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·Original Article·

Intraneuronal accumulation of $A\beta_{42}$ induces age-dependent slowing of neuronal transmission in *Drosophila*

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ABSTRACT

Beta amyloid ($A\beta_{42}$)-induced dysfunction and loss of synapses are believed to be major underlying mechanisms for the progressive loss of learning and memory abilities in Alzheimer's disease (AD). The vast majority of investigations on AD-related synaptic impairment focus on synaptic plasticity, especially the decline of long-term potentiation of synaptic transmission caused by extracellular $A\beta_{42}$. Changes in other aspects of synaptic and neuronal functions are less studied or undiscovered. Here, we report that intraneuronal accumulation of $A\beta_{42}$ induced an age-dependent slowing of neuronal transmission along pathways involving multiple synapses.

Keywords: neuronal transmission; synaptic dysfunction; latency; Alzheimer's disease; intraneuronal beta amyloid

INTRODUCTION

Synaptic loss in the frontal and temporal cortex of patients with Alzheimer's disease (AD) correlates with the degree of dementia[1-3]. Compared to plaques, tangles, and neuron death, synaptic loss is most closely correlated with the degree of dementia in AD patients[2]. The degree of dementia in these patients and synaptic density in AD mice correlate very well with the brain levels of soluble $A\beta_{42}$ and its oligomers[4-8]. Moreover, at nanomolar concentrations, soluble $A\beta_{42}$ oligomers inhibit long-term potentiation of synaptic transmission and disrupt cognitive function[9-11]. These discoveries promoted the conversion of the "amyloid cascade hypothesis" into a "toxic Aβ oligomers" hypothesis[12, 13]. In fact, the toxic oligomeric Aβ species have received much attention in related studies and the molecular mechanisms underlying the dysfunction and loss of neurons and synapses, especially the impairment of long-term potentiation, are also widely studied by the extracellular application of oligomeric Aβ[14-16].

However, synaptic plasticity is just one of the properties of synapses, and Aβ accumulates not only extracellularly but also intraneuronally[17-20]. Other aspects of synaptic transmission by degenerating synapses and neurons, and the behavior of neuronal circuits in the AD brain might be affected by either extracellular or intracellular accumulation of Aβ. Indeed, alterations of basic synaptic transmission[21-24], synaptic fatigue[25], synaptic release probability[26, 27], and brain seizure activity[14] in AD model

animals have been reported.

Previously, using the Gal4-UAS (upstream-activating sequence) method, we expressed human $A\beta_{42}$ in neurons of the giant fiber (GF) system and a subgroup of neurons elsewhere in the adult *Drosophila* brain. $A\beta_{42}$ was found to accumulate intraneuronally and cause a range of age-dependent functional and ultrastructural changes in the synapse[25, 27], including depletion of the readily-releasable pool of synaptic vesicles and reduction of synaptic vesicle release probability associated with loss of synaptic vesicle protein, presynaptic active zone structure, and protein. Here, we set out to determine the effects of intraneuronal $A\beta_{42}$ accumulation on neuronal transmission along pathways involving multiple synapses.

MATERIAL AND METHODS

Genetics

Drosophila stocks were cultured on standard medium at 23–25°C. After pupation, the adult flies were cultured on standard medium and entrained to a 12 h/12 h light/dark cycle at 28.5°C. The Cantonese S and w^{1118} strains were used as controls. The UAS transgenic lines used for expressing wild-type human $A\beta_{1\text{-}40}$ ($A\beta_{40}$) and either one ($A\beta_{42}$×1) or two copies ($A\beta_{42}$×2) of human $A\beta_{1\text{-}42}$ were generous gifts from Dr. Crowther[28]. In these lines, the recombinant Aβ DNAs were fused to a secretion signal of the *Drosophila necrotic* gene. [Gal4]A307[29] was used to drive the expression of Aβ in the GF system.

Electrophysiology

Evoked excitatory junctional potentials (EJPs) in the GF system were recorded intracellularly as previously reported[25] with some modifications (Fig. 1). During each experiment, an adult female fly at a specific age was mounted ventral side down on a slide with Tackiwax (Boekel Scientific, Feasterville, PA) under a dissecting microscope. The recording involved a reference electrode in the abdomen, two stimulating electrodes inserted into the eyes, and two recording electrodes (~1.0 MΩ). One recording electrode was inserted into a dorsal longitudinal flight muscle cell (DLM) to record the DLM EJPs, and the other into the contralateral tergotrochanter muscle cell (TTM) to record the TTM EJPs. The muscle cell identity was determined by electrode placement and verified by the recorded latency. Electrical stimulation (0.1 Hz, 10 pulses) generated by a Master-8 (AMPI, Jerusalem, Israel) stimulator with an ISO-flex stimulus isolator (AMPI) was delivered to both eyes. The stimulus intensity was 5–20 V with a duration of 0.2 ms, about 150% of the threshold stimulus intensity at 0.5 Hz. Electrical signals were recorded and amplified by an Axon Clamp 900A (Molecular Devices, Sunnyvale, CA) and digitized at 20 kHz with a Digidata 1440A (Molecular Devices). Data were collected and analyzed with pClamp software (version 10.0; Molecular Devices). All electrodes were glass and filled with 3 mol/L KCl. The recording environment temperature was 22°C.

ELISA Quantification of Aβ

Sixty fly heads (30 female and 30 male) per strain were homogenized thoroughly in cold ELISA sample buffer supplemented with a protease inhibitor cocktail (Calbiochem, La Jolla, CA), incubated on ice for 3 h, and then centrifuged at 4 000 g for 5 min. The supernatants were collected for ELISA assay using an $A\beta_{42}$ or $A\beta_{40}$ Human ELISA kit (Invitrogen, Frederick, MD) according to the manufacturer's instructions.

Real-time PCR Quantification of Aβ mRNA

Thirty fly heads (15 female and 15 male) per strain were homogenized thoroughly with 500 μL Trizol (Cat #: 3101-100, PUFEI, Shanghai) and total RNAs were extracted according to the manufacturer's instructions. Real-time PCR was performed with the 7500 Real Time PCR system (ABI, Foster, CA). Oligo dT was used for reverse transcription of $A\beta_{40}$ and $A\beta_{42}$ mRNAs. Since the $A\beta_{40}$ and $A\beta_{42}$ transgenes are identical except that the $A\beta_{42}$ transgene contains 6 extra nucleotides at the 3' end, the same pair of primers was used for the amplification of $A\beta_{42}$ or $A\beta_{40}$ cDNAs. The forward primer was 5'-ATG GCG AGC AAA GTC TCG ATC C-3', and the reverse was 5'-CAC CAC GCC GCC CAC CAT CAA G-3'.

Data Analysis and Statistics

Data are presented as mean ± SD. Student *t*-test was used for analysis. The criterion for a significant difference was $P < 0.05$.

RESULTS

The *Drosophila* GF system consists of a pair of GF neurons

in the brain, each of which sends one giant axon to the thorax to activate different muscle fibers *via* two pathways, the di-synaptic TTM and the tri-synaptic DLM pathways[30,31] (Fig. 1). We expressed wild-type human $A\beta_{42}$ or $A\beta_{40}$, each containing a secretion signal peptide in the N-terminal, in neurons of the GF system. Three groups of Aβ flies were generated: $A\beta_{42}$×2, $A\beta_{42}$×1, and $A\beta_{40}$×1, expressing two or one copy of wild-type $A\beta_{1-42}$, or one copy of $A\beta_{1-40}$, respectively.

EJPs in the DLM and TTM cells evoked by brain stimulation were simultaneously recorded in controls and flies expressing $A\beta_{1-42}$ or $A\beta_{1-40}$ at a range of ages (3–36 days post-eclosion). At ages <3 weeks, the latencies of evoked DLM and TTM EJPs were about 1.2 ms and 0.8 ms in all control and Aβ-expressing flies (Fig. 2A). At ages >3 weeks, the latencies of evoked DLM and TTM EJPs in Ctrl1, Ctrl2 and $A\beta_{40}$×1 flies were slightly but not significantly increased, suggesting that aging does not cause a significant slowdown of neurotransmission in wild-type flies and in flies expressing $A\beta_{40}$. However, the latencies in both $A\beta_{42}$×2 and $A\beta_{42}$×1 flies were significantly increased (Fig. 2B1, B2), and the increase appeared to be dosage-dependent (Fig. 2C1, C2), demonstrating a slowing of neurotransmission in both the TTM and DLP pathways.

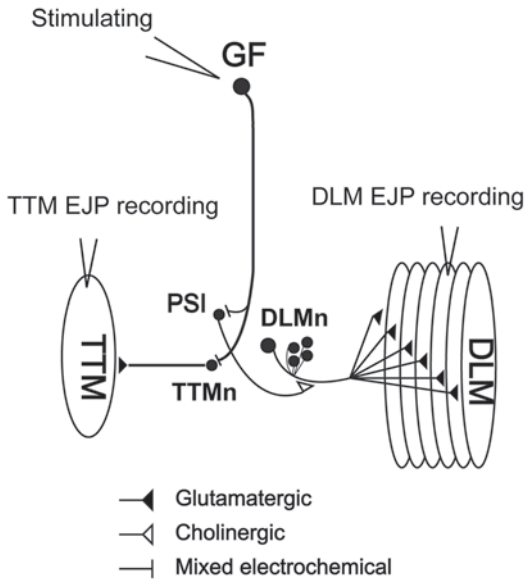

Fig. 1. Schematic diagram of the giant fiber (GF) system in adult *Drosophila.* The GF system contains a pair of GF neurons in the brain, each sending a giant axon to the thorax to activate the single ipsilateral TTM motor neuron *via* mixed electrochemical synapses, which in turn innervates the TTM muscle cell *via* a chemical synapse (the di-synaptic TTM pathway). The GF axon also activates the ipsilateral peripherally-synapsing interneuron (PSI) *via* mixed electrochemical synapses, which in turn innervates five contralateral DLM motor neurons, finally activating the DLM muscle cells *via* chemical synapses (the tri-synaptic DLM pathway). For clarity, only the GF system on one side is presented.

Moreover, in some $A\beta_{42}$×2 flies >3 weeks old, neither DLM nor TTM EJPs were evoked by brain stimulation (indicated by the dots above the dashed lines in Fig. 2B1, B2), demonstrating a loss of neurotransmission. Failure of neurotransmission in the TTM pathway (Fig. 2B2) was more frequent than in the DLM pathway (Fig. 2B1), suggesting that the TTM pathway is more susceptible to the intraneuronal accumulation of $A\beta_{42}$, although the TTM pathway is relatively shorter and contains fewer synapses.

To explain the differential impairment of neurotransmission in $A\beta_{42}$×2, $A\beta_{42}$×1, and $A\beta_{40}$×1 flies, we measured the brain levels of $A\beta_{40}$ and $A\beta_{42}$ in those flies by ELISA assay. The $A\beta_{42}$×2 flies accumulated much more Aβ than $A\beta_{42}$×1 and $A\beta_{40}$×1 flies, and $A\beta_{42}$×1 flies accumulated slightly but significantly more Aβ than $A\beta_{40}$×1 flies (Fig. 3A). We also quantified the relative mRNA level of Aβ in the three strains by real-time PCR and found that $A\beta_{42}$×2 flies had a significantly higher level of Aβ mRNA than $A\beta_{40}$×1 and $A\beta_{42}$×1 flies, with no significant difference between $A\beta_{40}$×1 and $A\beta_{42}$×1 flies (Fig. 3B).

Aβ mRNA and protein levels were positively correlated, but the correlation was not linear. $A\beta_{42}$×1 flies had a slightly but not significantly higher level of mRNA than $A\beta_{40}$×1 flies and accumulated 38% more Aβ (Fig. 3A, B), consistent with the finding that neurons in AD brains accumulate more $A\beta_{42}$ than $A\beta_{40}$[32]. This could be due to the fact that $A\beta_{42}$ is more hydrophobic, aggregates more easily, and has a higher affinity for the cell membrane, thereby readily accumulating in neurons. $A\beta_{42}$×2 flies had ~70% more mRNA than $A\beta_{42}$×1 flies, but accumulated ~180% more Aβ (Fig. 3). Currently, the exact reason for this phenomenon is not known.

DISCUSSION

During the aging process in $A\beta_{42}$-expressing flies, in which $A\beta_{42}$ accumulates intraneuronally in the GF system[25], we recorded an age-dependent increase in the latencies of

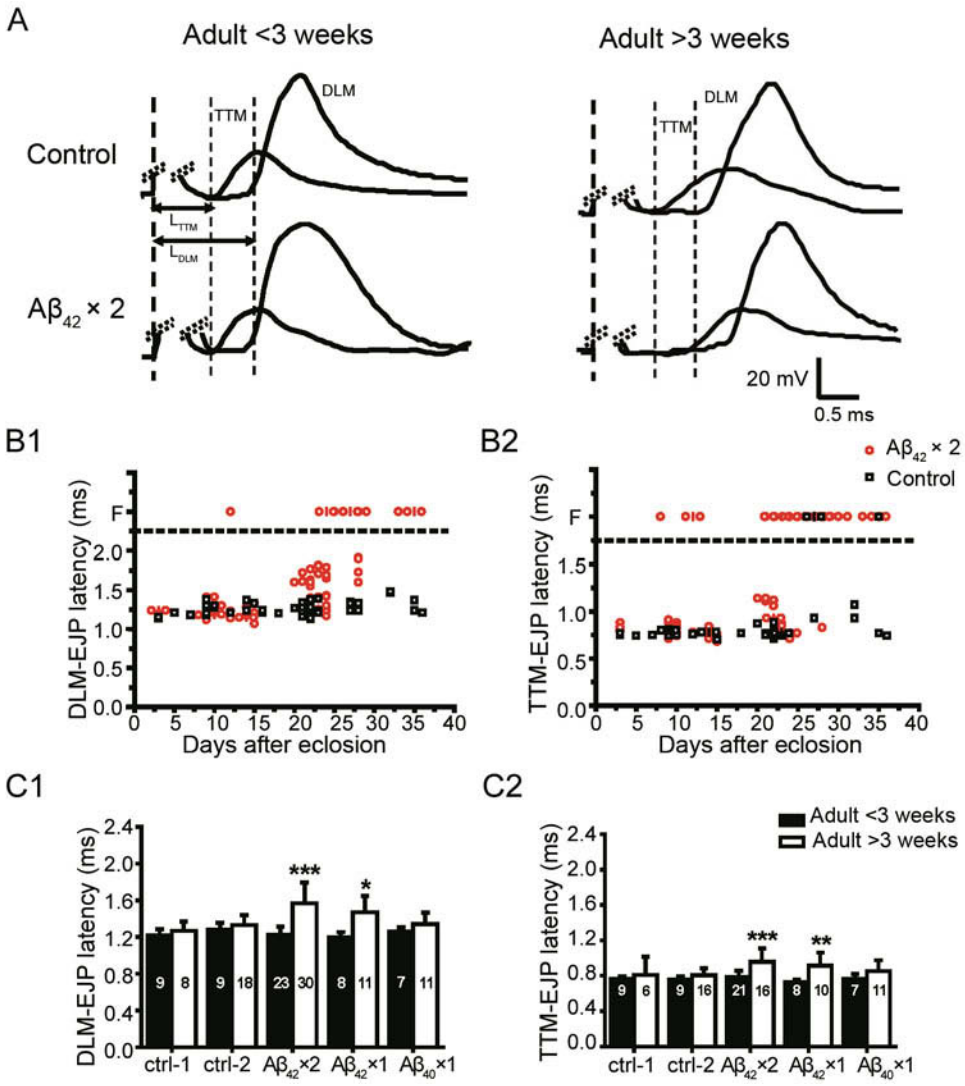

Fig. 2. Age-dependent slowing of neurotransmission along TTM and DLM pathways in $A\beta_{42}$-expressing flies. A: Representative brain stimulation-evoked TTM and DLM EJPs recorded simultaneously from control (upper) and Aβ42×2 flies (lower) at ages <3 weeks (left) and >3 weeks (right). Dashed vertical line 1 indicates the stimulus onset; lines 2 and 3 indicate the onsets of TTM and DLM EJPs respectively. For clarity, the stimulus artifacts were truncated. B: Dot plots of the latencies of DLM (B1) and TTM EJPs (B2) from control and $A\beta_{42}$×2 flies against age. Flies that displayed no DLM or TTM EJP response are plotted above the horizontal dashed "F" line. C: Based on age, the latencies of TTM and DLM EJPs from all five groups were divided into two groups and quantified. The number in each bar represents the number for each data point, Student's *t*-test. **P* <0.05, ***P* <0.01, ****P* <0.001.

DLM and TTM EJPs. The latencies of DLM and TTM EJPs, evoked by brain stimulation, reflect the time required for action potential initiation and propagation, and synaptic transmission along the DLM and TTM pathways. Thus, the increase in the latencies of DLM and TTM EJPs in $A\beta_{42}$-expressing flies demonstrates an age-dependent slowdown of neurotransmission along the two pathways. It is known that the timing of neurotransmission is critical for the processing and storage of information[33, 34]. A slowing of neurotransmission in neuronal pathways may contribute to cognitive impairment. Thus, besides impairment of synaptic transmission and synaptic plasticity, and imbalance of excitation and inhibition in the neuronal network[35], slowing of neurotransmission may also contribute to the cognitive deficits in AD.

Previously, we reported that intraneuronal Aβ

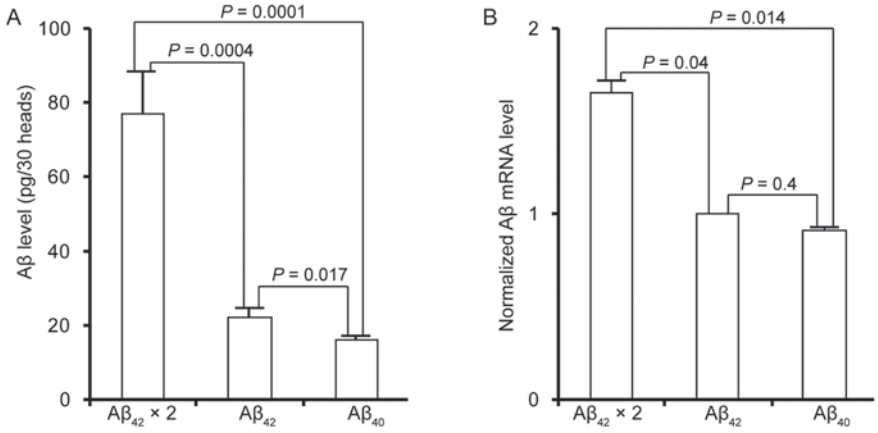

Fig. 3. Quantification of Aβ mRNA and protein levels in Aβ-expressing flies. ELISA quantification of Aβ protein (A) and relative quantification of Aβ mRNA by real-time PCR (B) in $A\beta_{42}$×2, $A\beta_{42}$×1 and $A\beta_{40}$×1 flies. *n* = 3 or 4 for each data point, Student's *t*-test.

accumulation causes age-dependent depletion of the readily-releasable pool of synaptic vesicles[27] and reduction of synaptic vesicle release probability, in association with loss of synaptic vesicle proteins—synapsin, presynaptic active zone voltage-gated calcium channels (VGCCs), and Bruchpilat (Brp) protein[27]. Synapsin regulates the number of synaptic vesicles available for release *via* exocytosis[36]; VGCCs control the release of synaptic vesicles by mediating calcium influx into the presynaptic terminal[37]; and Brp is a homolog of mammalian ELKS/CAST and a determinant of the structure and function of the presynaptic active zone where synaptic vesicles are released[38, 39]. The above presynaptic cellular and molecular changes induced by intraneuronal Aβ accumulation could cause an age-dependent slowdown and reduction of synaptic vesicle release in the synapses of the DLM and TTM pathways, and at least partially contribute to the slowing of neurotransmission along the two pathways.

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