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·Original Article·

Amyloid-β depresses excitatory cholinergic synaptic transmission in *Drosophila*

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Abstract: Objective Decline, disruption, or alterations of nicotinic cholinergic mechanisms contribute to cognitive dysfunctions like Alzheimer's disease (AD). Although amyloid-β (Aβ) aggregation is a pathological hallmark of AD, the mechanisms by which Aβ peptides modulate cholinergic synaptic transmission and memory loss remain obscure. This study was aimed to investigate the potential synaptic modulation by Aβ of the cholinergic synapses between olfactory receptor neurons and projection neurons (PNs) in the olfactory lobe of the fruit fly. **Methods** Cholinergic spontaneous and miniature excitatory postsynaptic current (mEPSC) were recorded with whole-cell patch clamp from PNs in *Drosophila* AD models expressing Aβ40, Aβ42, or Aβ42Arc peptides in neural tissue. **Results** In fly pupae (2 days before eclosion), overexpression of Aβ42 or Aβ42Arc, but not Aβ40, led to a significant decrease of mEPSC frequency, while overexpression of Aβ40, Aβ42, or Aβ42Arc had no significant effect on mEPSC amplitude. In contrast, Pavlovian olfactory associative learning and lifespan assays showed that both short-term memory and lifespan were decreased in the *Drosophila* models expressing Aβ40, Aβ42, or Aβ42Arc. **Conclusion** Both electrophysiological and behavioral results showed an effect of Aβ peptide on cholinergic synaptic transmission and suggest a possible mechanism by which Aβ peptides cause cholinergic neuron degeneration and the consequent memory loss.

Keywords: Aβ peptide; projection neurons; Alzheimer's disease; neurotoxicity; electrophysiolgy; cholinergic synaptic transmission

1 Introduction

Alzheimer's disease (AD), the most prevalent form of dementia, is an age-related, slowly progressive and degenerative brain disease. The classical histopathological lesions in the brain of an individual with AD are extracellular amyloid plaques and intracellular neurofibrillary tangles. The amyloid plaques are composed of amyloid-β (Aβ) peptides, which are produced by proteolytic cleavage of the transmembrane receptor amyloid precursor protein[1]. There is growing evidence that Aβ is central to the pathogenesis of AD[2]. In most animal models, elevated levels of Aβ expression and accumulation of oligomeric Aβ may contribute to synaptic failure and cognitive deficits[3].

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Besides the detrimental effects of Aβ on cognitive function, the decline, disruption, or alteration of nicotinic cholinergic mechanisms has also been proposed to contribute to AD[4]. It has been shown that the most well-recognized neuronal loss in AD is in the cholinergic system[5]; the degeneration of cholinergic neurons of the basal forebrain is one of the earliest pathological features of AD[5]; and the decline of cortical cholinergic activity also correlates with the severity of AD symptoms and with the intellectual deterioration observed in life[5]. According to the "cholinergic hypothesis" of geriatric memory dysfunction, the deterioration of cognitive function associated with AD dementia in the elderly is attributable to a decline in basal forebrain cholinergic neurotransmission[8]. Furthermore, although it is well-known that Aβ is toxic to cholinergic neurons, more data are needed to explore the relationship between the toxic effects of Aβ and cholinergic synaptic transmission.

In *Drosophila*, the olfactory system is important for identifying food sources, avoiding predators, and recognizing mating partners[9]. Odor information received by the olfactory receptor neurons (ORNs) in the antennae and the maxillary palps is relayed to projection neurons (PNs) in the antennal lobes, where axons of ORNs expressing the same odorant receptors make synapses with the dendrites of corresponding PNs in the glomeruli. After processing in the antennal lobes, olfactory information is relayed by PNs to the mushroom bodies and the protocerebrum. Like other excitatory neurons in the insect central nervous system (CNS), most of these PNs are cholinergic[15].

Although Aβ aggregation and cholinergic neuron degeneration are pathological hallmarks of AD, the complicated relationship between cholinergic synaptic transmissions in a behaviorally relevant neural circuit and the neurotoxicity of Aβ peptide remains unclear. Therefore, this study combined genetics, electrophysiological and behavioral approaches to address this question in *Drosophila* AD models, in order to enhance our understanding of the mechanisms by which Aβ peptides modulate cholinergic synaptic transmission.

2 Materials and methods

2.1 Transgenic fly lines Three DNA fragments containing the human genomic sequences encoding Aβ40, Aβ42 and Aβ42Arc were kindly provided by Dr. D. C. Crowther (Cambridge University, Cambridge, UK), who subcloned these fragments into *Drosophila* strains. The effects of Aβ expression in the fly CNS were investigated using the UAS/Gal4 activation system. Canton-S stock was maintained in the laboratory as the wild-type control.

2.2 Isolated brain preparation Experiments were performed on fly pupae two days before eclosion. Since making patch clamp recordings from the Aβ42 and Aβ42Arc strains is extremely difficult, we chose neurons from a pupal stage for analysis. The brains were prepared as previously described[16,17]. In summary, the entire brain, including the optic lobes with attached ommatidia, was removed from the head in extracellular saline, containing (in mmol/L): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO_4, and 20.7 $NaHCO_3$, aerated by mixed 95% O_2 and 5% CO_2. The osmolarity was adjusted to 250 mOsm and the pH to 7.25. Then the dissected brain was mounted in a recording chamber. Papain (20 U/mL activated by 1 mmol/L *L*-cysteine) was added to the recording saline to soften the connective tissue sheath surrounding the brain. Pipettes were targeted to PNs in the dorsal neuron cluster in the antennal lobe with the anterior of the brain facing up.

2.3 Electrophysiological recordings from PNs in *Drosophila* brain Recordings were made using micropipettes (10–14 MΩ) filled with internal solution containing (in mmol/L): 102 K-gluconate, 0.086 $CaCl_2$, 17 NaCl, 1.7 $MgCl_2$, 8.5 HEPES, and 0.94 EGTA. The osmolarity was adjusted to 235 mOsm and the pH to 7.25. The holding potential was −70 mV. The external solution contained (in mmol/L): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO_4, and 20.7 $NaHCO_3$. The osmolarity was adjusted to 250 mOsm and the pH to 7.25. Cholinergic miniature excitatory postsynaptic currents (mEPSCs) were recorded using the same internal solution and standard external solution supplemented with tetrodotoxin (TTX) (1 μmol/L) and picrotoxin (10 μmol/L). Chemical products

used to prepare external and internal solutions were purchased from Sigma-Aldrich Co. (St. Louis, MO).

All electrophysiological signals were acquired with an EPC10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany), filtered at 5 kHz using a built-in filter, and digitized at 5 kHz. Data analysis was performed with pClamp10 Clampfit software (Molecular Devices, Germany). Cholinergic spontaneous post-synaptic currents (sPSCs) and mEPSCs were detected using MiniAnalysis (Synaptosoft, Decatur, GA).

2.4 Biocytin staining and confocal imaging Biocytin (Sigma-Aldrich) was loaded into the soma and terminals of identified PNs during the whole-cell recording configuration for at least 30 min, and the morphology of the recorded cell was visualized by *post hoc* staining after incubation with 1:200 streptavidin-Cy3 (Molecular Devices) as previously described[18]. To visualize glomerular boundaries and the neuropil, brains were incubated in 1:10 mouse monoclonal nc82 antibody (Developmental Studies Hybridoma Bank, Iowa city, Iowa) and a secondary incubation with 1:200 anti-mouse Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Optical slices through the antennal lobes were taken on a Zeiss LSM 710 confocal microscope with a 20× objective.

2.5 Learning and memory assays Short-term olfactory associative memory tests were performed at 25°C and 70% relative humidity in an olfactory T-maze. The T-maze (General Valve Corp., Fairfield, NJ) is a previously described *Drosophila* olfactory learning and testing apparatus[17] and the odorants and conditioning parameters were used as described by Yin *et al.*[18]. Approximately 100 flies in each group were trained by exposure to electroshock paired with one odor of either 1.5‰ 3-octanol (OCT, purity 99%; Sigma-Aldrich) or 1‰ 4-methylcyclohexanol (MCH, 98%; Sigma-Aldrich). The preference index (PI) was calculated as follows:

$$PI = (N_{CS-} - N_{CS+})/(N_{CS-} + N_{CS+}) \times 100\%,$$

where CS represents the condition stimulus, N_{CS-} is the number of flies approaching the CS- odor and N_{CS+} is the number of flies approaching the CS+ odor. The average of the two PIs from the reciprocal experiments was taken as one complete PI. In order to avoid any possibility of odor bias, the presentation sequence of the two odors was reversed in order to rule out non-associative effects.

2.6 Survival assays Survival assays were performed as previously described[21]. Briefly, food vials containing 100 flies of each genotype were kept at 25°C and 70% humidity. The vials were changed every 2–3 days, and the number of dead flies was counted at each change. At least four vials were prepared for each genotype. Experiments were repeated three times. Survival curves were analyzed using Kaplan-Meier plots and log-rank statistical analysis.

2.7 Statistical analysis Data are presented as mean ± SEM. Unless otherwise indicated, statistical significance was assessed with one-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni test for multiple comparisons. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

3 Results

3.1 Effects of Aβ on cholinergic sPSCs of PNs in *Drosophila* PNs are located in the dorsal antennal lobe glomeruli with main branches projecting to the mushroom body and lateral horn, which are important in olfactory information processing. PNs were chosen for analysis because they are both cholinergic and cholinoceptive, receiving cholinergic synaptic input from olfactory receptor neurons and potentially lateral excitatory input[22]. Each PN was initially identified by its specific electrical activity and subsequently confirmed by its stereotyped morphology after biocytin staining (Fig. 1A). In these experiments, the majority of action potential (AP)-dependent synaptic currents were blocked by the noncompetitive nicotinic acetylcholine receptor antagonist mecamylamine hydrochloride (MCA).

Spontaneous activity patterns varied from neuron to neuron (Fig. 1B, C). The mean sPSC frequency was 13.8 ± 1.21 Hz in the control group and 11.48 ± 1.05 Hz in the Aβ40 group, but was markedly reduced to 8.91 ± 0.81 Hz in the Aβ42 group and 8.79 ± 0.99 Hz in the Aβ42Arc group (Fig. 1D; $P < 0.05$; $n = 10–12$). While the sPSC amplitude was 24.20 ± 3.81 pA in the control group and 18.34 ± 1.38 pA in the Aβ40 group, it was reduced to 15.01 ± 1.67 pA in the Aβ42 group and 15.42 ± 1.77 pA in Aβ42Arc group

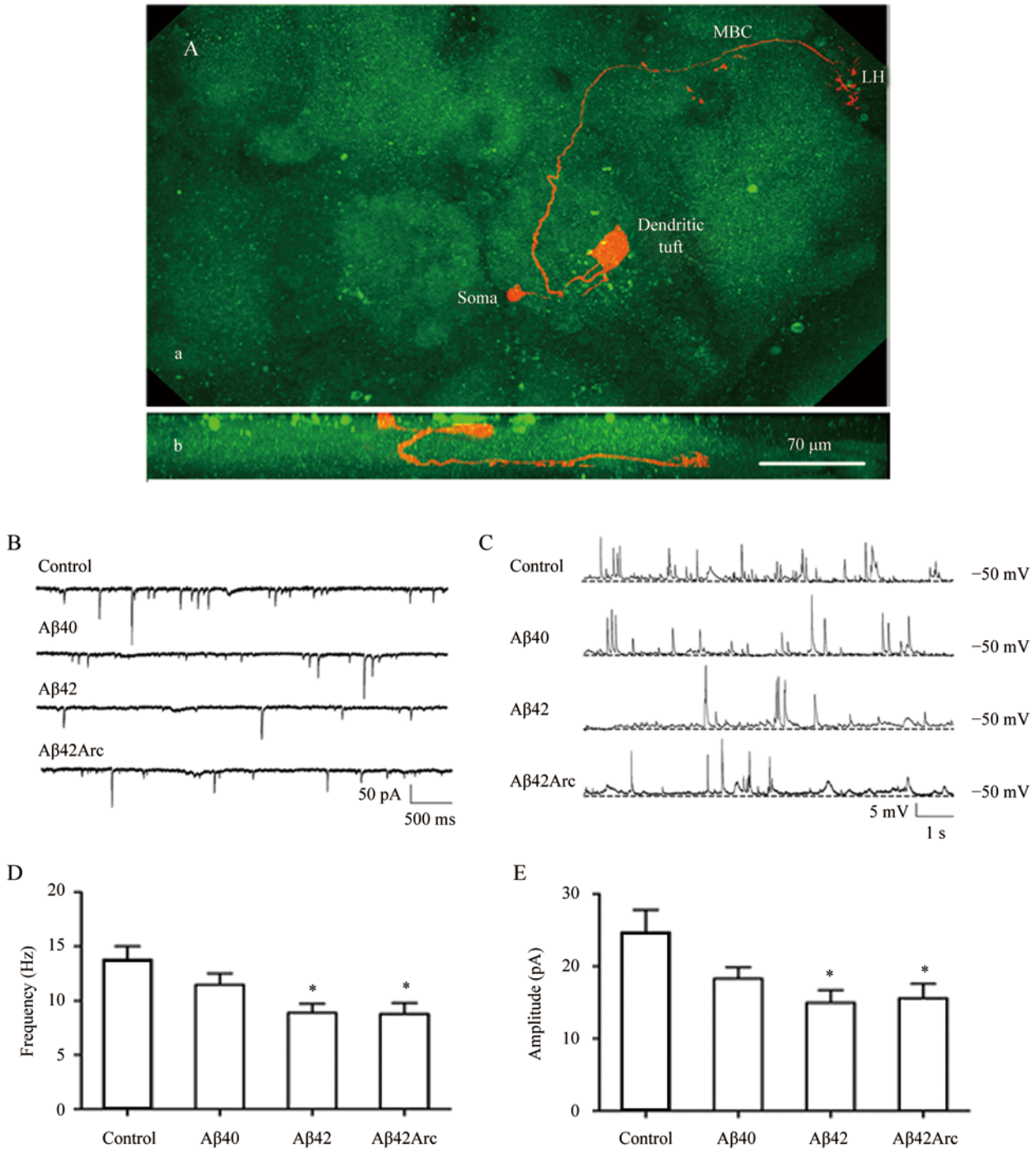

Fig. 1. A: Projection of a confocal stack showing the morphology of an antennal lobe projection neuron (PN, red) labeled with fluorescent-conjugated streptavidin. An antibody to *Drosophila* neuropil (nc82, green) defines the contours of the brain in the x-y plane (a) and in the z-level plane (b). MBC, mushroom body calyx; LH, lateral horn. Scale bar, 70 μm. 3D reconstruction of the PNs used 3D imaging software (BITPlan Imaris, Switzerland). B: Whole-cell voltage-clamp recordings of spontaneous post-synaptic currents (sPSCs) in PNs from Canton-S, Aβ40, Aβ42 and Aβ42Arc flies. C: Whole-cell current-clamp recordings of spontaneous activity in PNs from Canton-S, Aβ40, Aβ42 and Aβ42Arc flies. D and E: Mean sPSC frequency (D) and amplitude (E) (*P <0.05 *vs* Canton-S control; n = 10–12/group).

(Fig. 1E; P <0.05, n = 10–12). Therefore, Aβ42 and Aβ42Arc expression decreased the spontaneous cholinergic synaptic activity of PNs in *Drosophila* brain.

3.2 Effects of Aβ on cholinergic mEPSCs of PNs in *Drosophila* To explore the neurotoxicity of Aβ *in vivo*, we monitored sodium AP-independent mEPSCs in the antennal lobe PNs of whole brains isolated from fly pupae 2 days before eclosion. mEPSCs were recorded from Canton-S, Aβ40, Aβ42 and Aβ42Arc flies in the presence of TTX, a sodium channel blocker, and picrotoxin, a blocker of GABA receptors. These mEPSCs, which were totally blocked by 150 μmol/L MCA, were cholinergic

Fig. 2. Sodium action potential-independent synaptic currents mediated by nicotinic acetylcholine receptors. mEPSCs recorded from projection neurons were totally blocked by the noncompetitive nicotinic acetylcholine receptor antagonist mecamylamine (MCA) at 150 μmol/L. PTX, picrotoxin; TTX, tetrodotoxin.

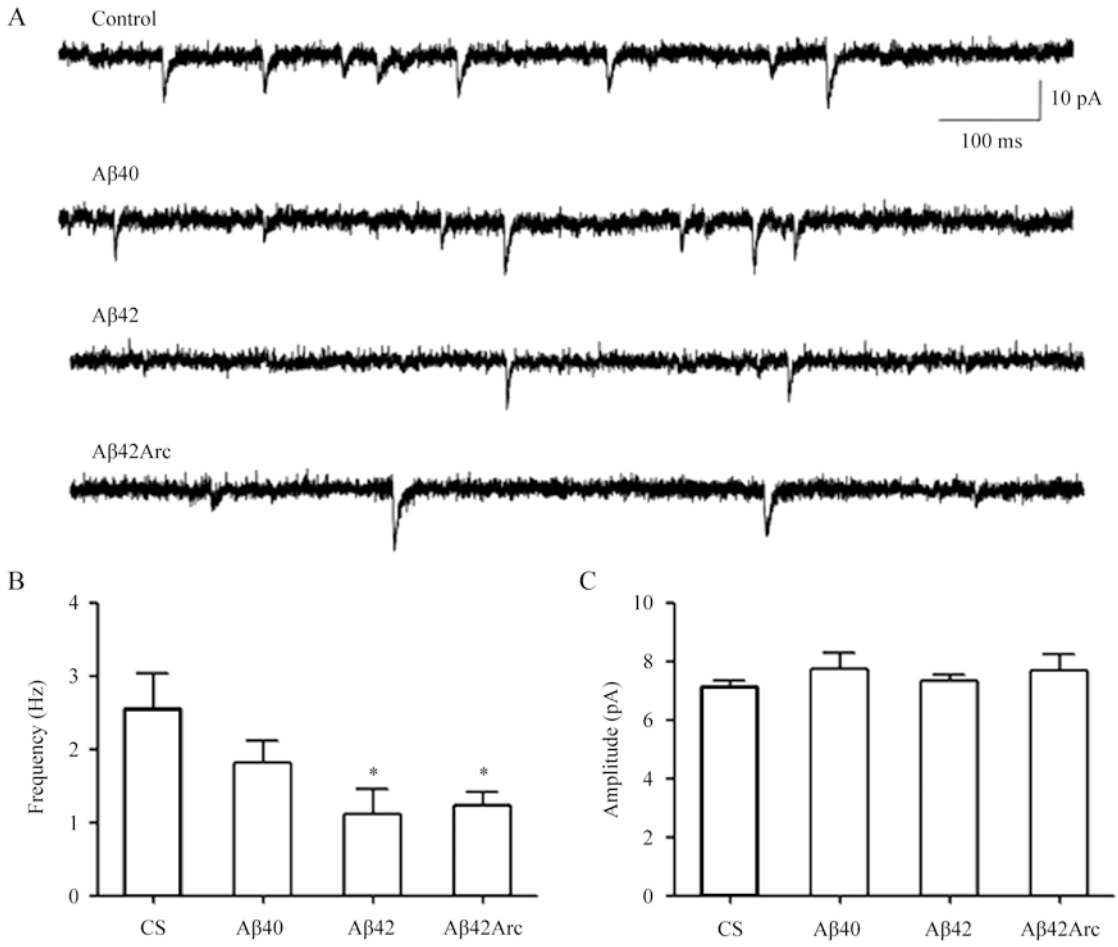

Fig. 3. The frequency, but not the amplitude, of cholinergic mEPSCs in projection neurons was affected by Aβ overexpression. A: Cholinergic mEPSCs recorded from single projection neurons after addition of TTX and PTX to the external solution. All synaptic currents were recorded at a holding potential of −70 mV. B: Quantification of mEPSC frequency in Canton-S (CS), Aβ40, Aβ42 and Aβ42Arc flies. The mEPSC frequency was reduced in Aβ42 and Aβ42Arc flies compared to that in Canton-S control flies (*P <0.05, n = 10–12). C: Quantification of mEPSC amplitude in Canton-S, Aβ40, Aβ42 and Aβ42Arc flies. There were no significant differences in mEPSC amplitude among the four groups (n = 10–12).

(Fig. 2). The mean mEPSC frequency was 2.56 ± 0.46 Hz in the control group and 1.82 ± 0.29 Hz in the Aβ40 group, but was reduced to 1.19 ± 0.33 Hz in the Aβ42 group and 1.22 ± 0.19 Hz in the Aβ42Arc group (Fig. 3B; P <0.05; n = 10–12), while the mEPSC amplitude of these four groups showed no significant difference (Fig. 3C). These data showed modulation of the mEPSC frequency of PNs by Aβ42 and Aβ42Arc, indicated their ability to regulate the mEPSC properties of neurons, and supported our hypothesis that Aβ peptides modulate the cholinergic input circuit, and thus, these Aβ peptides could potentially affect the formation of synaptic plasticity because of the close relationship between mEPSC and synaptic plasticity, and the relationship between cholinergic transmission and synaptic plasticity

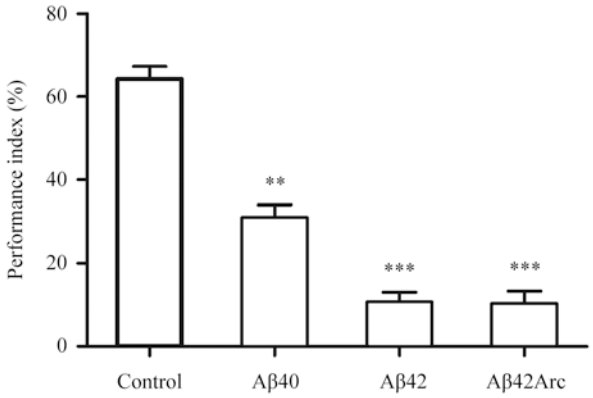

Fig. 4. Aβ induced immediate memory loss assayed by a Pavlovian olfactory associative learning paradigm. The learning ability of Canton-S, Aβ40, Aβ42 and Aβ42Arc flies is presented as mean ± SEM. The number of flies was 100/group, n = 3 trials (Tukey-Kramer; **P <0.01; ***P <0.001 compared to control).

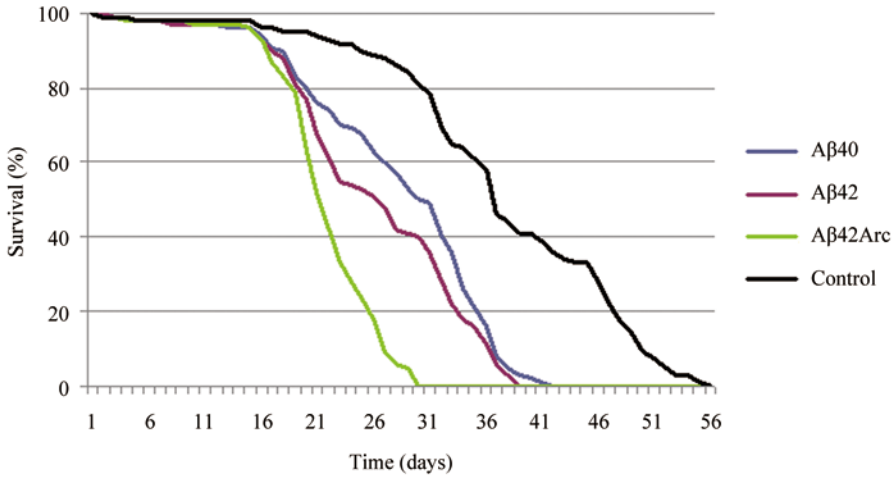

Fig. 5. Premature death caused by overexpression of Aβ in flies. The percentage survival of flies expressing Aβ40, Aβ42 and Aβ42Arc was plotted against age (days). The effect ranks as Aβ42Arc >Aβ42 >Aβ40 >Canton-S. Approximately 100 flies were analyzed for each genotype.

3.3 Short-term olfactory memory deficits induced by Aβ40, Aβ42 and Aβ42Arc The onset of memory deficits was measured in Canton-S, Aβ40, Aβ42 and Aβ42Arc flies (3 days old) by classical Pavlovian olfactory conditioning[19]. The PI of the olfactory learning and memory was calculated for the numbers of flies that chose the conditioned stimulus and flies that avoided the unconditioned stimulus (Fig. 4). According to this assay, the short-term memory of these genotypes ranked as follows: Canton-S >Aβ40 > Aβ42 >Aβ42Arc.

3.4 Shortened lifespan in Aβ40, Aβ42 and Aβ42Arc flies The average lifespan of Canton-S flies (65.9 days) was the longest, followed by Aβ40 (49.1 days) and Aβ42 (45.5 days). Aβ42Arc flies had the shortest lifespan (37.3 days). All these results demonstrated that the expression of Aβ led to a shortened lifespan, with the influence of the defect ranking Aβ42Arc >Aβ42 >Aβ40 >Canton-S (Fig. 5).

4 Discussion

AD manifests as a gradual decline of cognitive func-

tions such as learning and memory, which significantly correlates with synaptic loss[3,23-28]. Numerous results show that synaptic dysfunction occurs in the very early stages of many neurodegenerative diseases and precedes the accumulation of aberrant protein aggregates[24]. Despite numerous studies having documented brain amyloidosis in transgenic models of AD, data concerning changes to the cholinergic projection system in these animals are surprisingly scarce, especially data concerning Aβ overexpression in transgenic models. The overexpression of Aβ may inhibit acetylcholine release, which might, in turn account for the cognitive performance deficits often observed in these models. Before significant neurodegeneration and Aβ accumulation are evident[21,29,30], we used a combination of Aβ40, Aβ42, and Aβ42Arc transgenic fly models in the early stage with electrophysiological measurements of synaptic currents to determine the possible modulation of cholinergic synaptic transmission by Aβ. Acetylcholine is a major excitatory neurotransmitter in the fly CNS and the predominant form of fast excitatory transmission in embryonic *Drosophila* culture is mediated by nicotinic acetylcholine receptors[31]. Bungarotoxin-sensitive nicotinic acetylcholine receptors mediate fast excitatory synaptic transmissions in Kenyon cells in *Drosophila* and these receptors likely contribute to plasticity during olfactory associative learning[32]. In our study, mEPSCs recorded from PNs in *Drosophila* pupae were shown to be cholinergic as well (Fig. 2). We found that overexpression of Aβ42 and Aβ42Arc, but not Aβ40 led to a significant decrease of mEPSC frequency. In contrast, overexpression of Aβ40, Aβ42, and Aβ42Arc showed no significant mEPSC amplitude changes. The difference in mEPSC frequency suggests that the probability of release of acetylcholine from presynaptic terminals is different. Finally, Aβ42 and Aβ42Arc, but not Aβ40, play an important role in the depression of presynaptic cholinergic synaptic transmission, because of the close relationship between the frequency changes of mEPSCs and the activity of presynaptic ion channels.

AP-dependent regulation of transmission at central synapses plays a fundamental role in information processing in all animals. We recorded sPSCs in transgenic flies, and found that the frequency and amplitude differed among the groups (Fig. 1). Although we did not investigate whether the cholinergic synapses in *Drosophila* neurons exhibit synapse-specific changes in synaptic strength, like glutamatergic synapses in the vertebrate CNS, cholinergic synapses are potential sites of plasticity which may be important in regulating neuronal function in the fly CNS. Future studies will be necessary to determine whether the change in sPSC frequency reflects an alteration of presynaptic or postsynaptic excitability/inhibition, the probability of neurotransmitter release, the sensitivity of postsynaptic receptors, or some combination thereof.

Cholinergic innervation of the olfactory lobe has been implicated in memory formation and retrieval. Abundant reports show that overexpression of Aβ in AD patients has a variety effects on plasticity and memory. Consistent with electrophysiological analysis, Aβ42 and Aβ42Arc flies were particularly defective in short-term memory, the major clinical manifestation in patients at an early stage of AD. As Aβ40 flies showed defects in short-term memory at 3 days old, but not in cholinergic synaptic transmission 2 days before eclosion, there may be age-dependent defects and different neurotoxicity of specific Aβ peptides. While the molecular basis for this finding is not clear, the early decline of cognitive functions supports a primary role for synaptic dysfunction in these transgenic models. Lifespan analysis also showed that Aβ40, Aβ42 and Aβ42Arc flies are more likely to die prematurely than Canton-S flies. Such lethality resembles symptoms associated with human neurodegenerative disorders and has been used extensively to study neurodegeneration in flies. The decrease of mEPSC frequency correlated with behavioral defects found in our study implied that there might be a relationship between cholinergic synaptic transmission and neurotoxicity of Aβ peptide to memory[37,38].

In AD, prior to memory loss and histological changes, physiological dysfunction has already occurred. Our data showed that before behavioral changes were seen (at 3 days), recordings already showed depression of synaptic transmission (at 2 days before eclosion). The electrophysi-

ological and behavioral differences between the transgenic models (flies expressing Aβ40, Aβ42, or Aβ42Arc) were mainly the result of the differences in Aβ toxicity, which is a complex and multifaceted phenomenon that may be due to the assembly of multiple forms of Aβ. In healthier AD patients, Aβ40 peptide deposition is predominant, while in sporadic and most cases of familial AD, either the ratio of Aβ42 to Aβ40 is increased or the total concentration of Aβ42 is raised. Aβ42 aggregates more rapidly, therefore forming stable Aβ oligomers at an earlier time. Moreover, Aβ42 tends to form stable trimeric and/or tetrameric oligomers, whereas Aβ40 does not. Inherited missense mutations, such as human Aβ42 with the Arctic mutation (Aβ42Arc) that causes early-onset familial AD, strongly enhance oligomerization.

The cholinergic–cholinoceptive system plays an integral role in the vertebrate CNS. Cholinergic fibers are found in all cortical areas and layers, with their density differing from one area to another, and from one layer to another. Since the recording of central cholinergic synaptic currents has limitations in mammals, recent studies have provided insights into the process by intracellular recordings of cholinergic synaptic activity in *Drosophila* neurons that show evidence of Aβ expression and cholinergic synaptic modulation[16,32,49,50]. The preparations used in these studies are analogous to the "brain slice" and cell culture that have been widely used in investigations of the cellular mechanisms of synaptic transmission[16]. The transgenic *Drosophila* AD models and whole-brain recording techniques used here serve as an ideal platform to investigate the complex toxicity of Aβ *in situ*.

Using transgenic *Drosophila* AD models, we found that overexpression of Aβ42 and Aβ42Arc led to a significant decrease of cholinergic synaptic transmission in PNs of the antennal lobe and resulted in disrupted short-term memory and premature death. Surprisingly, the Aβ40 model showed significant behavioral changes only, and not depression of cholinergic synaptic transmission. This may be explained by the fact that different ages of flies were used for the electrophysiological and behavioral studies. All these results suggest that there might be a potential link between cholinergic synaptic transmission in a behaviorally relevant neural circuit and the neurotoxicity of specific Aβ peptides.

In summary, the present study demonstrated that Aβ induced a depression of excitatory cholinergic synaptic transmission and memory loss in *Drosophila*. These findings provide direct experimental methods for understanding the modulation of cholinergic synaptic neurons by Aβ. Further studies using methods such as gene expression or chemical intervention, will be necessary to explore the molecular and cellular basis of AD pathogenesis. In addition, a major challenge is to identify novel compounds that have optimal effects with respect to both the amyloid hypothesis and the cholinergic hypothesis of AD. Because fast excitatory transmission mediated by nicotinic acetylcholine receptors has also been reported in the mammalian hippocampus[51] and cortex[52], studies in this model system may also reveal genes that are important in regulating cholinergic transmission in mammals.

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