

Epidermal growth factor receptor is a preferred target for treating Amyloid- β -induced memory loss

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Current understanding of amyloid- β (A β) metabolism and toxicity provides an extensive list of potential targets for developing drugs for treating Alzheimer's disease. We took two independent approaches, including synaptic-plasticity-based analysis and behavioral screening of synthetic compounds, for identifying single compounds that are capable of rescuing the A β -induced memory loss in both transgenic fruit fly and transgenic mouse models. Two clinically available drugs and three synthetic compounds not only showed positive effects in behavioral tests but also antagonized the A β oligomers-induced activation of the epidermal growth factor receptor (EGFR). Such surprising converging outcomes from two parallel approaches lead us to conclude that EGFR is a preferred target for treating A β -induced memory loss.

Amyloid- β (A β) oligomers-induced memory loss is thought to be a hallmark of Alzheimer's disease (AD) progression (1–3). A β peptides are cleaved from a membrane protein APP via β - and γ -secretases' activities (4). They can be removed through activities of neprilysin, insulin-degradation enzyme, and possibly other mechanisms (5–7). A β is also able to bind with a large array of target proteins, such as EphB2, TNF-R1, RAGE1, and NMDA receptor and prion (8–12) to exert a wide range of effects, including synaptic transmission, protein transportation, mitochondrial functions, and others (13–15). Thus, there are a large number of potential targets for developing AD treatment based on the A β hypothesis, for example, the mechanisms either reducing the production of A β peptides or enhancing the degradation process. It remains, however, an open question as to whether some of these targets are, at the organism level, better suited for drug development than others. One important reason is that manipulating activities of such production and degradation enzymes may affect many other physiological proteins. Thus, reported failures in a number of A β -based drug efforts (16) stress the necessity of identifying such preferred targets.

To evaluate the possibility of finding preferred targets, we looked for overlapping and converging effects of identified targets with multiple independent approaches. First, following the direction of a synaptic-plasticity-based, mechanism-guided study, we continued to work on the signal transduction pathway of PI3-kinase that has been shown to mediate an A β -induced change in long-term synaptic depression as well as the A β -induced memory loss in A β 42-expressing *Drosophila*, which recapitulates several AD-like symptoms (17). These efforts led us to find A β 42 oligomers-induced activation of the epidermal growth factor receptor (EGFR) and the rescue of A β -induced memory loss in transgenic *Drosophila* and APP/PS1 double transgenic mouse models through treatments with clinically available EGFR inhibitors. Second, we worked to identify single compounds capable of rescuing A β -induced memory loss through large-scale behavioral screening with A β 42-expressing transgenic fruit flies, followed by a confirmative behavioral assay in APP/PS1 double transgenic mice with overloaded A β deposits. The behavioral screening included 2,000 synthetic compounds that are potential inhibitors of protein kinases (randomly selected on the basis of the backbone structures

of the chemicals). We were surprised to learn that four compounds rescued memory loss in both fly and mouse models, and three of them were capable of suppressing oligomeric A β 42-induced EGFR activation. The current work suggests that EGFR is an important factor that mediates A β 42 toxicity and inhibition of oligomeric A β 42-induced EGFR activation is an effective way to treat A β 42-induced memory loss.

Results

Ameliorating A β 42-Induced Memory Loss by Inhibition of EGFRs in Transgenic Fruit Flies. A β -induced memory loss is observed across a wide range of organisms including *Drosophila*, mice, and humans, suggesting a conserved underlying molecular mechanism (18, 19). In *Drosophila*, expression of a secretory form of human A β 42 in the brain recapitulates AD-like features, such as age-dependent accumulation of A β deposits, memory loss, and late-onset severe neurodegeneration (20, 21). Our earlier work reveals a role of PI3-kinase in A β 42-induced alteration in long-term depression and memory loss (17). To find receptors associated with this PI3-kinase effect, we first evaluated behavioral effects of overloaded insulin receptor (InR) or EGFR in A β 42-expressing *Drosophila* brains.

The severity of the memory loss induced by pan-neuronal expression of A β 42 (*elav-Gal4 > UAS-A β 42*) depended on the amount of expressed A β 42. Female adult transgenic flies (*elav-Gal4/+;UAS-A β 42/+*) were selected for the following genetic interaction study because of their lower expression level of A β 42 resulting from gene-dosage effects (20).

Memory was measured through a well-established aversive Pavlovian olfactory conditioning assay (22), in which fruit flies learn to avoid an odor paired with electric shock in training trials. In 5-d-old transgenic female flies, expression of A β 42 showed no detectable memory loss, and overexpressing InR or EGFR (*elav-Gal4/+;UAS-InR^{wt}/+* or *elav-Gal4/+;UAS-EGFR^{wt}/+*) had very little effect on memory performance (Fig. 1A). In contrast, coexpression of EGFR with A β 42 (*elav-Gal4/+;UAS-A β 42/+;UAS-EGFR^{wt}/+*) produced a synergistic effect in reducing the immediate memory, whereas coexpression of InR with A β 42 (*elav-Gal4/+;UAS-A β 42/UAS-InR^{wt}*) yielded a memory score similar to that found with

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Conflict of interest statement: Behaviorally effective compounds reported in this study are under patent filings entitled Methods and compositions for treating Alzheimer's disease.

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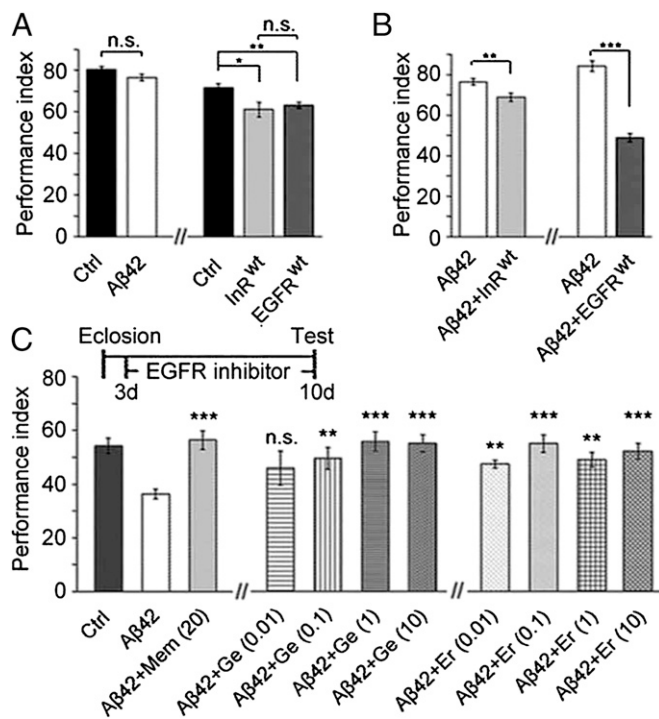


Fig. 1. Identification of the involvement of EGFR in Aβ42-induced immediate memory loss in *Drosophila*. (A) In 5-d-old adult female flies, no memory effects were observed in Aβ42-expressing flies, but slight defects were shown owing to pan-neuronal expression of either InR or EGFR (*elav/+;UAS-InR^{wt}/+* or *elav/+;UAS-EGFR^{wt}/+*). $n = 10$ –13 PIs. All data in this and following figures, unless otherwise indicated, are means \pm SEM, and for *t* test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant, $P > 0.05$. (B) Synergistic effect of EGFR, but not InR, on Aβ42-induced memory loss (*elav/+;UAS-Aβ42/+;UAS-EGFR^{wt}/+* and *elav/+;UAS-Aβ42/+;UAS-InR^{wt}*). $n = 14$ –29 PIs. (C) Rescue of Aβ42-induced memory loss. Drug-feeding paradigm for fruit flies is illustrated for this and following figures (Upper). The histograms represent immediate memory for effects of drug feeding (Lower). Drug concentrations (in $\mu\text{g}/\text{mL}$) are indicated in brackets and the control is treated with sucrose. Mem, memantine; Ge, gefitinib; Er, erlotinib. $n = 8$ PIs for each group.

overexpression of InR alone (Fig. 1B). Such genetic interaction data imply that increased EGFR activity, instead of InR, might be relevant in causing the Aβ42-induced memory loss.

To verify this genetic observation, we tested effects of two EGFR inhibitors, gefitinib (Ge) and erlotinib (Er), which are used in clinical cancer therapy (23). Both drugs inhibit the tyrosine kinase activity of EGFR by binding with the ATP-binding sites that are conserved between fruit flies and humans (24). For the purpose of seeing a stronger memory-loss phenotype, we selected 3-d-old male adults (*elav-Gal4/Y;UAS-Aβ42/+*) for drug treatment. Memory scores were measured at day 10 of posteclosion following 4 h of drug feeding each day for 7 consecutive days (17) (Fig. 1C and Fig. S1A). It was intriguing to note that memantine (Mem), the clinically available drug for AD treatment, was capable of preventing memory loss in Aβ42-expressing flies (Fig. 1C). Feeding of either EGFR inhibitor also prevented memory loss in 10-d-old Aβ42-expressing males over a range of concentrations (0.01, 0.1, 1, and 10 $\mu\text{g}/\text{mL}$; Fig. 1C). Memory was not affected in control flies fed with higher concentrations of either Ge (100 $\mu\text{g}/\text{mL}$) or Er (40 $\mu\text{g}/\text{mL}$) (Fig. S1B). Behavior data suggest that inhibition of EGFRs prevents the Aβ42-induced memory loss in *Drosophila*.

Rescuing Memory Loss by Oral Administration of an EGFR Inhibitor in APP/PS1 Double Transgenic Mice. To determine the general significance of such observations, we tested the effects of Ge, which penetrates the blood–brain barrier and has some effectiveness in

treating brain tumors (25, 26), in Tg(APP^{swe}.PSEN1dE9) double transgenic mice with the expression of two mutated AD-linked transgenes: a chimeric human amyloid beta (A4) precursor protein (APP^{swe}) and a “DeltaE9” mutant of human presenilin 1 (27). Extensive plaques are reported to be visible in early ages and the memory-loss phenotype is evident around 6–9 mo of age in double transgenic mice (27–31). The Morris water maze was used for the behavioral assay, in which mice learned to find a hidden platform (32). For drug treatment, we chose a very short period of feeding paradigm, including only a 7-d pretesting drug treatment followed with a 2-d adaption to the water maze environment and then 9 d of training and testing (Fig. 2A). We reasoned that such a short period of treatment is sufficient to reverse behavioral defects if the mechanism we identified is indeed a direct cause of the Aβ-induced memory loss. For this reason, we used 8-mo-old mice in which the memory-loss phenotype was evident.

Indeed, the treatment was sufficient in rescuing memory loss. Mice were trained four times a day over a period of 9 d. After training, the escape latency of wild-type (control) mice decreased (Fig. 2B–E, open circles), indicating that the mice learned the location of the hidden platform. However, the double transgenic mice did not improve their performance (Fig. 2B–E, filled circles). The memory loss of APP/PS1 mice was rescued after feeding with Ge in an optimal concentration (0.01 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$; Fig. 2D) and in a wide range of concentrations (Fig. 2C, D, F), which were hundreds, if not thousands, of times lower than that used for treating tumors in mice (26, 33). In contrast, with such a short period of treatment Mem was unable to improve the memory-loss phenotype (Fig. 2E), although it was reported to be effective with much longer treatment times (32, 34). Effects of Ge were also consistent in the plot of representative paths (Fig. 2G) and in quadrant occupancy times (Fig. 2H and I). Thus, inhibition of EGFR activity is capable of rescuing the Aβ-induced memory loss in mice.

Oligomeric Aβ42-Induced Activation of EGFRs. To determine molecular mechanisms underlying observed genetic and pharmacological effects of EGFR in Aβ-induced memory loss, we assayed the level of EGFR activation in the hippocampus region through Western blotting of p-EGFR^{Tyr1068}, the site for binding with Grb2 that leads to activation of MAPK (35). The p-EGFR level was significantly increased in the hippocampus of the double transgenic mice (Fig. 3). Importantly, the increased p-EGFR level was brought back to a level similar to the control after 18 d (the duration used for memory rescue) of Ge treatment (10 $\text{mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, Fig. 3), showing that elevated EGFR activity correlated well with the Aβ-induced memory loss.

To make sure that the EGFR activation was caused by expressed Aβ, we measured p-EGFR levels in cultured COS-7 cells, a cell line derived from monkey kidney with low background affinity with Aβ42 (12), transfected with human wild-type EGFR (EGFR^{wt}). Monomeric and oligomeric Aβ42 preparations were produced separately (36). We had no estimation of precise concentrations of each aggregation form (Fig. S2A). Western blotting showed that although expression levels of EGFRs were not affected after application of 25 $\mu\text{g}/\text{mL}$ Aβ42 from oligomeric preparation, the level of p-EGFR or activated EGFR was significantly elevated (Fig. 4A and Fig. S2B). In contrast, treatment with monomeric Aβ42 preparation had the opposite effect on EGFR activation (Fig. S2C). EGFR inhibitors, Er and Ge, suppressed both EGF- and Aβ42 oligomers-activated EGFR (Fig. 4B).

To gain further insights into such Aβ-induced activation of EGFR, we performed immunoprecipitation to determine whether endogenously produced Aβ42 could directly bind to EGFRs. COS-7 cells were cotransfected with genes encoding a secretory form of Aβ42 and a human EGFR^{wt}. Cell lysates were assayed for coimmunoprecipitation with rabbit anti-EGFR 48 h later. We found that both Aβ42 monomers and oligomers, probably dimers

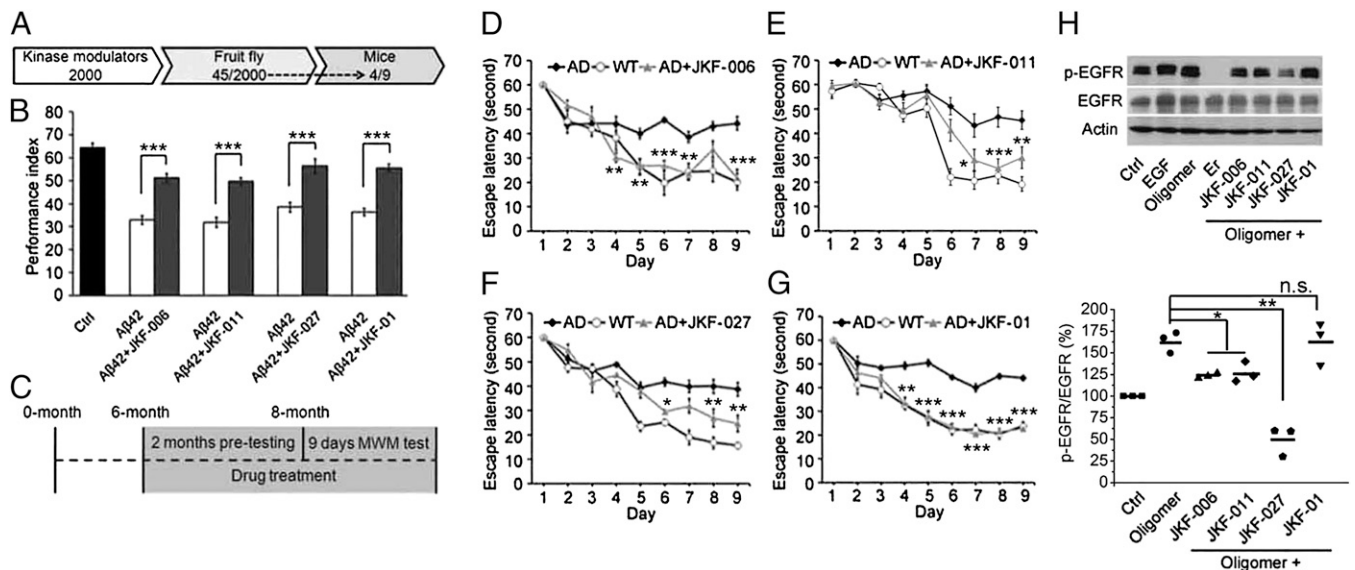


Fig. 5. Behavioral screening of synthetic compounds. (A) Schematic illustration of screening processes and summary of results. (B) Prevention of A β 42-induced memory loss in *Drosophila*. Effects of four representative compounds are shown. Concentrations: 50 μ g/mL, $n = 6-8$ Pls. (C) Drug feeding scheme for double transgenic mice. Six-month-old mice were subject to a 2-mo drug treatment and then to the MWM test. (D-G) Rescue of A β -induced memory loss in mice. Results of the four screened compounds as in B are shown. Concentrations (in milligrams per kilogram per day) are 58 for JKF-006, 14.4 for JKF-011, 55 for JKF-027, and 40 for JKF-01. $n = 6-9$. (H) Effects of four positive compounds (all in 100 μ g/mL) on 10 μ g/mL oligomeric A β 42-induced EGFR activation in COS-7 cells transfected with EGFR^{wt} plasmid. Except JKF-01, the other three compounds were capable of antagonizing the induced p-EGFR elevation. $n = 3$.

systems at every level in *Drosophila* might be favored in helping to identify drug-preferred and conserved targets.

Taken together, convergent outcomes from mechanism-guided study and behavioral screening of synthetic chemicals lead us to conclude that EGFR is a preferred target for treating A β -induced memory loss. Because inhibition of EGFR activity represents an efficient treatment for A β 42-induced deficits in transgenic animals, it would be of interest to see further effects of EGFR inhibitors as well as behaviorally screened chemicals in treatments of AD patients.

Materials and Methods

***Drosophila* Stock.** Human wild-type A β 42 transgenic flies (*UAS-A β 42*) used in this study have been previously described (21). *UAS-InR^{wt}* and *UAS-EGFR^{wt}* were obtained from the Bloomington *Drosophila* Stock Center. *elav-Gal4* is a laboratory stock. Flies were raised and maintained at room temperature (22–24 °C). All stock used for Pavlovian olfactory conditioning were equilibrated by five generations of out-cross to the isogenic line, *w¹¹¹⁸* (*isoCJ1*).

Pavlovian Olfactory Associative Immediate Memory. The training and testing procedures were the same as previously described (22). During one training session, a group of 100 flies was sequentially exposed for 60 s to two odors, 3-octanol (OCT, Fluka) or 4-methylcyclohexanol (MCH, Fluka), with 45 s of fresh air between presentations. Flies were subjected to foot-shock (1.5-s duration with 3.5-s interstimulus interval, 60 V) during exposure to the first odor (CS+) but not to the second (CS-). To measure “immediate memory” (also referred to as “learning”), flies were transferred immediately after training to the choice point of a T-maze and forced to choose between the two odors for 2 min. Then flies were trapped in their respective T-maze arms, anesthetized, and counted. A performance index (PI) was calculated from the distribution of this group of flies in the T-maze. A reciprocal group of flies was trained and tested by using OCT as the CS+ or MCH as the CS+, respectively. The so-called half-PIs, PI (OCT) and PI (MCH), were finally averaged and multiplied by 100, yielding an $n = 1$. A PI of 0 indicated a distribution of 50:50 (no learning), whereas a PI of 100 indicated “perfect learning”: 100% of the flies avoided the CS+ previously paired with foot shock. Control groups were age-matched to the experimental groups in each test.

Mouse Strains and Genotyping. Double transgenic mice that express a mutant chimeric mouse/human APP^{sw} and a mutant human presenilin 1 (Delta E9), both driven by the prion protein promoter, were purchased from The Jackson Laboratory [strain B6C3-Tg(APP^{sw}.PSEN1dE9)85Dbo/J]. Transgenic mice were derived from B6C3/Tg+ \times B6C3 crosses. Genotyping is done by PCR following

Jackson Laboratory protocols [primers for Tg(APP): 5' AAT AGA GAA CGG CAG GAG CA 3' and 5' GCC ATG AGG GCA CTA ATC AT 3'; primers for Tg(PSEN1): 5' AGG ACT GAC CAC TCG ACC AG 3' and 5' CGG GGG TCT AGT TCT GCA T 3']. Tg+ and their Tg- littermates were randomly assigned to various groups for drug treatment or vehicle control. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tsinghua University.

Morris Water Maze. The Morris water maze was performed as in previous reports (30, 32). A water tank (120 cm in diameter) was filled with room-temperature water (19–20 °C) that was made opaque with milk. A transparent platform (15 cm in diameter) was placed in the center of one of the four virtually divided quadrants and 2 cm below the water surface. Distal cues were provided in all experiments as spatial references. Mice swam until they found the platform and remained on it for at least 5 s. During the first day of the experiment (day 1), if a mouse did not find the platform within 60 s, it was gently guided to the platform and given the 5-s stay. From days 2–9, if animals did not find the platform within 60 s, they were given a nominal latency of 60 s. Four trials were performed each day. A video tracking system (Jiliang Software Technology) was used to record data.

Latency to find the platform (maximum of 60 s) was recorded for each trial and the four daily trials were averaged for statistical analysis. Averaged escape latencies of double transgenic mice (AD mice) and drug-treated AD mice on days 7–9 were calculated. Their differences were used to indicate the dosage effect of drug at different concentrations (Fig. 2F).

Probe Trial. The platform was removed on day 10, 1 d after the memory ability test. Mice were allowed to swim for 60 s. The time spent in each quadrant and path length were recorded.

Drug Treatment of Animals. Gefitinib and erlotinib were purchased from LC Laboratories. Memantine was bought from Sigma-Aldrich.

For drug feeding treatments for flies, all chemicals (gefinitinib, erlotinib, memantine, and synthetic compounds) were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at –20 °C. Flies were maintained at 29 °C after eclosion. Flies were starved for 3 h in empty vials and then fed with drugs, diluted in 4% sucrose (chemical concentrations are vol/vol for liquid and wt/vol for solid), for another 4 h. Flies were transferred to normal food after treatment. Drug feeding was carried out once each day during the treatment period.

For treating mice, all chemicals were dissolved in physiological saline containing 0.5% Tween-80. Drugs were prepared every week. Tg+ and Tg- mice received one daily dose of drugs or vehicle by intragastric administration. Except gefitinib and memantine, which were subjected to an 18-d procedure

with 8-mo-old mice, other drugs were used for a 2-mo treatment on 6-mo-old mice until the end of experiments. Drug treatments were carried out after the last trial every day during the testing period.

Statistical Analysis. Data were analyzed by *t* test or post hoc test following ANOVA (Origin version 8; OriginLab Corporation). Statistical results are presented as means \pm SEM. Western blotting results are shown as individual data points and means (horizontal lines). Asterisks indicates critical values (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001).

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