Blockade of EphA4 signaling ameliorates hippocampal synaptic dysfunctions in mouse models of Alzheimer's disease

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Alzheimer's disease (AD), characterized by cognitive decline, has emerged as a disease of synaptic failure. The present study reveals an unanticipated role of erythropoietin-producing hepatocellular A4 (EphA4) in mediating hippocampal synaptic dysfunctions in AD and demonstrates that blockade of the ligand-binding domain of EphA4 reverses synaptic impairment in AD mouse models. Enhanced EphA4 signaling was observed in the hippocampus of amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mouse model of AD, whereas soluble amyloid-β oligomers (Aβ), which contribute to synaptic loss in AD, induced EphA4 activation in rat hippocampal slices. EphA4 depletion in the CA1 region or interference with EphA4 function reversed the suppression of hippocampal long-term potentiation in APP/PS1 transgenic mice, suggesting that the postsynaptic EphA4 is responsible for mediating synaptic plasticity impairment in AD. Importantly, we identified a small-molecule rhynchophylline as a novel EphA4 inhibitor based on molecular docking studies. Rhynchophylline effectively blocked the EphA4-dependent signaling in hippocampal neurons, and oral administration of rhynchophylline reduced the EphA4 activity effectively in the hippocampus of APP/PS1 transgenic mice. More importantly, rhynchophylline administration restored the impaired long-term potentiation in transgenic mouse models of AD. These findings reveal a previously unidentified role of EphA4 in mediating AD-associated synaptic dysfunctions, suggesting that it is a new therapeutic target for this disease.

Abeta | receptor tyrosine kinase | ephrin | drug discovery

Cognitive impairment, regarded as an early manifestation of Alzheimer's disease (AD), is attributable to disruptions of synaptic functions which correlate with the severity of memory deficit in AD (1). Soluble amyloid-β peptide oligomers (Aβ), which are generated by the proteolytic cleavage of amyloid precursor protein (APP), are believed to be a major causative agent of synaptic impairment during AD progression (2). Thus, reversing Aβ-induced synaptic deficits is considered a promising therapeutic approach for alleviating cognitive impairment in AD (3).

Aβ binds to synaptic sites (4), resulting in synaptic loss and reduced glutamatergic synaptic transmission (5, 6). Aβ also rapidly impairs synaptic plasticity in the hippocampus; this includes the inhibition of long-term potentiation (LTP) (2) and facilitation of long-term depression (LTD) (7), which are major cellular mechanisms associated with learning and memory. Synaptic defects triggered by Aβ are mediated by the internalization and downregulation of both NMDA- and AMPA-type glutamate receptors (8, 9) together with a reduction of dendritic spines (6), where excitatory synapses are located. Therefore, identifying molecular targets that mediate the action of Aβ in synaptic depression in AD is crucial for the development of therapeutic interventions for AD. Interestingly, various cell surface receptors such as α7-nicotinic acetylcholine receptors, metabotropic glutamate receptors, insulin receptors, and the receptor tyrosine kinase, EphB2, are reported to mediate the action of Aβ at synapses (10).

The erythropoietin-producing hepatocellular (Eph) family of receptor tyrosine kinases is important for the regulation of synapse development and synaptic plasticity (11, 12). EphB enhances synapse development via its interaction with NMDA receptors (13), whereas EphA4, which is mainly expressed in the adult hippocampus, acts as a negative regulator of neurotransmission and hippocampal synaptic plasticity (14). EphA4 activation by its ligands, ephrins, triggers forward signaling (12) that leads to the retraction of dendritic spines via cyclin-dependent kinase 5 (Cdk5)-dependent RhoA activation and reduced cell adhesion (15–17). EphA4 also causes the removal of synaptic and surface AMPA receptors during homeostatic plasticity (18, 19). Interestingly, AD patients with only mild cognitive deficits exhibit deregulated EphB and EphA4 expression (20). Given that EphA4 activation results in dendritic spine loss and reduced AMPA receptor abundance (14, 19, 21), which are potential mechanisms that underlie synaptic dysfunctions in AD (6, 8), we investigated the possible link between EphA4 signaling and Aβ-induced synaptic failure.

The present study demonstrates that EphA4 mediates the Aβ-induced impairment of synaptic plasticity. Depletion of postsynaptic EphA4 or blockade of the activity of EphA4 through targeting its ligand-binding domain reversed the synaptic deficits in AD mouse models. Importantly, molecular

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docking analysis identified a small molecule, rhynchophylline (Rhy), as a candidate EphA4 inhibitor. Rhy rescued the impaired neurotransmission induced by Aβ as well as the LTP defects in the AD mouse models. Thus, the present findings not only reveal an important role of EphA4 in the pathogenesis of AD, but also identify a small-molecule inhibitor of EphA4 that can be further developed as a potential therapeutic intervention for AD.

Results

Aβ Stimulates EphA4 Activation in Neurons. To investigate if EphA4 is involved in synaptic dysfunctions upon AD progression, we examined the regulation of EphA4 protein and its activity in the hippocampus of AD mouse models. EphA4 was prominently detected in mouse hippocampal synaptosomal fractions, and its expression remained relatively unchanged upon development and aging (Fig. 1*A* and *SI Appendix*, Fig. S1 *A* and *B*). The tyrosine phosphorylation of EphA4 at residue 602 (P-Tyr EphA4), which reflects the autophosphorylation status of the receptor, was up-regulated in synaptosomal fractions of the mouse hippocampus from 6 to 11 mo (*SI Appendix*, Fig. S1 *A* and *B*). Interestingly, P-Tyr

EphA4 levels were elevated in the hippocampal synaptosomal fractions of an AD transgenic mouse model (APPswePS1de9, hereafter designated APP/PS1) at as early as 3 mo (Fig. 1 *A* and *B*). Soluble Aβ could be detected in 3-mo–old APP/PS1 mice (*SI Appendix*, Fig. S1*C*), whereas impaired synaptic plasticity was first observed in AD mouse models at ∼6 mo (22, 23). Therefore, the increase in EphA4 activity in the hippocampus of 3-mo–old APP/PS1 mice is concordant with the notion that EphA4 is a potential cellular target of Aβ that contributes to synaptic dysfunctions in AD.

Next, we examined whether EphA4 signaling in neurons is activated by Aβ. Both EphA4 tyrosine phosphorylation and clustering are required for maximal receptor activation (18, 24). Aβ increased the EphA4 tyrosine phosphorylation in acute rat hippocampal slices in a dose-dependent manner (Fig. 1*C* and *SI Appendix*, Fig. S1*D*); the increase was observed at as early as 1 h after treatment and remained high at 2 h (Fig. 1*D* and *SI Appendix*, Fig. S1*E*). Aβ also enhanced EphA4 clustering in cultured hippocampal neurons (Fig. 1 *E* and *F*) and enhanced the activation of Cdk5, a downstream target of EphA4 (*SI Appendix*, Fig. S2). Together, these results suggest that Aβ rapidly induces EphA4 activation and downstream signaling in hippocampal neurons.

EphA4 is a type I transmembrane protein with an N-terminal ectodomain comprising an ephrin-binding domain, a cysteine-rich region, and a fibronectin type III repeat domain (25). Interestingly, blockade of the extracellular ligand-binding domain of EphA4 with a 12-amino acid peptide inhibitor KYLPYWPVLSSL (KYL) (26), abolished the Aβ-stimulated EphA4 tyrosine phosphorylation (Fig. 1 *G* and *H*). These findings suggest that EphA4–ligand interaction is critical for Aβ-triggered EphA4 activation in hippocampal neurons. Interestingly, the ligand-binding domain of EphA4 was able to bind Aβ in vitro (*SI Appendix*, Fig. S3), raising the interesting possibility that EphA4 is a direct cellular target of Aβ.

Blockade of EphA4 Activation Prevents Aβ-Induced Synaptic Dysfunctions. Given the negative regulatory roles of EphA4 in synaptic transmission and plasticity (16, 19, 21), the ability of Aβ to activate EphA4 might contribute to the synaptic dysfunctions observed in AD. To test this hypothesis, the effect of KYL peptide on Aβ-induced dendritic spine loss was examined. Reduced spine density was observed in cultured hippocampal neurons after Aβ treatment for 24 h, whereas cotreatment with KYL peptide abolished the Aβ-triggered reduction of dendritic spines (Fig. 2 *A* and *B*). In addition to reducing the number of dendritic spines (27), Aβ also reduced neurotransmission in hippocampal neurons (6) as evidenced by the decreased frequency of AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs). Treating neurons with Aβ significantly increased the interevent interval, which is inversely proportional to frequency (Fig. 2 *C*–*F* and *SI Appendix*, Fig. S4). To verify that EphA4 activation is required for Aβ-mediated neurotransmission, EphA4 signaling was blocked by adding the unclustered extracellular domain of EphA4 (i.e., EphA4-Fc), which interacts with endogenous ephrin ligand and hence prevents EphA4 activation and forward signaling (16). EphA4-Fc similarly rescued the Aβ-induced synaptic depression and blocked the Aβ-induced increase of the interevent interval of mEPSCs (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S4*A*). The importance of EphA4 in mediating Aβ-stimulated synaptic dysfunction was further confirmed in $EphA4^{-/-}$ neurons. Aβ reduced mEPSC frequency by ∼40% in hippocampal neurons prepared from $EphA4^{+/+}$ mice, whereas the decrease was abolished in $EphA4^{-/-}$ hippocampal neurons (*SI Appendix*, Fig. S5). Intriguingly, blockade of EphA4 or Cdk5 signaling by [2,5-dimethylpyrrolyl benzoic acid (Cpd1)] (28) or [roscovitine (Ros)] (16), respectively, also attenuated the Aβ-stimulated reduction in neurotransmission (Fig. 2 *E* and *F* and *SI Appendix*, Fig. S4*B*). Taken together, these observations suggest that blockade of EphA4/Cdk5 signaling rescues the Aβ-induced suppression of neurotransmission.
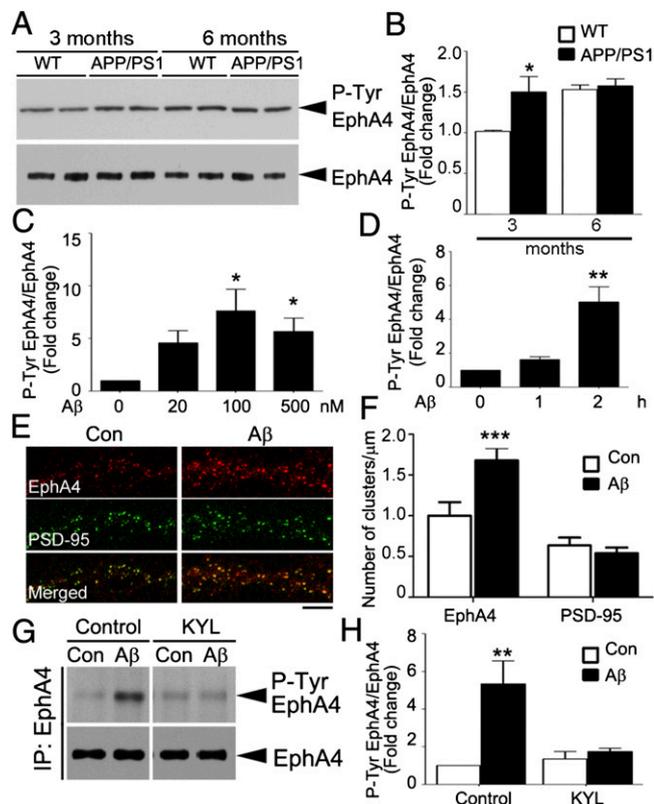

Fig. 1. Aβ stimulates the activation of EphA4 signaling. (*A* and *B*) Western blot of Tyr602 EphA4 phosphorylation (P-Tyr EphA4) in hippocampal synaptosomal fractions of WT and APP/PS1 mice. Quantification analysis (*$P <$ 0.05; one-way ANOVA, Student–Newman–Keuls test; $n ≥ 3$ hippocampi). (*C* and *D*) Aβ increased EphA4 tyrosine phosphorylation. Acute rat hippocampal slices were treated with Aβ at different concentrations for 2 h or 500 nM for various periods. EphA4 was immunoprecipitated, and subjected to Western blotting for P-Tyr EphA4. (*$P <$ 0.05, **$P <$ 0.01; one-way ANOVA, Student–Newman–Keuls test; $n ≥ 3$ slice samples.) (*E* and *F*) Aβ increased the number of EphA4 clusters. (*E*) Representative images. (Scale bar, 10 μm.) (*F*) Quantification analysis (***$P <$ 0.001, Student *t* test, $n = 15$ neurons per group). (*G* and *H*) Blockade of EphA4–ligand interaction abolished the Aβ-stimulated activation of EphA4. Acute rat hippocampal slices were pretreated with KYL peptide followed by Aβ. (*G*) EphA4 was immunoprecipitated and subjected to Western blot analysis for P-Tyr EphA4. (*H*) Fold change of P-Tyr EphA4/EphA4. [**$P <$ 0.01; one-way ANOVA followed by the Student–Newman–Keuls test ($n ≥ 3$ slice samples)].
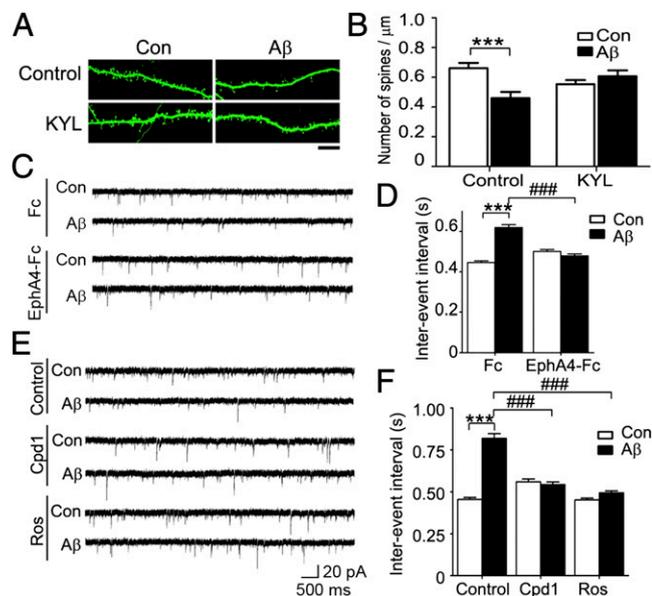
Fig. 2. Inhibition of EphA4 activity prevents Aβ-mediated neurotransmission impairment. (*A* and *B*) KYL peptide attenuated the Aβ-triggered reduction of dendritic spines in hippocampal neurons. (*A*) Representative images. (Scale bar, 10 μm.) (*B*) Quantification analysis [***P < 0.001; two-way ANOVA followed by the Bonferroni posttest (*n* ≥ 22 neurons for each condition)]. (*C* and *D*) Unclustered EphA4-Fc, which inhibits the EphA4 forward signaling, rescued the Aβ-triggered increase in the interevent interval of mEPSCs. (*C*) Representative mEPSC traces. (*D*) mEPSC interevent interval (mean ± SEM, *n* ≥ 39 neurons for each condition). (*E* and *F*) Small-molecule inhibitor for EphA4 (Cpd1) and Cdk5 (Ros) prevented the Aβ-induced increase in interevent interval. (*E*) Representative mEPSCs. (*F*) mEPSC interevent interval (mean ± SEM, *n* ≥ 22 neurons for each condition). (*D* and *F*) ***P < 0.001, ###P < 0.001; one-way ANOVA with Kruskal–Wallis test.

Blockade of EphA4 Signaling Reverses the Impaired Hippocampal Synaptic Plasticity in AD.

To evaluate the effect of EphA4-signaling blockade on Aβ-induced synaptic plasticity impairment, LTP was measured in the hippocampal Schaffer-collateral (SC) pathway in hippocampal slices upon Aβ treatment in the presence of EphA4 inhibitors (i.e., EphA4-Fc or KYL peptide). High-frequency stimulation (HFS) triggered a significant increase in the magnitude of SC–CA1 LTP, whereas LTP was inhibited in Aβ-treated slices (29, 30) (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S6). Pretreating slices with either EphA4-Fc (*SI Appendix*, Fig. S6) or KYL peptide (Fig. 3 *A* and *B*) prevented the Aβ-induced suppression of LTP. Treatment with EphA4-Fc or KYL peptide alone did not significantly affect the HFS-induced LTP (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S6). Taken together, these findings demonstrate that blocking EphA4-mediated signaling alleviates the Aβ-induced impairment in synaptic plasticity.

Next, we examined whether blockade of EphA4 signaling can rescue the impaired synaptic plasticity in AD mouse models. HFS-triggered hippocampal SC–CA1 LTP was impaired in 6- to 7-mo-old APP/PS1 mice compared with littermate controls (23) (Fig. 3 *C–F*). Blockade of EphA4 signaling in the brain for ∼3 wk by intracerebral infusion of KYL peptide restored the LTP formation in APP/PS1 mice (Fig. 3 *C* and *D*). APP/PS1 mice exhibited a lower slope of field excitatory postsynaptic potential (fEPSP) than the WT (i.e., vehicle control), whereas the decrease in LTP was restored in APP/PS1 mice infused with KYL. Similarly, depletion of EphA4 expression in the hippocampal CA1 region in APP/PS1 mice by lentiviral-based EphA4 shRNA alleviated the impaired LTP (Fig. 3 *E* and *F*). EphA4 knockdown in the hippocampal CA1 region partially rescued the impaired LTP of APP/PS1 mice compared with that of the mice infected with GFP-expressing virus. This partial rescue was likely because only a proportion of neurons in the CA1 region were infected

with the EphA4-RNAi–expressing virus (*SI Appendix*, Fig. S7). Thus, these findings show that inhibition of postsynaptic EphA4 function ameliorates the synaptic dysfunctions in APP/PS1 mice as evidenced by the restoration of synaptic transmission and rescue of LTP impairment. Accordingly, it would be of great interest to study whether blocking the EphA4 activity is a promising intervention strategy for AD.

Small-Molecule Inhibitor of EphA4 Identified by Virtual Screening.

Detailed structural analysis of the EphA4–ligand complex (25, 31, 32) provides a promising basis for the virtual screening of small molecules that specifically target the ligand-binding domain of EphA4. In particular, the uniqueness of the ligand-binding pocket in the ectodomain of EphA4 renders the receptor an ideal target for small-molecule screening (31, 33). Therefore, a virtual screening of an in-house traditional Chinese medicine
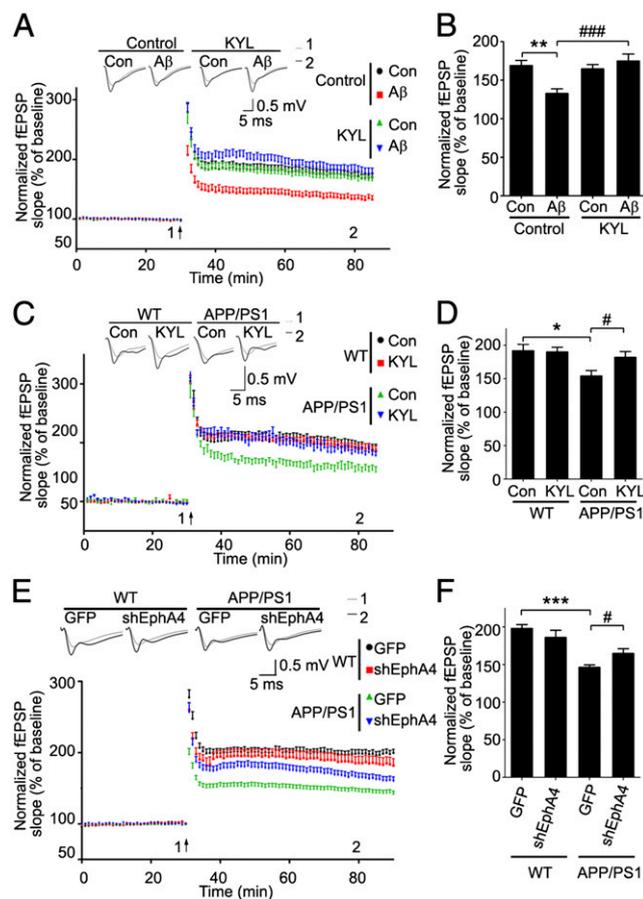

Fig. 3. Blockade of EphA4 signaling rescues the impairment of hippocampal LTP in AD models. (*A* and *B*) Blockade of EphA4 activity rescued the Aβ-induced reduction of LTP. Acute hippocampal slices were treated with Aβ in the presence of KYL peptide. (*C–F*) Blockade of postsynaptic EphA4 signaling rescued the LTP impairment in APP/PS1 mutant mice. WT and APP/PS1 mutant mice were infused with KYL peptide (*C* and *D*); or CA1 regions of WT and APP/PS1 mutant mice were injected with EphA4-shRNA (shEphA4) or GFP virus (*E* and *F*). (*A*, *C*, and *E*) Points represent averaged slopes of fEPSP normalized to baseline values (mean ± SEM). Trace recordings 5 min before (1) and 50 min after (2) LTP induction (arrow) are shown. Inset traces are representative fEPSPs recorded before (gray) and after (black) HFS. (*B*, *D*, and *F*) Quantification of mean fEPSP slopes in the last 10 min of the recording after LTP induction; *n* = ≥ 9 slices from 5 brains for each condition. (*B*) **P < 0.01, two-way ANOVA followed by the Bonferroni posttest; ###P < 0.001, one-way ANOVA followed by the Student–Newman–Keuls test. (*D*) *P < 0.05, #P < 0.05; one-way ANOVA followed by the Student–Newman–Keuls test. (*F*) ***P < 0.001, #P < 0.05; two-way ANOVA with Bonferroni posttest.

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database through molecular docking was performed to search for EphA4 inhibitors. Molecular docking was simulated between the extracellular domain of EphA4 and 225 compounds including a commercially available EphA4 inhibitor, Cpd1 (28), which was previously identified by high-throughput screening. A small molecule, Rhy (*SI Appendix*, Fig. S8*A*), was identified as one of the top three compounds that bind to EphA4 with the lowest docking energies. Rhy is the major alkaloid constituent of *Uncaria rhynchophylla* (Miq) Jack (UR), a Chinese medicinal herb commonly used in formulas targeting central nervous system diseases (34). Nonetheless, the clinical applications of Rhy in neurodegenerative diseases such as AD have not been investigated. The docking analysis demonstrates that Rhy provides a significantly lower docking energy (−9.0 kcal/mol) than Cpd1 (−6.5 kcal/mol), indicating that Rhy binds to EphA4 with higher affinity than Cpd1 (∼67-fold) (33). This strong binding affinity of Rhy may be attributable to its large interaction interface with the ligand-binding domain of human EphA4 (*SI Appendix*, Fig. S8*B*). Rhy notably forms extensive contacts with multiple hydrophobic residues on the ligand-binding channel of EphA4 (*SI Appendix*, Fig. S8*B*). Pulldown analysis revealed that biotinylated Rhy (Bio-Rhy) (*SI Appendix*, Fig. S8*A*) bound specifically to the extracellular domain of EphA4 but not with that of EphB2 (Fig. 4*A*). The effectiveness of Rhy as an EphA4 inhibitor was confirmed based on its ability to antagonize EphA4-dependent signaling and functions. Pretreating hippocampal neurons with Rhy reduced the ephrin-A1–induced tyrosine phosphorylation (Fig. 4 *B* and *C*) and clustering of EphA4 (Fig. 4*D* and *SI Appendix*, Fig. S9*A*). Both Rhy and Bio-Rhy but not its isomer isorhynchophylline (Iso-Rhy) effectively blocked the EphA4-dependent growth cone collapse (Fig. 4*E* and *SI Appendix*, Fig. S9*B*). Furthermore, Rhy pretreatment reduced the tyrosine phosphorylation of EphA4 induced by Aβ in acute mouse hippocampal slices (*SI Appendix*, Fig. S10). Importantly, oral administration of Rhy alleviated EphA4 tyrosine phosphorylation in the hippocampal synaptosomal fractions of APP/PS1 mice (Fig. 4 *F* and *G*), confirming that Rhy attenuates the increased EphA4 activation in the hippocampus of AD model mice.

Oral Administration of Rhy Reverses the Impairment of Hippocampal Synaptic Plasticity in AD Mouse Models. In light of the finding that blockade of EphA4 signaling ameliorates impairments in neurotransmission and synaptic plasticity in different AD models, the effects of Rhy on Aβ-induced synaptic deficits were further examined. We found that pretreating hippocampal neurons or acute hippocampal slices with Rhy rescued the Aβ-induced impairment of mEPSC and LTP. Aβ reduced the mEPSC frequency, whereas Rhy rescued the Aβ-induced reduction in mEPSC frequency (Fig. 5 *A* and *B* and *SI Appendix*, Fig. S11). Furthermore, pretreating acute hippocampal slices with Rhy prevented the suppression of LTP by Aβ, whereas treatment with Rhy alone did not affect hippocampal LTP (Fig. 5 *C* and *D*). Importantly, oral administration of Rhy (50 mg·kg^{-1}·d^{-1}) to ∼5-mo-old APP/PS1 mice for 3–4 wk alleviated the impaired synaptic plasticity (Fig. 5 *E* and *F*). Compared with WT mice, APP/PS1 mice exhibited decreased LTP in response to HFS. Rhy administration completely rescued the reduced LTP in APP/PS1 mice. Rhy also exhibited a similar effect in a dose-dependent manner in another AD mouse model, Tg2576 mice, which express high levels of the Swedish mutated form of human APP (*SI Appendix*, Fig. S12). Together, Rhy effectively abolished the deficits of neurotransmission induced by oligomeric Aβ and rescued the synaptic plasticity in AD mouse models.

Discussion

Emerging evidence indicates that synaptic loss and dysfunction, which are accompanied by neural network failure and cognitive decline in AD, may be the major causes of early AD development. Thus, the amelioration of synaptic dysfunction is a promising therapeutic approach for the treatment of cognitive decline in AD. The present findings demonstrate that EphA4 plays a key
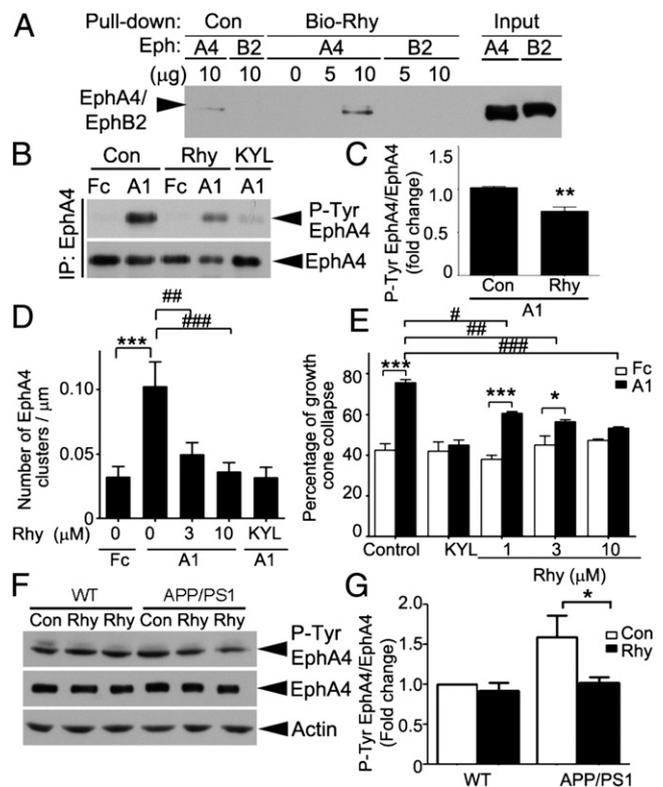

Fig. 4. Rhy is a small-molecule EphA4 inhibitor. (*A*) Rhy binds the extracellular domain of EphA4 but not that of EphB2. In vitro pulldown assay of recombinant EphA4-Fc or EphB2-Fc proteins with biotinylated Rhy (*n* = 3 experiments). (*B* and *C*) Rhy inhibited the ephrin-A (A1)-stimulated EphA4 tyrosine phosphorylation in rat cortical neurons. Lysate was immunoprecipitated with EphA4 antibody and subjected to Western blot analysis for P-Tyr. (*C*) Quantification analysis (mean ± SEM, **$P < 0.01$, Student *t* test, *n* = 5 neuronal cultures per group). (*D* and *E*) Rhy inhibited EphA4-dependent signaling and cellular functions. (*D*) Rhy abolished the ephrin-A1–induced EphA4 clustering. ***$P < 0.005$, ##$P < 0.01$, ###$P < 0.001$; one-way ANOVA followed by the Student–Newman–Keuls test; *n* ≥ 9 neurons. (*E*) Rhy inhibited ephrin-A1–stimulated growth cone collapse (mean ± SEM, ≥150 neurons for each group). *$P < 0.05$, ***$P < 0.001$, two-way ANOVA followed by the Bonferroni posttest; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, one-way ANOVA followed by the Student–Newman–Keuls test. (*F* and *G*) Rhy administration reduced EphA4 activation in the hippocampus of APP/PS1 mice. (*F*) Hippocampal synaptosomal fractions of APP/PS1 mice administered with Rhy were collected. (*G*) Fold change (mean ± SEM, *n* ≥ 3 mice, *$P < 0.05$, two-way ANOVA followed by the Bonferroni posttest).

role in mediating the synaptic dysfunctions in AD and suggest that it is a new therapeutic target for AD. Our results show that blockade of EphA4 activity by targeting its ligand-binding domain using multiple approaches including peptides and small molecules can rescue the impaired synaptic plasticity in AD mouse models. Thus, the development of small-molecule inhibitors targeting the ligand-binding domain of EphA4 might prove to be an effective disease-modifying treatment for AD.

Although Ephs are implicated in the regulation of synaptic functions and plasticity, the possibility of Eph family members being cellular targets of Aβ at synapses was only investigated recently (18, 35). EphB2 is down-regulated in AD and mediates Aβ-dependent synaptic dysfunctions (35), whereas the present study reveals that EphA4 is overactivated in AD, resulting in synaptic dysfunction. It is noteworthy that recent genome-wide association studies have identified a single-nucleotide polymorphism located proximal to the *EPHA4* (36) and *EPHA1* genes that is associated with late-onset AD (37).

Given that blockade of EphA4 signaling rescued the impairment of synaptic plasticity induced by Aβ and that depletion of
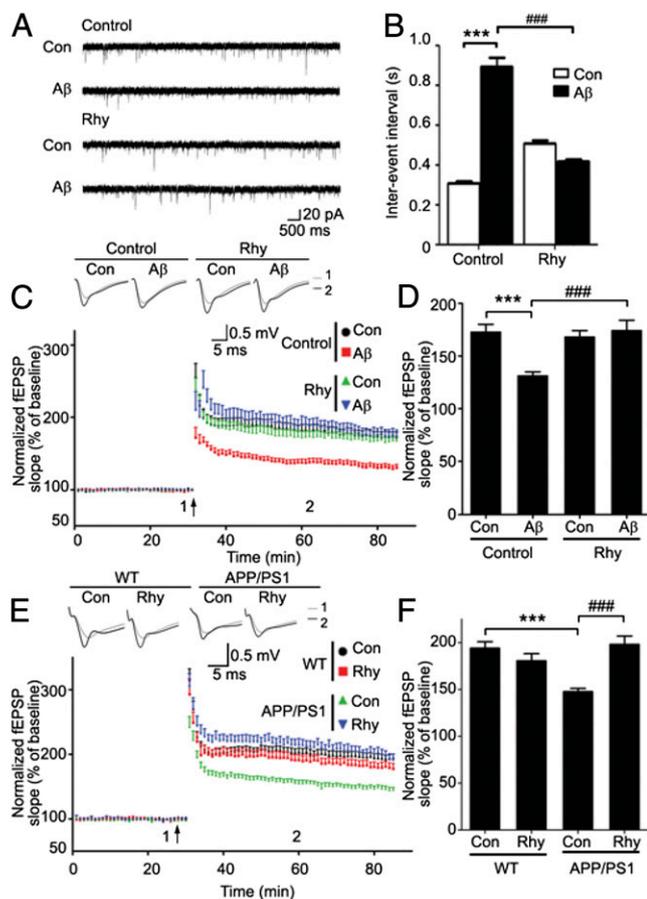
Fig. 5. Rhy rescues the Aβ-induced deficit in neurotransmission and LTP inhibition in AD mice. (*A* and *B*) Pretreatment with Rhy abolished the Aβ-induced reduction of neurotransmission in hippocampal neurons. (*A*) Representative mEPSC traces. (*B*) mEPSC interevent interval (mean ± SEM; $n \geq 19$ neurons for each group; ***$P < 0.001$, ###$P < 0.001$; one-way ANOVA followed by the Kruskal–Wallis test.) (*C* and *D*) Rhy prevented the Aβ-induced inhibition of LTP. Acute hippocampal slices were treated with Aβ in the presence of Rhy. (*C*) Averaged slopes of baseline-normalized fEPSP (mean ± SEM). (*D*) Quantification of mean fEPSP slopes during the last 10 min of the recording after LTP induction ($n \geq 10$ slices from 6 brains; ***$P < 0.001$, two-way ANOVA followed by the Bonferroni posttest; ###$P < 0.001$, one-way ANOVA followed by Student–Newman–Keuls test). (*E* and *F*) Rhy rescued the LTP impairment in APP/PS1 mutant mice. WT and APP/PS1 mutant mice were orally administered Rhy. (*E*) Averaged slopes of baseline-normalized fEPSP (mean ± SEM). (*F*) Quantification of mean fEPSP slopes during the last 10 min of the recording after LTP induction ($n \geq 8$ slices from 6 brains; ***$P < 0.001$, ###$P < 0.001$; two-way ANOVA followed by the Bonferroni posttest). (*C* and *E*) Trace recordings 5 min before (1) and 50 min after (2) LTP induction (arrow) are shown. Inset traces are examples of fEPSPs recorded before (gray) and after (black) HFS.

EphA4 in the CA1 region reversed LTP deficit in APP/PS1 mice, our findings suggest an important role for postsynaptic EphA4 in mediating the synaptotoxicity of Aβ. Postsynaptic EphA4 activation by astrocytic ephrin-A enables the activation of EphA4 forward signaling in the adult rodent hippocampus, resulting in dendritic spine loss as well as the removal of surface AMPA receptors, but not NMDA receptors (19, 21). EphA4 causes the retraction of dendritic spines, probably through actin cytoskeleton reorganization (16) or adhesion receptor regulation (15), and also triggers AMPA receptor degradation in a proteasome-dependent manner (19). Both dendritic spine reduction and AMPA receptor removal are critical factors that contribute to synaptic loss and dysfunction during AD progression (8, 27). Another interesting feature of EphA4 is that the receptor is able to trigger

reverse signaling in astrocytes via ephrin-A3 and modulate glutamate uptake by lowering glutamate transporters in glial cells (38). It is of interest to determine whether EphA4–ephrin-A3 reverse signaling is involved in synaptic dysfunctions in AD via the dysregulation of glutamate uptake, which has been reported in AD transgenic mice (39).

Reversing synaptic dysfunctions is a potential therapeutic strategy to treat cognitive decline in AD. The present study shows that Rhy and KYL peptide (40), which binds to the ligand-binding domain of EphA4, effectively alleviated Aβ-induced synaptic dysfunction and synaptic plasticity defects in AD transgenic mouse models. This suggests that blockade of EphA4 activity can be potentially developed as a therapeutic strategy for the treatment of AD. Although the 12-amino acid KYL peptide was able to block the EphA4 signaling effectively, there may be huge challenges to develop this peptide as a drug candidate, e.g., bioavailability. For the identified small-molecule EphA4 inhibitors, although their effectiveness was demonstrated in in vitro or cellular assays, the in vivo effects of these compounds on inhibiting EphA4 and the bioavailability of these agents have not been reported (28, 33, 41). The reduction of EphA4 activation in APP/PS1 mouse brains by oral administration of Rhy (Fig. 4 *F* and *G*) suggests that Rhy is able to pass the blood–brain barrier to exert its inhibitory effect on EphA4. Further pharmacokinetics study of Rhy is warranted to provide more evidence on the bioavailability of this small molecule. The mechanisms underlying the beneficial effect of Rhy in AD remain to be elucidated. Although Rhy has been suggested to be an NMDA antagonist (42) as well as a calcium channel blocker (43), a subsequent study revealed that Rhy neither binds to NMDA receptor nor inhibits the glutamate-induced Ca^{2+} influx (44). The present study identified Rhy as an EphA4 inhibitor through the structure-based in silico screening. Future characterization of the structural details of the binding interface of Rhy and EphA4 as well as other Eph members may facilitate the optimization of the structure of Rhy through chemical modifications; this may ultimately lead to the identification of new EphA4 inhibitors with greater affinity, specificity, and potency.

In conclusion, the present findings provide evidence that EphA4 is critical for mediating the impairment of synaptic plasticity in AD mouse models. Further understanding of the molecular and cellular mechanisms downstream of EphA4 may lead to the identification of new targets for AD therapy. Our findings also suggest that targeting EphA4 may be beneficial for the prevention and treatment of AD. Importantly, the ability of the small-molecule EphA4 inhibitor Rhy to alleviate synaptic impairment in AD models corroborates this intervention strategy.

Materials and Methods

For details, see *SI Materials and Methods*.

Preparation of β-Amyloid Oligomer and Ephrin. Oligomeric Aβ was prepared as described previously (45). Ephrin-A1–Fc was preclustered with goat anti-human Fc antibody (1:4.5) (16).

Virtual Screening of an In-House Traditional Chinese Medicine Database by Molecular Docking. AutoDock 4.0 was used to simulate docking between EphA4 (PDB code: 2WO2) and our in-house traditional Chinese medicine database containing 225 chemical compounds (46, 47).

Cell Culture and Animals. Primary cortical and hippocampal neurons were prepared from embryonic day 18–19 rat embryos (16). APP/PS1 (B6C3-Tg [APPswe, PSEN1dE9]85Dbo/J) double-transgenic mice were obtained from Jackson Laboratory. All of the transgenic mice and C57BL/6J mice were produced by the Animal Care Facility of The Hong Kong University of Science and Technology, and the experiments were approved by the Hong Kong University of Science and Technology Animal Ethics Committee and conducted in accordance with the Code of Practice Care and Use of Animals for Experimental Purposes of Hong Kong.

Synaptosomal Preparation, Immunoprecipitation, Western Blot Analysis, and Immunocytochemical and Confocal Microscopy. Hippocampal synaptosomes were prepared as described previously (48). Western blot analysis was performed as described previously (16). To examine the subcellular localizations of EphA4 and PSD-95 in Aβ-treated neurons, neurons were fixed with methanol for 20 min at −20 °C (16). Immunostaining was performed as described previously (49).

Cdk5 Kinase Assay, Rhy–EphA4 Binding Assay, EphA4 Clustering, Growth Cone Collapse Assay, and Dendritic Spine Analysis. The Cdk5/p35 kinase assay was performed as described in ref. 16. For the pulldown analysis, Bio-Rhy was bound to streptavidin magnetic beads; the beads were then incubated with recombinant EphA4-Fc or EphB2-Fc. For EphA4 clustering, neurons were pretreated with different concentrations of Rhy or KYL as a control, then with preclustered ephrin-A1 for another 15 min, and fixed (50). The growth cone collapse assay and dendritic spine analysis was performed as described previously (16, 50).

Electrophysiology. For mEPSC recordings, hippocampal neurons at ∼25–28 days in vitro were cotreated with or without Aβ together with the testing reagents for 24 h. For LTP recordings, the fEPSPs were recorded using a multi-electrode array system (MED64, Panasonic International, Inc.) as described previously (51).

Quantitative Analyses. Images from the same experiment were obtained using identical acquisition settings, and images were analyzed with Metamorph software (Meta Image Series 7.5, Universal Imaging Corp.) (16, 51). The investigators who collected and analyzed the electrophysiological and staining data were blinded to the genotype of the mice and treatment conditions. Results were obtained from at least three independent experiments. Error bars shown in the figures represent SEM. Statistical analyses were performed by GraphPad Prism.

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