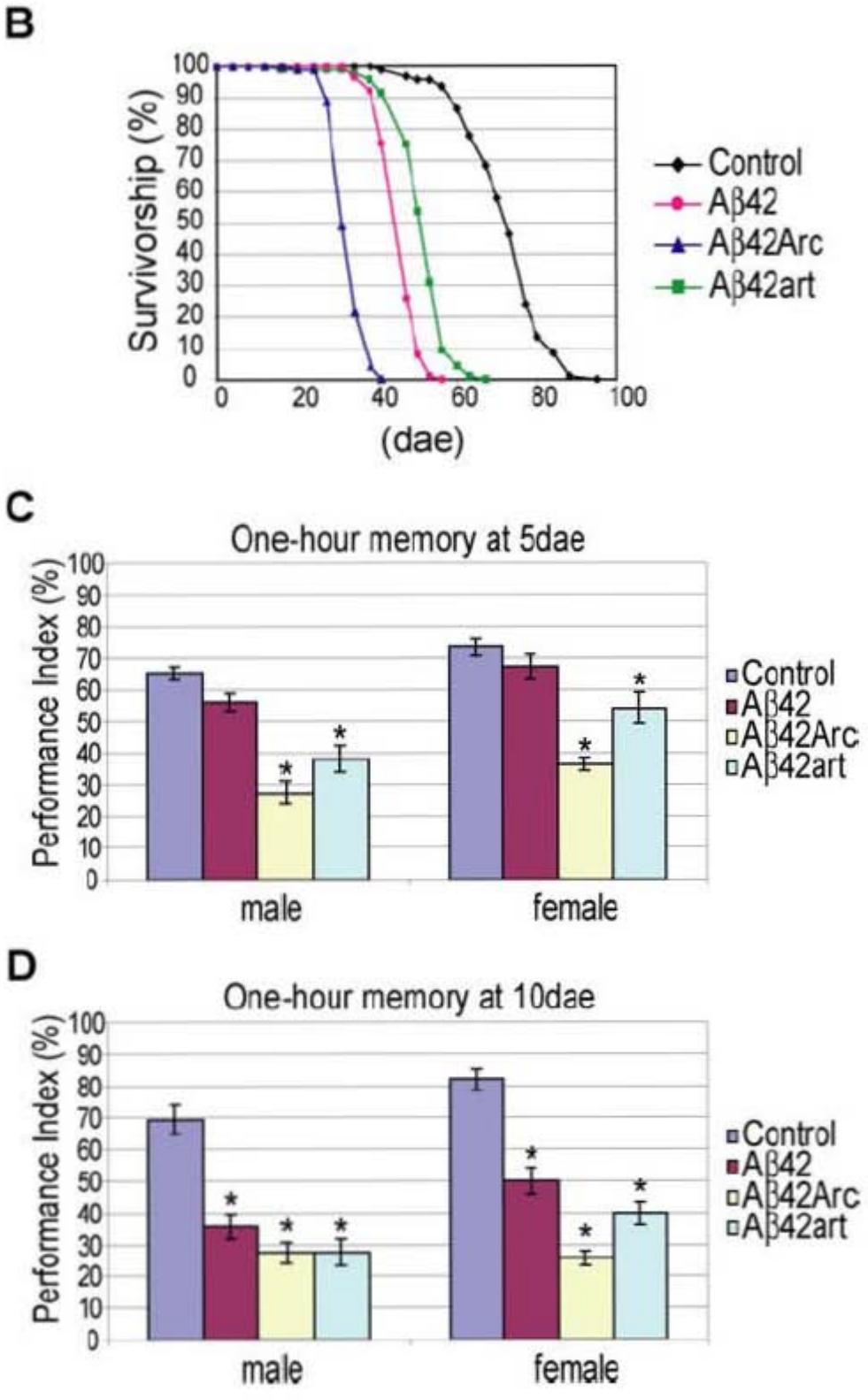


Figure 9, continued. Behavioral defects in Aβ42, Aβ42Arc, and Aβ42art flies.

Figure 9, continued. (A), Locomotor dysfunction. The percent of flies at the top (yellow), middle (magenta) or bottom (blue) of the vial at 10 seconds after knocking flies to the bottom are shown (average±SEM (n = 10)). (B), Premature death. The percent survivorship was plotted against the age (dae). (C) and (D), Memory defects. One hour memory was assessed by Pavlovian olfactory conditioning at 5 dae (C) and 10 dae (D). Asterisks indicate a significant difference from control (n = 6 or 8, $\alpha<0.05$, Tukey-Kramer significant difference). Average memory scores±SEM are shown. Data from Chiang for learning exp. and Iijima for climbing assay.

However, the onset of memory defects measured by Pavlovian olfactory classical conditioning (Tully and Quinn, 1985) did not follow this simple trend. This assay was conducted with younger flies (5 or 10 dae), before the flies developed locomotor defects (Figure 9A). Data obtained from male and female flies are presented separately, since expression of Aβ is higher in males than in females, as a result of dosage compensation. For 5 dae flies in both male and female groups, Aβ42Arc flies showed the most severe 1 hour memory defects (memory scores were measured 1 hour after the training session), and Aβ42art flies were also defective (Figure 9C). In contrast, memory in Aβ42 flies was indistinguishable from control flies (Figure 9C).

For 10 dae flies, all Aβ flies reached a similar level of memory defects in the male group (left panel in Figure 9D). In the female group, memory scores remained the lowest in Aβ42Arc flies, and both Aβ42art and Aβ42 flies showed similar defects (Figure 9D). Of note, learning scores were normal in both 10 dae Aβ42 and Aβ42art female flies, indicating that these flies were specifically defective in short-term memory, the major clinical manifestation observed in patients in the early stages of AD (Selkoe, 2002). The sensory motor activity of the flies, including sensing odors and electric shock, was

indistinguishable from controls at 10 dae (Table 1), indicating that the observed defects can be interpreted as learning and memory defects.

Genotype (females)	Shock reactivity (60 V)	Olfactory acuity (MCH, 10^{-3})	Olfactory acuity (OCT, 10^{-3})
Control	72 ± 4	45 ± 7	31 ± 7
Aβ42	80 ± 4	39 ± 7	24 ± 3
Aβ42Arc	77 ± 6	28 ± 6	29 ± 6
Aβ42art	73 ± 2	49 ± 5	27 ± 5

Genotype (males)	Shock reactivity (60 V)	Olfactory acuity (MCH, 10^{-3})	Olfactory acuity (OCT, 10^{-3})
Control	67 ± 5	46 ± 9	23 ± 5
Aβ42	69 ± 5	40 ± 3	21 ± 6
Aβ42Arc	67 ± 3	24 ± 4*	12 ± 4
Aβ42art	68 ± 5	57 ± 10*	17 ± 6

Table 1. Shock reactivity and olfactory acuity of transgenic flies at 10 dae. Aβ42, Aβ42Arc, and Aβ42art flies did not show any significant differences from control flies (n = 6, $\alpha<0.05$, Tukey-Kramer significant difference). Average scores±SEM are shown. In MCH olfactory acuity of males, there were no differences relative to controls, but Aβ42Arc and Aβ42art flies were significantly different from each other (*). Data from Chiang

Remarkably, Aβ42, Aβ42Arc, and Aβ42art each induced distinct pathologies. Neurodegeneration in the Aβ fly brains was observed as a vacuolar appearance both in the cell body and neuropil regions. To quantify the area lost in these regions, we focused on the mushroom body structure, in which the cell bodies (Kenyon cell body), dendrites (Calyxes), and axon bundles (Lobes) were easily identified (Heisenberg M., 2003). Our analysis revealed that, at 25 dae, Aβ42Arc fly brains showed more extensive cell loss than that in Aβ42 or Aβ42art brains (Figures 10A–F). However, the level of neuropil degeneration was greatest in Aβ42art flies (Figures 10A–F).

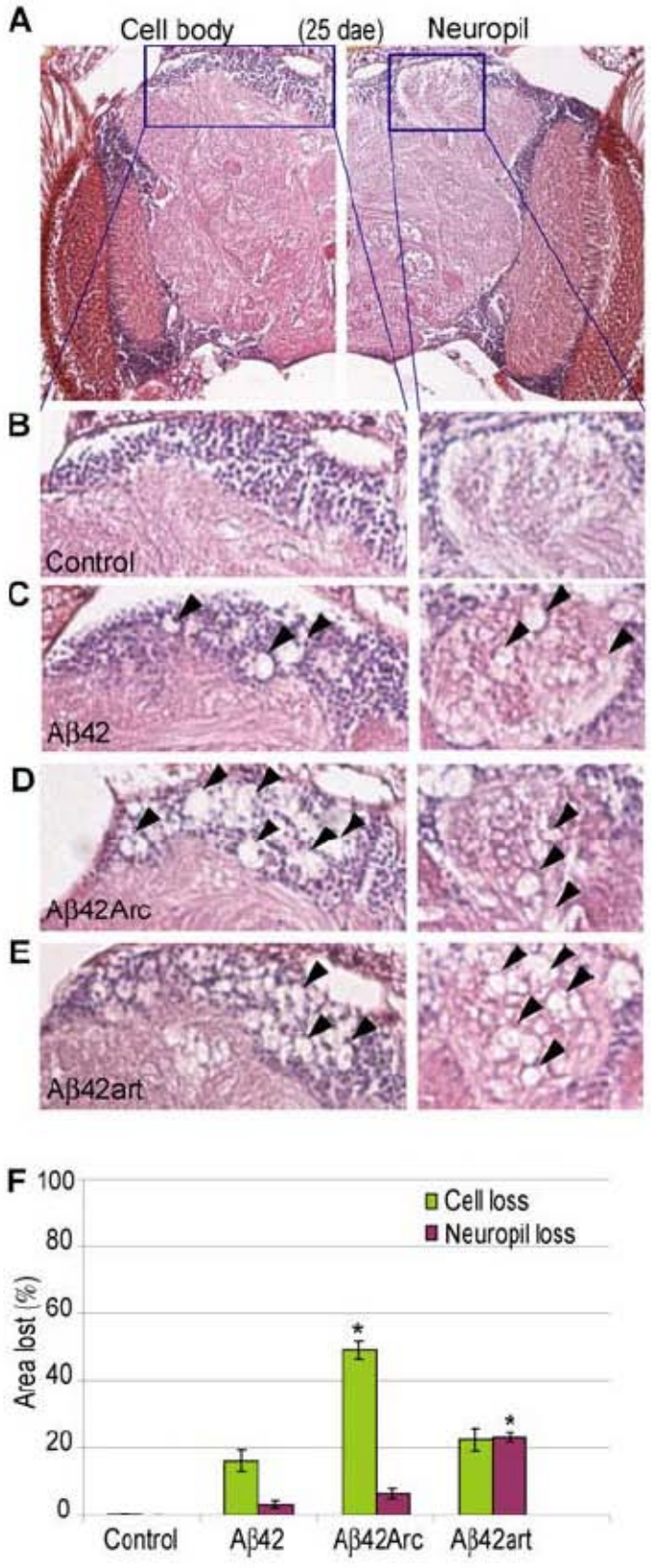

A
Cell body
(25 dae)
Neuropil
B
Control
C
Aβ42
D
Aβ42Arc
E
Aβ42art
F
100
80
60
40
20
0
Area lost (%)
Cell loss
Neuropil loss
*
*
Control
Aβ42
Aβ42Arc
Aβ42art

Figure 10.

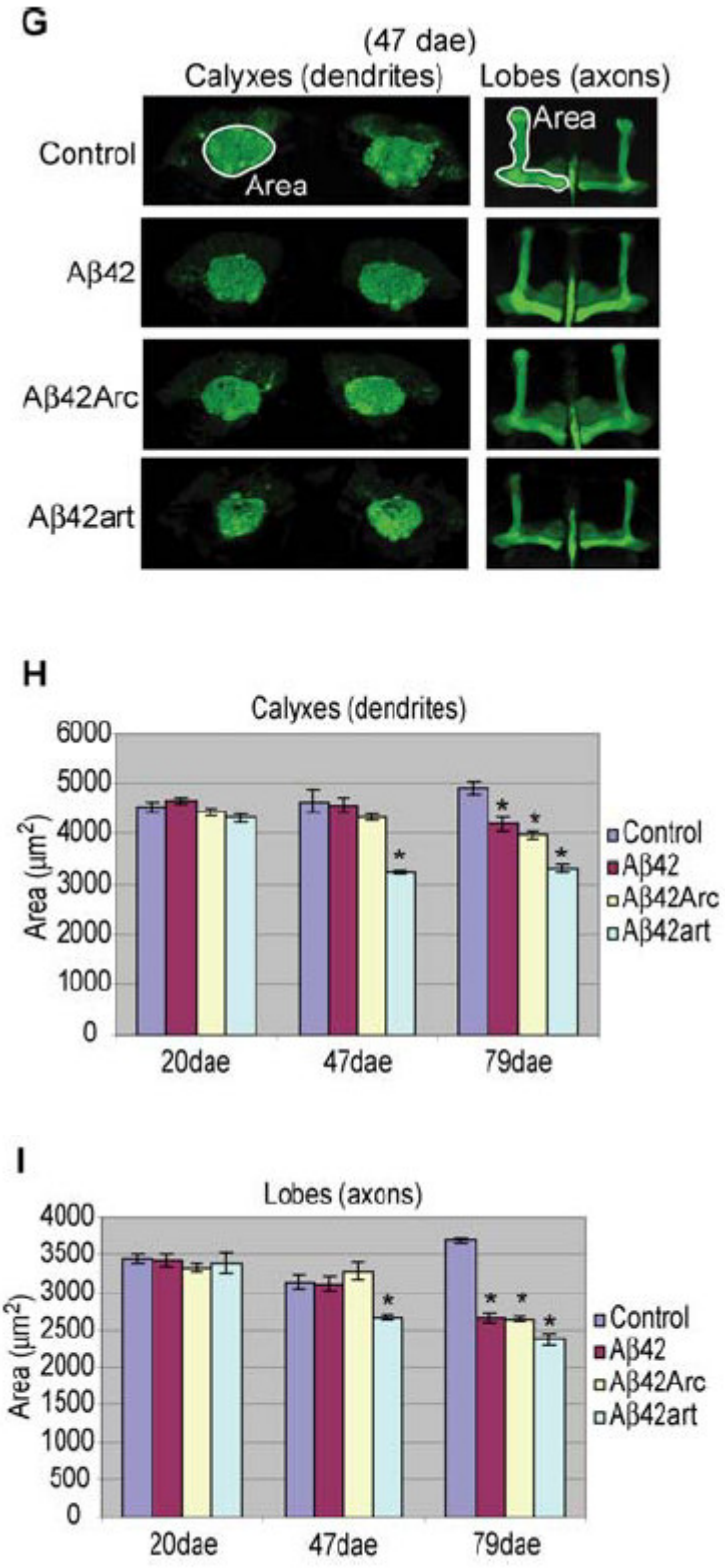


Figure 10, continued Cell body and neuropil degeneration in Aβ42, Aβ42Arc, and Aβ42art flies.

Figure 10, continued, A–E, Neurodegeneration in Aβ flies at 25 dae. The cell body and neuropil region in the mushroom body are enlarged. Arrowheads indicate neurodegeneration (C to E). F, Percentage of the area lost in the cell body (green) and neuropil (magenta) regions are shown as averages±SEM (n = 7–9 hemispheres). Asterisks indicate significant differences from Aβ42 ($P<0.05$, Student's t-test). G, Atrophy of Calyxes (dendrites) and Lobes (axons) in Aβ flies. H and I, Areas of Calyxes and Lobes were measured as indicated in (G) and presented as averages±SEM (n = 6 hemispheres). Asterisks indicate significant differences from control ($P<0.05$, Student's t-test). The ages of the flies are indicated at the bottom. Data from Chiang and Iijima

The enhanced neuropil degeneration observed in Aβ42art flies was further confirmed by confocal analysis. In this assay, each Aβ42 peptide was preferentially expressed in mushroom body neurons using the *OK107-gal4* driver, and the structure of dendrites (Calyxes) and axons (Lobes) was visualized by coexpressed *CD8-GFP*[26] (Figure 10G). Quantification of the size of these structures revealed that Aβ42art induced earlier onset and more severe atrophy in both the dendrites (Calyxes) and axons (Lobes) among all Aβ flies (Figure 10G–I). The severity of the atrophy in Aβ42 and Aβ42Arc was similar. Observed differences were not due to differences in brain size (Figure 11).

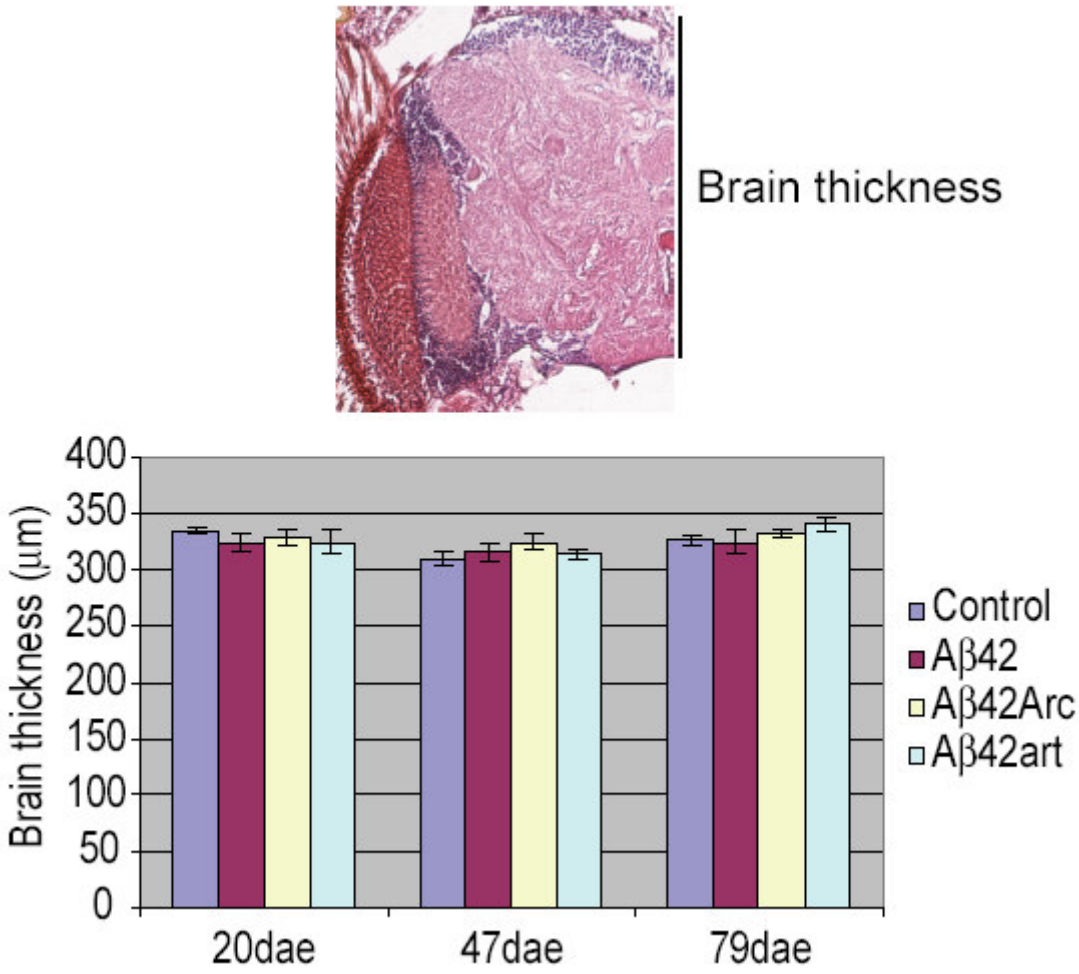


Figure 11. Brain sizes of control, Aβ42, Aβ42Arc, and Aβ42art flies were not significantly different. The thickness of fly brains was measured as indicated, and presented as average±SEM (n = 5 individual flies). The age of the flies is indicated at the bottom. Data from Chiang and Iijima

Immunostaining and Thioflavin S staining, which labels aggregated Aβ42, revealed that the degenerated structures in Aβ42, Aβ42Arc, and Aβ42art flies were closely correlated with the intraneuronal accumulation sites of each Aβ peptide. Aβ42Arc accumulated primarily in the cell soma as large deposits (Figure 12E, F, arrowheads in F), while Aβ42art was distributed primarily in the neurites (Figure 12G, H, arrows in H).

Aβ42 was detected both in the cell body and in the neurites, but to a lesser extent than the mutants (Figure 12C, D, arrowheads and arrows in D).

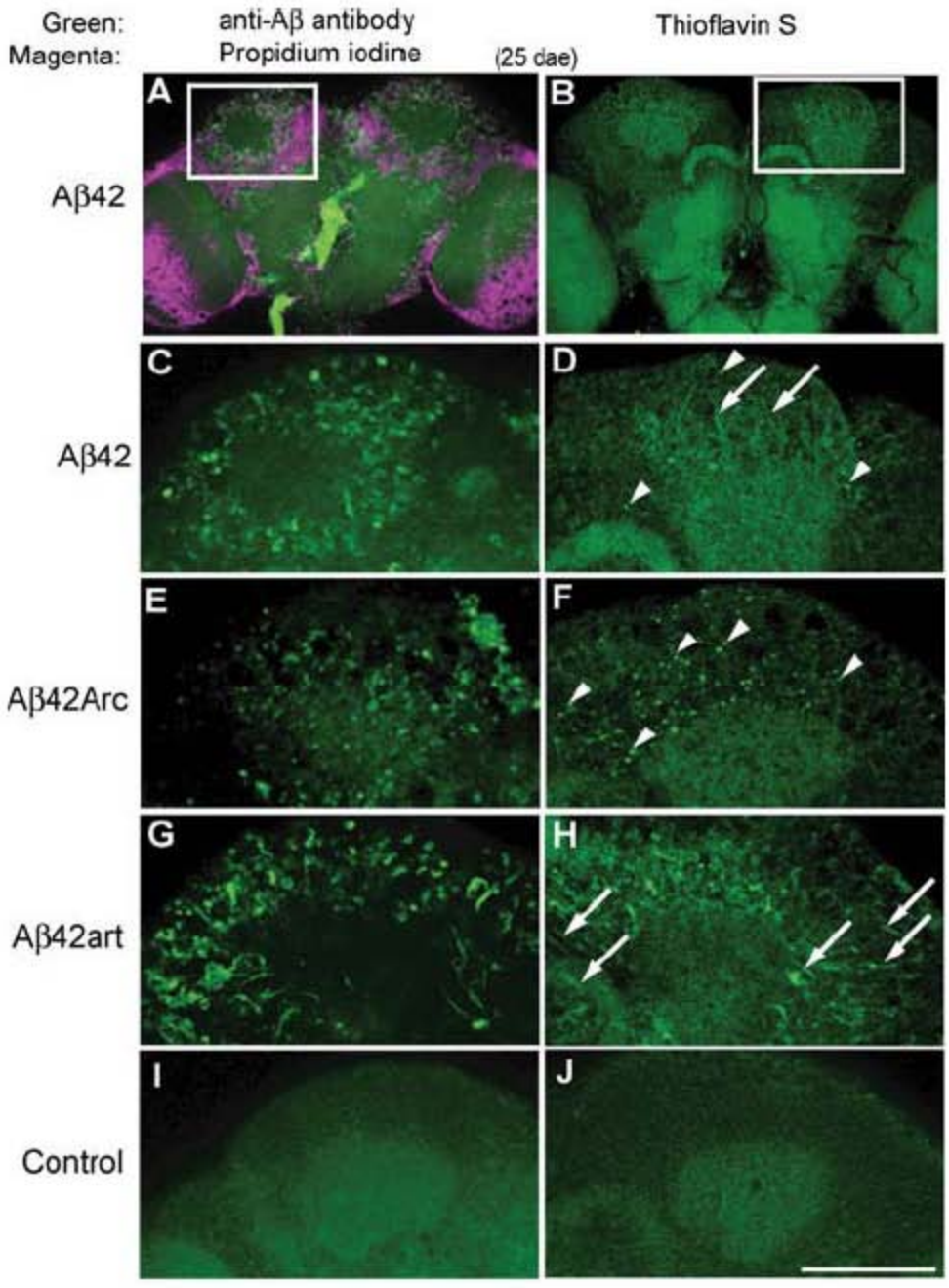


Figure 12.

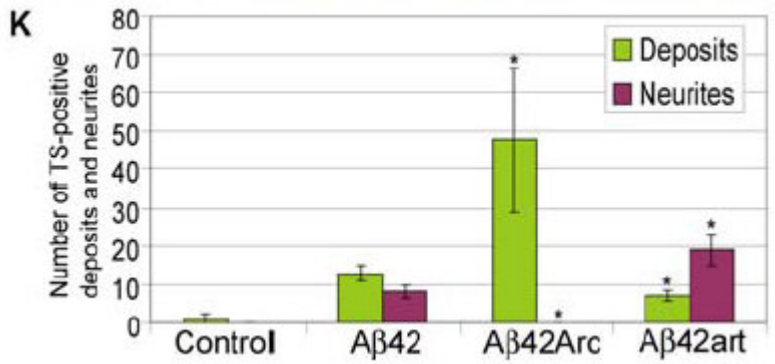


Figure 12, continued, Distribution and aggregation of Aβ42, Aβ42Arc, and Aβ42art peptides in fly brains. A,C,E,G and I, Immunostaining of brains of 25 dae flies with anti-Aβ antibody (green). In (A), nuclei were stained with propidium iodide (magenta). B,D,F,H and J, Thioflavin S staining of brains of 25 dae flies. Arrowheads and arrows indicate Thioflavin S-positive deposits and neurites, respectively. No signal was detected in the control (I, J). Scale bar in J: 50 µm. (C) and (D) are enlarged images of the boxed regions in (A) and (B), respectively. K, Numbers of TS-positive deposits and neurites were presented as averages±SD (n = 6 hemispheres). Asterisks indicate significant differences from Aβ42 ($P<0.05$, Student's t-test). Data from Chiang

Quantification of Thioflavin S-positive deposits and neurites in Aβ fly brains is shown in Figure 7K. No intraneuronal amyloid fibrils were observed by electron microscopy, suggesting that the majority of Thioflaiv S-positive aggregates did not contain amyloid fibril structures. These distinct patterns of neurodegeneration and Aβ accumulation were confirmed in several independent transgenic lines (data not shown).

Discussion:

This study highlights that the complex toxicities of Aβ42 are associated with different aggregation propensities *in vivo*. First, the increase in Aβ42 aggregation proneness associated with the pathogenic Arctic mutation (E22G) correlated with more severe detrimental effects on memory, locomotor ability, and lifespan than those caused by Aβ42 (Figure 5). These data are consistent with the fact that Aβ42Arc causes EOFAD (Nilsberth, et al., 2001), and indicates that aggregation proneness contributes to Aβ42 toxicity *in vivo*. Second, an artificial mutation (L17P) that decreased Aβ42 aggregation proneness suppressed the toxicities toward locomotor function and lifespan, but caused earlier onset of memory defects (Figure 5), showing that not all pathogenic effects of Aβ42 correlate directly with aggregation proneness. Third, the differences in aggregation tendencies of Aβ42 and derivatives correlated with qualitative shifts in pathology in the fly brain, exemplified by distinct neurodegeneration patterns accompanying the different accumulation profiles of Aβ42 peptides (Figure 6 and 7). Importantly, these differences are not due to a difference in genetic background (Ryman and Lamb, 2006), since these Aβ flies were generated in the same genetic background.

Under physiological conditions, the Aβ42 peptides are generated from amyloid precursor protein (APP) by β- and γ-secretases in the secretory pathway including, the transgolgi-network, endosome-system, and plasma membrane (Small and Gandy, 2006). Because *Drosophila* has no or very low endogeneous β-secretase activity (Greeve. et al., 2004), we used the artificial expression system to achieve high expression levels of Aβ42 in fly brains. In our transgenic flies, Aβ42 peptides were expressed in the ER and

distributed to the late secretory pathway compartments, axons, dendrites, and presynaptic terminals, as well as secreted from neurons (Figure 4).

Although the ER is not a major cellular site for Aβ generation under physiological conditions (Small SA, and Gandy S., 2006), several lines of evidence suggest that, under abnormal conditions, Aβ may be generated, retained, or recycled back to the ER and may induce ER stress (Busciglio., et al., 1993; Cook., et al., 1997; Lee., et al., 1998; Skovronsky., et al., 1998; Wild-Bode., et al., 1997; Cruz., et al., 2006). Our fly models may recapitulate neuronal dysfunction and degeneration induced by such abnormal intracellular metabolisms of Aβ42. It would be also important to examine the effects of the Arctic (E22G) and artificial (L17P) mutations on intracellular distribution and toxicities of Aβ42 generated from the full-length APP.

In summary, our results lead us to predict two issues. First, the partial prevention of Aβ42 amyloidgenesis by aggregation inhibitors may result in qualitative shifts in the pathogenic effects of Aβ42. Second, the tendency of Aβ42, a natively unfolded polypeptide consist primarily of random-coil structure in their native and soluble states (Kelly., 2005; Rochet and Lansbury., 2000), to aggregate may be affected by a combination of genetic (DeMattos., et al., 2004), environmental (Cherny., et al., 2001), and aging factors (Cohen., et al., 2006), and the resultant Aβ42 conformers or species may contribute to the heterogeneous pathogenesis of AD (Cummings., 2000). The existence of different ‘‘Aβ species’’ has been recently verified both *in vitro* (Petkova., et al., 2005) and *in vivo* (Meyer-Luehmann., et al., 2006).

CHAPTER 4

Distinctive Roles of different Beta-Amyloid 42 aggregates in modulation of synaptic functions

ABSTRACT

To determine how endogenously secreted Aβ42 aggregates regulate synaptic functions, we examined effects of Aβ42 at the neuromuscular junction (NMJ) of *Drosophila* larvae. Voltage-clamp recordings of synaptic transmission and optical analysis of vesicle recycling at presynaptic terminals show that expression of Aβ42 in neurons leads to a reduction of neurotransmitter release. However, expression of Aβ42 in postsynaptic muscle cells enhanced neurotransmitter release. Both effects are neutralized by Aβ antibody, suggesting a role for secreted Aβ42 peptides. Application of exogenously prepared Aβ42 oligomers leads to a reduction in synaptic responses, whereas mixed Aβ42 aggregates with mainly fibrils elicit an opposite effect by increasing synaptic transmission. Further analysis of long-term depression (LTD) confirms differential effects of different Aβ42 aggregates. Taken together, our data suggest that Aβ42 is secreted from neurons primarily as oligomers that inhibit neurotransmitter release and exert no effect on LTD, whereas larger sized aggregates, possibly fibrils, are major components secreted from muscle cells, which enhance synaptic transmission and LTD. Thus, different types of cells may secrete distinct forms of Aβ42 aggregates, leading to different modulation of synaptic functions.

INTRODUCTION

Accumulating evidence has lead to the hypothesis that toxicity of Aβ peptides, which are cleaved from APP by γ and β secretase activities, is largely conferred to its soluble aggregates (Mclean et al., 1999. Walsh et al., 2002. Haass and Selkoe, 2007). However, dystrophic neuritis is observed to surround large plaques of fibrillar Aβ in Alzheimer's Disease (AD) brains (Urbanc et al., 2002) and fibrillar Aβ deposits have been associated with synaptic abnormalities and breakage of neuronal braches in an AD mouse model (Tsai et al., 2004), suggesting fibrillar aggregates of Aβ might also contribute to neuronal injury.

In APP transgenic mice, Aβ accumulation leads to alterations in the expression and function of a range of molecules important for synaptic transmission and plasticity (Almeida et al., 2005; Chin et al., 2005; Hsieh et al., 2006). It is, however, interesting to note that Aβ effects on synaptic function are remarkably different in different brain regions. Recording from hippocampal CA1 neurons, the basal level of synaptic transmission is reduced while long-term potentiation (LTP) remains unaffected in the APP transgenic mice (Hsia et al., 1999. Palop et al., 2007). In contrast, recordings from the medial perforant pathway synapse within the dentate gyrus of the same transgenic mouse indicate that basal synaptic transmission is not affected but LTP is depressed (Palop et al., 2007).

On the basis of recordings from CA1 neurons, it has been proposed that activity-dependent release of Aβ forms part of a negative feedback mechanism to control neuronal hyperactivity (Kamenetz et al., 2003). However, this interpretation cannot

explain the occurrence of epileptic seizures in a large fraction of AD patients (Romanelli et al., 1990; Lozsadi and Larner., 2006), a population subject to Aβ over-production.

This seizure activity and studies of the dentate gyrus have raised a network perspective in which aberrant increases in network excitability and compensatory inhibitory mechanisms in the hippocampus is proposed to contribute to Aβ-induced neurological deficits (Graf et al., 1998, Palop et al., 2006, 2007). However, how accumulation of Aβ leads to aberrant increases in network excitability remains to be determined, particularly in light of the proposed role for Aβ in controlling hyperexcitability (Kamenetz et al., 2003). It is therefore critical to understand at cellular level why Aβ modifies synaptic functions differentially among different populations of neurons.

Powerful genetic tools available in *Drosophila* may facilitate such understanding. There is an APP-like (APPL) gene in *Drosophila* and the behavioral phenotype of APPL mutants can be rescued by expression of human APP (hAPP), suggesting conserved functions (Luo et al., 1992; Torroja et al., 1999). A recent publication claims that Aβ –like peptides are also cleaved from APPL, which can form amyloidogenic deposits and cause neurodegeneration in the Drosophila brain (Carmine-Simmen et al., 2008). Toxicity induced by human Aβ appears to be highly conserved across organisms, from *C. elegans* (Link 1995; Drake et al., 2003), *Drosophila* (Iijima et al., 2004; 2000), to mammals (Price et al., 1998). In *Drosophila*, expression of hAPP together with β secretase leads to age-dependent neurodegeneration and amyloid plaque formation (Greeve et al., 2004). Expression of hyperphosphorylated tau also triggers age-dependent neurodegeneration in *Drosophila* (Wittmann et al., 2001). In fact, *Drosophila* has been a

powerful genetic model for studying major age-dependent neurodegenerative diseases, including Parkinson's disease, Huntington's disease, and AD (Chan and Bonini, 2000; Marsh and Thompson, 2006; Ho et al., 2007).

In this study, we focus on the synaptic effects of Aβ42. Our previous works have shown that expression of secretary Aβ42 leads to age-dependent memory loss and severe neurodegeneration in *Drosophila* brain regions critical for memory formation (Iijima et al., 2004; 2008). To gain insights into cellular mechanisms of such effects, this report investigates how synaptic transmission and synaptic plasticity are affected by expression of Aβ42 peptides at the larval neuromuscular junction, the only preparation suitable for quantitative analysis of synaptic transmission at identifiable synapses in *Drosophila*.

Our analysis reveals that targeted expression of Aβ42 the presynaptic neuron induces a synaptic modulation that is opposite to that induced by expression in post-synaptic muscle cells at the same synapse. Further studies suggest that secreted different Aβ42 aggregates exert distinct modulation on synaptic functions.

RESULTS

Synaptic transmission was determined by excitatory junctional currents (EJCs) recorded via the two-electrode voltage-clamp method at the body-wall neuromuscular junction of third instar larvae (Jan and Jan, 1976)). Expression of the transgene encoding Aβ42 was mainly driven by either an *elav-Gal4* pan-neuronal expression driver or by a *G7-Gal4* muscle-specific expression driver. Additional drivers for expression were also used for confirmation. To control for non-specific effects of genetic background, all genotypes were backcrossed with an isogenic line w^{1118}*(isoCJ1)* for five generations.

Opposite synaptic effects resulting from neuronal versus muscle expression of Aβ42

Aβ42 immunoreactivity was detected at motor nerve terminals innervating larval body- wall muscle fiber 12 (for nomenclature, Johansen et al., 1989)with a well characterized antibody against N-terminal of Aβ peptide, 6E10, (see materials and methods) in *elav-Gal4/+; UAS-Aβ42/+* transgenic larvae (Figure 13A). The staining was also widely distributed in the larval ganglion within which motor neurons localized (not shown). In contrast, Aβ42 immunoreactivity was observed only in muscle fibers when driven by *G7-Gal4* (lower panels in Figure 13A). On the basis of this observation, all electrophysiological recordings were obtained from muscle 12 in body-wall segments 4 and 5.

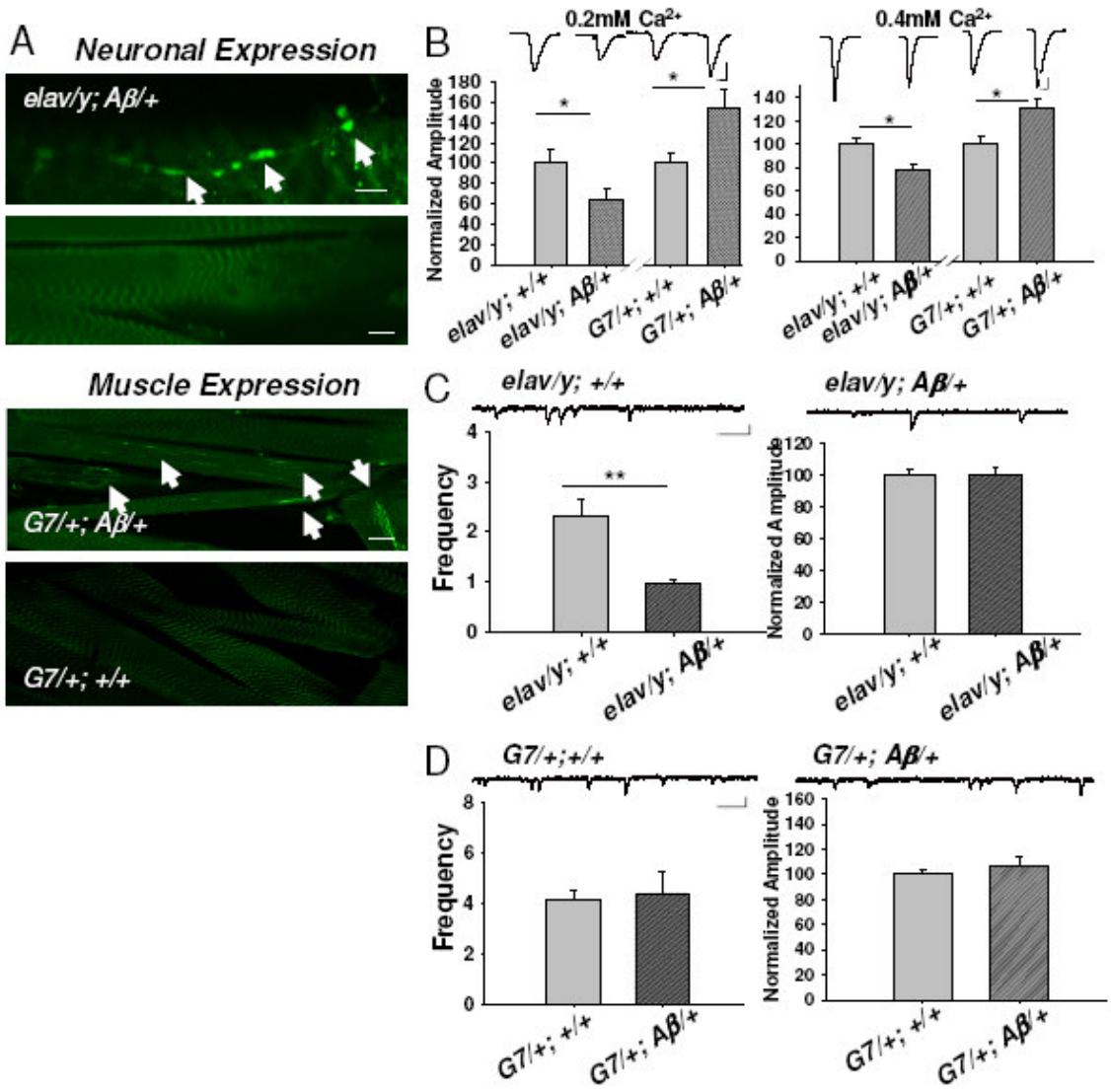


Figure 13. Opposite synaptic effects resulting from expression of Aβ42 in neurons versus in muscle cells. (A) Distribution of immunohistochemical staining of the Aβ42 peptide via antibody 6E10 in motor nerve terminals of *elav-Gal4/y; UAS-Aβ/+* (to save space in the figures nomenclature is shortened as *elav/y; Aβ/+*) larvae and in muscle cells of *G7/+; Aβ/+* larvae. Elav is a pan-neuronal driver while G7 is a muscle-specific driver. In top panel, arrows point to boutons of nerve terminals with positive anti-Aβ42 peptide staining. Scale bar: 15um. In lower panel, arrows point to fiber-like staining within muscle cells. Scale bar: 100um. (B) EJCs were depressed in *elav/y; Aβ/+* larvae while enhanced in *G7/+; Aβ/+* larvae. EJCs amplitude is normalized to the size of a respective control group (see material and methods). Ca^{2+} concentrations are as indicated. n=10, 11, 26, 24, 8, 8, 9, 10 for *elav/y; +/+*, *elav/y; Aβ/+*, *G7/+; +/+*, *G7/+; Aβ/+* at 0.2 and 0.4 mM Ca^{2+}, respectively. Scale: 5nA and 10nA (vertical) for 0.2 and 0.4mM Ca^{2+}, respectively, 7.5ms (horizontal). (C) and (D) Quantal analysis of effects of Aβ42 expression. Frequency of spontaneous mEJCs was significant lower in neuronal expression of Aβ42 (*elav/y; Aβ/+*) as compared to its control while the frequency was

Figure 13, continued. similar in muscle expression of Aβ42 (*G7/+; Aβ/+*). The amplitude of mEJCs remained unchanged in both cases. n=10, 9, 20, 11 for *elav/y; +/+*, *elav/y; Aβ/+*, *G7/+; +/+*, *G7/+; Aβ/+*, respectively. Scale: 0.4nA, 130ms. For all figures, t-test, * P<0.05, ** P<0.01, error bars are standard error.

Evoked EJCs were reduced in *elav-Gal4/+; UAS-Aβ42/+* larvae with expression of Aβ42 in neurons or presynaptic nerve terminals. This was true at a variety of external Ca^{2+} concentrations (Figure 13B). Surprisingly, EJC amplitude was increased significantly in *G7-Gal4/+; UAS-Aβ42/+* larvae with expression of Aβ42 in postsynaptic muscle cells (Figure 13B). Here, *elav-Gal4/+; +* and *G7/+; +* served as corresponding controls. EJC peak amplitude was similar between these two controls (10.4±1.5nA in *elav-Gal4/+; +* and 8.3±1nA in *G7/+; +* at 0.2 mM Ca^{2+}), which corresponds to data reported previously (Renden and Broadie 2003). Similar synaptic effects were observed in two independently isolated *UAS-Aβ42* lines. These results raised the question of how synaptic transmission would be effected if Aβ42 was expressed in neurons and muscle cells simultaneously. Driven by a universal promoter (Lawrence et al., 1996), *armadillo (arm)-Gal4*, EJC amplitude was reduced (39.1±2.2nA in *arm-Gal4/+; +/+*, 27.6±4nA in *arm-Gal4/+; UAS- Aβ42/+*). It appeared that Aβ42 expressed in neurons played a dominant role, which might reflect a stronger expression in neurons versus in muscle fibers.

In addition, we also examined effects of expressing Aβ40. There was no statistically significant difference observed in EJCs amplitude when comparing *elav-Gal4/+;UAS-Aβ40/+* and *G7-Gal4/+;UAS-Aβ40/+* with their controls. This is consistent with the previously reported observation in which Aβ40 expressed in the adult brain causes no neurodegeneration and only very mild learning defects even with much higher levels of

expression than Aβ42. Furthermore, there is no accumulation of oligomers or fibrils observed at such high level (Iijima et al., 2004). Therefore, it is not surprising to see no synaptic effects at the Aβ40 larval NMJ. In the following analysis, we focus on Aβ42.

Altered exocytosis rates

Analysis of spontaneous miniature EJCs (mEJCs) indicated that amplitudes of mEJCs remained unaltered in both cases (Figures 13C and 13D) while frequency of mEJCs was significantly decreased with neuronal expression of Aβ42 (Figure 13C), suggesting possible presynaptic effects. Both mini frequency and amplitude were not affected by muscle expression of Aβ42 (Fig. 13D) even though evoked ejcs were increased (Fig. 13B). Although mini analysis may provide hints on what happens at evoked synaptic transmission, there often is no direct causative relationship between these two. Therefore, we still can not rule out the possibility of presynaptic effects.

To confirm whether the observed effects were presynaptic, we examined exocytosis of synaptic vesicles at the nerve terminals through optical imaging analysis of evoked discharges of the fluorescent dye, FM1-43. FM1-43 was loaded into synaptic vesicles within boutons at which synapses are formed. This is a method commonly used in studying vesicle recycling (Kilic, 2002; Kuromi and Kidokoro, 1999). To achieve the dye loading, the neuromuscular preparation was treated with stimulation buffer containing a high potassium concentration (90 mM). The chosen loading regime showed no significant difference in fluorescence intensities of loaded dye at motor never terminals among different genotypes (Figure 14A). Loaded dye in boutons was unloaded using a stimulation buffer with 90 mM K^+ and 0.4 mM Ca^{2+}. The unloading rate in response to

stimulation was significantly reduced with neuronal expression of Aβ42 but increased with muscle expression (Figure 14B). This result confirmed that evoked release of synaptic vesicles was altered in opposite directions in larvae with expression of Aβ42 in presynaptic terminals versus in post-synaptic muscle cells. In addition, imaging data suggests that regardless of whether Aβ42 was expressed pre- or post-synaptically, the effect was always on presynaptic functions.

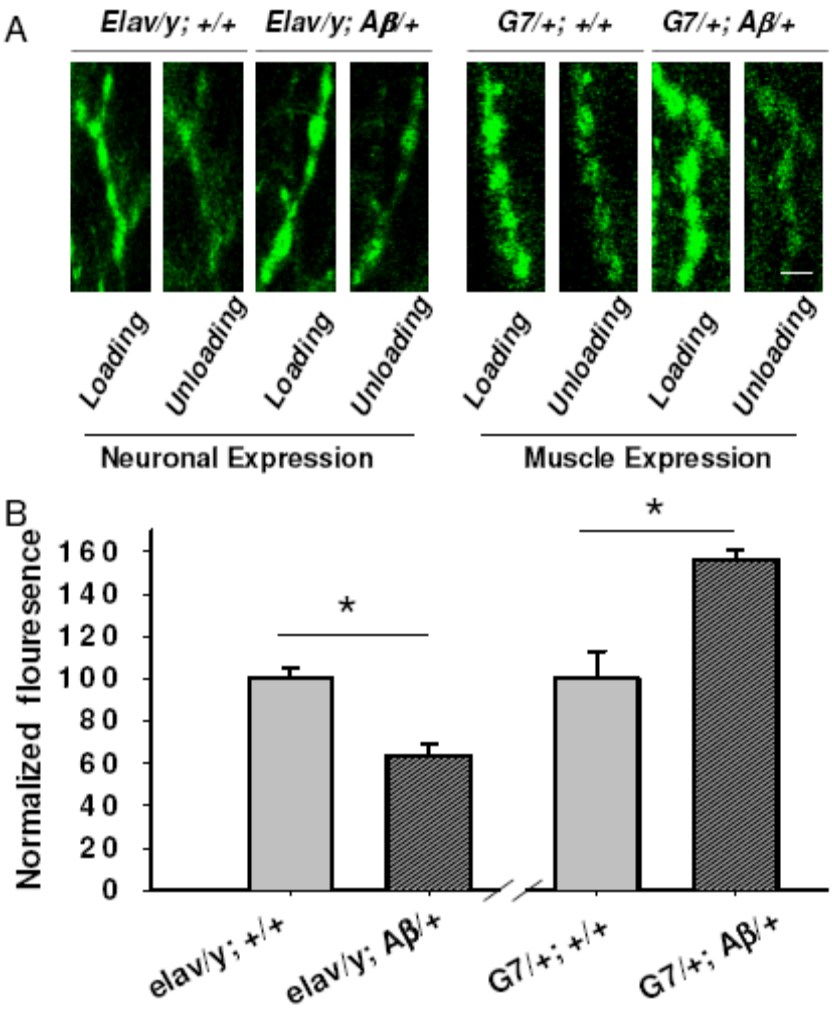


Figure 14. Exocytotic rates altered in opposite directions resulting from neuronal versus muscle expression of Aβ42 peptides. (A) Fluorescence imaging of motor nerve terminals stained with the FM1-43 dye. The dye was loaded into synaptic vesicles

Figure 14, continued, through a period (5mins) of high potassium depolarization-induced vesicle recycling processes and then was unloaded using mild stimulation (see materials and methods). Changes in the fluorescence intensity are proportional to exocytotic rates. Scale bar: 10um. (B) Statistical analysis of fluorescence intensity changes (normalized, see materials and methods) in different genotypes. n=4, 4, 5, 5 for *elav/y; Aβ/+*, *elav/y; +/+*, *G7/+; +/+*, *G7/+; Aβ/+*, respectively.

Distinct effects on long-term depression (LTD)

To further support the observation presented above, we examined LTD at the neuromuscular junction. We have shown previously that high frequency (30Hz) stimulation of motor axons can induce LTD that lasts up to one hour (Guo and Zhong, 2006). Our recordings showed that LTD was not affected by neuronal expression of Aβ42 in *elav/y; UAS-Aβ42* larvae (Figure 15A). In contrast, LTD was significantly enhanced in Aβ42 in *G7/+; UAS-Aβ42/+* larvae (Figure 15B). Again, we showed that expression of Aβ42 can lead to very different effects on LTD, depending on where Aβ42 is expressed. The enhancement of LTD by muscle expression of Aβ42 is also observed by using another muscle driver line, *C57-Gal4*, see Figure 15C.

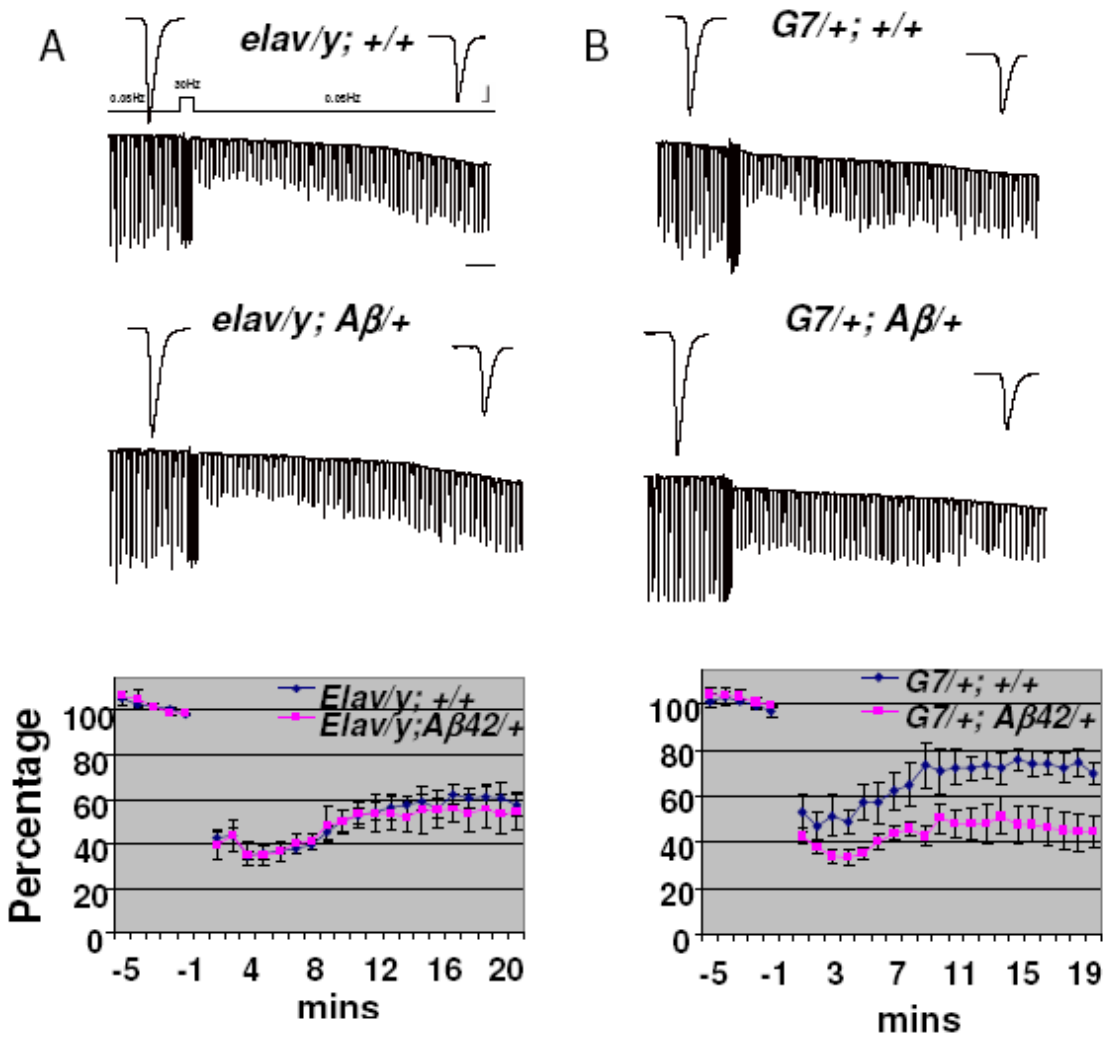


Figure 15. Enhancement of long-term depression (LTD) induced by expression of Aβ42 peptide in muscle cells. (A) No effects on LTD by neuronal expression of Aβ42 peptide in *elav/y; Aβ/+* larvae. Stimulation paradigm for induction of LTD is depicted. EJCs were recorded in 0.4mM Ca^{2+} saline. The horizontal bar represents 2 mins. (B) LTD is enhanced in *G7/+; Aβ/+* larvae with muscle expression of Aβ42 peptide. Same induction paradigm as in (A). (C) LTD is enhanced in *C57/A*b larvae with muscle expression of Ab peptide. For both (A), (B) and (C), representative EJCs traces for each genotypes are shown in top panels while bottom panels represent averaged EJC amplitude normalized to the basal level before titanic stimulation. n=4, 4, 5, 5, 5, 5 for *elav/y; +/+*, *elav/y; Aβ/+*, *G7/+; +/+*, *G7/+; Aβ/+*, *C57/+* and *C57/A*b Scale: 10nA (vertical), 7.5ms (horizontal).

C

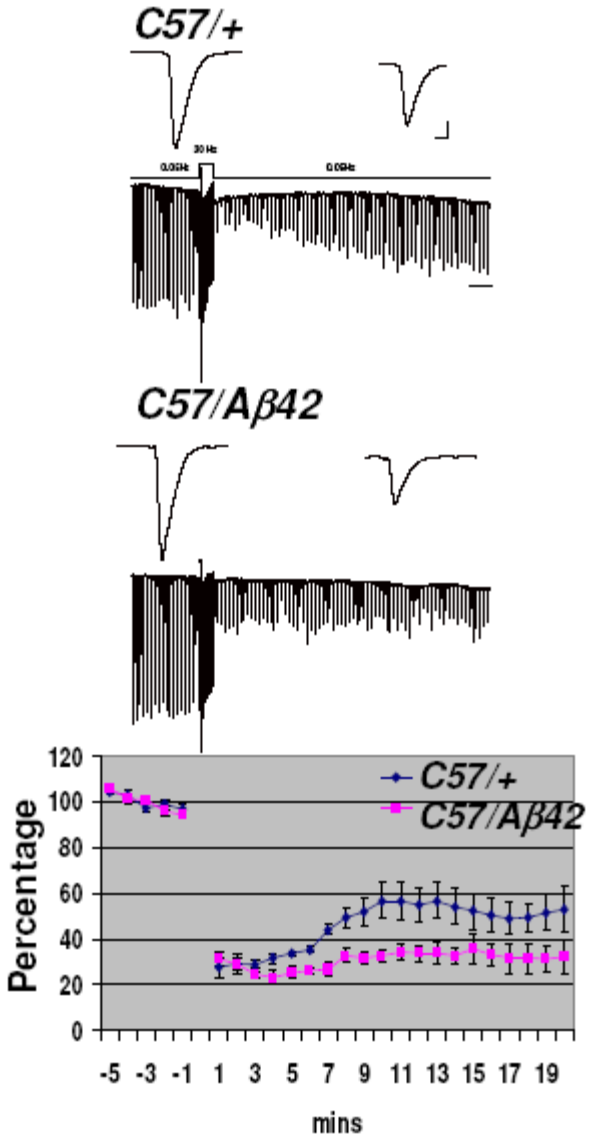


Figure 15, continued.

Opposite synaptic effects induced by Aβ42 oligomers versus fibrils

Since Aβ42 is targeted to the secretory pathway, it is possible that muscle cells expressing Aβ42 secrete Aβ42 that acts on presynaptic nerve terminals. To examine this possibility, an antibody against Aβ (6E10) was incubated with the neuromuscular junction preparation. Aβ42 effects were suppressed in both neuronal expression and

muscle expression (Figure 16), implying that the observed synaptic effects were caused by secreted Aβ42 no matter where it is expressed.

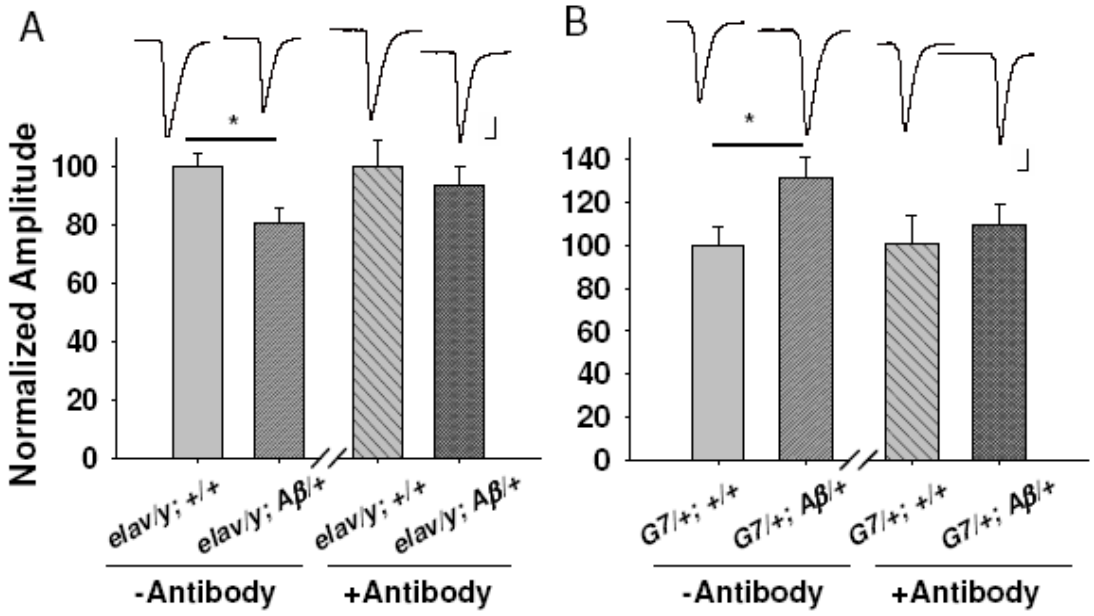


Figure 16. Application of Aβ peptide antibody reverses synaptic modification resulting from both neuronal and muscle expression of Aβ42. (A) and (B), Synaptic effects of incubation of Aβ42 antibody (0.5ug/ul) for 30mins in neuromuscular preparations of larvae with expression of Aβ42 in neurons (*elav/y; Aβ/+*) or in muscle cells (*G7/+; Aβ/+*). [Ca^{2+}] in saline is 0.4mM. n=14, 14, 13, 13, 10, 10, 8, 8 for *elav/y; +/+, elav/y; Aβ/+, G7/+; +/+, G7/+; Aβ/+* without and with antibody treatment, respectively. For this and all following figures, the scale bars are same as indicated before.

Such a conclusion, however, raised an obvious concern: how Aβ42 could produce opposite effects at the same presynaptic terminals. Considering that Aβ42 expressed in the adult *Drosophila* brain is highly aggregating but not Aβ40, we examined the idea that different Aβ42 aggregates were secreted from neurons versus from muscle fibers, which in turn, elicit differential synaptic effects.

We prepared oligomers as well as fibrils with synthetic Aβ42 from well-established protocols (Abad et al., 2006; Dahlgren et al., 2002; Lambert et al., 1998). Our western

blot analysis showed that dimer, trimer, and tetramer were produced when prepared with the oligomer protocol (see materials and methods) while a range of aggregates, including larger aggregates and fibrils (smear-like band on top in the right lane of Figure 17A), were formed with the fibril procedure (Figure 17A). Incubating the neuromuscular preparation with exogenously prepared fibrils (10 μM) for 30 minutes enhanced EJCs. In contrast, incubation with exogenously prepared oligomers (10μM) depressed EJCs (Figure 17B). This observation indicated that small Aβ42 oligomers inhibited synaptic transmission while larger oligomers or fibrils enhanced synaptic transmission.

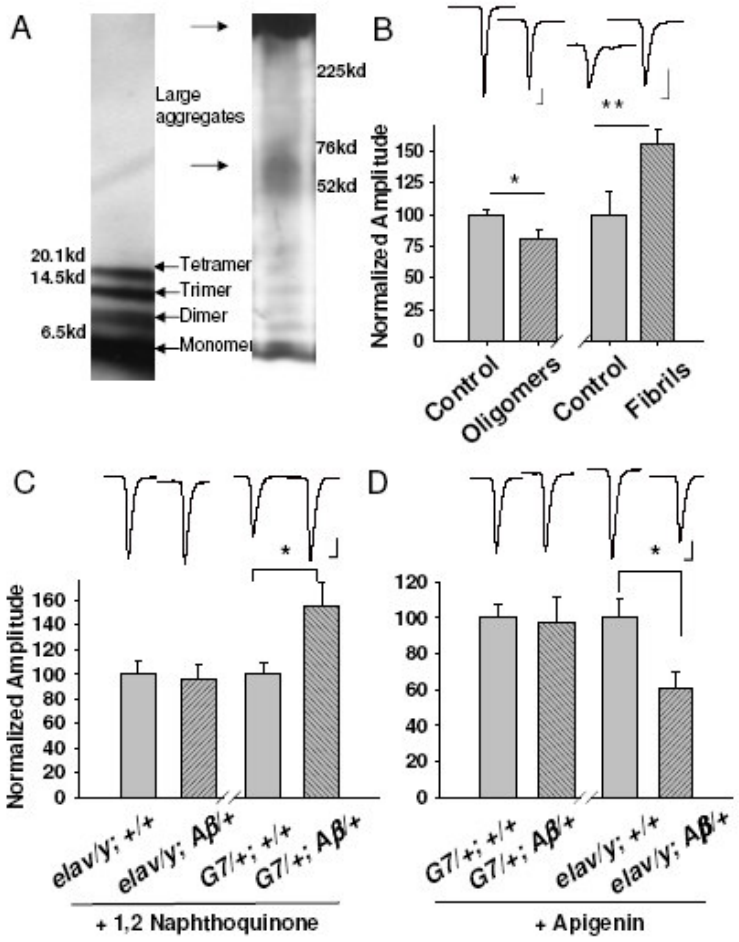


Figure 17.

Figure 17, continued. The change in synaptic transmission resulting from endogenous secretion of Aβ42 can be produced by exogenous application of synthetic oligomers or fibrils Aβ42 and reversed by drug application. (A) Shown are representative western blots of oligomeric (left) and fibrillar (right) Aβ42. (Left) Dimer, trimer and tetramer of Aβ42 in oligomer-forming condition, no any indication of higher molecular weight aggregates and smears. (Right) In fibril form condition, large Aβ42 aggregates with higher molecular weight including those remain in the well as pointed. (B) Application of synthetic oligomers and fibrils Aβ42 peptides (10uM) for 30mins depressed and enhanced the EJCs, respectively. Experiments were conducted in 0.4 and 0.2 mMCa^{2+} for oligomers and fibrils Aβ42 experiment, respectively. n=11, 11 for with and without application of oligomer Aβ42, respectively. *p<0.05, and n=8, 8 for with and without application of fibrils Aβ42, respectively. **p<0.02 (C) 30 mins incubation with 1,2 Naphthoquinone (0.15uM) rescued the synaptic transmission deficit in *elav/y; Aβ/+* larvae. n=6 for each genotype. *p<0.05. (D) Application of apegenin (15uM) for 30mins reversed the enhancement of EJCs in *G7/+; Aβ/+* larvae. n=4 for each genotype. *p<0.05.

Pharmacological analysis of Aβ42-dependent regulation of synaptic functions

Taken together, data obtained from genetically targeted expression of Aβ42 and from synthetic Aβ42 support a scenario in which small oligomers are secreted from motor nerve terminals that inhibit EJCs and exert no effect on LTD, whereas large oligomers or fibrils are secreted from muscle fibers that enhance EJCs and LTD. To advance this hypothesis, we examined pharmacological effects.

Drugs that inhibit fibrillization and oligomerization of Aβ peptides have been intensively studied and tested because of their potential for treatment of AD. Among them, apigenin and 1,2-naphthoquinone have been shown to inhibit fibrillization and oligomerization, respectively (Necula et al., 2007). In concordance, we found that oligomerization inhibitor, 1,2-naphthoquinone reversed the reduced EJCs seen with expression of Aβ42 in neurons but had no effect on synaptic transmission enhancement due to muscle expression of Aβ42 (Figure 17C). On the other hand, fibrillization

inhibitor, apigenin, neutralized enhanced EJCs caused by expression of Aβ42 in muscle but could not reverse the synaptic transmission depressed by expression of Aβ42 in the neuron (Figure 17D). Furthermore, apigenin completely suppressed enhanced LTD (Figure 18A) in larvae with muscle expression of Aβ42 but 1,2-naphthoquinone had little effect on enhanced LTD (Figure 18B).

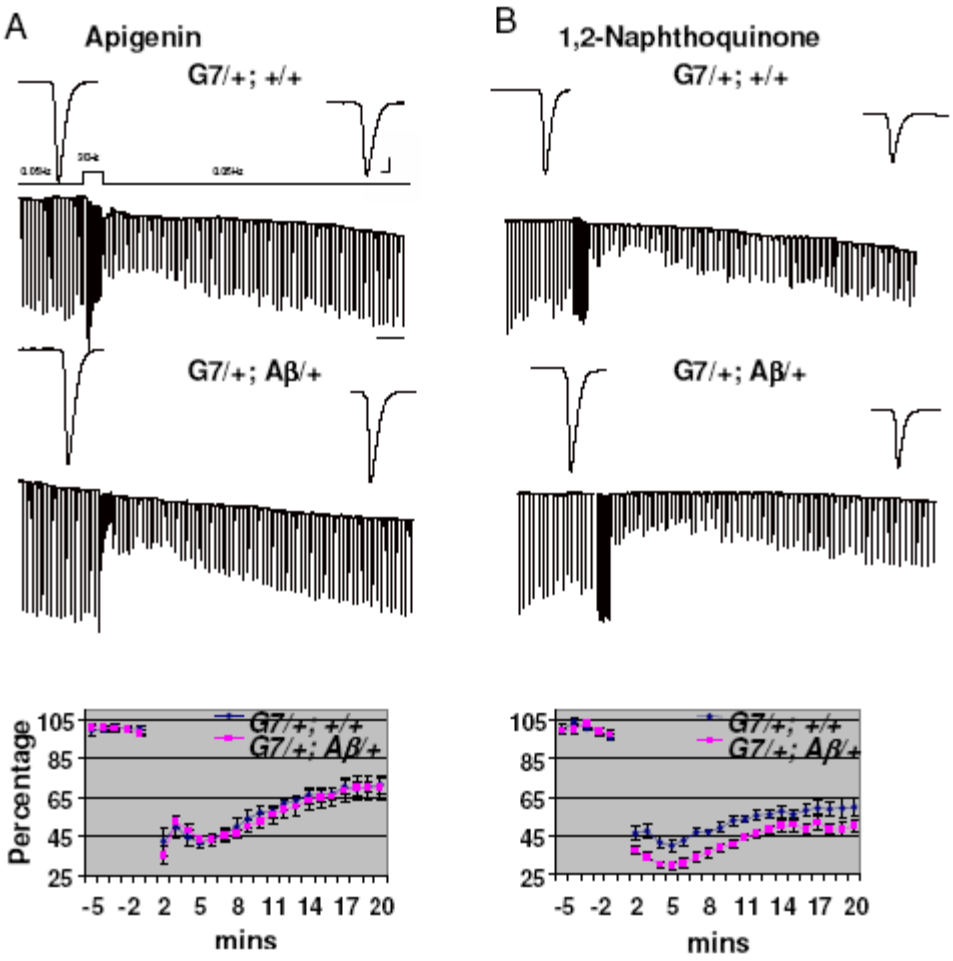


Figure 18. Inhibition of fibrilization reverses enhanced LTD. Effects of pre-incubation of fibrilization inhibitor, Apigenin (A), and oligomerization inhibitor, 1,2 Naphthoquinone (B) with *G7/+; Aβ/+* larvae. LTD induction paradigm is as indicated. Representative EJCs traces are shown in top panels. EJCs were recorded at 0.4mM Ca^{2+}. Normalized EJCs were plotted against time for statistical analysis (n=6 for each group).

To visualize whether different aggregates are indeed formed from targeted expression of Aβ42, we performed thioflavin-S staining; a fluorescence dye that has been used to stain Aβ fibrils which are primarily composed of β sheets (Urbanc et al., 2002). Confocal images showed positive thioflavan-S staining in muscle fibers that express Aβ42 (arrows in Figure 19). We found an average of 6 thioflavan-S positive spots in each muscle fiber in *G7/+; Aβ/+* larvae, while no signal was observed in motor nerve terminals of larvae that express Aβ42 in neurons. The thioflavan-S staining pattern in muscle fibers was similar to our immunostaining results (see arrows in Figure 13A in muscle expression). This observation supports the notion that fibrils were formed in muscle cells expression of Aβ42 but not in motor neurons.

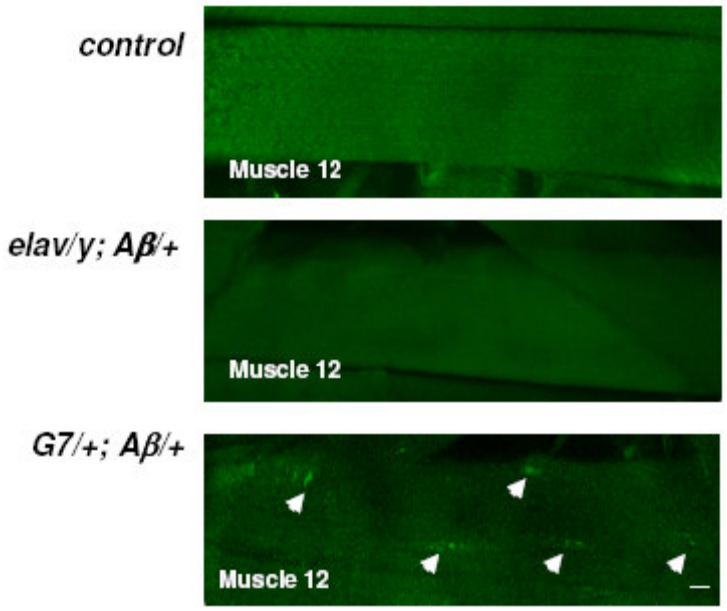


Figure 19. Endogenous Aβ42 fibrils revealed by thioflavin-S staining in muscle fibers of *G7/+; Aβ/+* larvae. Thioflavin-S-positive staining was detected in the muscle cells in *G7/+; Aβ/+* larvae, but not in the ctrl and *elav/y; Aβ/+* larvae. Arrow heads point to fiber-like staining within muscle cells. Scale: 10μm

DISCUSSION

In the current study, we focused our analysis on the effects of Aβ peptides. We demonstrated that expression of Aβ42 could lead to two different types of synaptic modulation at the same synapses, depending on which cells expressed Aβ42. Neuronal expression induced a reduction in EJC amplitude and had no effects on LTD. In contrast, muscle expression enhanced EJC as well as LTD. We then showed, using synthetic peptide, that exogenously-prepared small Aβ42 oligomers modulated EJC amplitude in a manner similar to neuronal expression while exogenously-prepared large Aβ42 aggregates, mainly fibrils, exerted effects similar to muscle expression. Although exogenously-prepared Aβ42 fibrils also contained less striking bands of small Aβ42 oligomers, enhanced synaptic effects is likely resulted from larger aggregates or fibrils. This conclusion is well supported by the observation that pharmacologically disruption of fibrilization, but not oligomization, leading to inhibition of synaptic effects elicited by muscle expression of Aβ42.

On basis of these observations, we were led to the conclusion that reduced synaptic transmission elicited by neuronal expression of Aβ42 is primarily mediated by released small oligomers (either dimer, trimer or tetramer) while enhanced synaptic transmission and LTP induced by muscle expression of Aβ42 are resulted from the release of large aggregates, such as fibrils. Thus, this *in vivo* study indicates that Aβ42 aggregates distinctively within different population of cells and this difference manifests in differential physiological functions.

In vitro studies of cultured vertebrate neurons have, in fact, shown that exogenously applied Aβ oligomers, protofibrils, or fibrils can produce qualitatively distinct effects on

neuronal activities and cell death (Dahlgren et al., 2002; Fu et al., 2006; Liu and Schubert, 1998; Wang et al., 2002; Ye et al., 2004). In hAPP transgenic mice, it has been shown that excitatory synaptic transmission was reduced in the hippocampus (Kamenetz et al., 2003; Hsieh et al., 2006) while synaptic activity at inhibitory synapses was increased (Palop et al., 2007). Our *in vivo* observation at the *Drosophila* neuromuscular junction not only indicates that distinct synaptic modulations resulting from Aβ42 oligomers versus fibrils are pathological, but also provides a plausible mechanism: different sizes of aggregates are formed in distinctive cell types.

Such an idea lends an intuitive explanation to apparently paradoxical observations. While Aβ peptide is shown to serve as a negative regulator of neuronal activity, seizure activities have been reported in both AD patients and in the AD mouse model (Romanelli et al., 1990; Lozsadi DA and Larner AJ., 2006; Palop et al., 2007). It is possible that Aβ indeed inhibits neuronal activity in CA1 neurons but might up-regulate synaptic activity in other populations of neurons. This may be responsible for the observed seizure activity.

Chapter 5

PI3 kinase signaling is involved in Aβ-induced memory loss in *Drosophila*

ABSTRACT

Multiple intracellular signal transduction pathways are altered in brain tissues of Alzheimer's disease (AD) patients. *In vivo* genetic modeling may be an effective approach in facilitating efforts to identify pathways crucial for AD pathogenesis. In particular, to gain insights into AD-related memory loss, we depart our investigation from synaptic plasticity, i.e. altered long-term depression resulted from expression of β-amyloid peptide (Aβ) in *Drosophila*. This transgenic fruit fly has been reported to recapitulate many AD features, including age-dependent memory loss, neurodegeneration, and accumulation of Aβ aggregates. Our data reveal that altered synaptic plasticity by expression of Aβ42 is through disrupting phosphoinositide 3-kinase (PI3K) signaling pathway. This finding leads us to discover that genetic silencing or pharmacological inhibition of PI3K function suppresses Aβ42 aggregation and rescues memory loss in Aβ42 transgenic fly. In addition, manipulate PI3K activity can not improve Aβ42-induced late-onset neurodegeneration suggesting the independent mechanisms underlying different pathological phenotypes.

Introduction

It remains to be a challenge to gain insights into pathogenesis of Alzheimer's disease (AD) and to develop novel treatment on basis of such understanding. Genetic study of early onset familial AD provides causative link between AD and the Amyloid β-peptide 42 (Aβ42) (Tanzi and Bertram, 2005), derived from proteolytic cleavage of the amyloid precursor protein (APP). Extensive efforts have been devoted to define molecular mechanisms underlying production and degradation of Aβ peptides. Yet, biochemical events that mediate Aβ42 toxicity in leading to memory loss and neurodegeneration are not well understood.

Analysis of *Drosophila* AD models may facilitate our understanding of such mechanisms. To date, all genetic modeling studies of Aβ pathology are achieved from overexpression of human mutant APP genes or direct overexpression of the human Aβ42 peptide (Götz and Ittner, 2008) because even endogenous mouse Aβ peptides exhibit little toxicity (De Strooper et al., 1995; Johnstone et al., 1991). In Drosophila, there is an APP-Like (APPL) gene (Torroja et al., 1999) and a newly published report claims that a larger sized peptides can be cleaved from APPL, which are able to form amyloidogenic deposits and causes neurodegeneration (Carmine-Simmen K et al., 2008). Since human APP or Aβ42 produces similar pathologic phenotypes across a wide range of organisms, from invertebrates to mammals, molecular basis mediating Aβ peptides toxicity is likely conserved (Luo et al., 1992; Torroja et al., 1999; Carmine-Simmen K et al., 2008).

Proteolytic cleavage of APP yields not only Aβ peptides but also APPα and the intracellular fragment (Esler and Wolfe, 2001.). The intracellular fragment is reported to regulate gene expression and its overexpression leads to neurodegeneration in mice (Cao

and Sudhof 2001; Kim et al. 2003). The large APPα fragment is also reported to have neural protective effects (Turner et al., 2003; Ring et al., 2007). To focus on Aβ toxicity, it is beneficial to study effects of expression of Aβ42 alone.

Expression of a secretory form of Aβ42 in the *Drosophila* brain recapitulates many features of AD, including age-dependent memory loss, massive neurodegeneration, accumulation of Aβ42 oligomers, and accumulation of fibril deposits (Iijima et al., 2004). Moreover, Aβ42 artic mutation associated with familiar AD enhances pathological phenotypes (iijima et al., 2008). Secreted Aβ42 also regulates glutamate-dependent synaptic transmission in a manner similar to that observed in vertebrates (Chiang et al., 2009).

In the current study, we devote our attention to Aβ42-induced memory loss. Since many learning and memory mutations identified in *Drosophila* alter synaptic transmission and plasticity at the larval neuromuscular junction (NMJ) (Keshishian et al., 1996), we departed our study from electrophysiological recordings at the NMJ, the only preparation in *Drosophila* suitable for quantitative analysis of synaptic transmission at identifiable synapses. Such efforts lead to a finding as to that phosphoinositide 3-kinase (PI3K) is altered at the basal level as well as in stimulated by insulin. Such alteration contributes defects in plasticity and memory loss.

Results

The two-electrode voltage-clamp method was used to record synaptic transmission at the larval body wall NMJ, which has been extensively characterized (Zhong and Wu, 1991). Various forms of synaptic plasticity can be elicited via different stimuli (Zhong and Wu, 1991; Guo and Zhong, 2006), including short-term facilitation, post-tetanic potentiation, and long-term depression (LTD). In particular, we have reported that LTD is altered resulting from expression of Aβ42 postsynaptically in muscle cells while presynaptic-neuronal expression affects synaptic transmission differently (Chiang et al., 2009). Since it is known that and we know that protein kinas B (Akt) is involved in LTD induction (Guo and Zhong 2006), we began to investigate whether Aβ42-induced LTD enhancement is related to the pathway.

Altered basal and insulin-stimulated PI3 kinase activity

With a 30s tetanic stimulation (see the methods; Guo and Zhong, 2006), LTD was reliably elicited and enhancement of LTD was evident in larvae with targeted expression of Aβ42 in muscle cells (*G7/+; UAS-Aβ42/+*) (Figure 20A). G7 is a muscle specific Gal4 driver (Renden and Broadie, 2003). Since Akt is at down stream of PI3 Kinase (PI3K), we wanted to test PI3K effects first. This was achieved via application of a pharmacological inhibitor of PI3K, wortmannin, at a10nM concentration to the larval NMJ. The enhanced LTD returned to the control level in larvae expressing Aβ42 (Figure 20B), suggesting Aβ42-induced enhancement of LTD is resulted from an elevated PI3K activity.

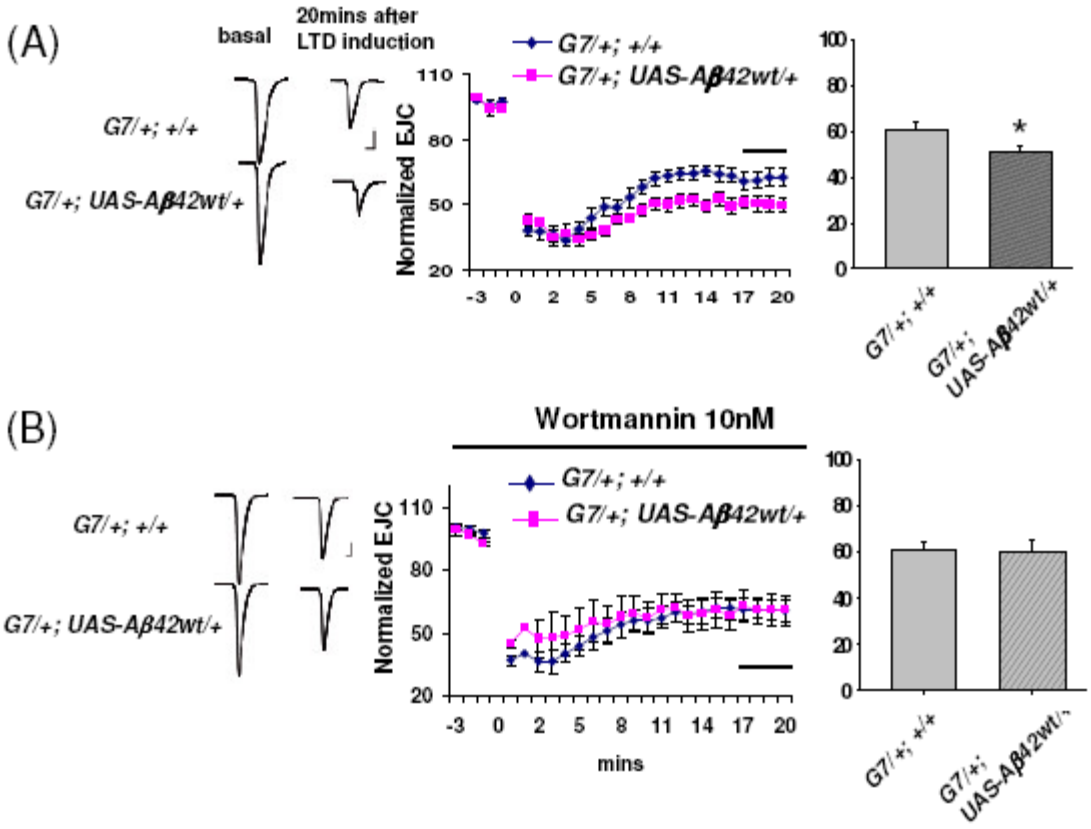


Figure 20. The enhanced LTD by Aβ42 is reversed by wortmannin application in NMJ. (A) LTD was enhanced in *G7/+; Aβ42t/+*. EJCs were recorded in 0.4mM Ca^{2+} saline. See material and methods for the protocol used to induce LTD. Representative EJCs traces for each genotypes are shown in top panels while bottom panels represent averaged EJC amplitude normalized to the basal level before titanic stimulation. n=5. (B) After 10nM wortmannin 30mins treatment, the enhance LTD in *G7/+; Aβ42/+* was reversed to the same level as control group. n=6 for each groups. n=6. Scale: 10nA (vertical), 7.5ms (horizontal). *=p<0.05.

PI3K activity is elevated by expression of Aβ42

To confirm this observation, we assayed PI3K activity more directly. PI3K can be activated via various extracellular signaling molecules, with insulin being best characterized (Barker and Houlston, 2003). Activated PI3K phosphorylates two major targets, Pi3,4P and Pi3,4,5P. These phosphorylated lipids can recruit proteins that contain

lipid-binding domain to the cell membrane and then trigger the downstream signaling, such as activation of Akt. We performed immunostaining to quantify the Pi3,4P and Pi3,4,5P levels in an attempt to determine how the basal and stimulated PI3K activity were affected by expression of Aβ42. Such assay has been routinely used (Aikawa and Martin, 2003; Fu et al., 2007; Berman et al., 2008). In *Drosophila*, there are insulin-like peptides and their respective receptors. Vertebrate insulin is capable of stimulating these receptors (Gorczyca et al., 1993). We focused on larval ganglion cells for the tissue is suitable for treatment, such as application of insulin (Hannan et al., 2006).

Immunostaining with an antibody specific to Pi3,4P revealed that PI3K activity was indeed altered in a manner consistent with electrophysiological data. Figure 21A1 shows confocal images of immunostaining of ganglia cells. Single cells with strong florescence and well-defined boundary were chosen for measuring the ratio of florescent intensities with peripheral over the center (see the top panel in Figure 21A2). A higher ratio indicates stronger immunoreactivity and therefore more Pi3,4P stained, so the higher PI3K activity.

As revealed in Figures 21A, the basal level of PI3K activity was significantly elevated in larval ganglion cells with pan-neuronal expression of Aβ42. Insulin was able to stimulate PI3K activity in phosphorylation of Pi3,4P in controls, but was unable to increase PI3K further in larvae with expression of Aβ42. Application of PI3K inhibitor, wortmannin, suppressed the Aβ42-induced increase in PI3K activity (Figures 21A1 and 2.). A similar result was obtained with immunostaining of Pi3,4,5P (Figures 21B1 and 2). Thus, neuronal expression of Aβ42 leads to elevated basal PI3K activity, but retard insulin-stimulated PI3K activity. This elevated PI3K produces an enhanced LTD

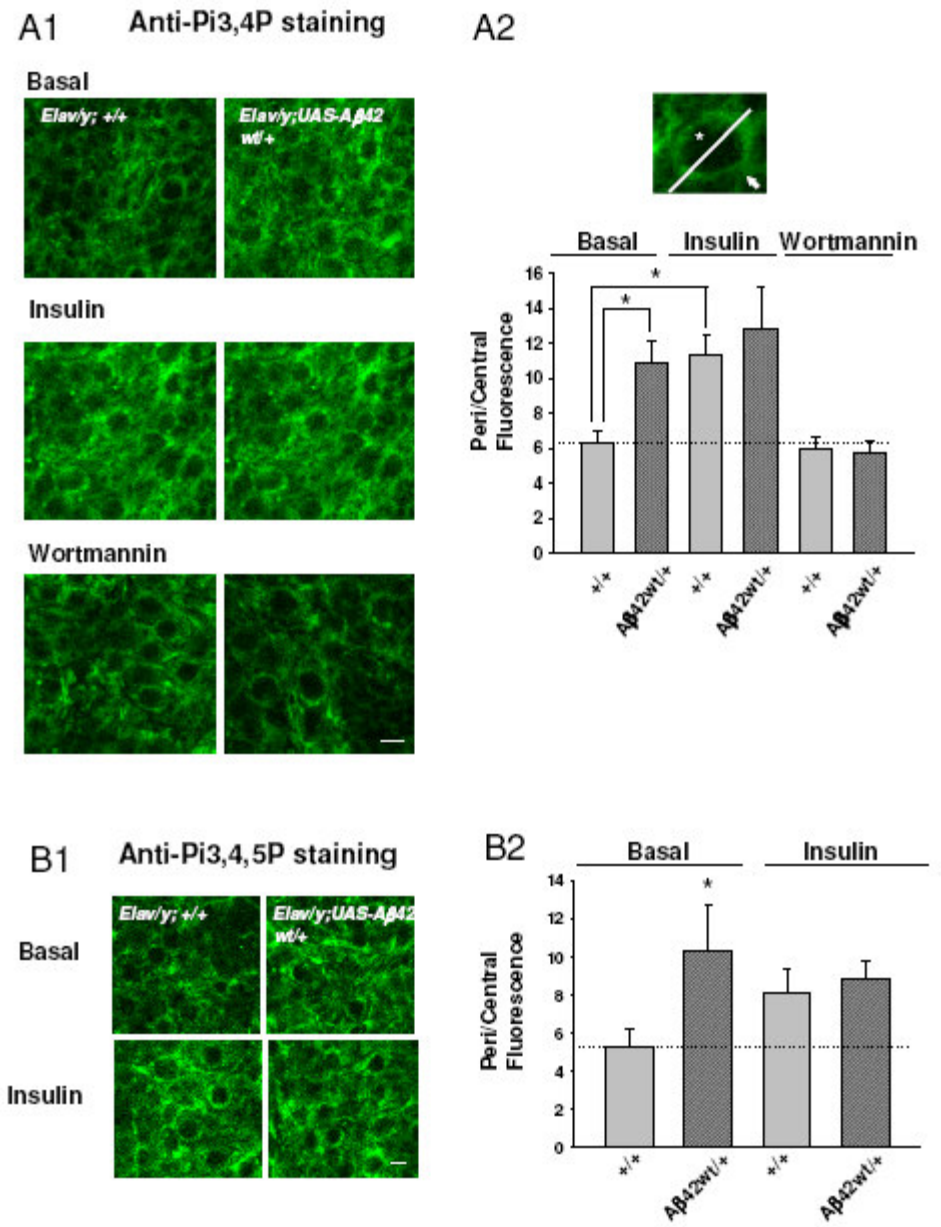


Figure 21. Aβ42 larvae showed insulin-resistant phenotype. Larvae were incubated with the indicated drug for 30 mins in HL3 buffer with 0.4mM Ca^{2+}, then stained with antibody that specific against PI3,4P (A) and PI3,4,5P (B). (A1) and (B1) is a representative picture. (A2) and (B2) is the statistical results. For relatively statistics, numbers were normalized to ctrl in animals not expressing Aβ42. (A2) is without normalization. (A2 TOP) The relative fluorescence in plasma membrane was calculated as a ratio between the plasma membrane fluorescence intensity (arrow) and the average cytosolic fluorescence intensity (star). 5 cells with positive signals from each larva were

chosen for the statistical analysis. For Pi3,4P staining, N=30, 15, 15 cells for basal, insulin and wortmannin treatment. For Pi3,4,5P, N=15 cells for all condition. Bar 10um

Aβ42 caused behavior damage is improved by PI3K reduction

Recovery in the LTD phenotype with the pharmacological treatment led us to examine the role of PI3K in Aβ42-induced memory loss. Young (1-2 days after eclosion) transgenic fruit flies with pan-neuronal expression of Aβ42 (*elav/Y; UAS-Aβ42/+*) had a normal immediate memory (measured 3min after training) (see Iijima et al., 2004; 2008), but this immediate memory became significantly lower in comparison to controls 7 days later after eclosion (Figure 22A). This age-dependent memory loss was rescued through expression of additional RNAi, knocking down the regulatory subunit of PI3K, Dp60 (*elav/Y; UAS-Aβ42/+;UAS-dp60RNAi/+*). The significance of this observation is strengthened by subsequent analysis of PTEN effects. PTEN is a phosphatase that dephosphorylates PI3K-phosphorylated lipids (Endersby and Baker, 2008).

Overexpression of PTEN is equivalent to inhibition of PI3K activity. The rescue of memory loss was also observed with additional overexpression of PTEN in transgenic fruit flies, *elav/Y; UAS-Aβ42/+;UAS-dpten/+* (Figure 22B). Conversely, Aβ42-induced memory loss was enhanced with expression of a dominant-negative PTEN, $DPTEN^{C124S}$ which is a catalytically inactive mutant of PTEN, in transgenic fruit flies *elav/+; UAS-Aβ42/+;UAS-dpten^{c124s}/+* (Figure 22C). Of note female fruit flies were used in this particular experiment for females show a weaker memory loss phenotype so that the enhanced memory loss is easier to be revealed. The weaker memory phenotype is resulted from gene-dosage effect: The elav-Gal4 driver is located in X chromosome and

female shows less expression of Gal4 and therefore less Aβ42, which produces a weaker phenotype (Iijima et al., 2004).

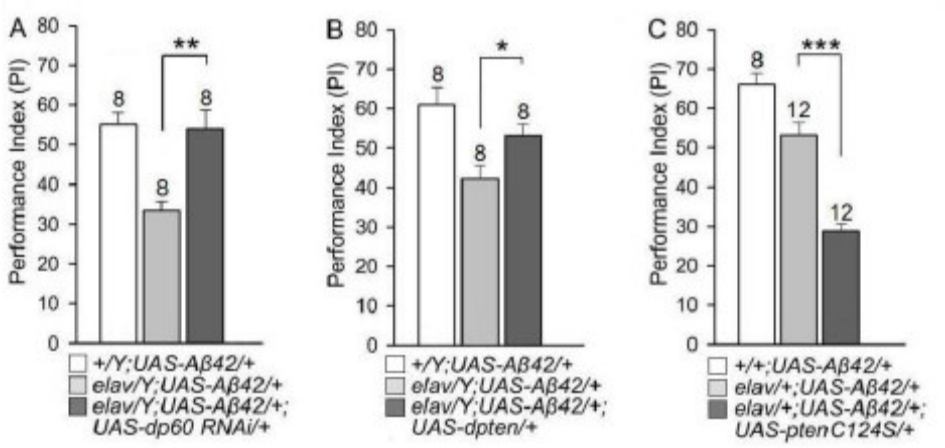


Figure 22. Reduction of PI3K signaling pathway improve the memory damage cause Aβ42. Memory was assessed by using Pavlovian olfactory conditioning in 5dae Aβ42 transgenic flies. Reduce PI3K activity by expression of P60 SiRNA (A) or PTEN (B), the memory deficit caused by expressed Aβ42 is corrected. Expressed PTEN c124s in Aβ42 transgenic flies will enhance the learning deficit (C). Number above each bar presents N number. *=p<0.05. **=p<0.01 and ***=p<0.001. Data from Wang

We further showed that overexpression of either p60 RNAi or PTEN alone had no effects on memory performance, but overexpression of $PTEN^{C124S}$ showed a very mild

some reduction in memory scores (Figure 23)

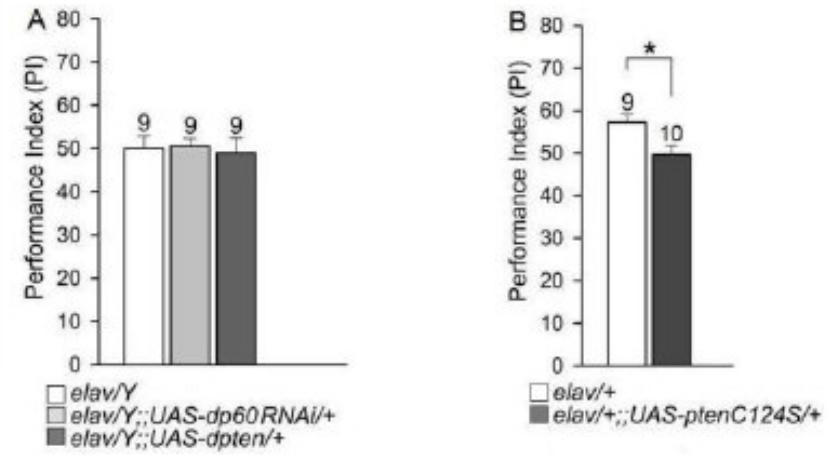


Figure 23. The effects of PI3K signaling pathway on memory change. Memory was assessed by using Pavlovian olfactory conditioning in 5dae transgenic flies. No change of memory with reduction of PI3K activity by expression of P60 SiRNA (A). PTEN c124s transgenic flies showed the learning deficit (B). Number above each bar presents N number. *=p<0.05. Data from Wang

Further analysis suggested that there is an age-dependent effect on the amelioration of the memory effect by reduction of PI3K. We tested the Aβ42-expressing flies on the age of 10 days and 15days. The experiment data showed there is mild but significant improvement of memory in Aβ42-expressing flies. (Figure 24).

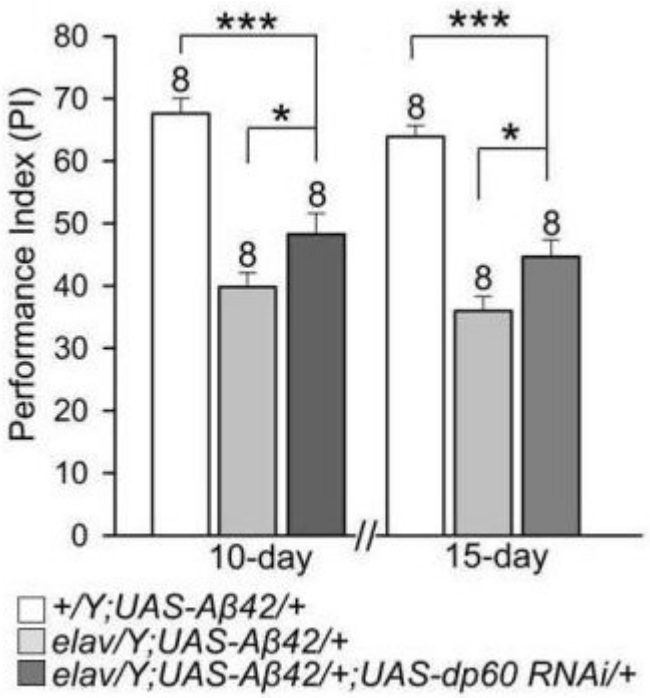


Figure 24. The effects of PI3K signaling pathway on memory change is age-dependent. Memory was assessed by using Pavlovian olfactory conditioning in 10, 15 dae transgenic flies. Number above each bar presents N number. *=p<0.05 and ***=p<0.001. Data from Wang

Besides memory deficit, we also tried to examine if reduced PI3K would also benefit locomotor ability. From our previous study in the Aβ42 transgenic flies and also patients who suffered from AD both showed severe locomotion damage (Iijima, et al., 2004; Hebert, et al., 2008). To test if reducing PI3K is also able to improve locomotion ability in the Aβ42 transgenic flies, we performed a locomotion test. The data showed that the locomotion deficit was age-dependent; older Aβ42 flies displayed more severe locomotion damage (Figure 25). However, when Aβ42 transgenic flies with reduction of PI3K, the locomotion performance was improved. Also the improvement is age-dependent, the older flies the less be improved.

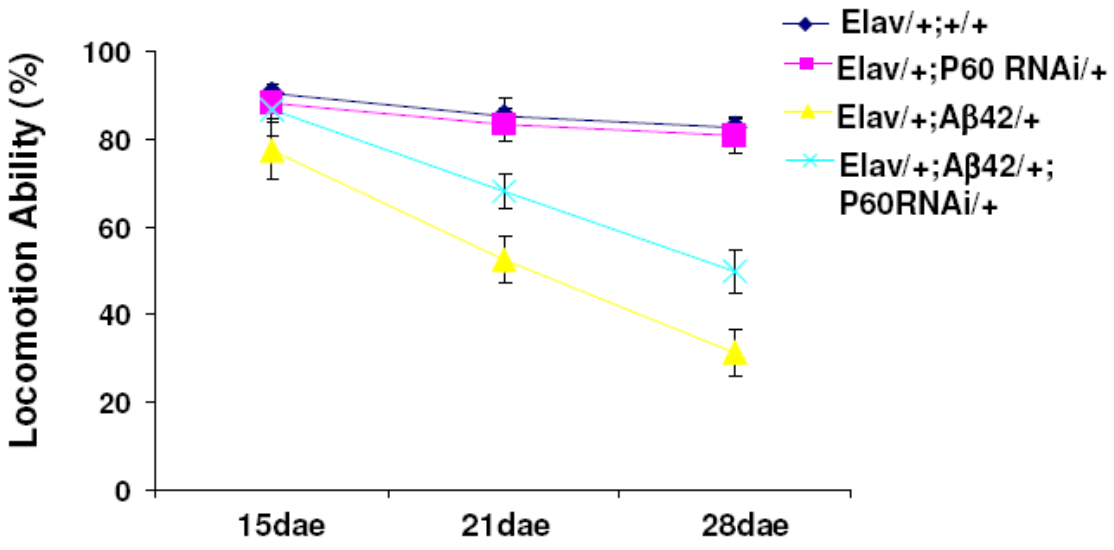


Figure 25. Locomotion damage is improved in Aβ42 flies with PI3K reduction. Express P60 RNAi improved the locomotion damaged caused by Aβ42. Flies were test on the date indicated on the X axel. N=3 for each experiment.

Oral administration wortmannin is able to recover the behavior damage in Aβ42 transgenic flies.

On basis of results presented above, we wanted to see whether feeding fruit flies with pharmacological inhibitors of PI3K could prevent the memory loss. Drug (wortmannin) feeding was begun with two-days old fruit flies, with 3 hours each day for consecutive 7 days. The memory score was determined on day 10 after eclosion. Memory was much improved with Aβ42-expressing fruit flies (*elav/Y; UAS-Aβ42/+*) when 100nM wortmannin was fed each day but not with 25nM concentration. (Figure 26A).

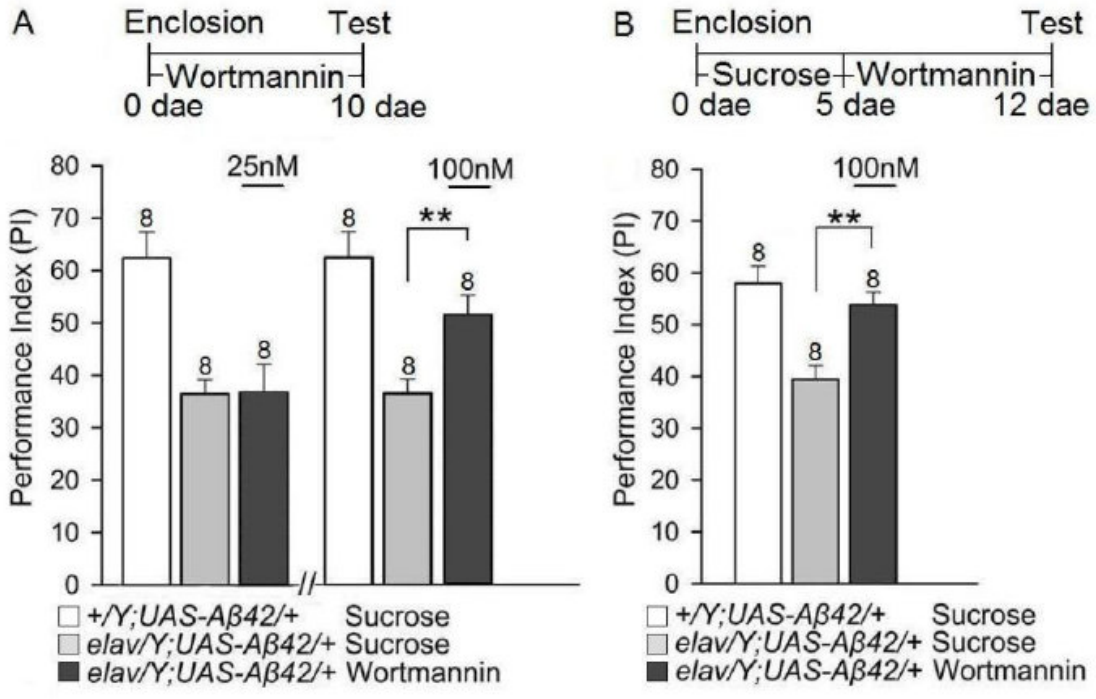


Figure 26. The memory deficit caused by Aβ42 is cured by feeding PI3K inhibitor. Application of PI3K inhibitor rescued the memory deficit in Aβ42 flies. The rescue effect is dose dependent, 25 and 100 nM wortmannin was used for this study (A). Feeding the Aβ42 flies with wortmannin after memory already decay is still able to recover the behavior deficit (B). Upper panel shows the design paradigm used in this experiment. Bottom panel shows the experiment result. **=P<0.01. Data from Xie

For 2-days-old Aβ42-expressing fruit flies, their memory is still normal. We wonder whether the drug treatment can improve memory at a time memory scores are already significantly lower, such as at day 5 after eclosion. We fed fruit flies with 100nM wortmannin for additional 7 days and at day 12 after eclosion memory was assayed. There was still significant memory improvement in Aβ42-expression transgenic fruit flies. (Figure 26B)

To strength our finding that drug feeding is able to improve the memory decay, besides feeding Aβ42-expressing flies with wortmannin, we also used LY294002,

another PI3K inhibitor, to do the experiment. Our data showed that both wortmannin and LY294002 is able to rescue Aβ42 mediate memory decay. (Figure 27).

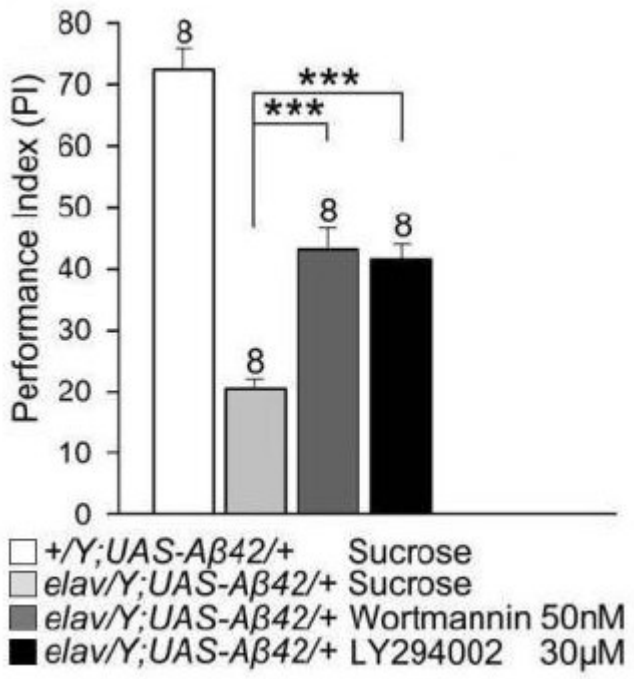


Figure 27. The memory deficit caused by Aβ42 is corrected by feeding two different PI3K inhibitors. Application of PI3K inhibitor both wortmannin, 50nM and LY294002, 30uM rescued the memory deficit in Aβ42 flies. ***=P<0.001. Data from Xie

Reduced PI3K has no effect on neurodegeneration in Aβ42 flies.

Obviously, we wanted to determine whether Aβ42-induced neurodegeneration was also rescued by inhibition of PI3K. As visualized in florescent images of 50-day-old brains in Figure 31**,** there were many black holes within the brain, which are indication of neurodegeration. We measured the ratio of the area of empty holes to the area of the whole cell body region and use it to indicate the level of neurodegenration (Iijima et al., 2008). We found that there was no difference between Aβ42-expressing fruit flies and

Aβ42 fruit flies with PI3K reduction (Figure 28). Thus, inhibition of PI3K improved Aβ42-induce memory loss but had no effect on Aβ42-induced neurodegeneration.

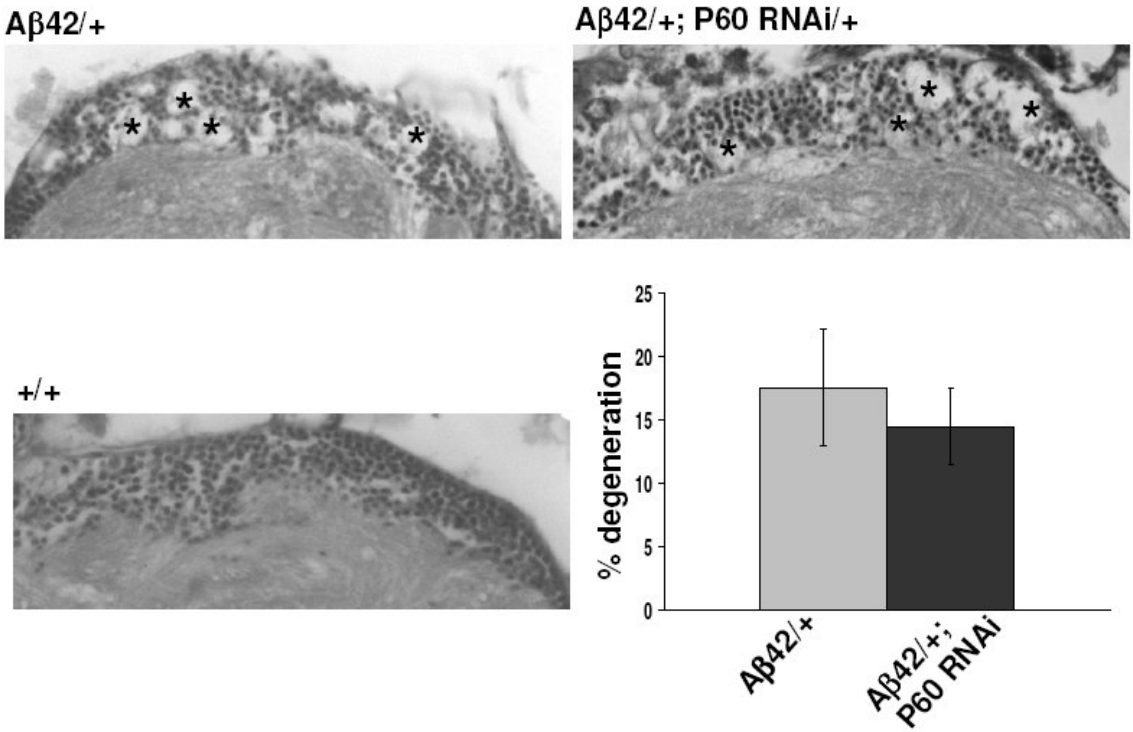


Figure 28. Cell degeneration is not improved in PI3K reduction Aβ42 flies. The cell body region in the 40dae female flies is used to quantify the severity of degeneration. (A) The representative imagines. Arrow points to the degeneration. (B) Percentage of the area lost in the cell body regions are shown as averages ±SEM. N=6 for each genotype.

Reduced PI3K is able to reduce Aβ42 oligomers formation in Aβ42 transgenic flies.

To gain insights into how PI3K activity regulates Aβ42-induced LTD enhancement and memory loss, we investigated whether Aβ42 aggregation is affected. Aβ oligomers have recently become the focus and are considered as the major pathological species of Aβ peptides (Haass and Selkoe, 2007). In particular, a number of experiments demonstrated that Aβ oligomers can causes learning defects in mice (Lesné et al., 2006; Shankar et al., 2008).

Whole brain lyses were used to perform the western blot. Here we found that the ratio of oligomers to monomer was significant lower in Aβ42-expressing trangenic fruit flies with additional expression of p60-RNAi to silencing PI3K activity (Figure 29A). The reduction in dimmer and trimmer is moderate but significant for 15-days-old fruit flies. As a control, we showed that the level of Aβ42 monomers was similar between different genotypes (Figure 30). A similar reduction was also observed with overexpression of PTEN (Figure 29A) as well as with 10-days drug feeding (Figure 29B).

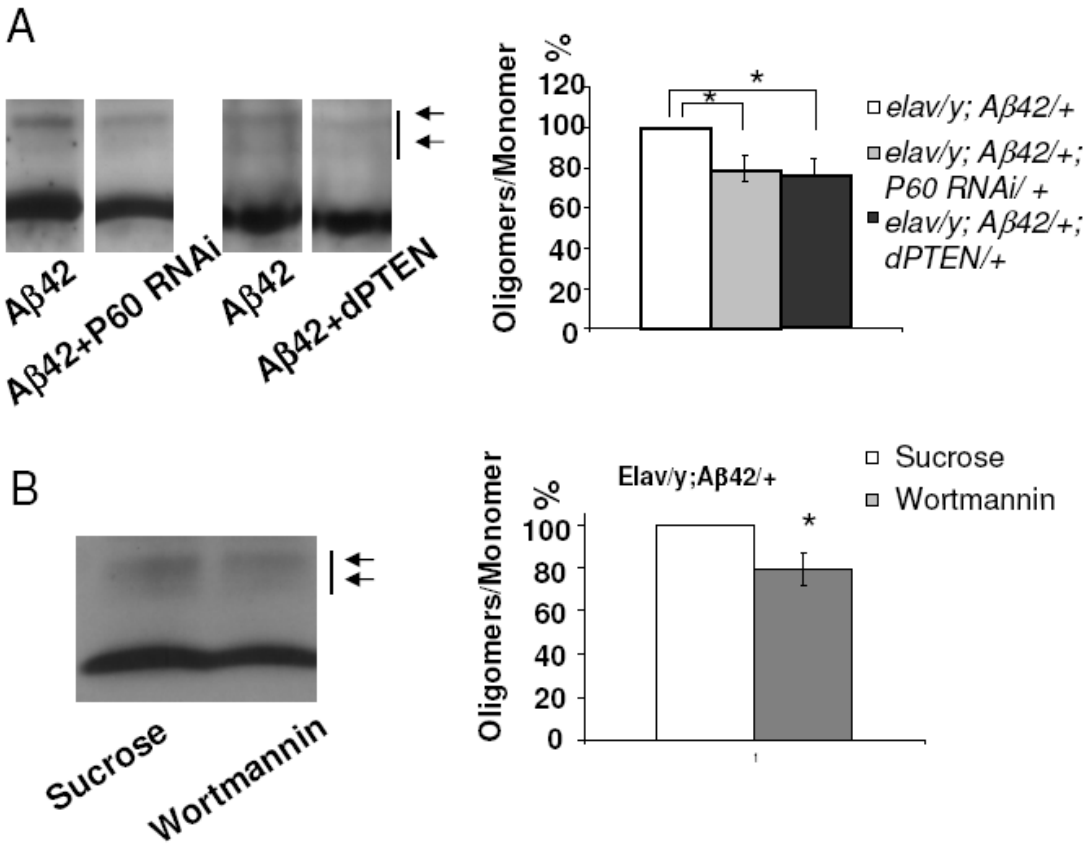


Figure 29.

Figure 29, continued. Less Aβ42 oligomers showed in Aβ42 flies with PI3K reduction. (A) Whole brain lysates were used to run western assay. The experiment was done on 15dae flies. N=3 for each genotypes. (B) Apply PI3K inhibitor, wortmannin, to

5dae Aβ42 flies for 7days, showed a significant reduction of Aβ42 oligomers formation. N=3 foe each genotypes. Left is the representative result. Right is the quantative result. Arrows point to oligomers.

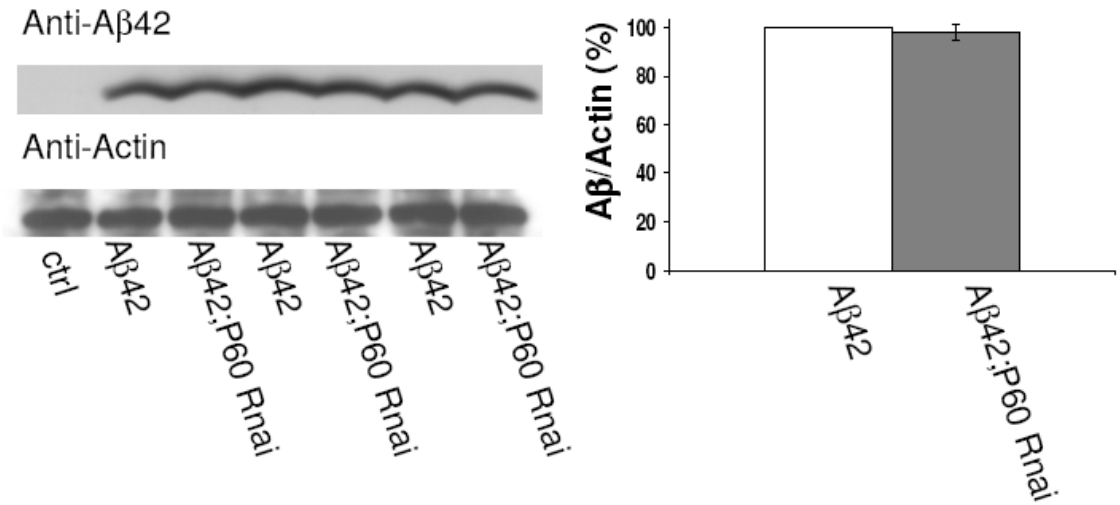


Figure 30. No change of Aβ42 monomers showed in Aβ42 flies with PI3K reduction. Whole brain lysates were used to run western assay. The experiment was done on 15dae flies. N=3 for each genotypes.

The aggregation of Aβ42 is suppressed by reducing PI3K

Next, we examined the deposits of Aβ42 fibril aggregates. We have shown before that such deposits accumulate with age and can be revealed by Thioflavan-S staining (Iijima et al., 2004; 2008). We focused our observation around the mushroom body region for it shows severe Aβ42-induced neurodegeneration and intense Aβ42 deposits.

Confocal images of Thioflavan-S staining repeated previously reported observation: The Aβ42 deposits (*elav/Y; UAS- Aβ42/+*) grow large in sizes and in numbers with aging (Figure 31). We classified the deposits on basis of size and compared them at different ages (15 and 50 days old). In younger fruit flies (15days), Aβ42 deposits are primarily

small deposits and this number was dramatically reduced in the Aβ42-expressing brain with inhibition of PI3K activity (*elav/Y; UAS- Aβ42/+;UAS-dp60RNAi*) resulted from expression of dp60-RNAi (Figure 31A). In older fruit flies, large sized deposits were more abundant. Deposits were significantly reduced in all categories but more striking in large sized (Figure 31A). This observation was verified by treating fruit flies with PI3K inhibitor wortmannin for 10 days and a similar reduction in small sized deposits was observed in Aβ42-expressing fruit flies fed with drug in comparison with those fed with sucrose (Figure 31B). The magnitude of this reduction in fibril deposits are much more profound than that observed in oligomers.

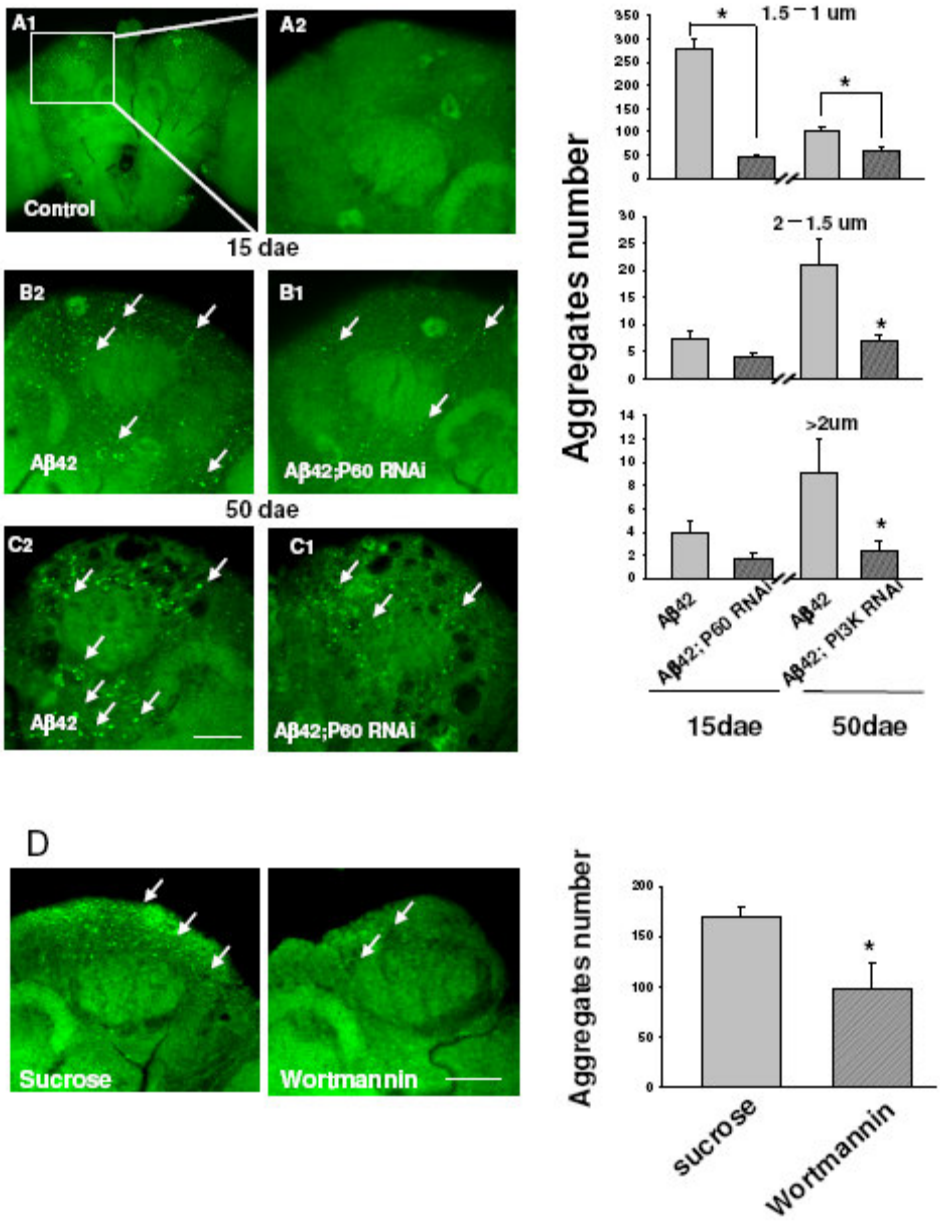


Figure 31. Reduced PI3K in Aβ42 flies leads to less Aβ42 fibrils in the fly brain. (A) Thioflavin S (TS) staining of brains in different aged flies. Arrows indicate TS-positive deposits. No signal was detected in the control (B). Scale bar in 25 um. (B, C, D, E) are enlarged images of the boxed regions in (A), respectively. Arrow indicated TS positive signaling. Right pannel, Numbers of TS-positive deposits were presented as averages (n = 4 for 15 dae and n=3 for 60 dae).

Discussion

In this study, we began with electrophysiological analysis of how Aβ42 affected long-term depression (LTD) in an attempt to gain insights into Aβ42-induced memory loss. We found that pharmacological inhibition of PI3K activity was capable of returning enhanced LTD in Aβ42-expressing larvae to the control level, suggesting that an elevated PI3K is responsible for Aβ42-induced LTD enhancement. This result was confirmed from more direct assay of PI3K activity through immunostaining of PI3K lipid substrates, which revealed an increased basal level activity but retarded insulin-stimulated PI3K activity in Aβ42-expressing fruit flies. Further behavioral tests demonstrated that this altered PI3K activity contribute the Aβ42-induced memory loss for genetic and pharmacological inhibition of PI3K activity rescues memory loss. This conclusion was supported also by manipulating PTEN activity, a phosphatase antagonizing PI3K function. Further analysis of Aβ42 aggregation showed that the small oligomers and fibril deposits were reduced because of inhibition of PI3K activity in Aβ42-expressing fruit flies. Thus, this study identifies the PI3K pathway as an important regulatory mechanism in Aβ42 aggregation and memory loss.

Role of PI3K in Aβ42-induced memory loss

AD has been notoriously known as “brain diabetes” for brain tissues from AD patients are known to be insulin resistant (Craft et al., 1998; Zhao and Townsend, 2008). A growing body of evidence indicates importance of the insulin-activated pathways in progression of AD, including studies of animal models and direct observations from patients (Haugabook et al., 2001; Pei et al., 2003; Rickle et al., 2006). However, the role

of PI3K signaling pathway, the classical pathway being activated through insulin receptors, in the Aβ pathogenesis is still not well defined. On one hand, Akt activity is increased and PTEN activity is reduced in the temporal cortex of the post-mortem AD brain (Pei et al., 2003; Rickle et al., 2006). On the other hand, although PI3K activity in the AD brain is reduced in the soluble pool, no difference is found in the particulate pool in the frontal cortex (Rickle et al., 2004). Moreover, *in vitro* experiments suggest that increased PI3K signaling activity is able to reduce Aβ toxicity and thus improve cell viability (Nakagami, 2004; Lee et al., 2008).

Results from this study support the notion that altered PI3K activity contributes to Aβ42-mediated toxicity and therefore to AD pathogenesis. The nerve tissues in Aβ42-expressing ganglia also did not respond to insulin stimulation much because of the basal level of PI3K was already elevated to a level similar to the stimulated in controls. The lack of response to insulin stimulation results from the basal elevation of PI3K activity in Aβ42-expressing cells making this signaling pathway in these cells can not be regulated by insulin properly. Such insulin dysregulation makes these cells lose the sensitivity to insulin stimulation is becoming insulin-resistant like.

The consequence of rising PI3K activity by Aβ42 will lead to synaptic dysfunction and behavior damage. The notion that intensified PI3K results in behavior damage is further strength by the finding that flies with increase PI3K activity by expression of PTEN mutant, *elav/+;UAS-dpten*^{c124s}*/+*, have a lower memory performance score. The described phenotypes results from expression of Aβ42 lead to increase PI3K activity is reversed by the genetic or pharmacological inhibition of PI3K. In addition to recover the

behavior damage, further analysis showed that reduction of PI3K in Aβ42 expressing fruit flies is also able to suppress the Aβ42 aggregation.

While our data is consistent with the recent finding that Aβ42 oligomers stimulus PI3K activity (Bhaskar et al., 2009) and reduce PI3K activity in a rat by application of wortmannin improve the special memory (Dash et al., 2002), our data suggested that there is a reciprocal relation between PI3K and Aβ42 (Figure 32). Accumulated Aβ42 aggregates will increase the PI3K activity leading to memory damage. Such memory decay is reversed by reducing the activity of PI3K. On the other hand, the requirement of PI3K activity for Aβ42 aggregation suggests that increase of PI3K promotes the Aβ42 aggregates. Example of reciprocal relation to amplify a cellular signaling effect has been reported (Zhao et al., 2008).

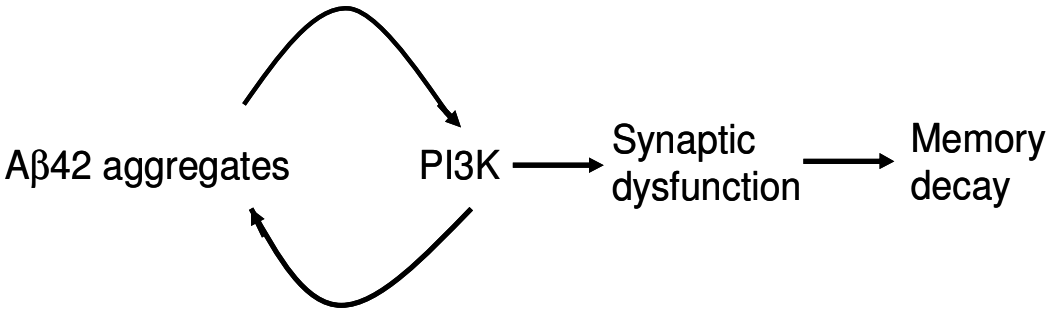


Figure 32. A reciprocal model in demonstrating the relationship between Aβ42 and PI3K. Aβ42 aggregates increase the activity of PI3K. The Aβ42 aggregates will affect synaptic function through PI3K signaling leading to behavioral deficiencies. The PI3K activity, however, is required to regulate Aβ42 aggregation.
.

How could this model to contribute to disease pathogenesis? One can envision a number of possibilities. For example, increase of PI3K activity results in the lack of response to insulin stimulus will disrupt the regulation of glucose circulation. Abnormal of glycogen circulation in the brain could lead to dementia (Zhao and Townsend, 2008).

Alternatively, as the results of reciprocal relation the Aβ42 aggregation and PI3K signaling will be produced constitutively. Accumulation of Aβ42 aggregation and constantly activation of PI3K is becoming neurotoxic and leading to memory damage as shown in many studies and this work (Haass and Selkoe, 2007; Zhao et al., 2008; Bhaskar et al., 2009).

Independent mechanisms for memory loss and neurodegeneration?

A cascade hypothesis suggests that synaptic dysfunction/alteration, early event of AD, leading to memory deficit and subsequently causing neurodegneration (Haass and Selkoe, 2007). Base on this hypothesis an interesting but remain to be determinate is are the memory loss and neurodegenration the same mechanism or different mechanisms. Due to the observation that hAPP mouse model can not recapitulate significant neuronal death as in AD patient in the brain this question has been less addressed. Surprisingly, massive neuronal loss is observed in our Aβ42 fly model (Iijima et al., 2004; 2008). Both memory loss and neurodegeneration are shown in our Aβ42 fly model. These findings enable us to address this question in our fly model.

As our data shown, inhibition of PI3K improved Aβ42-induce age-dependant memory loss but had no effect on Aβ42-mediate age-dependant neurodegeneration. The reason why reduction of PI3K only rescued Aβ42-mediated age-dependant memory loss but not neuronal death is not known. However, one explanation for this discrimination is there is another mechanism besides PI3K that involves in Aβ42 induced neuronal death. We speculate that this cell death signaling is activated through constitutive stimulation by Aβ42 oligomers accumulation. In our western blot, only about less than 30% reduction of

Aβ42 oligomers in Aβ42 fly with PI3K inhibition. There is still about more than 70% of Aβ42 oligomers accumulated in the Aβ42 fly brain. During the aged the rest of Aβ42 oligomers constantly stimulate the death signaling and the activation of this signaling is resulting in cell death.

This speculation implicates two issues. First, the accumulation of Aβ42 causing functional and structure change in the brain, might results from two different cellular signaling both activated by Aβ42. Second, it may not be enough to test memory recovery as an only read-out to evaluate the drugs that design for cure AD.

Chapter 6

General Discussion

The goal of this thesis project is to understand the toxicity of Aβ42, a major factor that contributes to Alzheimer's disease (AD). First, we established a useful model that can recapitulate most of the AD phenotypes observed in the AD mouse model. The finding of the diverse effect of Aβ toxicity from a cellular level to a behavioral level explains the complexity of disease progress. Manipulated PI3K activity is able to change the toxicity, and an aggregation property of Aβ42 provides a therapeutic treatment for the disease.

In the following discussion, I will summarize the significant findings in our AD fly model and their potential implication in AD pathogenesis.

1. AD fruitfly model recapitulated phenotypes in the AD patient and AD mouse model

In this section, I will list some aspects in the AD fly model that recapitulate AD phenotypes and also point out the significance of these findings in terms of AD pathogenesis.

First, secreted Aβ42 is taken up by glia cells. A secretory signal peptide was fused to the Aβ42 peptide N-terminal targeting to the secretory pathway (Iijima et al., 2004). The secretion of Aβ42 was confirmed by electron-microscopy, demonstrating that this peptide is distributed in the endoplasmic reticulum (ER) and Golgi (Iijima et al., 2008). Notably, observing Aβ42, driven by *elav-gal4*, in the glia cells suggests that secreted Aβ42 from neurons is uptaken by glia cells. This finding supports a hypothesis that activated glia

cells clear up the extracellular Aβ peptides through phagocytosis in the brain of the AD mouse or AD patient (Webster et al., 2001; Guénette, 2003; Takata et al., 2007).

Second, cognitive damage is preceded by the death of neurons. The progression of the disease in AD flies also supports the observations on the AD mouse model in which learning and memory deficiencies are preceded by cells beginning to degenerate, which highlights a hypothesis that AD can be initiated from synaptic failure (Selkoe, 2002). There involves a neuronal functional failure first, followed by cell death.

Third, oligomers, but not fibrils, correlate with synaptic dysfunction and memory decay. The memory deficits of the AD patient and AD mouse model do not correlate well with the burden of senile plaque deposits in the brain. Memory deficit precedes senile plaque buildups in the brain of the AD mouse model. On the other hand, the early detection of an Aβ oligomer correlates with early synaptic dysfunction and memory damage. This observation leads to a hypothesis that an Aβ oligomer is responsible for memory deficits, as reviewed by Haass and Selkoe (2007). In our AD fly model, we found that age-dependent memory loss appeared before the presence of Aβ42 fibrils. In addition, there was an early detection of Aβ42 oligomers in the brains of AD flies. Although we cannot conclude that the memory damage in these AD flies directly resulted from the accumulation of Aβ42 oligomers in the brain, our findings support the notion that there is no correlation between behavioral damage and the accumulation of fibrils.

Finally, massive cell degeneration in the brains of the AD flies is consistent with the findings in the brains of the AD patients reviewed by Iijima and Iijima-Ando (2008). Therefore, the AD fly model provides a better opportunity than the hAPP mouse model to study the mechanisms of Aβ42 causing cell death *in vivo*.

2. The effects of Aβ aggregate from synaptic function to behavior

A wealth of data from many different laboratories suggests that Aβ has diverse effects on cellular and/or molecular signaling that lead to neuronal dysfunction (Small et al., 2001). Recent studies and our data suggest that the diverse effects of Aβ might be due to its aggregation propensity.

In this section, I will discuss the data from this work and also from other labs that supports this hypothesis, as well as how changes in Aβ aggregation lead to neuronal dysfunction and its implications for disease pathogenesis. Since my thesis project is focused on synaptic function and behavior, I will first discuss how Aβ aggregates affect synaptic function, then how these changes lead to behavior change, from the cellular to the behavioral level.

2.1 Aβ peptides affect synaptic function

It has been proposed that AD is a synaptic failure disease and an Aβ peptide initiates the synaptic dysfunction (Selkoe, 2002).

Synapse loss and synaptic dysfunction, which result in cognitive damage, are considered to be early symptoms of dementia. A functional change in the early progression of the disease is believed to lead to structural change in the disease's later progress.

Evidence that Aβ initiates functional and structural change in the synapse

The short-term application of Aβ oligomers, created either by artificial synthesis or natural secretion, to hippocampus slice cultures changes synaptic transmission and

disrupts synaptic plasticity (Haass and Selkoe, 2007). Furthermore, injecting Aβ aggregates into animals shows abnormal extracellular field potential, suggesting that Aβ can initiate synaptic dysfunction (Walsh et al., 2002; Szegedi et al., 2005; Lesne´ et al., 2006; Shankar et al., 2008).

At the anatomic level, there is a correlation of synaptic loss and cognitive deficit. As the disease progresses, the button number (in both cholinergic and glutaminergic neurons) dramatically decreases (Hu et al., 2003; Bell et al., 2007). In addition, dystrophic neurite is observed around plaque formation (Dong et al., 2007).

Another piece of evidence that supports the idea of Aβ changing synaptic function is the decrease in the number of *N*-methyl *D*-aspartate (NMDA) receptors. Over-expression of hAPP promotes the endocytosis of NMDA receptors (Hsieh et al., 2006). This phenotype has been proposed as one of the mechanisms that changes the synaptic plasticity in the AD mouse model.

Abnormal brain activity observed in the early stage of AD and MCI patients

Although a conclusive diagnosis of AD requires a postmortem autopsy, behavioral changes and cognitive deficiencies can be used to estimate the disease's progression. When examined by electroencephalography (EEG), both patients in the early stage of AD and individuals with mild cognitive impairment (MCI) show abnormal brain activity (Jackson and Snyder, 2008).

It has been shown that a lower delta power spectrum is associated with memory damage in the MCI patients. In addition, a diminished alpha power spectrum is observed in the AD patients as well as MCI patients (Kwak et al., 2006; Liddell et al., 2007).

The reason to study MCI is that an MCI patient often shows the same phenotypes as AD patients, such as memory loss and gradual cognitive damage (Collie and Maruff, 2000; Petersen et al., 2001). In addition, for an MCI patient, the transition rate from MCI to AD is between 10–54 percent, compared to 0.2–2.3 percent, which is the conversion rate of a healthy individual in the same age group to AD (Petersen et al., 1999, 2001).

2.2 Synaptic dysfunction in the Aβ42 transgenic fly observed in larval NMJ

Our data, in Chapter 4, shows that the effect of endogenous expression of Aβ42 on synaptic function depends on Aβ42 aggregates. Different Aβ aggregates have distinct effects on synaptic functioning. Aβ42 oligomers from neurons that suppress the synaptic function are consistent with the finding that *in vitro* Aβ42 oligomers' application disrupts synaptic function in the vertebrate hippocampus slice cultures. Aβ42 fibrils from muscle have the opposite effect of Aβ42 oligomers on synaptic function. Secreted Aβ42 fibrils increase the synaptic transmission. This finding is consistent with *in vitro* studies (Dahlgren et al., 2002; Fu et al., 2006; Liu and Schubert, 1998; Wang et al., 2002; Ye et al., 2004). Furthermore, injecting Aβ42 fibrils into a rat hippocampus region shows an increase of NMDA receptor activity (Szegedi et al., 2005).

Recordings in the neocortex of primary cell cultures also show that Aβ fibrils enhance neuronal activity, increasing the excitatory postsynaptic potential (EPSP) and membrane depolarization. Notably, this study further demonstrates that different Aβ aggregates affect different glutamine receptors (Ye et al., 2004). This notion is further strengthened by our *in vivo* findings that different aggregations affect synaptic function differently.

Possible mechanisms in the regulation of the synaptic function in *Drosophila* NMJ by Aβ42

One explanation for the opposing regulation of synaptic function by Aβ oligomers and fibrils is that these aggregates alter the exocytosis of presynaptic vesicles. In fact, in hippocampal neuronal cultures and also neuronal cell line studies, it has been shown that Aβ fibrils increase exocytosis (Liu and Schubert, 1998).

Previous work from our group has shown that there is a decreased frequency of mEJC after inducing LTD in the *Drosophila* larval NMJ, suggesting that the induction of LTD in this preparation is through presynaptic modulation (Guo and Zhong, 2006). This finding supports our conclusion that Aβ aggregates affect the presynaptic mechanism of synaptic transmission, since we found Aβ fibrils affect the LTD response with the same preparation.

We speculate that dysregulation of calcium concentration might play a role in Aβ42 regulated synaptic function (Bezprozvanny and Mattson, 2008). Our data showed that Aβ42 fibrils do not change mEJC amplitude and frequency, illustrating that the quantal size and the number of postsynaptic receptors are not affected. One of the reasons that Aβ42 fibrils change the EJC but not mEJCs could be that Aβ fibrils dysregulate calcium concentration. The influx of calcium increases the intracellular calcium concentration, which can initiate the neurotransmitter release through multiple mechanisms. The increase of intracellular calcium by Aβ42 fibrils has been well documented (Blanchard et al., 2004). It has been shown that the application of Aβ42 fibrils will increase intracellular calcium through the AMPA receptor in the mouse neuronal cell line. Although we do not have direct evidence showing that the intracellular calcium is

affected by Aβ42, our studies here and also studies from other labs suggest that calcium dysregulation may play a role.

Neuronal activity changes in AD and also in the AD mouse model

One proposed mechanism by which Aβ suppresses neuronal activity conflicts with recent findings that AD patients and AD mice models have epileptic seizures and abnormally higher cell activity (Romanelli et al., 1990; Lozsadi and Larner, 2006). A study on seizure activity employing the AD mouse model proposed another mechanism: abnormal excitatory neuronal activity due to the accumulation of Aβ. Accumulated Aβ can initiate a compensatory inhibitory mechanism to neutralize over-excitation (Palop et al., 2007). Although this hypothesis explains the seizure activity in AD, the exact cause of the abnormal excitatory neuronal activity has not yet been addressed.

Electrophysiological recordings from hippocampus slice cultures also showed inconsistent results. The hAPP-J20 mice—those with hAPP carrying the Swedish and Indiana FAD mutations—show abnormal synaptic transmission but a normal LTP in the CA1 region (Palop et al., 2007). Another study using the hAPP swe mice (human Swedish mutant APP transgenic mice) showed the decrease of synaptic transmission and disruption of LTP in the CA1 region (Kamenetz et al., 2003). Applying artificial Aβ peptides increased the LTP level in the hippocampus slice cultures (Wu et al., 1995).

Our *in vivo* findings show that different Aβ aggregates affects synaptic function differently, providing a possible explanation for such contradictory findings. Cell activity can be affected differently depending on the different Aβ aggregation. In fact, our findings in the *Drosophila* larval NMJ show that Aβ fibrils increase the cells' activity,

leading us to develop a hypothesis that the seizure activity might be through Aβ fibrils. This hypothesis has been confirmed in the recent study. One group using hAPP seizure mice showed that the application of Aβ fibrils can reproduce the same phenotypes using patch-clamp recordings in hAPP swe mice showing seizure activity (Society for Neuroscience, 2008). They concluded, "Fibrillar β-amyloid is associated with epileptic seizures in APPswe/PS1de9 mice and changes excitability in cortical and hippocampal neurons."

The same conclusion they draw from the study of vertebrate is the one we have determined from the AD fly model, further providing a good example that research of invertebrate can be very useful to interpret some aspects of diseases' phenotypes.

The implication of this finding: Aβ42 as a cell activity balancer

Our finding provides a possible explanation for the physiological function of Aβ42. While Aβ42 oligomers mainly suppress the synaptic function, Aβ42 fibrils increase the synaptic function. We speculate that Aβ42 may serve as a balancer to stabilize the synaptic activity. In addition to the negative feedback model (Kamanetz, 2003), our study suggests that there is another mechanism that Aβ42 will bring up the neuronal activity.

We propose that the accumulation of extracellular Aβ42 oligomers will bring down the neuronal activity. Decreased neuronal activity will reduce Aβ42 oligomers' secretion from neurons. The accumulated Aβ42 oligomers will gradually aggregate into higher aggregation forms, such as Aβ42 fibrils. The increase of Aβ42 fibrils will increase the neuronal activity. After the neuronal activity resumes, Aβ42 oligomers are secreted again to regulate neuronal activity.

2.3 Aβ and changes in behavior: Different Aβ aggregates have distinct effects on the pathogenesis of the disease

In this section, I will expand our findings to the behavioral level and discuss how different aggregations have different effects on disease pathogenesis, not only in our study but also in studies from other labs.

Study from *C. elegans*:

Fay and colleagues developed many Aβ42 transgenic *C. elegans* mutant lines. First, they used thioflavin-S staining to assay the propensity of aggregation in each mutant line. Most of the Ab42 mutant lines have less aggregation than transgenic wild-type Aβ42 *C. elegans*. In their findings, one of the mutant lines that suppressed the aggregation of Aβ42 showed different toxicity from a Aβ42 wild-type animal (Fay et al., 1998).

Study from *Drosophila*:

Luheshi and colleagues developed different Aβ42 transgenic fly lines with different Aβ42 mutations. Seventeen mutants with different aggregation propensity were created. Their read-outs concerned longevity and locomotion ability. They found that there is a positive correlation between aggregation and behavioral outcome: the more Aβ42 aggregates, the more severe the damage towards both lifespan and locomotion (Luheshi et al., 2007).

Another study, by Iijima and colleagues, used transgenic lines that contained Aβ42 and Aβ40 separately; Aβ42 tends to have more aggregates than Aβ40. They showed that behavioral outcome did not simply follow the trends of aggregation. Their data showed

that there is no difference in learning decay between these two species. Both of them showed the same deficiencies. However, only Aβ42 flies showed signs of decline in the locomotion and lifespan test. These results suggest that the aggregative tendency does not simply reflect the toxicity (Iijima et al., 2004).

Study in current work:

To further examine the relation of Aβ42 toxicity, such as neurodegeneration and memory loss, and the propensity to aggregate, we developed different Aβ42 lines that have different levels of propensity to aggregate, as mentioned in Chapter 3. Aβ42arc, a familial AD mutation, promoted the aggregation of Aβ42. Aβ42art, an artificial mutation, suppressed the aggregation of Aβ42. The experiments were set up in order to observe the toxic effects of these various mutants.

In the flies expressing Aβ42arc, there was quick memory decay, a severe locomotion deficit and a shorter lifespan, while Aβ42art also showed quick memory decay but suffered less damage to locomotive capabilities and had a prolonged lifespan. These results were all compared to Aβ42wt. Thus our data suggests that different aggregation propensities of Aβ42 modify qualitatively the pathogenicity of A*β*42 *in vivo*.

These works suggest that the varieties of Aβ42 toxicity are contributed to by different aggregation forms of Aβ42.

Implication of our study

Our data and also data from other lab studies demonstrate that different aggregation propensities of Aβ42 contribute to the diverse effects of Aβ42. In addition, this idea

suggests a notion that an aggregation inhibitor that partially prevents Aβ42 amyloidgenesis may result in qualitative shifts in the pathogenic effects. Altogether, studies conducted by this work and also evidence provided from other laboratories provide a mechanism to explain the diverse effects of Aβ42 on behavior change.

From the cellular to behavioral level, we clearly show that different aggregations of Aβ42 have distinct effects on cell function and molecular pathways. This finding offers a new way to interpret the progression of the disease. The severity of disease progression is contributed to by the different aggregation properties of Aβ peptides.

3. A treatment for the AD fly model: The role of PI3K in the disease's pathogenesis

In concluding that the diversity of the toxic effect of Aβ42 is from different aggregations, we have been led to find a possible mechanism that is able to reduce the aggregation propensity of Aβ42 and reverse its toxicity.

Our finding is that a reduction of PI3K suppresses the Aβ42 aggregation, reverses the enhancement of LTD and recovers the behavioral deficit caused by the expression of Aβ42, demonstrating that PI3K is involved in Aβ42 pathogenesis. Our data suggests that there is a feedback loop between Aβ42 and PI3K.

This feedback loop model demonstrates how PI3K is involved in Aβ42 pathogenesis. Initially, Aβ42 will form aggregates, and those Aβ42 aggregates will promote the activity of PI3K. Increased activity of PI3K will mediate the Aβ42 toxicity to cause synaptic dysfunction, leading to behavioral effects. However, the activity of PI3K is required to regulate Aβ42 aggregation.

The implication of this model in disease pathogenesis is discussed in Chapter 4. In this section, I will try to discuss the potential mechanisms by which PI3K regulates Aβ42 aggregation.

Although we did not have any evidence to explain why PI3K activity is required for maintaining Aβ42 aggregates, two mechanisms could explain this. One of the potential mechanisms is that downstream lipid products of PI3K are required to form Aβ42 aggregates. A body of evidence from *in vitro* study showed that Aβ peptides will interact with lipid rafts on the cell membrane and, therefore, increase the Aβ aggregation (Vestergaard et al., 2008). Lipids such as cholesterol, ganglioside and phospholipid have been used to study the interaction with Aβ peptides and enhance the Aβ aggregation (Schneider et al., 2006; Hung et al., 2008; Okada et al., 2008). Although, currently, there is no direct evidence that demonstrates the interaction of Aβ with lipid products of PI3K, one still cannot exclude the possibility that those lipid products might help to at least maintain the Aβ aggregates from degradation.

The other mechanism would be increased PI3K activities leading to decreased degradation activity intracellularlly. It has been shown *in vitro* that increasing type 1 PI3K (the same subtype we studied here) activity by either feeding cells with synthetic lipids (dipalmitoyl phosphatidylinositol 3,4-bisphosphate and dipalmitoyl phosphatidylinositol 3,4,5-triphosphate) or by stimulating the enzymatic activity by interleukin-13 decreases the macroautophagy and increases the accumulation of intracellular proteins (Petiot et al., 2000). The possible mechanism is that activating PI3K will lead to an activation of PKB and target rapamycin (TOR), which leads to inhibition of autophagy (Sarkar and Rubinsztein, 2008) and increases protein accumulation.

In conclusion, AD has been a mystery for more than 100 years, a complex disease that is caused by a complicated protein, Aβ. This thesis project reveals one aspect of AD, the effect of Aβ42 aggregations in the progress of AD in an animal model, and offers a therapeutic method by utilizing the role of PI3K in Aβ42 pathogenesis for future therapeutic strategies. We believe that gains a greater understanding the intricacies of Aβ42 aggregation, as was the goal of this project, can strongly contribute to understanding the pathogenesis of AD.

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