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

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Current understanding of amyloid-β (Aβ) 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 Aβ-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 Aβ-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 Aβ-induced memory loss.

Amyloid-β (Aβ) oligomers–induced memory loss is thought to be a hallmark of Alzheimer’s disease (AD) progression (1–3). Aβ 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). Aβ 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 Aβ hypothesis, for example, the mechanisms either reducing the production of Aβ 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 Aβ-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 Aβ-induced change in long-term synaptic depression as well as the Aβ-induced memory loss in Aβ42-expressing Drosophila, which recapitulates several AD-like symptoms (17). These efforts led us to find Aβ42 oligomers–induced activation of the epidermal growth factor receptor (EGFR) and the rescue of Aβ-induced memory loss in transgenic Drosophila and APP/PS1 double transgenic mouse models through clinically available EGFR inhibitors. Second, we worked to identify single compounds capable of rescuing Aβ-induced memory loss through large-scale behavioral screening with Aβ42-expressing transgenic fruit flies, followed by a confirmative behavioral assay in APP/PS1 double transgenic mice with overloaded Aβ 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 Aβ42-induced EGFR activation. The current work suggests that EGFR is an important factor that mediates Aβ42 toxicity and inhibition of oligomeric Aβ42-induced EGFR activation is an effective way to treat Aβ42-induced memory loss.

Results

Ameliorating Aβ42-Induced Memory Loss by Inhibition of EGFRs in Transgenic Fruit Flies. Aβ-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 Aβ42 in the brain recapitulates AD-like features, such as age-dependent accumulation of Aβ deposits, memory loss, and late-onset severe neurodegeneration (20, 21). Our earlier work reveals a role of PI3-kinase in Aβ42-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 Aβ42-expressing Drosophila brains. The severity of the memory loss induced by pan-neuronal expression of Aβ42 (elav-Gal4 > UAS-Aβ42) depended on the amount of expressed Aβ42. Female adult transgenic flies (elav-Gal4/+; UAS-Aβ42/) were selected for the following genetic interaction study because of their lower expression level of Aβ42 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 Aβ42 showed no detectable memory loss, and overexpressing InR or EGFR (elav-Gal4/+; UAS-InR147e3/+ or elav-Gal4+/+; UAS-EGFR121e3/+ or UAS-EGFR121e3i10e7/+ or UAS-EGFR121e3i10e7i7e7) had very little effect on memory performance (Fig. 1L). In contrast, coexpression of EGFR with Aβ42 (elav-Gal4+/+; UAS-Aβ42+/+; UAS-EGFR121e3i10e7i7e7) produced a synergistic effect in reducing the immediate memory, whereas coexpression of InR with Aβ42 (elav-Gal4+/+; UAS-Aβ42/ UAS-InR147e3) yielded a memory score similar to that found with Aβ42.

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

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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-old male adults (elavGal4/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 reversing memory loss at a concentration of 10 mg·kg⁻¹·d⁻¹, 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 pathways (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-EGFRY1068 (Fig. S2) and in a wide range of concentrations (Fig. 2C, D, F), which were hundreds of times lower than that used for treating tumors in mice (26, 33). 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 mg·kg⁻¹·d⁻¹; Fig. 2D) and in a wide range of concentrations (Fig. 2C, D, F), which were hundreds 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 pathways (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.

**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(APPsw.PSEN1dE9) double transgenic mice with the expression of two mutated AD-linked transgenes: a chimeric human amyloid beta (A4) precursor protein (APPsw) 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. 2D). 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 mg·kg⁻¹·d⁻¹; Fig. 2D) and in a wide range of concentrations (Fig. 2C, D, F), which were hundreds 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 pathways (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.
according to the molecular weight, were pulled down with EGFRβ3 (Fig. 4C).

Taken together, these results obtained from mechanism-guided study support the hypothesis that EGFR functions as a cell membrane receptor of Aβ peptides, and the Aβ oligomers-induced activation of EGFR plays a critical role in leading to memory loss.

Behaviorally Identified Effective Synthetic Compounds Antagonize Oligomeric Aβ42-Induced EGFR Activation. In parallel with the mechanism-guided study presented above, we performed behavioral screening of 2,000 synthetic compounds (purchased from TimTec LLC) with structures that are presumably targeted to protein kinase activities. The drug feeding scheme for fruit flies is indicated in Fig. 1C. Only male flies were selected for 7-d drug treatment and then subjected to behavioral assay at day 10 after eclosion. Going through an initial n = 2 screening followed with n > 6 confirmation, 45 synthetic compounds were found to be effective in rescuing memory loss in Aβ42 transgenic flies. Among them, nine were tested in double transgenic mice, and four compounds were indicated to have positive results after 2 mo of treatment (6–8 mo of age). The summary of behavioral screening and testing is depicted in Fig. 5A. Behavioral data are provided for these four compounds related to rescuing memory loss in flies (Fig. 5B) and mice (Fig. 5C–G). We were amazed to find that three compounds, designated as JKF-006, JKF-011, and JKF-027 (Table S1 shows their structures), not only showed effective results in rescuing memory loss, but also significantly antagonized a 10 μg/mL oligomeric Aβ42-induced phosphorylation of human EGFR expressed in COS-7 cells (Fig. 5H).

Discussion

Data presented above demonstrate that overactivation of EGFRs plays a critical role in causing Aβ-induced memory loss. We first showed that expression of additional EGFRs exacerbated memory loss, whereas inhibition of EGFR activity through oral administration of two different drugs rescued memory loss in Aβ42-expressing fruit flies. One would expect that reduced EGFR activity through either genetic manipulation or pharmacological treatment should prevent or slow Aβ42-induced memory loss. Genetic manipulation, such as the use of a loss-of-function mutation or RNAi knockdown, however, was not adopted in the study because of potential complications during development. Second, we showed that the level of p-EGFR (activated EGFR) was elevated in the hippocampal tissues of APP/PS1 double transgenic mice and this increase was suppressed by feeding Ge. Treatment with this EGFR inhibitor completely rescued impaired memory in mice. It is worth noting that a dose as low as 0.01 mg·kg⁻¹·d⁻¹ is behaviorally effective in mice, whereas the rescuing effect is moderate with higher doses, such as 40 mg·kg⁻¹·d⁻¹ (Fig. S3). The effective low dose suggests that drug treatment is highly specific to EGFRs. Therefore, suppression of EGFR activity, but not other proteins’ activities, is more likely responsible for the rescued memory. The ineffectiveness of Ge at higher doses suggests that an appropriate level of EGFR activity is critical in maintaining normal physiological function. Alternatively, such ineffectiveness might result from the unidentified side effects of EGFR inhibitors on other protein targets at higher doses. Thus, enhanced EGFR activity appears to be a conserved mechanism in mediating Aβ-induced memory loss in both Drosophila and the mouse.
The EGFR pathway functions to enhance the differentiation, maturation, and survival of a variety of cell types (37). Accumulating evidence also indicates that the EGFR pathway can regulate neuronal plasticity by changing the intracellular Ca\(^{2+}\) concentration or glutamate release in postmitotic neurons (38, 39). In human patients, it is well studied that an increased level of \(egfr\) expression is closely related to tumorigenesis (40); however, the relationship between the EGFR pathway and AD is not clear. Previous reports showed that presenilin and \(A\beta\) could regulate the expression and metabolism of EGFR, which suggested the involvement of EGFR in the AD process (15, 41, 42). In our study, enhanced EGFR activity likely resulted from \(A\beta\) oligomers-dependent activation, possibly through direct binding. We have shown that oligomeric \(A\beta_{42}\) peptides were capable of stimulating EGFR activities, whereas monomeric \(A\beta_{42}\) expressed opposite effects. In combination with observations of elevated EGFR activities in the hippocampus and the rescuing effects of EGFR inhibitors on memory loss, we propose that \(A\beta\) oligomers-induced EGFR activation results in memory loss. However, which forms of oligomers lead to such activation remains to be determined. Immunoprecipitation assays suggested a potential binding between \(A\beta\) oligomers and EGFRs. However, we should point out that there is no direct evidence showing whether such binding leads to activation of EGFRs and, in particular, how oligomers bind with the receptors. It is interesting to note that \(A\beta\) oligomers also bind with EphB2, another receptor tyrosine kinase, and such binding is reported to play a critical role in mediating memory loss in another mouse model of AD (8).

The memory loss dependent on an oligomeric \(A\beta\)-induced activation of EGFR may reflect an acute toxic effect of \(A\beta\), which might be independent of synaptic and neuronal degeneration. To gain insights into the nature of observed EGFR effects, we purposely shifted the drug feeding paradigm of Ge to a short-period treatment. Only 18 d (the shortest we tested) of drug treatment was sufficient to rescue memory loss of 8-mo-old APP/PS1 mice, which had severe memory loss but few morphological changes in the brain. In the current study, behavior results of Ge treatments in the mouse model contrasted sharply with those of Mem, which had no memory-rescuing effects with the short-period treatment. However, in other reports, Mem was effective after oral administration for several months (32, 34). We also noted that treating APP/PS1 mice with Ge for 18 d did not significantly influence the \(A\beta\) aggregation (oligomers and amyloid plaques) level in the brain (Fig. S4). These observations lead us to hypothesize that EGFR hyperactivity-dependent memory loss is due to acute effects of \(A\beta\) oligomers. It is known that \(A\beta\) oligomers are capable of disrupting long-term synaptic plasticity (1) and the downstream signaling pathways of EGFR, such as PI3-kinase and Ras, which are important in regulating long-term synaptic plasticity (17, 37, 43).

This acute role of \(A\beta\) oligomers could be a major mechanism causing \(A\beta\) oligomers-dependent memory loss. We were surprised to find that three out of four behavior-screening identified compounds were capable of suppressing the oligomeric \(A\beta\)-induced activation of EGFRs. One possible explanation for such a high ratio is that EGFR activation might be a major component leading to \(A\beta_{42}\)-induced memory loss in 10-d-old fruit flies. Such mechanisms, however, might be more complex in mice because of their genomic complexity and longer progression toward developing memory deficits. As a result, mouse-based drug screening would yield a much lower ratio in converging to one component. Simpler
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 Akt-induced memory loss. Because inhibition of EGFR activity represents an efficient treatment for Akt-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 Akt42 transgenic flies (UAS-Akt42) used in this study have been previously described (21). UAS-Inh**β**42** and** UAS-**EGFR** 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, **w**1118 (isoC11).

**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 mouse APPsw and a mutant human presenilin 1 (Delta E9), both driven by the prion protein promoter, were purchased from The Jackson Laboratory [strain B6C3-Tg(APPsw.PSEN1ΔE9)85Dbo]. Transgenic mice were derived from B6C3Tg+ × 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 CACTG 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 (gefitinib, 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 volume/volume for liquid and weight/volume 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 FEBS Lett or Stock Center, Jackson Laboratory, and Center 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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