

# Overexpression of Neprilysin Reduces Alzheimer Amyloid- $\beta$ 42 ( $A\beta$ 42)-induced Neuron Loss and Intraneuronal $A\beta$ 42 Deposits but Causes a Reduction in cAMP-responsive Element-binding Protein-mediated Transcription, Age-dependent Axon Pathology, and Premature Death in *Drosophila*\*

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The amyloid- $\beta$ 42 ( $A\beta$ 42) peptide has been suggested to play a causative role in Alzheimer disease (AD). Neprilysin (NEP) is one of the rate-limiting  $A\beta$ -degrading enzymes, and its enhancement ameliorates extracellular amyloid pathology, synaptic dysfunction, and memory defects in mouse models of  $A\beta$  amyloidosis. In addition to the extracellular  $A\beta$ , intraneuronal  $A\beta$ 42 may contribute to AD pathogenesis. However, the protective effects of neuronal NEP expression on intraneuronal  $A\beta$ 42 accumulation and neurodegeneration remain elusive. In contrast, sustained NEP activation may be detrimental because NEP can degrade many physiological peptides, but its consequences in the brain are not fully understood. Using transgenic *Drosophila* expressing human NEP and  $A\beta$ 42, we demonstrated that NEP efficiently suppressed the formation of intraneuronal  $A\beta$ 42 deposits and  $A\beta$ 42-induced neuron loss. However, neuronal NEP overexpression reduced cAMP-responsive element-binding protein-mediated transcription, caused age-dependent axon degeneration, and shortened the life span of the flies. Interestingly, the mRNA levels of endogenous fly NEP genes and phosphoramidon-sensitive NEP activity declined during aging in fly brains, as observed in mammals. Taken together, these data suggest both the protective and detrimental effects of chronically high NEP activity in the brain. Down-regula-

tion of NEP activity in aging brains may be an evolutionarily conserved phenomenon, which could predispose humans to developing late-onset AD.

Alzheimer disease (AD)<sup>3</sup> is a progressive neurodegenerative disease defined by two protein deposits in autopsied brains: extracellular amyloid plaques composed of the 40- or 42-amino acid  $\beta$ -amyloid peptides ( $A\beta$ 40 or  $A\beta$ 42), and intracellular neurofibrillary tangles composed of abnormally hyperphosphorylated microtubule-associated protein Tau (1).  $A\beta$  peptides are physiological metabolites of the amyloid precursor protein (APP) resulting from sequential cleavage by  $\beta$ -secretase and  $\gamma$ -secretase complex, whose catalytic subunits are presenilin 1 (PS1) and presenilin 2 (PS2) (2). Molecular genetic studies of early onset familial AD patients have identified causative mutations in *APP*, *PS1*, and *PS2* genes, and these mutations promote  $A\beta$ 42 production, aggregation, and stability against clearance (3). Thus, the increased  $A\beta$ 42 levels in the brain are believed to initiate AD pathogenesis.

The mechanisms by which  $A\beta$ 42 reaches pathological levels in the brains of late-onset AD (LOAD) patients are not well understood. The steady state level of  $A\beta$ 42 in the brain reflects the balance between production and clearance, and a reduction in clearance activity would raise  $A\beta$ 42 levels. A deficiency in neprilysin (NEP), a major rate-limiting  $A\beta$ -degrading enzyme (4, 5), accelerates formation of extracellular amyloid deposits (6), amyloid angiopathy (6), synaptic dysfunctions (7), and memory defects (7) caused by human  $A\beta$  in transgenic mice. In LOAD patients, NEP mRNA and pro-

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<sup>3</sup> The abbreviations used are: AD, Alzheimer disease;  $A\beta$ ,  $\beta$ -amyloid peptide; NEP, neprilysin; LOAD, late onset Alzheimer disease; TS, thioflavin-S; CREB, cAMP-responsive element binding protein; dn, dominant negative; d, *Drosophila*; PBS, phosphate-buffered saline; APP, amyloid precursor protein; ER, endoplasmic reticulum; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; dae, day-after-eclosion; CRE, cAMP-responsive element; TBP, TATA-binding protein.

tein levels are selectively reduced in brain regions highly vulnerable to AD pathology (8), and NEP levels and activity decrease in human and rodent brains with aging (9–12), suggesting that reduction in NEP levels may contribute to the onset and/or progression of LOAD.

Activation of NEP could be a potential disease-modifying therapy for AD. Transgenic (13, 14), viral (15, 16), or *ex vivo* delivery (17) of NEP to brains of APP transgenic mice reduces extracellular amyloid deposits, synaptic dysfunction, and premature death. Intraneuronal accumulations of A $\beta$ 42 have also been detected in AD brains (18, 19) and may contribute to AD pathogenesis. Intraneuronal A $\beta$  is highly toxic in cultured neurons (20, 21), and transgenic *Drosophila* expressing human A $\beta$ 42 peptides in the secretory pathway of neurons exhibited age-dependent behavioral defects and neurodegeneration with intraneuronal A $\beta$ 42 accumulation (22, 23). Some transgenic mice overproducing A $\beta$  accumulate A $\beta$  intraneuronally, prior to extracellular amyloid pathologies (24–26). These intraneuronal A $\beta$  accumulations were correlated with synaptic dysfunction (25), memory defects (27, 28), and neurodegeneration (29–31). However, little is known about the effects of NEP on intraneuronal A $\beta$ 42 accumulation and toxicity.

In contrast to the potential benefits of enhanced A $\beta$  clearance, sustained NEP activation may be detrimental (32), because NEP can degrade a wide range of circulating peptides, including enkephalin, atrial natriuretic peptide, endothelin, and substance P (33). Although transgenic mice expressing high levels of human NEP do not show detectable adverse effects (13), potential side effects of a chronic increase in NEP activity have not been fully established.

In this study, we demonstrated that expression of human NEP in transgenic A $\beta$ 42 fly brains effectively reduced A $\beta$ 42 levels, intraneuronal A $\beta$ 42 deposits, and neuron loss. However, chronic NEP activity attenuated CREB-mediated transcription measured by an *in vivo* CRE-luciferase reporter, caused age-dependent axon degeneration, and shortened the life span of the flies. Interestingly, mRNA levels and activity of endogenous fly NEPs in brains reduced with aging as observed in humans.

## EXPERIMENTAL PROCEDURES

**Establishment of Transgenic Fly Lines, Genetics, and Culture Conditions**—The cDNA constructs for human NEP and an inactive mutant NEP were obtained from Drs. T. Saido and K. Shirotani (RIKEN, Japan). These cDNAs were cloned into the pUAST *Drosophila* transformation vector and microinjected into fly embryos of the *w<sup>1118</sup>* (*isoC1*) genotype. Several transgenic lines for each NEP construct were established. Transgenic A $\beta$ 42 flies were established as described (22, 23). The transgenic *UAS-CD8::GFP;OK107* line was a kind gift from Dr. L. Luo. The *elav-GAL4<sup>C155</sup>* flies were obtained from the Bloomington *Drosophila* Stock Center (Indiana University). When flies were raised at 25 °C throughout development, the expression of NEP, but not NEPmut, caused infertile phenotypes, including wet body and folded wing, presumably because of its ability to degrade circulating endogenous fly peptides (data not shown). Thus, flies were placed at 18 °C during development to reduce the activity of Gal4 and transgene (NEP and A $\beta$ 42) expression, and were maintained at 25 °C, under conditions of

70% humidity and a 12:12-h light:dark cycle after eclosion. This treatment dramatically prevented developmental defects induced by NEP, but it was sufficient to induce A $\beta$ 42-induced behavioral defects and neurodegeneration in adult flies. The transgenic UAS-dCREB2b (dnCREB) and CRE-luciferase reporter (CRE-Luc) lines were described previously (34). Because the expression of dnCREB caused infertile phenotypes when raised at 25 °C (data not shown), flies were placed at 18 °C during development and were maintained at 27 °C, under conditions of 70% humidity and a 12:12-h light:dark cycle after eclosion.

**Western Blotting**—To examine the expression levels of NEP, NEPmut, and dCREB2 proteins, heads from flies at 1–2 days-after-eclosion (dae) were homogenized in Tris-glycine sample buffer (Invitrogen) and centrifuged at 13,000 rpm for 10 min, and the supernatants were separated on 10% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). The membranes were blocked with 5% nonfat dry milk (Nestlé) and blotted with the anti-NEP antibody (Novocastra) or anti-dCREB2 antibody (a kind gift from Dr. J. Yin). For sequential extractions of A $\beta$ 42, 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  $\times$  *g* for 1 h, and supernatants were collected (SDS-soluble fraction). SDS-insoluble pellets were further homogenized in 70% formic acid (Sigma) followed by centrifugation at 13,000 rpm for 20 min, and the supernatants were collected (formic acid fraction). Formic acid was evaporated by SpeedVac (Savant, SC100), and protein was resuspended in dimethyl sulfoxide (Sigma). Protein extracts were separated on 10–20% Tris-Tricine gels (Invitrogen) and transferred to nitrocellulose membranes. The membranes were boiled in phosphate-buffered saline (PBS) for 3 min, blocked with 5% nonfat dry milk, and blotted with the 6E10 antibody (Signet).

**Neprilysin Enzymatic Assay**—Around 30 fly heads from the appropriate genotype and age were homogenized in 25 mM Tris-HCl, pH 7.4, and centrifuged at 1,000 rpm for 5 min. The enzymatic activity of the supernatant was assayed using glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide (Sigma) as a substrate in the presence or absence of phosphoramidon (Sigma), as described previously (35). Recombinant human neprilysin was purchased from R & D Systems. Protein levels were quantified using Micro BCA protein assay kit (Pierce).

**Whole-mount Immunostaining and Thioflavine S Staining**—Transgenic fly brains expressing NEP or NEPmut in the mushroom body structure were dissected in cold PBS, fixed in PBS containing 4% paraformaldehyde (Electron Microscopy Sciences), 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 boiled in citrate buffer (Chemicon) for 5 min and stained with a mouse monoclonal anti-NEP antibody (Novocastra) followed by detection with biotin-XX goat anti-mouse IgG and streptavidin-Texas Red conjugate (Molecular Probes). The dendritic and axonal structures of the mushroom body neurons were visualized with the co-expressed CD8-GFP signal. The brains were analyzed using a confocal microscope (Carl Zeiss LSM

510). For thioflavin S (TS) staining, the brains were permeabilized and incubated in 50% EtOH containing 0.1% TS (Sigma) overnight. After washing in 50% EtOH and PBS, the brains were analyzed using a confocal microscope. The numbers of TS-positive deposits were quantified from six hemispheres from three flies per genotype. The fluorescence intensity in Kenyon cell regions in the same images was measured using NIH image.

**Quantification of Neurodegeneration**—Heads were fixed in 4% paraformaldehyde, processed to embed in paraffin blocks, and sectioned at a thickness of 6  $\mu\text{m}$ . Sections were placed on slides, stained with hematoxylin and eosin (Vector Laboratories), and examined by bright field microscopy. To quantify neurodegeneration in the cell body and neuropil, images of the sections were captured, and the areas of the vacuoles in the cell body or neuropil regions were measured using Photoshop (Adobe Systems). The ratio of lost area in the cell body region was calculated by dividing the sum of the vacuole areas by the total area of the cell body region.

**Survival Assay**—Food vials containing 25 flies were placed on their sides at 25 °C, under conditions of 70% humidity and a 12:12-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.

**Transmission 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 °C. Thin sections (100 nm) of neuropil regions were collected on nickel grids (100 mesh, Veco-EMS). Grids were then rinsed in 10 drops of distilled water and air-dried. Thin sections were stained for 5 min in 3% uranyl acetate dissolved in 30% ethanol and then rinsed in distilled water.

**Luciferase Assay**—*In vivo* luciferase activity was recorded from flies as described (34). Briefly, CRE-Luc flies were loaded in 96-well plates filled with agar containing sucrose and luciferin (Biosynth) and covered with PCR caps and clear plastic covers (Topseal, PerkinElmer Life Sciences) that had holes punched in them. Activity was measured by using a Topcount microplate scintillation and luminescence counter (PerkinElmer Life Sciences) under a 12:12-h light:dark cycle. Experiments were repeated more than three times, and the representative data are shown.

**Quantitative Real Time PCR Analysis**—For each sample, 200–300 flies were collected and frozen at 2, 10, and 25 dae. Heads were mechanically isolated, and total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol with an additional centrifugation step (11,000  $\times g$  for 10 min) to remove cuticle membranes prior to the addition of chloroform. Total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen), and the resulting

cDNA was used as a template for PCR on a 7500 fast real time PCR system (Applied Biosystems). The average threshold cycle value ( $C_t$ ) was calculated from five replicates per sample. Expression of NEP1–5 was standardized relative to TBP. Relative expression values were determined by the  $\Delta\Delta C_t$  method according to the formula  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t$  is the difference in  $\Delta C_t$  at different time points (where d is day) ( $\Delta C_{tx,2d} - \Delta C_{tx,10d}$ , or  $\Delta C_{tx,2d} - \Delta C_{tx,25d}$ ), and  $\Delta C_{tx}$  is the difference in threshold cycles for target ( $\Delta C_{t_{NEP1-5}}$ ) and reference ( $\Delta C_{t_{TBP}}$ ) according to quantitative PCR Analysis User Bulletin (Applied Biosystems). To confirm the specificity of the PCR, PCR products were electrophoresed on agarose gels. Primers were designed using PrimerExpress 3.0 (Applied Biosystems) to detect NEP1–5 and TBP as follows: NEP1, GATGACGCAGG-GCGAGAA (forward) and TGGGCGTAGTTGAGAAAG-AACA (reverse); NEP2, CCGCAGATGGGCTGAGAA (forward) and TGCACGCCGGTAGTAATACG (reverse); NEP3, GTCCAGCCGCACCAAAAA (forward) and CCATTGATTGCAGGAATATCCA (reverse); NEP4, ACGTCTCGAACT-CCGTTGA (forward) and AGGAATGTGTGGCATATGT-CATG (reverse); NEP5, GCACCCACCTGGAGAACCT (forward) and GGCACAGTGATTGCATTCTGA (reverse); and TBP, GCGGCTGTGATTATGCGAAT (forward) and AGGG-AAACCGAGCTTTTGGGA (reverse).

## RESULTS

**Establishment of Human NEP and Mutant NEP (NEPmut) Transgenic Flies**—To determine the effects of neuronal expression of human NEP on intraneuronally expressed A $\beta$ 42-induced pathology and toxicity in the brain, we established transgenic flies expressing human NEP utilizing the Gal4-UAS system (36). We also created transgenic lines carrying an inactive mutant form of human NEP (NEPmut) with an amino acid substitution of Glu at 585 to Val in the zinc-binding motif of neprilysin, as controls (37, 38). The NEP or NEPmut proteins were expressed in all fly neurons under the control of the pan-neuronal *elav-Gal4* driver, and expression levels in fly heads were determined by Western blotting (Fig. 1A). The flies carrying *elav-Gal4* driver alone were used as a control. In this study, we used primarily the NEP3 and NEP45 lines for wild-type human NEP and the NEPmut18 and NEPmut30 lines for inactive mutant NEP, because the levels of expression were comparable. To achieve higher levels of expression of NEP and NEPmut in flies, we prepared double transgenic flies carrying NEP3 and NEP45 (NEP3+45) or NEP18 and NEP30 (NEPmut18+30). Head lysates from NEP flies (NEP45 and NEP3+45) showed ~3-fold more catalytic activity toward the fluorogenic NEP substrate, glutaryl-Ala-Ala-Phe-4-methoxy-2-naphthylamide, compared with control flies (Fig. 1B). In contrast, lysates from NEPmut fly brains (NEPmut18+30) show basal NEP activity comparable with that in control flies (Fig. 1B). These results demonstrate that human NEP protein expressed in the fly brain forms an active enzyme.

**Preferential Localization of NEP and NEPmut Proteins in Axons and Dendrites**—NEP proteins are detected in axons and dendrites in mammalian neurons (39, 40). To examine the neuronal distribution patterns of human NEP and NEPmut proteins, we used the *OK107-gal4* driver to specifically express the

proteins in the mushroom body structure, in which the cell bodies (Kenyon cell bodies), dendrites (calyxes), and axons (lobes) of Kenyon cells are readily identifiable (41) (Fig. 2). Mushroom bodies are paired structures formed from ~2,500 cells in each hemisphere, which play a central role in olfactory

learning and memory in flies. The mushroom body calyxes, located just beneath the mushroom body cell bodies (Kenyon cell bodies) in the dorsal posterior brain, delineate the dendritic fields of the intrinsic mushroom body neurons. The axons of mushroom body neurons (lobes) extend anteriorly and branch into the dorsally projecting lobes (42).

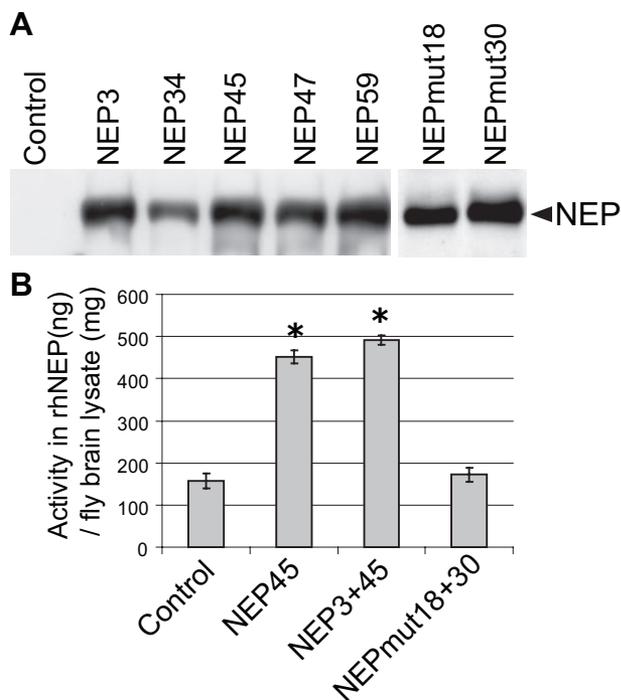
Whole-mount immunostaining with an anti-NEP antibody strongly detected NEP and NEPmut proteins in the axons and dendrites (Fig. 2), indicating that human NEP proteins expressed in fly neurons exhibit a cellular localization similar to that in mammalian neurons. These results suggest that the cellular mechanism that localizes NEP proteins to neurites may be conserved between fly and human.

**Reduced Brain A $\beta$ 42 Levels and TS-positive Deposits by NEP, but not NEPmut, Expression**—To determine whether overexpression of human NEP can reduce the intraneuronal accumulation of A $\beta$ 42, we expressed NEP or NEPmut in transgenic A $\beta$ 42 fly brains. In this model, A $\beta$ 42 peptides were expressed in the endoplasmic reticulum (ER), and they were detected in the Golgi apparatus, lysosomes, dendrites, axons, and presynaptic terminals by immunoelectron microscopy (immuno-EM) (23). In fly brains, monomeric A $\beta$ 42 peptides were detected as 4-kDa signals in both detergent-soluble (extracted with RIPA containing 1% SDS) and detergent-insoluble fractions (extracted with 70% formic acid) by Western blotting (22). Expression of NEP, but not NEPmut, significantly reduced the level of A $\beta$ 42 both in detergent-soluble and -insoluble fractions in aged A $\beta$ 42 brains. However, quantification of A $\beta$ 42 signals showed that there was a significant level of residual A $\beta$ 42 in both fractions even in flies carrying two copies of the NEP transgene (Fig. 3). NEP did not change the level of a co-expressed CD8::GFP fusion protein (Fig. 2), confirming the specificity of NEP activity on A $\beta$ 42 levels.

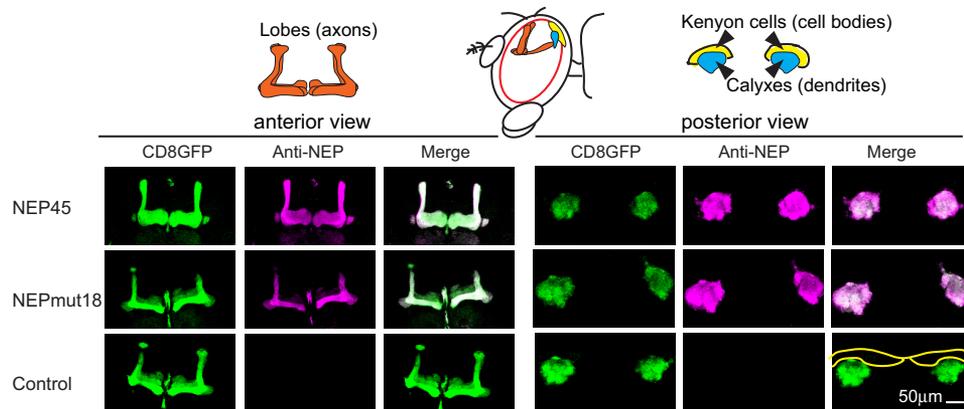
We tested whether neuronal expression of NEP can prevent formation of intraneuronal TS-positive A $\beta$ 42 deposits in the cell body regions. Quantification of whole mount TS staining revealed that TS-positive A $\beta$ 42 deposits were dramatically reduced by NEP, but not NEPmut, expression (Fig. 4, A–E).

These results demonstrate that neuronal overexpression of NEP significantly reduced accumulation and deposition of intraneuronally expressed A $\beta$ 42 in the fly brain. Similar results were obtained in another independent A $\beta$ 42 transgenic line (data not shown).

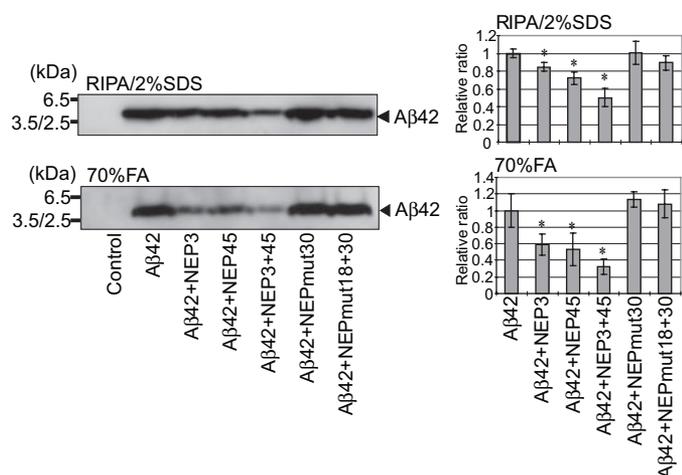
**Suppression of A $\beta$ 42-induced Neuron Loss by NEP, but not NEPmut, Expression**—Expression of A $\beta$ 42, but not A $\beta$ 40, caused age-dependent neuron loss, which could be identified by the appearance of vacuoles in the cell body regions of fly brains (22). We examined the effect of neuronal expression of NEP on this phenotype (Fig. 5, A–D). Quantification of the area covered by vacuoles revealed that



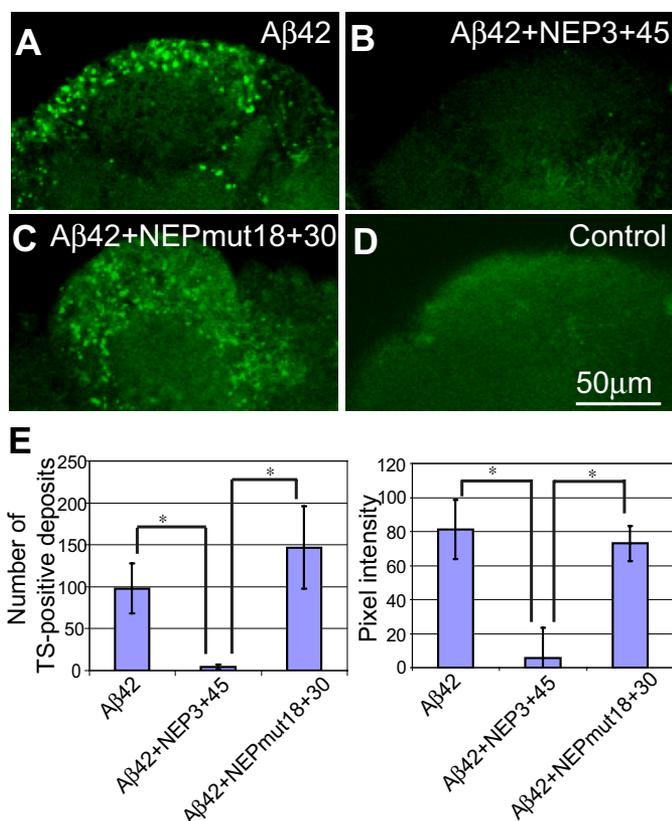
**FIGURE 1. Characterization of expression levels and activity of human NEP and NEPmut in fly brains.** A, expression levels of NEP or NEPmut in independent transgenic lines were compared by Western blotting. Equal amounts of proteins were loaded. B, NEP, but not NEPmut, expressed in fly brains exhibited phosphoramidon-sensitive endopeptidase activity. Arbitrary fluorescence units for each sample were compared with a standard curve prepared using recombinant human neprilysin (rhNEP) to determine the NEP-specific activity per mg of protein. The neprilysin activity was equivalent to  $157 \pm 17$ ,  $492 \pm 12$ , or  $172 \pm 16$  ng of recombinant human NEP/mg fly head protein in control, NEP45, NEP3+45, and NEPmut18+30, respectively. Bars indicate averages  $\pm$  S.E. ( $n = 3$  or  $4$ ). The asterisk indicates significant difference from control ( $p < 5 \times 10^{-9}$ , Student's *t* test).



**FIGURE 2. Axonal and dendritic localization of NEP and NEPmut in fly brain neurons.** NEP or NEPmut proteins were expressed in mushroom body structures, where Kenyon cell body regions, axon bundles (Lobes), and dendrites (Calyxes) can be easily identified, using the OK107-Gal4 driver. Lobes and calyxes were labeled by co-expressed membrane targeting mCD8::GFP fusion proteins (green). Immunostaining with an anti-human NEP antibody (Anti-NEP, magenta) reveals preferential localization of NEP or NEPmut in axons (lobes) and dendrites (calyxes) (Merge, white). Control brains without NEP transgene do not show any signal from human NEP antibody, indicating the specificity of antibody. The cell body region is outlined in yellow. A schematic of a sagittal view of mushroom body structure is shown at the top.

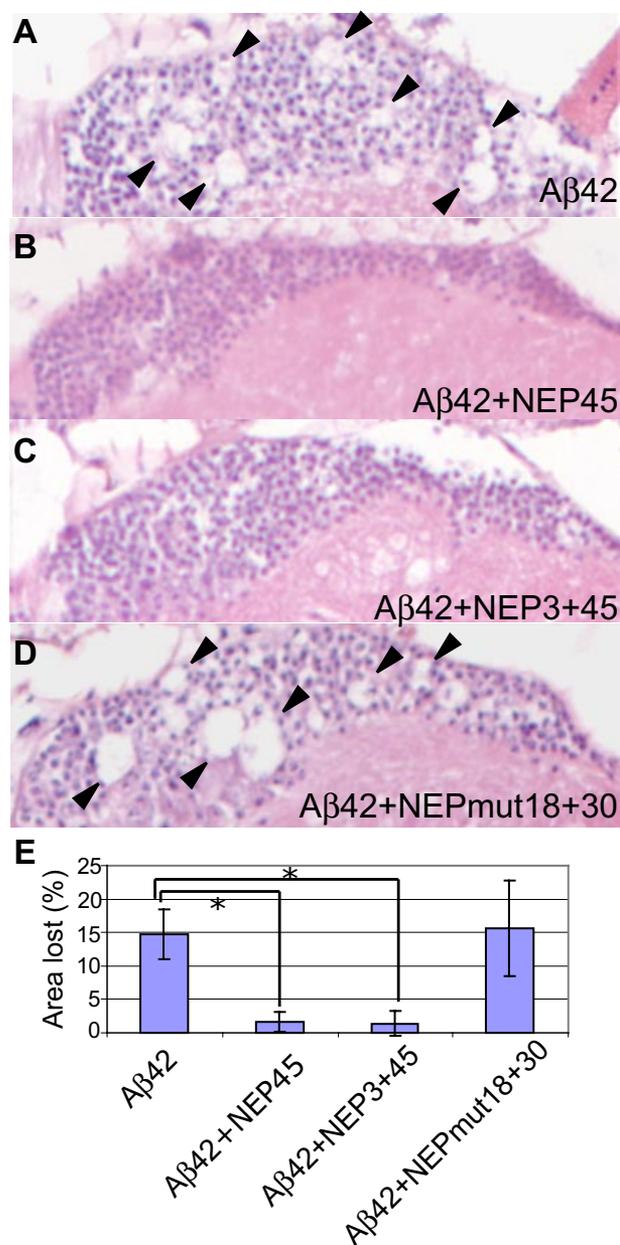


**FIGURE 3. Reduced accumulation of Aβ42 by co-expression of NEP in fly brains.** Aβ42 in fly brains at 28 dae in detergent-soluble (top panel) and -insoluble fractions (bottom panel) were detected by Western blotting. Co-expression of Aβ42 with NEP (Aβ42 + NEP3, Aβ42 + NEP45, and Aβ42 + NEP3 + 45) but not NEPmut (Aβ42 + NEPmut30 and Aβ42 + NEPmut18 + 30) reduced Aβ42 levels in both fractions. Representative blots are shown on the left, and means ± S.E. from three experiments are plotted on the right. The asterisk indicates significant difference from control ( $p < 0.005$ , Student's  $t$  test,  $n = 3$ ).



**FIGURE 4. Reduced intraneuronal TS-positive Aβ42 deposits by co-expression of NEP.** TS staining of Kenyon cell body regions in brains expressing Aβ42 (A), Aβ42 and NEP3 + 45 (B), or Aβ42 and NEPmut18 + 30 (C) at 35 dae is shown. No TS-positive signal was detected in the age-matched nontransgenic control brains (D). Scale bar in D indicates 50 μm. E, numbers of TS-positive deposits (left panel) and signal intensity (right panel) in a hemisphere are presented as averages ± S.D. ( $n = 6$  hemispheres). Asterisks indicate significant differences from Aβ42 + NEP3 + 45 ( $p < 0.05$ , Student's  $t$  test).

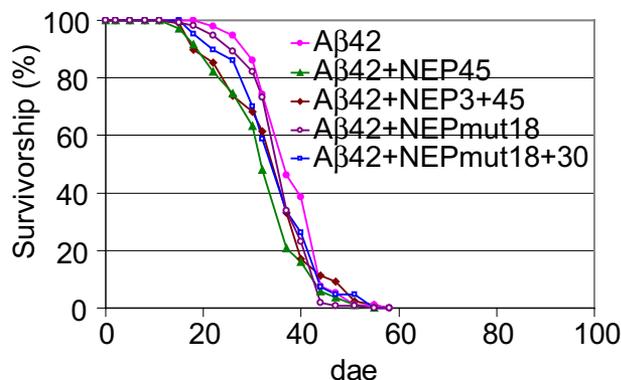
NEP, but not NEPmut, dramatically suppressed neuron loss (Fig. 5E), suggesting that the NEP-sensitive Aβ42 population contributes to neuron loss in Aβ42 fly brains. The rescue effects



**FIGURE 5. Suppression of Aβ42-induced neuron loss by neuronal expression of NEP.** A–D, Kenyon cell body regions in flies expressing Aβ42 alone (A, Aβ42), Aβ42, and one copy of NEP (B, Aβ42 + NEP45), Aβ42, and two copies of NEP (C, Aβ42 + NEP3 + 45), or Aβ42 and two copies of NEPmut (D, Aβ42 + NEPmut18 + 30) at 28 dae. Arrowheads indicate vacuoles, which represent neuron loss in the brain (A–D). E, percentages of the area lost in the cell body regions are shown as averages ± S.D. ( $n = 7–9$  hemispheres). Asterisks indicate significant differences from Aβ42 alone ( $p < 0.00005$ , Student's  $t$  test).

of NEP were confirmed in another independent Aβ42 transgenic line (data not shown).

**No Rescue of Aβ42-induced Premature Death by NEP Expression**—Expression of Aβ42 in neurons causes premature death of flies (22). We found that neuronal NEP expression did not rescue this phenotype (average life spans, 42, 38, 37, 40, and 41 dae for Aβ42, Aβ42+NEP3, Aβ42+NEP45, Aβ42+NEP3+45, and Aβ42+NEPmut18+30 flies, respectively) (Fig. 6), whereas neuron loss was greatly suppressed in NEP flies (Fig. 5). These results suggest that the residual frac-

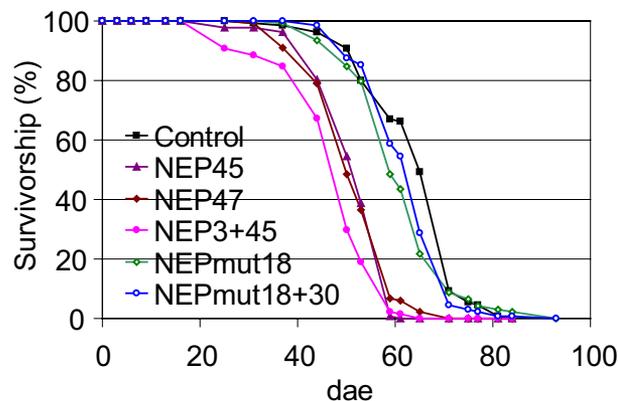


**FIGURE 6. No rescue of A $\beta$ 42-induced premature death by neuronal overexpression of NEP.** The percent survivorship of flies expressing A $\beta$ 42 alone (A $\beta$ 42), A $\beta$ 42 and one copy of NEP (A $\beta$ 42+NEP45), A $\beta$ 42 and two copies of NEP (A $\beta$ 42+NEP3+45), A $\beta$ 42 and one copy of NEPmut (A $\beta$ 42+NEPmut18), or A $\beta$ 42 and two copies of NEPmut (A $\beta$ 42+NEPmut18+30) in neurons were plotted against the age (dae). Approximately 100 flies were analyzed for each genotype.

tion of A $\beta$ 42 may play a role in premature death in our A $\beta$ 42 fly models (Fig. 3). Additionally, the detrimental effects of NEP expression may contribute to this phenotype (see below). Similar results were obtained in another independent A $\beta$ 42 transgenic line (data not shown).

**Shortened Life Span of Flies with Neuronal NEP Overexpression**—NEP is capable of degrading many physiological peptides (33), and sustained activation of NEP in neurons may have detrimental effects. We found that neuronal expression of NEP significantly shortened the life span of flies relative to controls (average life spans, 65, 52, 52, 48, 68, and 63 dae for control, NEP45, NEP47, NEP3+45, NEPmut18, and NEPmut18+30 flies, respectively, Fig. 7). Importantly, the expression of NEPmut did not show this detrimental effect, suggesting that not merely protein overexpression but NEP activity is responsible for this premature death phenotype. Although NEP transgenic flies (average life spans, 52, 52, and 48 dae for NEP45, NEP47, and NEP3+45, respectively) lived significantly longer than A $\beta$ 42 flies (average life span, 42 dae), the detrimental effect of NEP may partly explain why NEP expression failed to rescue A $\beta$ 42-induced premature death.

**Age-dependent Neuropil Degeneration and Axon Pathology Induced by NEP Expression**—To gain more insight into the potential detrimental effects of neuronal NEP overexpression, we examined the brains of aged (56 dae) NEP flies. We detected neurodegeneration in the neuropil regions, including superior lateral protocerebrum, as indicated by the many vacuoles in NEP fly brains (Fig. 8A, arrowheads in pink region), compared with NEPmut and control brains (Fig. 8, C and D, respectively). This degeneration is age-dependent, because no vacuole was observed in young (8 dae) fly brains overexpressing NEP (Fig. 8B). Quantification of the area covered by vacuoles is shown in Fig. 8E. The degeneration was limited to neuropil regions, and no significant cell body loss was observed in NEP flies (Fig. 8A, no vacuoles in the cell body region (purple area)), which is consistent with the preferential distribution of NEP in axons and dendrites (Fig. 2). Moreover, despite a broad expression of NEP in fly brain neurons by the pan-neuronal *elav-Gal4* driver, the degeneration was observed in specific neuropil regions such



**FIGURE 7. Premature death caused by neuronal overexpression of NEP in flies.** The percent survivorship of flies expressing one copy of NEP (NEP45 or NEP47), two copies of NEP (NEP3+45), one copy of NEPmut (NEPmut18), or two copies of NEPmut (NEPmut18+30) in neurons was plotted against the age (dae). Approximately 100 flies were analyzed for each genotype.

as superior lateral protocerebrum, suggesting that certain types of neurons are more vulnerable to excessive NEP activity. At the ultrastructural level, highly swollen axons were observed in NEP fly brains (Fig. 8, F and G, asterisks) but not in control brains (Fig. 8, H and I, arrows indicate normal axons). Taken together, our results demonstrate that chronic NEP expression in fly brains causes age-dependent axon pathology in certain types of neurons.

**Reduction in CREB-mediated Transcription Induced by Neuronal NEP Overexpression**—What is a potential mechanism(s) underlying the observed detrimental effects of NEP? Because NEP can degrade many physiological peptides, its artificial overexpression would disrupt diverse signaling pathways activated by NEP-sensitive peptides in fly brains. Several neuropeptides are known to stimulate G-protein-coupling receptors and activate CRE-binding protein family transcription factor, which is an evolutionary conserved key mediator of stimulus-induced transcription of pro-survival factors (43). We hypothesized that NEP overexpression may reduce CREB-mediated transcription in flies. CREB activity can be measured using transgenic flies carrying the luciferase reporter gene fused to a promoter region containing the CRE sequence (34). As described previously, CREB activity oscillates in a circadian-dependent manner (Fig. 9, control) (34). In flies expressing NEP in neurons, CREB activity was lower than that in control flies at all time points, whereas a reduction in CREB activity was not observed in flies with NEPmut expression (Fig. 9). Neurodegeneration was not observed in flies with NEP overexpression at this age (3–8 dae, Fig. 8).

**Premature Death Induced by a Reduction in CREB Activity in Neurons**—To test the hypothesis that detrimental effects of NEP expression is, in part, attributable to reduced CREB activity in neurons, we analyzed the effect of neuronal overexpression of the dCREB2-b repressor isoform (dnCREB) (44, 45). The dnCREB proteins were expressed in all fly neurons under the control of the pan-neuronal *elav-Gal4* driver (Fig. 10A). As expected, dnCREB expression in neurons reduced CREB activity in flies (Fig. 10B). We found that neuronal expression of dnCREB significantly shortened the life span of flies (average life span, 36 dae) relative to controls (average life span, 48 dae)

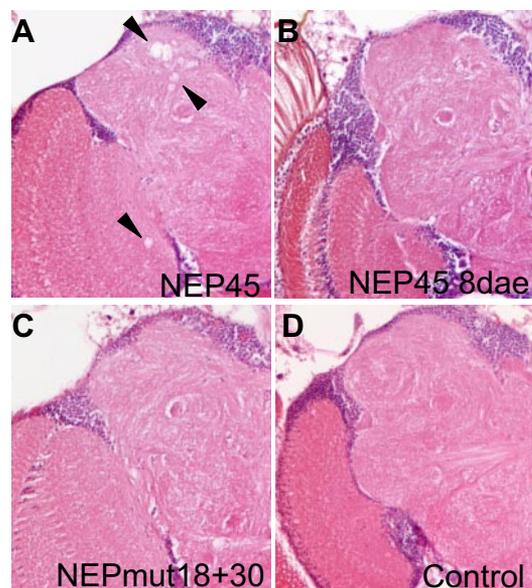


FIGURE 8. Age-dependent neuropil degeneration and axonal pathology caused by neuronal overexpression of NEP in fly brains. A–D, neuropil degeneration was detected in flies expressing NEP (A, NEP45, arrowheads) but

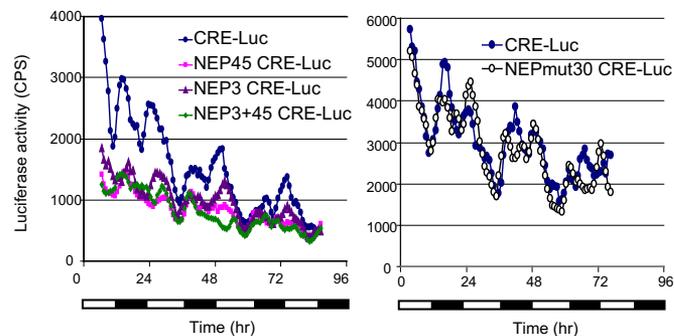
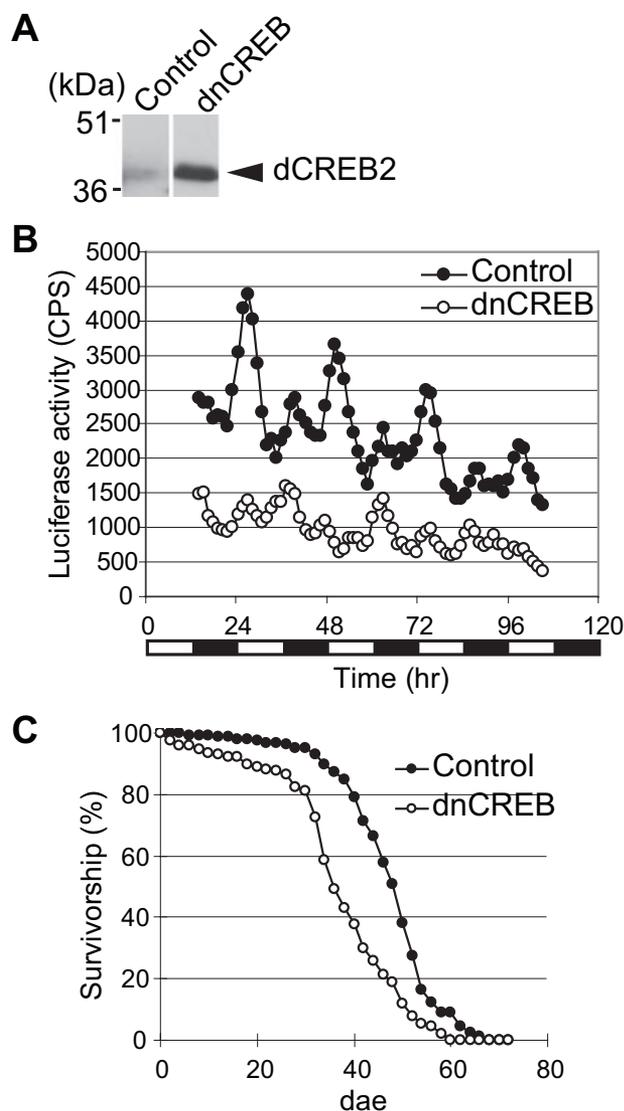


FIGURE 9. Reduction in CREB activity by neuronal overexpression of NEP in fly brains. Left, CRE-Luc signal (photon counts/s, CPS) from control flies (CRE-Luc, blue circles) and flies expressing one copy of NEP (NEP45 CRE-Luc, pink squares, or NEP3 CRE-Luc, purple triangles) or two copies of NEP (NEP3 + 45 CRE-Luc, green diamonds) were plotted against the Zietgeber time (ZT). Right, CRE-Luc signal from control flies (CRE-Luc, blue circles) and flies expressing NEPmut (NEPmut30 CRE-Luc, open circles) were plotted against the Zietgeber time (ZT). Luciferase activity was recorded under a 12:12-h light:dark cycle (indicated by the white/black bars). An average of more than 20 flies was assayed for each time point.

(Fig. 10C). Similar results were obtained from several independent transgenic lines for dnCREB (data not shown). In contrast, neurodegeneration was not prominent in dnCREB fly brains (data not shown), suggesting that NEP-induced neurodegeneration is conferred by other mechanism(s). Taken together, these results indicate that premature death induced by chronic NEP expression may be explained by a reduction in CREB activity in neurons.

*Age-dependent Reduction in mRNA Levels of NEP Family Genes and Phosphoramidon-sensitive NEP Activity in Fly Brains*—Although the underlying biological mechanisms are not well understood, age-dependent reductions in NEP mRNA levels have been reported in human and rodent brains (9–12, 46, 47). We were motivated to test whether a similar phenomenon can be seen in the fly brain. In the *Drosophila* genome, five NEP family genes (NEP1–5) have been identified. NEP1 and NEP3 are the fly homologues of human NEP and endothelin-converting enzyme 1 (ECE1), rate-limiting  $\beta$ -degrading enzymes in the mouse brain, respectively. We compared the expression level of NEP genes in fly heads at 2, 10, and 25 dae by quantitative reverse transcription-PCR. The mRNA levels of all NEPs, with the exception of NEP2, showed an age-dependent decrease (NEP1, -3, and -5 showed approximate 40% reductions and NEP4 showed an approximate 20% reduction at 25 dae; Fig. 11, A, C, D, and E). We also detected that phosphoramidon-sensitive NEP activity in 26 dae (old) fly heads showed a 40% reduction compared with that in 2 dae (young) (Fig. 11F). These results suggest that down-regulation of NEP transcription and activity with aging in brains may be an evolutionarily conserved phenomenon across animal phyla.

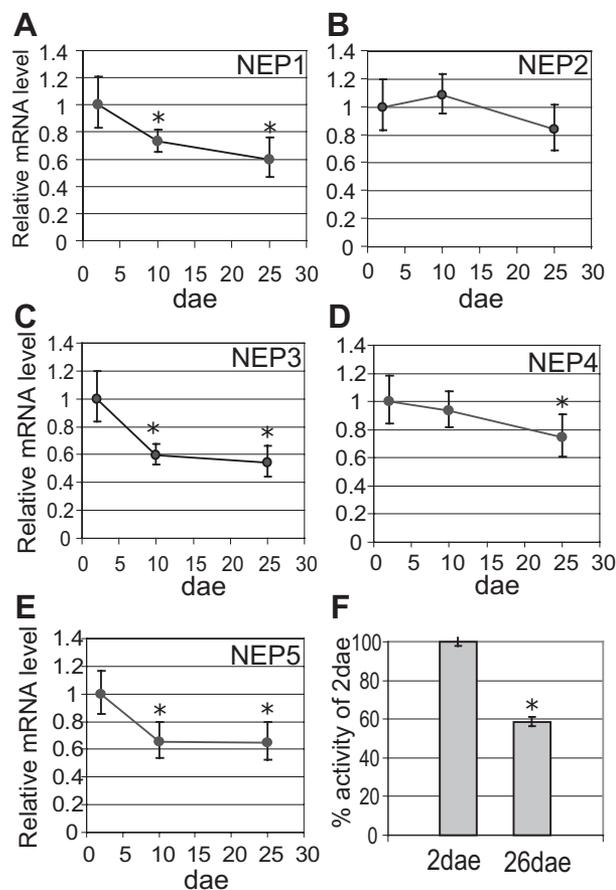
not in flies with two copies of NEPmut (C, NEPmut18+30), or control flies without NEP transgene (D), at 28 dae. Neurophil degeneration was not observed in flies expressing NEP at 8 dae (B, NEP45 8 dae). E, areas lost in neuropil regions were measured and are presented as means  $\pm$  S.D. ( $n = 6$ –10 hemispheres). Asterisks indicate significant differences from control ( $p < 0.005$ , Student's *t* test). F–I, axon pathology in fly brains with neuronal NEP overexpression (F and G, asterisks indicate swollen axons). No such pathology was observed in control fly brains (H and I, arrows indicate normal axons). Scale bars are 10  $\mu$ m in F and H and 5  $\mu$ m in G and I.



**FIGURE 10. Age-dependent premature death caused by neuronal overexpression of dnCREB in fly neurons.** *A*, endogenous dCREB2 and overexpressed dnCREB were detected with anti-dCREB2 in fly heads. Western blotting by anti-dCREB2 antibody in flies carrying *elav-gal4* driver only (*Control*) or with overexpression of dnCREB in neurons (*dnCREB*). Equal amounts of proteins were loaded. *B*, reduction in CREB activity by neuronal overexpression of dnCREB. The CRE-Luc signal from control flies (filled circles) and that from flies expressing dnCREB (open circles) were plotted against the Zietgeber time (ZT). Luciferase activity was recorded under a 12:12-h light:dark cycle (indicated by the white/black bars). An average of more than 11 flies was shown for each time point. *C*, premature death caused by dnCREB overexpression in neurons. The percent survivorship of flies expressing dnCREB in neurons (open circles) or control flies (filled circles) was plotted against the age (dae). Approximately 150 flies were analyzed for each genotype.

## DISCUSSION

Emerging evidence suggests that intraneuronal accumulation of A $\beta$ 42 may be an early event in AD pathogenesis and can be an important therapeutic target (48). In this study, we demonstrated that neuronal expression of human NEP reduced intraneuronal accumulation of A $\beta$ 42 and suppressed A $\beta$ 42-induced neuron loss in transgenic *Drosophila* brains. However, our results also showed that chronic neuronal expression of NEP reduced CREB-mediated transcription, caused age-dependent axon degeneration, and shortened the life span of the flies.



**FIGURE 11. Age-dependent reduction in NEP expression and activity in fly brains.** *A–E*, NEP1–5 mRNA levels were analyzed at 2, 10, and 25 dae using quantitative reverse transcription-PCR. Levels of mRNA relative to 2 dae are shown (averages  $\pm$  S.E. ( $n = 5$ )). Asterisks indicate significant differences compared with 2 dae ( $p < 0.001$ , Student's *t* test). *F*, phosphoramidon-sensitive endopeptidase activity in fly brains at 2 and 26 dae. Bars indicate averages  $\pm$  S.E. ( $n = 5$ ). The asterisk indicates significant difference from 2 dae ( $p < 1.0 \times 10^{-7}$ , Student's *t* test).

Mechanisms underlying A $\beta$ 42 accumulation within neurons are not well understood. In AD patients or transgenic AD model mice brains, A $\beta$  has been detected in several organelles, including the ER (49), Golgi apparatus (49), endosomal-lysosomal system (50), as well as multivesicular bodies (51), autophagolysosomes (52), mitochondria (53), and nuclei (21). Under normal conditions, the endosome system is a major cellular compartment for A $\beta$  generation (54). However, evidence suggests that A $\beta$  can be generated intracellularly from the secretory pathway (55). For example, A $\beta$ 42, but not A $\beta$ 40, can be produced in the ER (56–59). In addition, A $\beta$  secreted into the extracellular space can be taken up by neurons and internalized into intracellular pools (60). Thus, under abnormal conditions, A $\beta$  might be generated, retained, or recycled back to several intracellular locations, thus facilitating the pathological intraneuronal accumulation (61).

NEP is a type 2 membrane protein identified as a major rate-limiting A $\beta$ -degrading enzyme in the brain (4, 5). NEP is a member of the neutral endopeptidase family, which exhibits maximum catalytic activity in neutral pH environments (33). Thus, NEP would appear to degrade A $\beta$  most efficiently in the extracellular space. However, recent reports have shown that

not only extracellular but also intraneuronal A $\beta$  levels are reduced by NEP overexpression in primary cortical neurons, presumably by degradation in the secretory pathway (62). Moreover, a potential intracellular role for NEP in A $\beta$  catabolism *in vivo* has recently been suggested from analysis of the half-life of interstitial fluid A $\beta$  in NEP knock-out mice (6).

In our transgenic A $\beta$ 42 flies, A $\beta$ 42 peptides were expressed in the ER and localized to the late secretory pathway compartments, axons, dendrites, and presynaptic termini, as well as secreted from neurons (23). Here, we demonstrated that, in this fly model, neuronal overexpression of human NEP significantly reduced brain A $\beta$ 42 levels and intraneuronal TS-positive deposits (Fig. 3) and also significantly suppressed A $\beta$ 42-induced neuron loss (Fig. 4). Our results suggest that up-regulation of neuronal NEP activity is protective against intraneuronal A $\beta$ 42 accumulation and neuron loss induced by intracellularly generated A $\beta$ 42.

Despite high expression levels of NEP, a significant amount of A $\beta$ 42 was still detected by Western blotting in A $\beta$ 42 fly brains (Fig. 3). In contrast, neuronal overexpression of NEP significantly reduced TS-positive deposits of intraneuronally expressed A $\beta$ 42 in the fly brain (Fig. 4). These results suggest that residual A $\beta$ 42 in fly brains with NEP overexpression may not form TS-positive aggregates. What is the nature of this NEP-insensitive fraction of A $\beta$ 42? In this fly model, all A $\beta$ 42 is generated in the ER and distributed into the secretory pathway (23). Some A $\beta$ 42 may be transported into cellular compartments where NEP cannot act because of the unfavorable pH environment. In addition, A $\beta$ 42 peptides with particular misfolded structures may be resistant to NEP degradation. In fact, pathogenic mutations in A $\beta$  make it resistant to proteolytic degradation by NEP *in vitro* (63). Interestingly, our recent results suggest that the particular aggregation propensities of A $\beta$ 42 influence the cellular localization of A $\beta$ 42 in this fly model (23), suggesting that the misfolding properties of A $\beta$ 42 may affect its sensitivity to NEP by altering the cellular localization. We hypothesize that this NEP-insensitive population of A $\beta$ 42 may contribute to the premature death phenotype, which was not rescued by NEP overexpression in our fly models. Several A $\beta$ -degrading enzymes are likely to contribute to A $\beta$  clearance in the brain by complementing each other in a subcellular, cell type, and/or brain region-specific manner (32). Insulin-degrading enzyme and ECE1 have been shown to degrade intracellular A $\beta$ 42 in cultured cells (64, 65), suggesting that activation of these A $\beta$ -degrading enzymes together with NEP may additively suppress intraneuronal A $\beta$ 42-induced toxicity. Further analysis, including the effect of other A $\beta$ -degrading enzymes, will be required to address the nature of the NEP-insensitive A $\beta$ 42 fraction in our fly models.

NEP is involved in the metabolism of a number of regulatory peptides in the mammalian nervous, cardiovascular, and immune systems, and up- or down-regulation of NEP can lead to a range of pathological conditions. For example, down-regulation of NEP has been detected in a number of distinct cancers (66), and up-regulation has been detected in gliomas (67). Thus, although activation of NEP could be a potential disease-modifying therapy for AD (68), the potential side effects of

chronic overexpression of NEP in the brain need to be carefully evaluated.

We demonstrated here that transgenic overexpression of human NEP in neurons shortened the life span of flies and caused age-dependent axon degeneration in the brain. Moreover, we found that NEP overexpression in neurons decreased CREB-mediated transcription in the fly (Fig. 9), and reducing CREB activity in neurons was sufficient to cause premature death (Fig. 10). These data suggest that a reduction in CREB-mediated transcription underlies premature death induced by NEP overexpression. However, it is possible that premature death and neurodegeneration induced by NEP expression is because of a consequence of misexpression in brain regions that do not usually express NEP, and enhancement of endogenous NEP may not cause these effects.

Contrary to the detrimental effects of neuronal NEP expression in flies, it has been shown that transgenic mice expressing NEP under the control of the neuron-specific CaM kinase II promoter are healthy for up to 14 months without obvious brain pathology (13). This may be one of the reasons why premature death in A $\beta$ 42 flies was not rescued by NEP overexpression, although it is reported that NEP expression decreased premature death in human APP transgenic mice (13). What is a possible explanation for this discrepancy? In flies, dCREB2, a fly homologue of mammalian CREB and cAMP-response element modulator, plays a central role in CREB-mediated transcription (34), and NEP-sensitive peptides play a dominant role in homeostatic CREB activation (Fig. 9). In contrast, mice have several CREB family members, including cAMP-response element modulator with redundant functions, and signaling mechanisms other than the NEP-sensitive pathway(s) may exist to activate CREB-mediated transcription. Because CREB function is also known to be vital in mice (69), the lack of a severe detrimental effect in NEP transgenic mice may be due to a complex compensation mechanism for CREB-mediated transcription in mammalian brains. It would be important to examine whether CREB-mediated transcription is altered in NEP transgenic mice.

NEP expression and activity are complexly regulated by many factors that have been implicated in AD, including somatostatin, estrogen, exercise, environmental enrichment, oxidative stress, hypoxic and ischemic conditions, and aging (70). Aging is the most significant risk factor for the development of LOAD, and down-regulation of NEP and other A $\beta$ -degrading enzymes in human and rodent brains have been hypothesized to be involved in the increased risk for LOAD (68). In the *Drosophila* genome, five NEP family genes (NEP1–5) have been identified. Overexpression of *Drosophila* NEP2 (dNEP2), a fly homologue of human NEP2, has been shown to ameliorate A $\beta$ 42-induced retinal degeneration (71), although unlike human NEP, NEP2 is a secreted protein (72). By quantitative reverse transcription-PCR analysis, we found that the mRNA levels of all fly NEPs, with the exception of NEP2, decreased with age in the fly brain (Fig. 11). Of particular interest, this trend is prominent for NEP1 and NEP3, whose mammalian homologues are NEP and ECE1, respectively.

In summary, we have demonstrated the protective and detrimental aspects of chronically high NEP activity in the fly

brain. Moreover, we observed age-dependent reduction in the mRNA levels and activity of NEP in *Drosophila* brains. Elucidation of the physiological mechanism(s) underlying age-dependent down-regulation of NEP in flies, which is a powerful genetic model system, will facilitate our understanding of brain aging and may lead to the discovery of novel therapeutics in AD.

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## Yin and Yang of Enhanced Neprilysin Activity in the Brain

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