



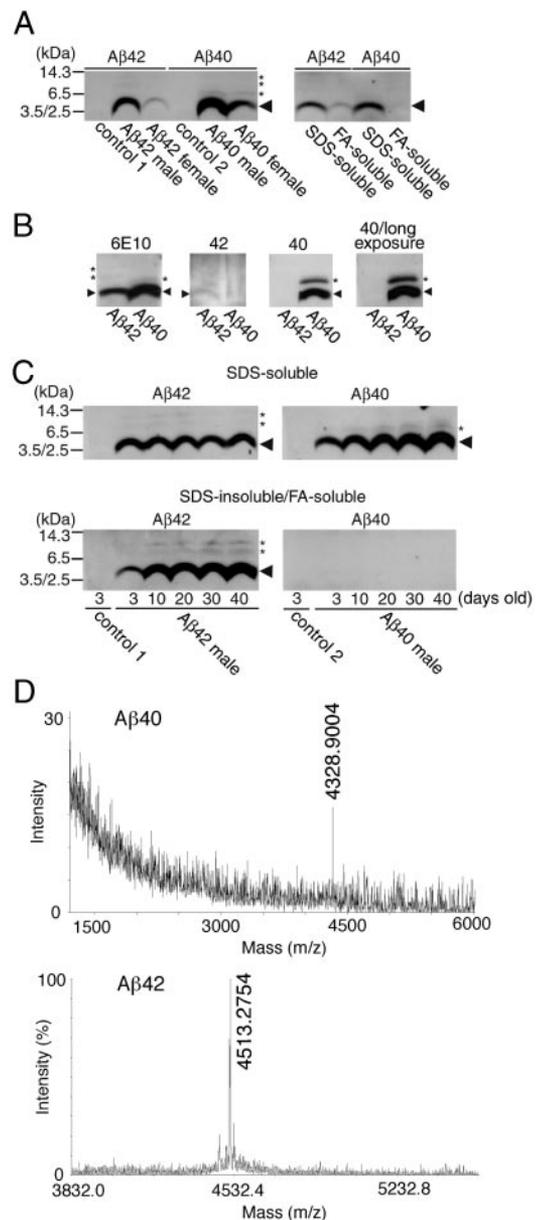
**Climbing Assay.** Twenty flies were placed in a plastic vial and gently tapped to the bottom. The number of flies at the top of the vial was counted after 18 s of climbing under red light (Kodak, GBX-2, Safelight Filter). The data shown represent results from a cohort of flies tested serially for 40–60 days. The experiment was repeated more than three times.

**Survival Assay.** Twenty to 30 flies were placed in a food vial. Each vial was kept on its side at 25°C, 70% humidity, under a 12-h light–dark cycle. Food vials were changed every 2–3 days, and the dead flies were counted at that time. At least 100 flies were prepared for each genotype, and the experiments were carried out more than three times.

**Anatomical Study.** To detect neurodegeneration, fixed and permeabilized fly brains were stained with NBD C6-ceramide, followed by counterstaining with propidium iodide (Molecular Probes) (28). Samples were cleared by incubation in FocusClear solution (PacGen, Vancouver) and viewed with a Zeiss LSM 510 confocal microscope with a  $\times 40$  C-Apochromat water immersion objective lens. To detect amyloid deposits, fixed and permeabilized brains were treated with 10% formic acid (Acros Organics, Fairlawn, NJ), followed by immunostaining with a mouse monoclonal anti-A $\beta$  antibody (Chemicon). ThioflavinS staining was performed following Fay *et al.* (29). Transmission electron microscope analysis was performed as described (30).

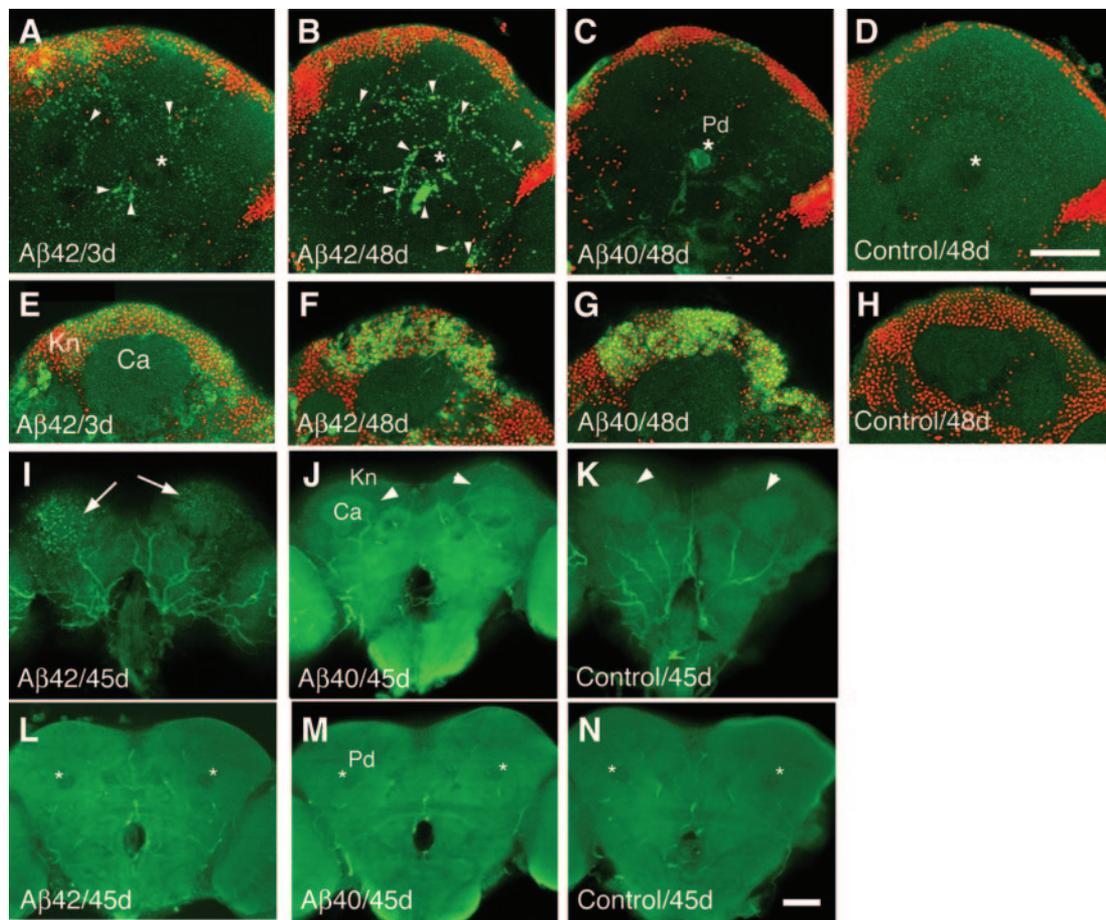
## Results

**Expression and Accumulation of A $\beta$ 40 and A $\beta$ 42 in Transgenic Fly Brains.** Each A $\beta$ 40 or A $\beta$ 42 peptide was fused to the rat pre-proenkephalin signal peptide at the N terminus to ensure secretion of A $\beta$  peptides once expressed (24). The A $\beta$  fusion constructs have been shown to produce secreted A $\beta$  peptides when expressed in human embryonic kidney cells (24) or in *Drosophila* S2 cells (16). The A $\beta$  peptides were targeted to express in all neurons in *Drosophila* by using the GAL4-UAS system (23) (driven by the pan-neuronal *elav-GAL4<sup>c155</sup>* driver; see *Methods*). Several lines of evidence suggested that A $\beta$ 40 or A $\beta$ 42 was produced appropriately in the fly brain. First, an A $\beta$  signal of the correct size of 4 kDa was readily detected in Western blots of flies that express either A $\beta$ 40 or A $\beta$ 42 but not in controls (Fig. 1*A Left*, arrowhead). Putative oligomeric forms of A $\beta$  peptides were also observed, including a 6-kDa band in A $\beta$ 40 flies (as better shown in Fig. 1*B*, asterisk), and 8- and 12-kDa bands in A $\beta$ 42 flies (as better shown in Fig. 1*C*, asterisks). Second, the A $\beta$  peptides were correctly cleaved from the fused signal peptide, as indicated by the precise molecular weight of A $\beta$ 40 and A $\beta$ 42 measured by mass spectrometry (Fig. 1*D*;  $M_r = 4328.9004$  for A $\beta$ 40 and 4513.2754 Da for A $\beta$ 42). Third, the intactness of the C-terminal end of both A $\beta$ 40 and A $\beta$ 42 was further confirmed by Western blotting with each C-terminal end-specific antibody (Fig. 1*B*). Finally, the age-dependent accumulation of A $\beta$  peptides showed biophysical features similar to previous characterization (31–33), i.e., A $\beta$ 40 was accumulated in the SDS-soluble fraction (Fig. 1*C Upper Right*), whereas A $\beta$ 42 accumulated in the SDS-insoluble/FA-soluble fraction (Fig. 1*C Lower Left*). From Western blot analysis of a 3-day-old head (Fig. 1*A Left*), we estimated that the expression level of peptides ( $R =$  signal intensity compared to A $\beta$ 42 males,  $n = 3$ ) was significantly higher for A $\beta$ 40 ( $R = 3.51 \pm 0.53$  for males and  $R = 1.42 \pm 0.40$  for females) than for A $\beta$ 42 ( $R = 1.00$  for males and  $R = 0.19 \pm 0.04$  for females), even after taking into account the insoluble fraction (Fig. 1*A Right*, arrowhead). The higher expression level of A $\beta$  peptides in male than in female flies can be partly explained by the effect of gene dosage compensation, because the GAL4 promoter is located on the X chromosome.



**Fig. 1.** Expression and accumulation profiling of A $\beta$  in transgenic fly heads. (A) Expression levels of A $\beta$  peptides in SDS-soluble (Left) and FA fraction (Right) at 3 days old. (B) Confirmation of the intactness of A $\beta$ 40 or A $\beta$ 42. 6E10 recognizes the common part of A $\beta$ 40 and A $\beta$ 42, whereas the 40 or 42 antibody is specific to each C terminus. (C) Age-dependent accumulation of A $\beta$  peptides in SDS-soluble (Upper) and FA fraction (Lower). Arrowheads, monomeric A $\beta$ ; asterisks, putative oligomeric forms. (D) Mass spectrometric analysis of A $\beta$  peptide from A $\beta$ 40 (Upper) or A $\beta$ 42 transgenic fly heads (Lower).

**Formation of Amyloid Deposits in A $\beta$ 42 but Not in A $\beta$ 40 Flies.** To determine whether expressed and accumulated A $\beta$  peptides form A $\beta$  deposits in the fly brain, we performed whole-mount immunohistochemical staining. In the neuropil region, 48-day-old A $\beta$ 42 fly brains showed the presence of abundant amyloid deposits (Fig. 2*B*, arrowheads), and both the number and size of the deposits were increased during aging (comparing Fig. 2*A* and *B*, arrowheads). In contrast, such clear deposits were not observed in A $\beta$ 40 or control brains (Fig. 2*C* and *D*). Importantly, the staining signal observed in A $\beta$ 40 brain (Fig. 2*C*, asterisk) is not an A $\beta$  deposit but the expression of A $\beta$ 40 in peduncle structure, which is the axon bundle of mushroom body neurons



**Fig. 2.** Detection of A $\beta$  deposits in fly brain. (A–H) Whole-mount A $\beta$  immunostaining (green) and nuclear staining (red) in the neuropil region (A–D) and Kenyon cell layer (E–H). Arrowheads, deposited A $\beta$ 42 (A and B); asterisks, the peduncle structure, an axon bundle of Kenyon cells. (I–N) ThioflavinS staining in the Kenyon cell (I–K) and neuropil regions (L–N). ThioflavinS-positive deposits were detected in A $\beta$ 42 flies (I, arrows) but not in A $\beta$ 40 or control (J and K, arrowheads). The fiber structures seen in I–K are tracheas. Pd, peduncle; Kn, Kenyon cell layer; Ca, calyx; the dendritic structure of Kenyon cells. [Bar (D, H, and N) = 50  $\mu$ m.]

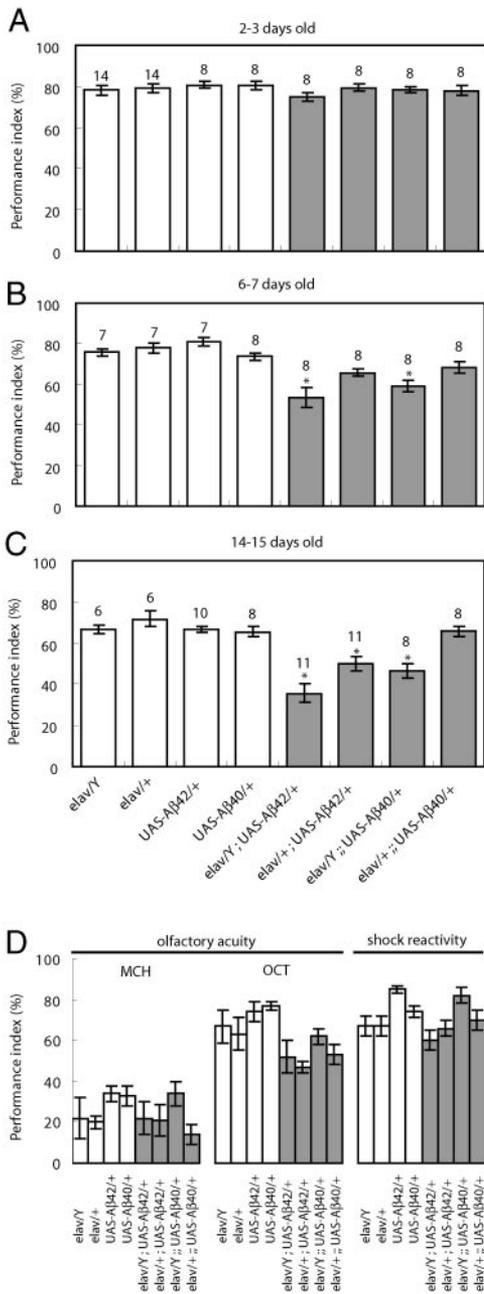
(Kenyon cells). In the Kenyon cell body region, strong A $\beta$  staining was observed in both A $\beta$ 40 and A $\beta$ 42 brains (Fig. 2 E–G) but not in control (Fig. 2H), confirming the specificity of the antibody.

We also performed thioflavinS staining to label the A $\beta$  deposits containing amyloid fibril structures. The thioflavinS-positive deposits can be observed in the Kenyon cell body region of A $\beta$ 42 brains (Fig. 2I, arrows) but not in A $\beta$ 40 or control brains (Fig. 2J and K, arrowheads). In contrast, no thioflavinS staining was detected in the neuropil region (Fig. 2L–N) even in A $\beta$ 42 fly brains, which had abundant immunopositive A $\beta$  deposits as shown above (comparing Fig. 2B and L). We further analyzed the A $\beta$ 42 brains in both the Kenyon cell body and the neuropil regions by transmission electron microscopy; however, there was no evidence of clear amyloid fibril structure in both regions. These results suggest that observed A $\beta$ 42 deposits in our fly brain are mainly diffused A $\beta$  deposits without clear amyloid fibril core structures, although some of the deposits were stained by thioflavinS. In summary, both A $\beta$ 40 and -42 peptides accumulated during aging (see Fig. 1C); however, only A $\beta$ 42 peptides could form diffused amyloid deposits in the fly brains.

**Age-Dependent Olfactory Learning Defects Induced by A $\beta$ 40 and A $\beta$ 42.** Learning and memory of these flies were tested by using a Pavlovian olfactory learning assay (26). For 2- to 3-day-old adult flies, no significant defect was observed in either A $\beta$ 40 or A $\beta$ 42 flies (Fig. 3A). Both A $\beta$ 40 and A $\beta$ 42 flies began to show

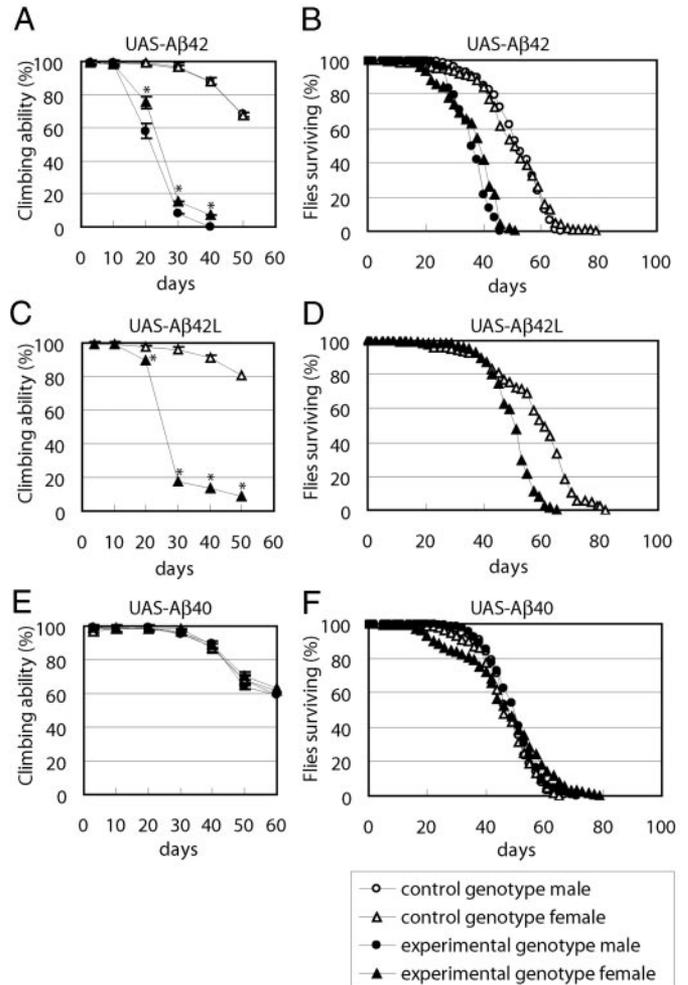
a subtle but statistically significant learning defect at 6–7 days old (Fig. 3B, asterisks). This decline became more obvious for 14- to 15-day-old flies (Fig. 3C, asterisks). The defect was greater in male flies than in females, consistent with higher expression of peptides in male flies (see Fig. 1A and text). Controls did not show the sex difference at any time point (Fig. 3A–C, compare *elav/Y* and *elav/+*). For A $\beta$ 40 flies, learning defects were observed only in males but not in females (Fig. 3A–C), suggesting a much higher level of A $\beta$ 40 than A $\beta$ 42 is required to affect learning ability (see Fig. 1A and text). We also examined odor avoidance and electric-shock reactivity, two sensorimotor activities necessary for performing the learning task. There was no significant difference among all groups for 14- to 15-day-old flies for shock reactivity and avoidance of the odor methylcyclohexanol (Fig. 3D). Avoidance of octanol is slightly lower in flies expressing either A $\beta$ 40 or A $\beta$ 42 as compared to the *UAS-A $\beta$ 42/+* and *UAS-A $\beta$ 40/+* controls but not significant compared to the *elav* controls (Fig. 3D). This slight difference should not contribute to observed learning defects, because A $\beta$ 40 female flies showed a normal learning score (Fig. 3A–C, *elav/+;UAS-A $\beta$ 40/+*). That learning was normal in A $\beta$ 40 female flies also suggests that progressive learning defects are a result of A $\beta$  toxicity rather than of genetic background or stress imposed by the expression of peptides.

**Climbing Disability and Shortened Life Span in A $\beta$ 42 but Not in A $\beta$ 40 Flies.** A $\beta$ 42 flies started to show locomotor dysfunction after 3 weeks of age. The climbing ability of flies in response to light



**Fig. 3.** Progressive loss of learning ability in  $A\beta$  flies assayed by a Pavlovian olfactory associative learning paradigm. (A–C) Learning abilities at 2–3 (A), 6–7 (B), and 14–15 days old (C) are presented in mean  $\pm$  SEM. The numbers of experiments are indicated on top of the bars. Asterisks show statistical difference from controls [ $\alpha < 0.05$ , Tukey–Kramer honestly significant difference (HSD)]. (D) No statistical difference in olfactory acuity and shock reactivity between experimental genotype and appropriate control genotypes at 14–15 days old ( $n = 8$ ; except  $n = 6$  for octanol olfactory acuity for *elav/Y;UAS-Aβ42/+* and shock reactivity for *UAS-Aβ40/+*) at the level of  $\alpha = 0.05$  (Tukey–Kramer HSD).

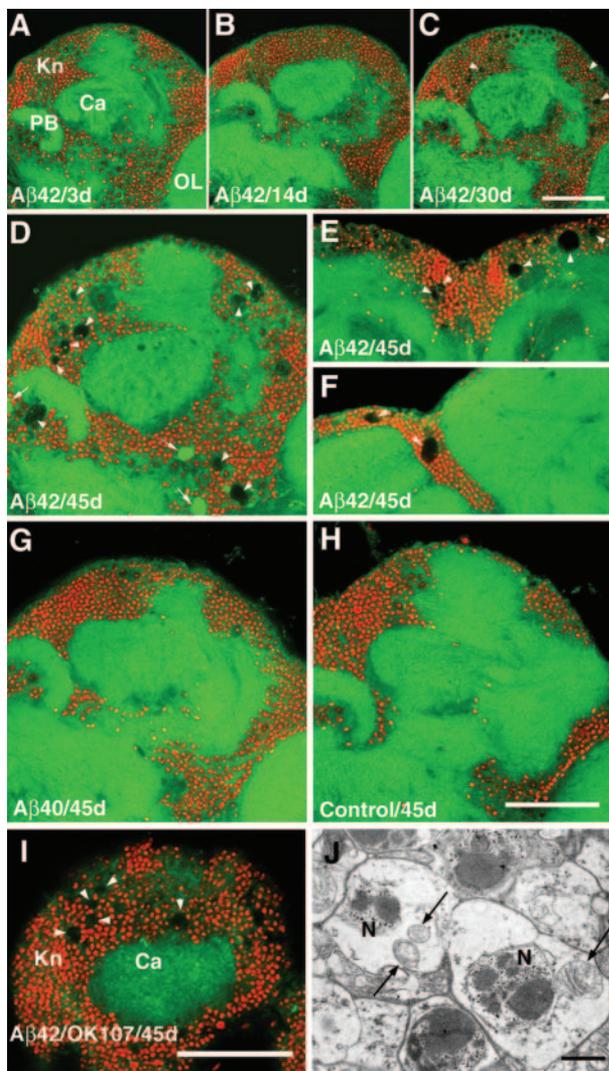
tapping (34) began to decline significantly after 20 days in  $A\beta42$  flies but not in  $A\beta40$  flies (Figs. 4 A and E). The presence of alterations in motor activity prevents us from examining learning ability after 3 weeks. Even older  $A\beta42$  flies stayed at the bottom of the vial and could not climb up the wall. Accompanying this locomotor defect, the life span of  $A\beta42$  flies was also much shorter, whereas  $A\beta40$  flies were not affected (Figs. 4 B and F). To confirm the effect of  $A\beta42$ , we examined another indepen-



**Fig. 4.** Progressive climbing disability and shortened life span in  $A\beta42$  flies. (A, C, and E) Climbing ability in  $A\beta42$  flies (A and C, asterisks,  $P < 0.001$ , Student's  $t$  test) and  $A\beta40$  flies (E). The SDs of 10 trials are within the symbols. (B, D, and F) Survival rate of  $A\beta42$  flies (B and D) and  $A\beta40$  flies (F).

ently isolated  $A\beta42$  line, which has a lower level of  $A\beta42$  expression (*UAS-Aβ42L*). Similar results were obtained regarding both climbing ability and life span (Fig. 4 C and D). We could examine only females of this line, because both *UAS-Aβ42L* and *elav-GAL4<sup>ci155</sup>* transgenes are located on the X chromosome.

**Late-Onset Progressive Neurodegeneration Caused by  $A\beta42$  but Not by  $A\beta40$ .** Anatomical analysis by confocal microscope revealed extensive neurodegeneration in aged  $A\beta42$  but not in  $A\beta40$  flies. In 45-day-old  $A\beta42$  flies, we observed severe neuronal loss, as indicated by the number of vacuoles in the Kenyon cell layer (Fig. 5D, arrowheads), a brain region crucial for olfactory learning (35–37). Degeneration was also seen in other brain regions (Figs. 5 E and F). In contrast, age-matched  $A\beta40$  or control flies did not show obvious cell loss (Figs. 5 G and H). To eliminate the possibility that observed neurodegeneration is a nonspecific effect due to fly death, we analyzed 55-day-old  $A\beta40$  or control flies. We did not see much degeneration in either group of brains (see Table 1, which is published as supporting information on the PNAS web site). To determine the time of onset of degeneration, we examined 3-, 14-, and 30-day-old  $A\beta42$  fly brains. There was no detectable abnormality in 3- and 14- day-olds (Figs. 5 A and B), whereas a small amount of cell loss started to appear in 30-day-old brains (Fig. 5C, arrowheads; see also Table 1). The



**Fig. 5.** Late-onset progressive neurodegeneration in  $A\beta_{42}$  brains. (A–H) Progressive neuronal loss occurred in  $A\beta_{42}$  (A–F, arrowheads) but not in  $A\beta_{40}$  or control brains (G and H). (A–D, G, and H) The Kenyon cell region. (E) Medial brain. (F) Lateral brain. Green, neuropil structure; red, nuclei. Arrows in D indicate the aggregates, presumably amyloid deposits. Kn, Kenyon cell layer; Ca, calyx; PB, protocerebral bridge; OL, optic lobe. [Bars (C and H) = 50  $\mu\text{m}$ .] (I) Neuronal loss induced by different Gal4 line, OK107. (Bar = 50  $\mu\text{m}$ .) (J) Ultrastructural analysis of degenerating neurons with digested cytoplasm (electron-lucent) and swollen mitochondria (arrows). N, nucleus. (Bar = 1  $\mu\text{m}$ .)

average number and area of vacuoles are summarized in Table 1. Similar results were also observed when using a different Gal4 driver, OK107, which drives peptide expression preferentially in the mushroom body structure (Fig. 5J, arrowheads; data not shown). Cell death in the Kenyon cell layer of  $A\beta_{42}$  flies was analyzed in the ultrastructural level. The degenerating neurons were readily identified, and vacuoles were detected as cell loss. The majority of dying neurons showed the typical features of necrotic-type cell death: digested cytoplasm (electron-lucent) with swollen mitochondria (Fig. 5J, arrows), whereas nuclei were relatively intact (Fig. 5J, indicated by N). Besides  $A\beta$  depositions, another characteristic lesion in AD patient brains is intracellularly formed protein aggregates called NFTs. The major component of NFTs is hyperphosphorylated  $\tau$  protein, which is assembled into paired helical filament (PHF) structure (38–40). The pathological interaction between  $A\beta$  depositions and NFT formation remains to be elucidated, because none of the AD

mouse models carrying abundant amyloid deposits developed NFTs (6, 7, 9–12, 14, 15). Therefore, we were motivated to determine whether accumulation of  $A\beta_{42}$  leads to the formation of NFT and/or PHF structure with fly endogenous  $\tau$  protein (41). PHF  $\tau$  was not detected by either immunoblotting or electron microscopy in  $A\beta_{42}$  fly head tissues.

## Discussion

In this study, we have established accumulation of either  $A\beta_{40}$  or  $A\beta_{42}$  peptides in the *Drosophila* brain induces progressive learning defects, but only  $A\beta_{42}$  is capable of causing the formation of diffused  $A\beta$  deposits, locomotor dysfunction, neurodegeneration, and premature death. It is remarkable to note that in an organism with a life span of 2–3 months, accumulation of  $A\beta_{42}$  induces the sequential progression of pathological symptoms resembling those in mouse AD models (6–15) and AD patients (1, 42). Intriguingly, the onset of learning defects by  $A\beta_{42}$  occurs much earlier than that of degeneration in the flies, similar to that observed in mouse AD models and AD patients (7, 8, 43–46). Furthermore, that both  $A\beta_{40}$  and  $A\beta_{42}$  affect learning but only  $A\beta_{42}$  causes degeneration leads to the speculation that neuronal dysfunction and neurodegeneration may be mediated by different mechanisms.

We have concluded that most amyloid deposits in  $A\beta_{42}$  fly brains are not cored (mature) plaques but diffused (immature) deposits, because we could not detect clear amyloid fibrils at the ultrastructural level. The lack of mature plaques may be due to the short life span of  $A\beta_{42}$  flies (within 50 days) and/or the absence of potential cofactors needed to form cored plaques in the fly brain. On the other hand, this result indicates that the cored plaque formation is not necessary to induce any of the pathological phenotypes observed in the  $A\beta_{42}$  flies.

$A\beta_{42}$  flies exhibit severe neurodegeneration in the absence of cored plaques containing clear amyloid fibril structures and the formation of NFTs. This has a parallel in studies that show polyglutamine- (47) or  $\tau$ -induced (48, 49) neurodegeneration can be dissociated from the formation of nuclear inclusion or NFTs, respectively. These facts support the notion that an ordered prefibrillar oligomer, or protofibril, but not the fibrillar form, may be responsible for cell death (50).

It has been reported that cognitive defects and  $A\beta$  deposits were not well correlated in AD patients (43, 46). In AD mouse models, the development of synaptic dysfunction and/or behavioral deficits precedes the formation of amyloid deposits (13, 51, 52).

These facts are reminiscent of that  $A\beta_{40}$  flies showed learning defects without amyloid deposits. As for the  $A\beta_{42}$  flies, we cannot conclude whether the deposits contribute to behavioral defects, because the 3-day-old flies already developed small amounts of deposits. Recently, soluble oligomeric forms of  $A\beta$  peptides have been suggested to be responsible for synaptic dysfunctions (53). We detected putative oligomeric forms of both  $A\beta_{40}$  and  $A\beta_{42}$  in the fly brain (Fig. 1A), whereas the pathological roles of these oligomers remain elusive.

## Conclusion

This study strongly supports the idea that excessive accumulation of  $A\beta_{42}$  is sufficient to cause memory defects and neurodegeneration resembling AD and suggests that the molecular basis underlying  $A\beta$  toxicity is conserved over different organisms. Our  $A\beta$  flies may serve as a model for the genetic and pharmacological screening system for AD therapeutics targeting  $A\beta$ -induced neurotoxicity and  $A\beta$  clearance, as well as for the understanding of the molecular and cellular basis of AD pathogenesis.

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