Downregulation of RBO-PI4KIII α Facilitates A β ₄₂ Secretion and Ameliorates Neural Deficits in A β ₄₂-Expressing *Drosophila*

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Phosphoinositides and their metabolizing enzymes are involved in A β ₄₂ metabolism and Alzheimer's disease pathogenesis. In yeast and mammals, Eighty-five requiring 3 (EFR3), whose *Drosophila* homolog is Rolling Blackout (RBO), forms a plasma membrane-localized protein complex with phosphatidylinositol-4-kinase Type III α (PI4KIII α) and a scaffold protein to tightly control the level of plasmalemmal phosphatidylinositol-4-phosphate (PI₄P). Here, we report that RBO binds to *Drosophila* PI4KIII α , and that in an A β ₄₂-expressing *Drosophila* model, separate genetic reduction of PI4KIII α and RBO, or pharmacological inhibition of PI4KIII α ameliorated synaptic transmission deficit, climbing ability decline, premature death, and reduced neuronal accumulation of A β ₄₂. Moreover, we found that RBO-PI4KIII α downregulation increased neuronal A β ₄₂ release and that PI₄P facilitated the assembly or oligomerization of A β ₄₂ in/on liposomes. These results indicate that RBO-PI4KIII α downregulation facilitates neuronal A β ₄₂ release and consequently reduces neuronal A β ₄₂ accumulation likely via decreasing A β ₄₂ assembly in/on plasma membrane. This study suggests the RBO-PI4KIII α complex as a potential therapeutic target and PI4KIII α inhibitors as drug candidates for Alzheimer's disease treatment.

Key words: A β ₄₂ secretion; *Drosophila*; intraneuronal A β ₄₂; neural deficits; phosphoinositides; PI4KIII α

Significance Statement

Phosphoinositides and their metabolizing enzymes are involved in A β ₄₂ metabolism and Alzheimer's disease pathogenesis. Here, in an A β ₄₂-expressing *Drosophila* model, we discovered and studied the beneficial role of downregulating RBO or its interacting protein PI4KIII α —a protein that tightly controls the plasmalemmal level of PI₄P—against the defects caused by A β ₄₂ expression. Mechanistically, RBO-PI4KIII α downregulation reduced neuronal A β ₄₂ accumulation, and interestingly increased neuronal A β ₄₂ release. This study suggests the RBO-PI4KIII α complex as a novel therapeutic target, and PI4KIII α inhibitors as new drug candidates.

Introduction

Cerebral accumulation of A β plays an important role in Alzheimer's disease (AD) pathogenesis (Hardy and Selkoe, 2002). In

brains of AD patients, A β accumulates both extracellularly and intracellularly. Extracellular A β accumulates as diffuse deposits and fibrillary plaques. Although its toxicity has been widely studied, no clinical trial on the removal of extracellular A β or inhibition of A β production has so far succeeded. In recent years,

Received Nov. 17, 2016; revised March 12, 2017; accepted March 31, 2017.

F.-D.H. conceived the project, designed, and participated in all experiments except the cell culture studies; X.Z. designed and participated in experiments of flies, cells, WB, co-IP, and liposome; W.-A.W. firstly obtained the quantitative data of fly behavior; L.-X.J. studied the effect of compounds on A β ₄₂ secretion, secretases' activity, and APP expression; B.-Z.Z. for WB and co-IP, B.-Z.Z. and Q.-Y.L. for purification of genetic background; F.-D.H., X.Z., and N.L. for paper preparation.

This work was supported by National Key Basic Research Program of China Grant 2013CB530900 and National Natural Science Foundation of China Grants 81371400, 81571101, and 81071026. We thank Dr. M. Ho, Dr. J. Li, and Dr. Y. Hu for critical reading; J. Peng, A.Z. Chen, Dr. W.X. Li, and Q.Y. Li for technical support.

F.-D.H. has shareholding of a company possessing part of the intellectual property raised in this study. The remaining authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.3567-16.2017

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neuronal accumulation of A β has been found to be involved in synaptic deficits, amyloid plaque formation, and cell death (Wirhth et al., 2004; LaFerla et al., 2007; Gouras et al., 2010). In the brains of AD patients, oligomeric A β , possibly the most deleterious form of A β to synaptic and cognitive functions (Lambert et al., 1998; Walsh and Selkoe, 2007; Selkoe, 2008), is found to be associated with lipid membranes but is rarely detectable in the CSF and interstitial fluid (Yang et al., 2013; Hong et al., 2014; Savage et al., 2014), indicative of predominant membrane and intraneuronal localization. Consistently, presenilin-2-containing γ -secretase is found to be mainly localized at late endosomes/lysosomes and generates a prominent pool of intracellular A β_{42} ; disease-causing mutations in presenilin-2 could further increase the A β_{42} level in this pool. Moreover, a subset of FAD mutants in presenilin-1, whose wild-type form is more broadly distributed in the cell, phenocopies presenilin-2 and shifts their localization to late endosomes/lysosomes. This study clearly demonstrates the intracellular production and accumulation of A β_{42} (Sannerud et al., 2016). Thus, reducing the oligomerization and neuronal accumulation of A β is of therapeutic potential.

Phosphoinositides, the well-known second messengers, have been widely reported to be involved in AD pathogenesis (Arancio, 2008; Di Paolo and Kim, 2011). In APP (β amyloid precursor protein) transgenic mice, genetic reduction of synaptotagmin-1, which converts PI $_{4,5}$ P into PI $_4$ P, suppresses synaptic and behavioral impairments (McIntire et al., 2012; Zhu et al., 2013). In patients, changes in the plasma levels of certain phospholipids have been found to correlate with the conversion from normal aging to either amnesic mild cognitive impairment or early AD (Mapstone et al., 2014); moreover, depletion of phosphoinositol (PI) and PI $_{4,5}$ P, as well as a reciprocal elevation of PIP was observed along with disease progression in the parietal cortex of AD patients (Zhu et al., 2015). Mechanistically, the relationship between certain phosphoinositides and A β has been studied. PI and PI $_{4,5}$ P facilitate A β assembly in/on lipid membrane, and A β_{42} disrupts PI $_{4,5}$ P metabolism and PI $_3$ P kinase signaling (McLaurin and Chakrabarty, 1997; Berman et al., 2008; Chiang et al., 2010). However, whether phosphoinositides and their metabolizing enzymes can affect AD pathogenesis via influencing neuronal accumulation of A β has yet to be explored.

Drosophila has been used to model AD by pan-neuronal expression of A β_{42} . These flies show intraneuronal A β accumulation and behavioral deficits (Iijima et al., 2004; Crowther et al., 2005; Iijima-Ando et al., 2008). By expressing A β_{42} in the *Drosophila* giant fiber (GF) pathway, we developed another model, in which in addition to neuronal accumulation of A β and motor defects, age-dependent synaptic transmission failure is evident and easy to record (Zhao et al., 2010; Huang et al., 2013; Lin et al., 2014; Han et al., 2015; Liu et al., 2015), providing a convenient platform for testing the role of candidate genes in neuronal accumulation of A β and associated synaptic deficits, and for performing genetic screening for modifiers of the those neural deficits. Using this model, we found the potential role of RBO and PI4KIII α in AD pathogenesis. RBO has homologs in yeast and mammals, named as EFR3, which forms a plasma membrane localized protein complex with PI4KIII α and a scaffold protein to control the levels of plasmalemmal PI $_4$ P and PI $_{4,5}$ P, particularly PI $_4$ P (Faulkner et al., 1998; Huang et al., 2004; Baird et al., 2008; Hammond et al., 2012; Nakatsu et al., 2012). Here we present the effects of genetic reduction of RBO-PI4KIII α , or pharmacological inhibition of PI4KIII α on the defects in our fly model, and the potential mechanism.

Materials and Methods

Animal strains and genetics. *P{UAS-A β_{arc} }*, *P{UAS-A β_{42} }* (a gift from Dr. Crowther, Cambridge University), *P{UAS-dTAU}* (Mershin et al., 2004), *P{UAS-shibire^{ts1}3}* (Kitamoto, 2001), and *P{UAS-mCD8-GFP}* (lab stock) transgenes lines were crossed to *[GAL4]A307* or *P{GAL4-ninaE.GMR}12* (lab stock) line to generate flies expressing arctic mutant A β_{42} , wild-type A β_{42} , *Drosophila* tau, a temperature-sensitive dominant negative form of Dynamin, a RBO protein with mutation in the putative lipase catalytic site, and mCD8-GFP in the GF system, respectively. Other lines used are *rbo^{ts1}* (missense mutation, lab stock), *rbo²* (null allele, lab stock), *P{RBO^{S358A}}* (a transgenic line containing a *rbo* transgene generated by subcloning the genomic DNA of endogenous *rbo* gene with PCR-mediated point mutation to express the mutant RBO(S358A) under the control of *rbo*'s own promoter) (Vijayakrishnan et al., 2010), *itpr-83A^{sv35}* (nonsense mutation of IP $_3$ receptor, also known as *itpr^{sv35}*), a chromosomal deficiency line covering the *PI4KIII α* gene, *pi4k^{def/+}* (Df(1)ED6579, P{3'.RS5 + 3.3'}ED6579), and *PI4KIII α nonsense mutants* (*PI4KIII α ^{G527}* and *PI4KIII α ^{G786}*, gift from Dr. Schubach, Princeton University). Before use, all these transgenic and mutant flies were separately backcrossed to an isogenic control line *w¹¹¹⁸* for at least 5 generations. The fly strains used in this study are listed as follows: wild-type: *w¹¹¹⁸* (isogenic line); ctrl: *[GAL4]A307/+; rbo^{ts1/+}; [GAL4]A307/rbo^{ts1}; rbo^{2/+}; [GAL4]A307/rbo²; A β_{arc} : *[GAL4]A307/P{UAS-A β_{arc} }; A β_{arc} -*rbo^{ts1/+}; [GAL4]A307/rbo^{ts1}/P{UAS-A β_{arc} }; A β_{arc} -*rbo^{2/+}; [GAL4]A307/rbo²/P{UAS-A β_{arc} }; A β_{42} : *[GAL4]A307/P{UAS-A β_{42} }; P{UAS-A β_{42} }/+; A β_{42} -*rbo^{ts1/+}; [GAL4]A307/rbo^{ts1}/P{UAS-A β_{42} }; P{UAS-A β_{42} }/+; A β_{42} -*rbo^{2/+}; [GAL4]A307/rbo²/P{UAS-A β_{42} }; P{UAS-A β_{42} }/+; GMR: *P{GAL4-ninaE.GMR}12/+; GMR; A β_{arc} : *P{UAS-A β_{arc} }/P{GAL4-ninaE.GMR}12; GMR; A β_{arc} -*rbo^{ts1/+}; P{UAS-A β_{arc} }; rbo²/P{GAL4-ninaE.GMR}12; GMR; A β_{arc} -*rbo^{ts1/+}; P{GAL4-ninaE.GMR}12/rbo^{ts1}/P{UAS-A β_{arc} }; rbo²; dTAU: *[GAL4]A307/P{UAS-dTAU}; dTAU-*rbo^{ts1/+}; [GAL4]A307/rbo^{ts1}/P{UAS-dTAU}; dTAU-*rbo^{2/+}; [GAL4]A307/rbo²/P{UAS-dTAU}; SHIBIRE^{ts1}: *[GAL4]A307/+; P{UAS-shi^{ts1}}/+; A β_{arc} -SHIBIRE^{ts1}: *[GAL4]A307/P{UAS-A β_{arc} }; P{UAS-shi^{ts1}}/+; A β_{arc} -RBO^{S358A}: *[GAL4]A307/P{UAS-A β_{arc} }; P{RBO^{S358A}}/+; A β_{arc} -*itpr^{sv35}: [GAL4]A307/P{UAS-A β_{arc} }; itpr-83A^{sv35}/+; A β_{arc} -*rbo^{ts1/+}: [GAL4]A307/rbo^{ts1}/P{UAS-A β_{arc} }; rbo²; pi4k^{def/+}: Df(1)ED6579, P{3'.RS5 + 3.3'}ED6579/+; [GAL4]A307/+; pi4k^{G527/+}: *PI4KIII α ^{G527}/+; [GAL4]A307/+; A β_{arc} -pi4k^{def/+}: Df(1)ED6579, P{3'.RS5 + 3.3'}ED6579/+; [GAL4]A307/P{UAS-A β_{arc} }; A β_{arc} -pi4k^{G786/+}: *PI4KIII α ^{G786}/+; [GAL4]A307/P{UAS-A β_{arc} };*********************

Fly climbing and longevity assays. We used an automatic system for a highly replicable examination of the fly climbing ability (Liu et al., 2015). Briefly, for each group of flies, 30 flies were equally divided and separately transferred into three testing vials. After being tapped down to the bottom of the testing vials, flies were allowed to climb up along the walls of vertically placed testing vials, and were videotaped. The height of each fly in each vial at the fifth second was measured for evaluating the fly's climbing ability.

For longevity analysis, 200 flies (100 female and 100 male) of each genotype were equally separated into 10 vials containing standard fly food and dry yeast, and cultured at 25°C. Food vials were changed every 3 d, and dead flies were counted at that time. Survival rates were analyzed with the SPSS 11 Kaplan–Meier software (IBM).

Electrophysiology test of synaptic transmission failure in flies. Evoked excitatory junctional potential (EJP) in the GF system was recorded intracellularly as described previously (Zhao et al., 2010). Briefly, an adult female fly at certain age was mounted ventral side down on a glass slide with tackiwax (Boeckel Scientific) under a dissection microscope. Before the recording, a reference electrode was inserted into the abdomen, two stimulating electrodes into the two eyes, and a recording electrode into one dorsal longitudinal muscular cell. Intracellular penetration of the recording electrode into the muscle was monitored by a sudden potential drop of 40–70 mV. Square wave electrical stimulation (0.2 ms pulse width, 100 Hz, 50 pulses) was applied to both eyes at the intensity of 5–20 V (~150% of the threshold stimulation intensity). The signal of the elicited EJPs, whose success rate reflects the well-being of synaptic function, was recorded and amplified by Axonal clamp 900A (Molecular Devices) and digitized at 10 kHz by Digidata 1440A (Molecular Devices).

Data were collected and analyzed with the pClamp software (version 10.0; Molecular Devices). All electrodes were glass electrodes and filled with 0.1 M KCl. The recording environment temperature was 25°C.

Toluidine blue staining of *Drosophila* eye. Fly heads, with their mouthparts removed, were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C, after which the preparations were postfixed in 2% OsO₄ in the same buffer for 2 h at 4°C, dehydrated with increasing concentrations of ethyl alcohol, and embedded in Epon 812. Leica UC6 ultramicrotome was used to cut fly eyes transversely into 2- μ m-thick sections. Sections were stained with toluidine blue and visualized with NIKON E600FN Neurolucida light microscope. Rhabdomere number per ommatidium was analyzed with Stereo Investigator software (MBF Bioscience, RRID: SCR_002526).

Immunoprecipitation and immunoblot. A total of 300 fly heads of the designated genotype were collected and homogenized with Tris buffer containing 1% NP40 and protease inhibitors. The lysates were subjected to immunoprecipitation or immunoblot with a custom mouse monoclonal antibody against RBO and a custom rabbit polyclonal antibody against *Drosophila* PI4KIII α (anti-RBO was generated in collaboration with Abmart (Shanghai) using the RBO fragment 251a.a.-500a.a. as the epitope; anti-PI4KIII α was generated in collaboration with Abgent (Suzhou) using the peptide NH₂-KRSNRSKRLQYQKDSYC-CONH₂ as the epitope).

To compare the binding affinity of wild-type RBO versus temperature-sensitive mutant RBO to PI4KIII α using immunoprecipitation, the lysates were first immunoprecipitated using anti-RBO. The precipitates of each experiment group were then subjected to immunoblot to determine their relative RBO protein levels via quantifying gray value of RBO's bands. Subsequently, another immunoblot was performed, in which the input of each precipitate was adjusted accordingly to guarantee similar RBO protein levels. Finally, the relative PI4KIII α protein levels normalized against RBO protein levels in each precipitate were compared. The primary antibody used in the immunoblot of APP was 6E10 (RRID: AB_2565327).

Staining and imaging in flies. A β staining in fly CNS was performed as described previously (Zhao et al., 2010). Briefly, the whole CNS of flies, including the brain and ventral ganglion, was dissected out in cold PBS and fixed with 4% PFA in PBS for ~45 min. Preparations were washed with PBS for 30 min, treated with formic acid (70% in water) for 45 min to reexpose the epitope, washed repetitively with 5% BSA in PBS solution supplemented with 0.25% Triton, incubated with primary antibody (6E10, 1:100, RRID: AB_2565327) at 4°C for 10–12 h, washed with PBS again, and finally incubated with cy3-conjugated secondary anti-mouse antibody (Jackson ImmunoResearch Laboratories, 1:200, RRID: AB_2340813) at room temperature for 2 h. Images were taken under Nikon A1R-A1 confocal microscope; the genotypes of fly CNS were blind to the imager.

ELISA of A β ₄₂ in fly CNS. To analyze A β ₄₂ level in fly CNS, intact brains of 25 flies per strain were dissected out in cold PBS and placed immediately into 50 μ l cold ELISA sample buffer supplemented with cocktail protease inhibitor (Calbiochem). Brains were homogenized thoroughly, incubated at room temperature for 4 h. After centrifuge at 4000 $\times$ g and at 4°C for 10 min, the supernatant was collected. ELISA for A β ₄₂ level in fly CNS was performed using the A β ₄₂ Human ELISA Kit (Invitrogen, catalog #KHB3441) according to the manufacturer's instructions. The sensitivity of the kit is <10 pg/ml. A total of 3 μ l of the supernatant was diluted to 60 μ l with dilution buffer, and 50 μ l of the diluent was used for ELISA.

RT-PCR analysis. Trizol was used to extract total RNA, and a conventional two-step RT-PCR method was used to quantify the transcription level of genes. The primers used are listed as follows: *rbo*: 5'-GCGCCGT-GCGTCCACTATCT-3' and 5'-ATGGCAGCGCTCCACACAA-3'; *pi4kIIIa*: 5'-AAACAGAACCCTCAGGTGTC-3' and 5'-AGCTCAAGGAT-CAGATTGCG-3'; *Efr3a*: 5'-TGGAATCGGGGAACCAAAG-3' and 5'-AAGACTCCAGCACTGTCCG-3'; *PI4KA*: 5'-TTTCAACACGGTC-CTGTCAC-3' and 5'-ACGCCTTCGAACACCTTCAA-3'; and A β ₄₂: 5'-ATGGCGAGCAAAGTCTCGATC-3' and 5'-CGCAATCACCACGC-CGCCCCAC-3'.

Culture of dissected A β -expressing larvae or APP-expressing HEK293T cells. Cleaned and sterilized wandering third-instar larvae were dissected

along the dorsal middle line in Schneider's culture medium (Sigma-Aldrich, CAS #S9895). The tracheal, gut, and fat body were removed with caution. The dissected larvae were washed with medium and transferred into a 2 ml centrifuge tube containing 150 μ l Schneider's culture medium supplemented with gentamycin (20 mg/ml) and 1.2 mM Ca²⁺. The tubes, each containing 5 dissected larvae, were placed in a humid and dark environment at 25°C for 8 h. Then 100 μ l culture medium from each tube was used for ELISA quantification of A β ₄₂.

HEK293T cells stably transfected with human APP were cultured in DMEM (Hyclone) supplemented with 10% FBS (Invitrogen), penicillin-streptomycin, and 100 μ g/ml G418. For RNA interference, cells were transfected with plasmid pSUPER. basic encoding shRNA for targeted genes using lipofectamine 2000 (Invitrogen). The targeting sequences of shRNA were 5'-GGTTATTGAAATTCGAACT-3' and 5'-TGCTCATT AGCAGTAAAGA-3' for *Efr3a* and *PI4KA*, respectively. The knocking down efficacy of the shRNAs was ~20% for *Efr3a* and 30% for *PI4KA* (and data not shown). Experiments were performed 2 d after transfection. For quantification of secreted A β ₄₂, fresh-changed culture medium was examined for A β ₄₂ concentration after culturing cells for 12 h using ELISA. ELISA for A β ₄₂ level in culture mediums was performed using the A β ₄₂ Human ELISA Kit (Invitrogen, catalog #KHB3441) according to the manufacturer's instructions.

Assay of α -, β -, and γ -secretases' activities. The enzymatic activities of α -, β -, and γ -secretases were measured as previously described (Wang et al., 2015). Briefly, cells were first homogenized and centrifuged, and secretases in the pellet were resolubilized. The α -secretase activity was assayed in 100 mM sodium acetate, pH 7.0, with 2 μ g fluorogenic substrate (Merck, 565767), whereas β -secretase activity assayed in 100 mM sodium acetate, pH 4.5, with 2 μ g fluorogenic substrate (Merck, 565758) and γ -secretase activity assayed in 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, and 0.25% CHAPSO (w/v) with 8 μ g fluorogenic substrate (Merck, 565764). After incubation at 37°C, reactions were centrifuged at 13,200 rpm for 15 min at 4°C and then placed on ice. Supernatants were transferred to a transparent 96-well plate (Nunc, Nunc), the fluorescence intensities were measured with an excitation wavelength at 340 nm and an emission wavelength at 490 nm for α -secretase, 350 and 490 nm for β -secretase, and 355 and 440 nm for γ -secretase.

Phenylarsine oxide (PAO) application and toxicity examination. For PAO application to HEK293 cells, dissected *Drosophila* larva, living larva, and adult flies, a stock solution of 10 mM PAO was made by resolving PAO powder (Sigma-Aldrich, catalog #637-03-6) in DMSO and then diluted with the corresponding culture medium to the wanted concentrations. Although PAO at different concentrations was used to treat HEK293 cells or *Drosophila*, the final concentration of DMSO was adjusted to identical level to ensure the experimental results were not influenced by DMSO variation. PAO application to living *Drosophila* started from embryonic stage; PAO was mixed into the culture medium and stored at 4°C for no more than a week before use; when flies consumed food, they took in PAO in the meantime.

To test the toxicity of PAO in adult flies, we cultured control flies with fly food containing 50, 100, 200, 300, 400, and 600 μ M PAO and found that PAO treatment at $\leq$ 200 μ M neither changed the eclosion ratio of pupa nor altered the climbing ability of control flies. Then 25, 50, 100, and 150 μ M PAO were chosen for formal experiments.

To test the toxicity of PAO on dissected *Drosophila* larvae, 50, 100, 200, 300, 400, and 500 nM PAO were applied to the culture medium overnight. We found that CNS neurons and garland cells in the larvae treated with PAO at 300 nM and above turned white, reflecting damage. Then 50, 100, and 150 nM PAO were chosen for formal experiments.

To test the toxicity of PAO on cultured HEK293T cells, 50, 100, 200, 300, 400, and 500 nM PAO were applied to the culture medium of confluent cells. We found that PAO at $\geq$ 250 nM killed most of the cells after 12 h incubation, whereas PAO at $\leq$ 150 nM did not show evident toxicity after 12 h treatment, according to MTT tests. Then 25, 50, 100, and 150 nM PAO were used to treat HEK293T cells.

Studying the regulation of A β ₄₂ aggregation in liposome by PI₄P. Based on the procedures described previously (Tashima et al., 2004), we prepared liposome solutions containing synthetic monomeric A β ₄₂ and PI₄P, or PIP₂ at certain concentrations. The lipid composition of the

liposomes is similar to that found in the cerebral cortex membranes (CCM-lipid) (Tashima et al., 2004) containing phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin 1 (SM1), and cholesterol (Ch), but without monosialoganglioside (GM1), cereoside, and plasmalogen (see Table 2).

To prepare the monomeric $A\beta_{42}$ we used in this experiment, we disaggregated synthetic $A\beta_{42}$ by following the protocol described by Zagorski et al. (1999) as follows:

1. Add distilled trifluoroacetic acid (TFA) into a glass tube containing synthetic $A\beta_{42}$ powder (GL Biochem) at an ~1:1 ratio (mg:ml), followed by sonication with a KUDOS ultrasonic instrument (model SK250HP) at room temperature for ~15 min, during which additional portions (1 ml) of TFA are added until the peptide dissolves completely (complete solubilization is judged by the absence of precipitate after centrifugation).
2. Remove TFA by N_2 gas (after which the peptide coats the glass walls of the tube as a waxy layer), the residue trace amount of TFA is removed by addition of distilled hexafluoro-2-propanol (HFIP), sonication, and removal of TFA with N_2 gas. The latter is repeated 3 times.
3. The residual HFIP is removed by vacuum (<1 mmHg, 2 h). After removal of TFA, a concentrated stock solution (1.0 mg/ml) prepared in HFIP is stable for several months.

To prepare the liposomes used in this experiment, we used the following protocol:

1. Dissolve each lipid in chloroform at the concentration of 1 mg/ml to make a stock solution for each lipid. PC, PS, PE, SM1, and Ch were purchased from Sigma. PI, PI $_4$ P, and PI $_{4,5}$ P were purchased from Echelon Bioscience.
2. Mix the lipids according to Table 2. For example, to make the control liposome containing only $A\beta_{42}$, mix 113 μ l PC, 68 μ l PS, 198 μ l PE, 87 μ l SM1, 57 μ l Ch, and 45 μ l $A\beta_{42}$ together in a glass tube; to make liposomes containing $A\beta_{42}$ and PI (20 μ M in final liposome solution), an additional 17 μ l PI is mixed in.
3. Remove the HFIP and chloroform by lyophilization (<1 mPar, 2 h) using FreeZone^{2.5} (Labconco).
4. Resuspend the precipitate by adding 1.0 ml buffer (5 mM Tris, 100 mM NaCl, pH 7.0) followed by sonication at room temperature for 30 min.
5. Stay at 4°C for 48 h.
6. Immunoblot analysis of the liposome solutions.

Data analysis and statistics. SPSS software (IBM, RRID: SCR_002865) was used for data analysis. The data were presented as mean $\pm$ SEM if not specified elsewhere.

Results

Genetic method identifies RBO as a modifier of $A\beta$'s effects

Adult fruit flies expressing wild-type or arctic-mutant $A\beta$ ($A\beta_{42}$ and $A\beta_{arc}$ flies) in neurons of the GF pathway exhibit neuronal $A\beta$ accumulation, age-dependent increase in synaptic transmission failure, decline of flight and climbing ability, as well as premature death (Zhao et al., 2010; Liu et al., 2015). To identify modifiers of $A\beta$'s effects, heterozygous mutations, transgenes, and chromosome deficiencies (Bloomington deficiency kits) were individually introduced into $A\beta_{arc}$ flies. The modifying effect was first examined by the climbing assay; then those can influence climbing ability were further tested for age-dependent synaptic transmission failure. A candidate suppressor of $A\beta$'s effects, for example, was isolated if its loss-of-function mutation can ameliorate the age-dependent decline in climbing ability, and can slow down the age-dependent increase in synaptic transmission failure. Modifiers isolated from deficiencies of the third chromosome were reported previously (Liu et al., 2015). Loss-of-function mutations of *rolling blackout* (rbo^{ts1} and rbo^2), which were isolated as suppressors (data not shown), are investigated in this study.

RBO insufficiency suppresses neural deficits in flies expressing $A\beta_{42}$

The effect of *rbo* mutations on the defects induced by the expression of arctic mutant $A\beta_{42}$ were further studied with four groups of flies cultured at 25°C: control (ctrl), $rbo^{ts1/+}$ or $rbo^{2/+}$ heterozygotes (*rbo*), $A\beta_{arc}$ -expressing flies ($A\beta_{arc}$), $rbo^{ts1/+}$ or $rbo^{2/+}$ heterozygotes expressing $A\beta_{arc}$ ($A\beta_{arc}$ -*rbo*) (for genotypes of these strains, see the list of fly strains in Materials and Methods).

Synaptic transmission was examined by intracellular recording of EJPs in the dorsal longitudinal muscle fibers under high-frequency brain stimulation (100 Hz, 50 pulses). The success rates of elicited EJPs in all groups were indistinguishable on the 3–7th and 15–17th days after eclosion (Fig. 1A,B). On the 25–27th and 31–35th days, however, success rate in $A\beta_{arc}$ flies became lower than that in control flies (Fig. 1A,B), confirming our previous results that $A\beta_{arc}$ expression causes age-dependent synaptic transmission impairment (Zhao et al., 2010; Huang et al., 2013). The success rates in $A\beta_{arc}$ -*rbo* flies were higher than those in $A\beta_{arc}$ flies, although lower than those in control and *rbo* flies, which did not differ much from each other (Fig. 1A,B). This result demonstrates that *rbo* insufficiency ameliorated the age-dependent synaptic failure. The transgenic flies and *rbo* mutants used for generating the 4 groups of flies had been backcrossed to an isogenic wild-type line for at least 5 generations. Therefore, it is unlikely that the difference in genetic background had contributed to the improved synaptic transmission in $A\beta_{arc}$ -*rbo* flies. Because homozygous null mutation of *rbo* causes embryonic lethality (Faulkner et al., 1998), its effect on $A\beta_{arc}$ -induced synaptic failure could not be examined.

The motor ability was evaluated with climbing assay. Climbing ability was examined on the 3rd, 16th, 26th, and 31st day after eclosion. On the 3rd and 16th day, the climbing abilities of the 4 groups were similar (Fig. 1C). On the 26th and 31st day, $A\beta_{arc}$ -*rbo* flies climbed significantly higher than $A\beta_{arc}$ flies, although not as high as control and *rbo* flies (Fig. 1C). Lifespan study showed that $A\beta_{arc}$ -*rbo* flies lived longer than $A\beta_{arc}$ flies, although shorter than control and *rbo* flies (Fig. 1D). The same conclusion can be made by comparing the mean lifespan values among the 4 groups (Table 1). These results are consistent with the findings on synaptic transmission. The lifespan of $A\beta_{arc}$ flies in this study was longer than that in our previous study (Zhao et al., 2010). The major reason is that the flies in this study were cultured at 25°C, rather than at 29°C. Higher temperature may facilitate $A\beta_{arc}$ expression.

We further investigated the effects of *rbo* insufficiency on the synaptic transmission, climbing ability, and lifespan of flies expressing wild-type $A\beta_{42}$ and found even better improvement (Fig. 2A–D). We also investigated the effect of *rbo* insufficiency on the loss of photoreceptor cells caused by the expression of $A\beta_{arc}$ in the retina, and found a beneficial effect too (Fig. 2E,F), showing that the rescue effect of *rbo* insufficiency is not limited to the GF pathway.

The protection of *rbo* mutations against the $A\beta_{42}$ toxicity could not be ascribed to a general effect against neuronal accumulation of toxic proteins because *rbo* insufficiency could not ameliorate the motor defect and premature death of flies overexpressing *Drosophila tau* (Fig. 3A,B).

RBO is enriched in synapses and functions in endocytosis (Huang et al., 2004, 2006; Vijayakrishnan et al., 2009), and the rbo^{ts1} hemizygotes exhibit similar defects in synaptic transmission, bulk endocytosis, and motor function as *shibire^{ts1}* homozygotes at 37°C (restrictive temperature) (Huang et al., 2006; Vijayakrishnan et al., 2009, 2010). Nevertheless, the protective

Figure 1. *rbo* mutations suppress the neural deficits in Aβ_{arc}-expressing flies. Representative traces (**A**) and quantification (**B**) of elicited EJPs in four groups of flies at different ages. The first two groups of flies did not express Aβ_{arc} and were classified as “non-Aβ flies,” whereas the other two groups expressed Aβ_{arc} and were classified as “Aβ flies.” Notably, insufficiency of *rbo* suppressed the age-dependent synaptic failure in Aβ flies. Minimum *n* = 6 flies per bar, one-way ANOVA using LSD test for day 25–27, and Dunnett’s test for day 31–35. **C**, Insufficiency of *rbo* ameliorated the age-dependent decline of the climbing ability in Aβ_{arc}-expressing flies. Minimum *n* = 30 flies per bar, one-way ANOVA using LSD test for day 26, and Dunnett’s test for day 31. **D**, Insufficiency of *rbo* significantly prolonged the lifespan of Aβ_{arc} flies. *n* = 200 flies for each line, log-rank test, compared with Aβ_{arc}, *p* for Aβ_{arc}-*rbo*^{ts1/+} was 2.3727E-12, *p* for Aβ_{arc}-*rbo*^{2/+} was 1.1833E-30; compared with ctrl, *p* for Aβ_{arc}-*rbo*^{ts1/+} was 2.3727E-12, *p* for Aβ_{arc}-*rbo*^{2/+} was 1.1833E-30. **p* < 0.05. ***p* < 0.01. ****p* < 0.001.

Table 1. Mean lifespan of flies and comparison of means^a

Fly strain	Mean lifespan (days)	<i>p</i> value compared with ctrl flies	<i>p</i> value compared with Aβ _{arc} flies
ctrl	47.5	NA	<0.001
<i>rbo</i> ^{ts1}	46.2	0.201	<0.001
<i>rbo</i> ²	54.3	<0.001	<0.001
Aβ _{arc}	27.0	<0.001	NA
Aβ _{arc} - <i>rbo</i> ^{ts1/+}	39.9	<0.001	<0.001
Aβ _{arc} - <i>rbo</i> ^{2/+}	36.8	<0.001	<0.001

^a*p* values were obtained from Fisher’s LSD test.

effect of *rbo* insufficiency in Aβ_{arc}-expressing flies could not be ascribed to a general effect of mutations in synaptic proteins or endocytosis-related process because introducing dominant *shibire*^{ts1} mutation into Aβ_{arc}-expressing flies could not attenuate the motor defect and premature death (Fig. 3C,D).

Although RBO might be a putative DAG lipase (Huang et al., 2004), and the activity of DAG lipase was reported to be increased in the hippocampus of AD patients and an animal model (Farooqui et al., 1988, 1991), RBO might not regulate Aβ_{arc} toxicity by acting as a lipase because introduction of a genomic DNA transgene *rbo*^{S358A} containing the promoter of *rbo* gene to express a RBO protein with mutation at the putative catalytic site, which could rescue the lethality caused by *rbo* depletion (Vijaykrishnan et al., 2010), into Aβ_{arc}-expressing flies could not change the premature death (Fig. 3E).

PI4KIII α insufficiency suppresses the deficits in Aβ_{arc} flies

The RBO homologs in yeast and mouse recruit PI4KIII α and form a complex with it on the plasma membrane to control the

plasmalemmal level of PI₄P (Baird et al., 2008; Nakatsu et al., 2012). Consistent with this, we detected the coimmunoprecipitation between RBO and *Drosophila* PI4KIII α (encoded by *PI4KIII α*) (Fig. 4A). In addition, we found that removing one copy of *rbo* gene (*rbo*^{2/+}) significantly reduced the levels of both RBO and PI4KIII α protein in ctrl and Aβ_{arc}-expressing flies (Fig. 4B). *rbo*^{ts1} mutation did not significantly reduce PI4KIII α ’s level but weakened their interaction (Fig. 4B,C). Notably, neither *rbo* mutations changed the *PI4KIII α* transcription level (Fig. 4D). These data indicate that *rbo* mutations reduced the amount of PI4KIII α or membrane-localized PI4KIII α and presumably reduced the plasmalemmal level of PI₄P.

To test whether PI4KIII α plays a similar role in Aβ_{arc} expression-induced deficits, we separately introduced a chromosomal deficiency (deletion of a *PI4KIII α* -containing DNA segment of a chromosome, *pi4k*^{def/+}) and a nonsense mutation of *PI4KIII α* (*pi4k*^{GS27/+}) (Yan et al., 2011) into Aβ_{arc}-expressing flies. Indeed, both *PI4KIII α* mutations suppressed the Aβ_{arc}-induced deficits in synaptic transmission, motor function, and lifespan (Fig. 5A,C,D). Consistently, feeding Aβ_{arc} flies with PAO, a cell well-known membrane-permeable inhibitor of PI4KIII α (Balla et al., 2005, 2008; Hammond et al., 2012), also markedly ameliorated these defects in a dose-dependent manner (Fig. 5B,E).

The suppression of the neural deficits by downregulating RBO/PI4KIII α could not attribute to an attenuation of calcium release mediated by the receptor of inositol triphosphate (IP₃R) because introducing a nonsense mutation of the single IP₃R-encoding gene into Aβ_{arc} flies could not attenuate the synaptic failure (data not shown) and premature death (Fig. 3F).

Figure 2. Insufficiency of *rbo* suppresses neural deficits in *Aβ*₄₂-expressing flies, and the death of photoreceptor cells caused by the expression of *Aβ*_{arc} in the retina. The representative EJP recordings (**A**) and quantification of success rates of elicited EJPs (**B**) in ctrl, *rbo*^{ts1/rbo}², *Aβ*₄₂, *Aβ*₄₂-*rbo*^{ts1/+}, and *Aβ*₄₂-*rbo*^{2/+} flies at different stages of age. Minimum *n* = 6 flies per bar, one-way ANOVA using LSD test for day 37–39. **C**, Insufficiency of *rbo* ameliorated the age-dependent decline of the climbing ability in *Aβ*₄₂-expressing flies. *n* = 30 flies per arm, one-way ANOVA using Dunnett's test for day 37. **D**, Insufficiency of *rbo* significantly prolonged the lifespan of *Aβ*₄₂ flies (log-rank test, *p* < 0.01), but the lifespans of *Aβ*₄₂-*rbo*^{ts1/+} and *Aβ*₄₂-*rbo*^{2/+} flies were not different from that of ctrl flies. *n* = 200 flies per line, compared with *Aβ*₄₂, *p* for *Aβ*₄₂-*rbo*^{ts1/+} was 2.8220E-18, *p* for *Aβ*₄₂-*rbo*^{2/+} was 3.0199E-28; compared with ctrl, *p* for *Aβ*₄₂-*rbo*^{ts1/+} was 0.2173, *p* for *Aβ*₄₂-*rbo*^{2/+} was 0.0569. **E**, Representative pictures of transverse sections from the eyes of in GMR, GMR;*Aβ*_{arc}, GMR;*Aβ*_{arc}-*rbo*^{2/+}, and GMR;*Aβ*_{arc}-*rbo*^{ts1/+} flies at the age of 30 d. **F**, Quantification of survival rhabdomeres per ommatidium section, *n* > 200 ommatidia from 2 or 3 animals for each strain. One-way ANOVA using LSD test. **p* < 0.05. ***p* < 0.01.

Downregulation of RBO-PI4KIII α reduces neuronal *Aβ* accumulation

Previously, we determined that wild-type and mutant *Aβ*₄₂ expressed in the GF pathway accumulates in neurons in *Aβ*₄₂-expressing flies in an age-dependent fashion based on the localization of *Aβ* deposits along the GF pathway (Zhao et al., 2010; Huang et al., 2013; Lin et al., 2014; Han et al., 2015; Liu et al., 2015). Here, we further confirmed *Aβ*'s neuronal accumulation in this model by introducing another transgene *uas-mCD8-gfp*. The expression of mCD8-GFP, which targets the plasma membrane of the cell body and processes of fly neurons, was driven by the same driver as that of *Aβ*_{arc}, so that the *Aβ*_{arc}-expressing neurons could be labeled with GFP. Confocal imaging revealed that *Aβ* immunostaining signal in aged flies generally colocalized with neuronal GFP (Fig. 6A; the GFP imaging is not ideal because of formic acid treatment), demonstrating that *Aβ*_{arc} predominantly accumulates in neurons in this fly model.

To investigate whether RBO-PI4KIII α insufficiency affects neuronal *Aβ*_{arc} accumulation, we immunostained *Aβ* in *Aβ*_{arc}, *Aβ*_{arc}-*rbo*, and *Aβ*_{arc}-*PI4KIIIα* flies at the age of 25 d when the neural defects in *Aβ*_{arc} flies became prominent. The *Aβ*-immunoreactive signals in *Aβ*_{arc}-*rbo* and *Aβ*_{arc}-*PI4KIIIα* flies were weaker than that in *Aβ*_{arc} flies (Fig. 6B). Consistently, ELISA quantification revealed a remarkable reduction of *Aβ*₄₂ in the brains of *Aβ*_{arc}-*rbo*, *Aβ*_{arc}-*PI4KIIIα*, and PAO-treated flies (Fig. 6C,D). Thus, RBO/PI4KIII α insufficiency and PI4KIII α inhibition reduced neuronal *Aβ* accumulation in flies expressing *Aβ*_{arc}. Notably, neither *PI4KIIIα* nor *rbo* mutations reduced the transcription of *Aβ*_{arc} in *Aβ*_{arc}-expressing flies at different ages (Fig. 6E).

Downregulation of RBO/PI4KIII α facilitates *Aβ*₄₂ secretion

Downregulation of RBO-PI4KIII α presumably reduced the plasmalemmal level of PI₄P, and the plasmalemmal level of PI_{4,5}P was

Figure 3. The effects of *rbo*, *shibire*, and *itpr* mutations as well as *rbo*^{S358A} expression on the motor defect or premature death induced by dTau or A β _{arc}. Expression of *Drosophila* tau in the GF pathway produced aged-dependent decline of climbing ability (A) and premature death (B). *rbo* insufficiency worsened these deficits. Minimum $n = 30$ per strain for climbing assay, one-way ANOVA using LSD test; $n = 200$ per strain for longevity assay, log-rank test; compared with ctrl, p for dTAU is 1.7072E-5; compared with dTAU, p for dTAU-*rbo*^{ts1/+} is 1.1173E-4, p for dTAU-*rbo*^{2/+} is 0.5831. Expression of dominant SHIBIRE^{ts1} mutation neither improved the climbing ability (C) nor expanded the lifespan (D) of A β _{arc} flies. Minimum $n = 30$ per strain for climbing assay, one-way ANOVA using LSD test; $n = 200$ per strain for longevity assay, log-rank test; compared with ctrl, p for SHIBIRE^{ts1} is 0.0004, p for A β _{arc} is 0.0001; compared with A β _{arc}, p for A β _{arc}-SHIBIRE^{ts1} is 0.4895. Neither the expression of RBO^{S358A} (E) nor the mutation of *itpr* (F) expanded the lifespan of A β _{arc} flies. $n = 200$ per strain for longevity assay, log-rank test; compared with A β _{arc}, p for A β _{arc}-RBO^{S358A} is 0.4996, p for A β _{arc}-*itpr*^{sv35} is 0.0470. ** $p < 0.01$.

reported to inversely correlate with the cellular secretion of A β ₄₂ (Landman et al., 2006). It is conceivable that downregulation of RBO-PI4KIII α may facilitate A β ₄₂ secretion and thereafter help reduce neuronal A β ₄₂ accumulation. To test whether a change in A β ₄₂ release contributes to the reduced neuronal accumulation of A β in PAO-treated and RBO/PI4KIII α insufficiency flies, we developed a semi-*in vivo* analysis method to test A β ₄₂ release. The A β ₄₂ expressed in our flies was fused to a secretion signal peptide and could be released into extracellular spaces (Crowther et al., 2005), or further released out of the CNS. A β _{arc}-expressing third-instar larvae were dissected and cultured in Schneider's culture medium, and the A β ₄₂ released into the medium was quantified by ELISA. Indeed, PAO treatment increased A β ₄₂ concentration in the medium in a dose-dependent manner (Fig. 7A), indicating that PI4KIII α inhibition facilitates the A β ₄₂ release. Similarly, relatively higher A β ₄₂ concentrations were found in the medium culturing the dissected A β _{arc}-*rbo* and A β _{arc}-PI4KIII α larvae (Fig. 7B, C).

We also tested whether this facilitation applies to APP-derived A β ₄₂ by examining A β ₄₂ release from cultured HEK293T cells stably overexpressing human APP (APP-HEK293T cells). Indeed, PAO similarly increased A β ₄₂ concentration in the medium (Fig. 7D). A1, another specific inhibitor of PI4KIII α (Bojjireddy et al., 2014), which is structurally different from PAO, also facilitated A β ₄₂ secretion from APP-HEK293T cells (Fig. 7E). Knock-down of Efr3a (encoding a human homolog of RBO) and PI4KA (encoding PI4KIII α) significantly increased the medium A β ₄₂ as well, but to a lesser extent (Fig. 7F). Notably, PAO neither changed the enzymatic activities of α -, β -, and γ -secretase (Fig. 7G) nor altered the protein level of APP (Fig. 7H). PAO at the concentration of ≤ 150 nM unlikely inhibited the activities of tyrosine phosphatase, receptor endocytosis, and glucose transport (Le Cabec and Maridonneau-Parini, 1995). We have tried to investigate the effect of PAO on the intracellular level of A β ₄₂ in APP-HEK293T cells by ELISA, yet intracellular A β ₄₂ was undetectable, likely due to a rapid degradation mediated by N-end rule pathway (Brower et al., 2013).

Figure 4. Physical interactions between RBO and PI4KIII α . **A**, Representative immunoblot showing the coimmunoprecipitation of RBO and PI4KIII α . w^{1118} flies were used. $n = 3$ replicates. **B**, Representative immunoblot (left) and normalized quantification of RBO and PI4KIII α protein levels (middle and right) in *rbo* heterozygotes and control flies without or with the expression of $A\beta_{arc}$. $n = 7$ per bar, one-way ANOVA using Dunnett's test. **C**, Representative immunoblot (left) and normalized quantification (right) of PI4KIII α protein coimmunoprecipitated with the mutant RBO protein (mRBO, encoded by *rbo*^{ts1}), compared with that with the wild-type RBO (wtRBO). $n = 4$ per bar, paired *t* test. **D**, Normalized quantification of the *PI4KIII α* mRNA levels in $A\beta_{arc}$ $A\beta_{arc}$ $rbo^{ts1/+}$, and $A\beta_{arc}$ $rbo^{2/+}$ larva and adult flies. Minimum $n = 5$ per bar, one-way ANOVA using Dunnett's test. * $p < 0.05$.

PI₄P facilitates A β ₄₂ assembly or oligomerization in/on liposomes

Previously, PI and PI_{4,5}P were reported to facilitate A β ₄₂ assembly or aggregation in phospholipid membranes at neutral pH condition (McLaurin and Chakrabarty, 1997; McLaurin et al., 1998). Given the role of RBO/Efr3a-PI4KIII α in the metabolism of phosphoinositides, especially PI₄P, it may affect A β assembly or oligomerization in/on the plasma membrane. To test this possibility, we examined the effect of PI, PI₄P, and PI_{4,5}P on the oligomerization of monomeric A β ₄₂ in liposomes with lipid composition similar to that found in the cerebral cortex membranes at neutral pH condition (Table 2). PI, PI₄P, and PI_{4,5}P each only constitute a small percentage of total lipids in the liposomes (Table 2). As shown in Figure 8A, PI₄P in liposomes enhanced the assembly or oligomerization of A β ₄₂ in a dose-dependent manner. Moreover, PI₄P appeared to be the most efficient enhancer compared with PI and PI_{4,5}P (Fig. 8B). This is consistent with a previous finding that among the three forms of the headgroups of phosphoinositides (i.e., the monophosphorylated, diphosphorylated, and triphosphorylated inositol ring, the inositol-1,4-phosphate form appears to be the strongest inducer of β -structure in A β ₄₂ at neutral pH condition (McLaurin et al., 1998).

Discussion

Consistent with previous studies (Zhao et al., 2010; Han et al., 2015), this study demonstrates that expression of A β ₄₂ in the GF pathway of *Drosophila* leads to age-dependent neuronal A β ₄₂ accumulation, synaptic failure, and other neural deficits. Genetic reduction of RBO and PI4KIII α , or pharmaceutical inhibition of PI4KIII α , can reduce neuronal A β ₄₂ accumulation and suppress the associated phenotypic changes. The yeast and mammal homologs of RBO are membrane-localized proteins that recruit cytosolic PI4KIII α to form a complex that tightly controls the

plasmalemmal level of PI₄P (Faulkner et al., 1998; Huang et al., 2004; Baird et al., 2008; Nakatsu et al., 2012). In *Drosophila*, RBO is also a plasma membrane protein (Faulkner et al., 1998; Huang et al., 2004), which forms a complex with PI4KIII α and presumably controls the plasmalemmal level of PI₄P. PI₄P can facilitate A β ₄₂ assembly or oligomerization in/on liposomes with lipid composition similar to that of human cerebral cortex membranes. A β ₄₂ oligomers, compared with A β ₄₂ monomers, have a much higher affinity to plasma membrane and are much more difficult to leave the cells (Narayan et al., 2013; Sarkar et al., 2013). Therefore, downregulation of RBO/Efr3-PI4KIII α presumably decreased the level of PI₄P and oligomeric A β ₄₂ in/on the plasma membrane, hence allowed more A β ₄₂ release into the extracellular space. Consistently, genetic downregulation of RBO-PI4KIII α and chemical inhibition of PI4KIII α 's enzymatic activity facilitated cellular A β ₄₂ release.

Downregulation of RBO-PI4KIII α facilitated cellular A β ₄₂ release, but both immunostaining and ELISA revealed a reduction of A β ₄₂ level in the CNS of A β ₄₂-expressing flies. The reason might be that the A β ₄₂ released from neurons was at least partially further released out of the CNS, as was reflected by the ELISA results of A β ₄₂ in the medium culturing the dissected A β ₄₂-expressing larvae.

In the A β ₄₂-expressing transgenic flies, A β ₄₂ is released by γ -secretase-independent processing of a secretion signal peptide (Crowther et al., 2005). In contrast, A β ₄₂ from HEK293-APP cells is produced through sequential cleavage of APP by β - and γ -secretase. Despite all the differences, the A β ₄₂ release from HEK293-APP cells was also facilitated by inhibiting PI4KIII α 's enzymatic activity and by knocking down PI4KIII α or a human RBO homolog Efr3a, without changing APP level or APP-processing secretases' activities. These results suggest that plasmalemmal PI₄P acts on the APP-derived A β ₄₂ similarly as it does

Figure 5. Genetic reduction or pharmaceutical inhibition of PI4KIII α suppresses neural deficits in $A\beta_{arc}$ -expressing flies. The representative EJP recordings in ctrl, $pi4k^{def/+}$, $pi4k^{GS27/+}$, $A\beta_{arc}$, $A\beta_{arc}-pi4k^{def/+}$, $A\beta_{arc}-pi4k^{GS27/+}$ flies of 31–35 d (**A**) and in $A\beta_{arc}$ flies of 31–35 d treated at different doses of PAO (**B**). The synaptic failure, motor defect, and premature death in $A\beta_{arc}$ -expressing flies were attenuated by introducing a chromosome deficiency covering $PI4KIII\alpha$ (**A**, **C**), and a nonsense mutation of $PI4KIII\alpha$ (**A**, **D**), and by feeding with PAO (**E**, **F**). For clarity, the lifespan curves of control and $A\beta_{arc}$ flies were presented separately in **E** (bottom). Minimum $n = 6$ flies per bar for synaptic failure test, one-way ANOVA using Dunnett's test. Minimum $n = 30$ flies per bar for climbing assay, t test; $n = 100$ flies per line for longevity assays in **C** and **D**. Compared with $A\beta_{arc}$ p for $PI4KIII\alpha^{def/+}$ is 5.5337E-10, p for $PI4KIII\alpha^{GS27/+}$ is 5.5708E-22; $n = 200$ flies per line for longevity assays in **E**, log-rank test, compared with $A\beta_{arc}$ 0 μ M, p for $A\beta_{arc}$ 50 μ M is 3.3300E-8, p for $A\beta_{arc}$ 100 μ M is 1.9794E-10, p for $A\beta_{arc}$ 150 μ M is 1.7064E-22. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Figure 6. Downregulation of RBO/PI4KIII α reduces intraneuronal $A\beta$ accumulation. **A**, Whole-mount $A\beta$ staining of the ventral ganglion in control (top row) and $A\beta_{arc}$ flies (middle row). In both flies, the expression of mCD8-GFP was driven by the same driver [*Gal4*]/*A307*. Bottom row represents an enlarged view of the region defined by the square in the middle row. Notably, the $A\beta$ immunosignal in the ventral ganglion of $A\beta_{arc}$ flies generally colocalized with mCD8-GFP. Representative confocal images of whole-mount $A\beta$ staining of the ventral ganglion (**B**), and normalized ELISA quantification of CNS $A\beta_{42}$ level in $A\beta_{arc}$, $A\beta_{arc}-rbo^{ts1/+}$, $A\beta_{arc}-rbo^{2/+}$, $A\beta_{arc}-pi4k^{def/+}$, and $A\beta_{arc}-pi4k^{GS27/+}$ flies (**C**). Minimum $n = 3$ replicates per bar, unpaired t test. **D**, Normalized ELISA quantification of CNS $A\beta$ levels in $A\beta_{arc}$ flies with and without PAO treatment. $n = 3$ replicates per bar, paired t test. **E**, Quantification of the $A\beta_{arc}$ mRNA level in $A\beta_{arc}$ -expressing flies at different ages. Minimum $n = 3$, paired t test. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

on the $A\beta_{42}$ in the transgenic flies. That is to say, HEK293-APP cells may also undergo PI_4P -facilitated oligomerization or assembly of $A\beta_{42}$ on the plasma membrane. There are two origins of plasmalemmal $A\beta_{42}$: (1) the production and release of a portion of APP-derived $A\beta_{42}$ occur at the plasma membrane (Haass et al., 2012; Sannerud et al., 2016); and (2) some APP-derived $A\beta_{42}$ generated intracellularly might also be transferred to the plasma membrane by membrane trafficking (Rajendran et al., 2006; Annunziata et al., 2013). On the plasma membrane, the conformation of all $A\beta_{42}$, no matter produced locally or secreted by other cells, could be affected similarly by PI_4P . Therefore, downregulation of RBO-PI4KIII α produced similar effect on $A\beta_{42}$ release from HEK293-APP cells. Nevertheless, we could not exclude

other possible ways for downregulation of RBO-PI4KIII α to regulate cellular release of $A\beta_{42}$ in both $A\beta_{42}$ -expressing fly neurons and HEK293-APP cells.

$A\beta$ accumulates both extracellularly and intracellularly in AD brains. For many years, there has been much focus on the prevention and removal of extracellular amyloid deposits in both patients and animal models. However, neither the inhibition of $A\beta$ production by using γ - and β -secretase inhibitors (Doody et al., 2013) (www.alzforum.org) nor the removal of extracellular $A\beta$ by immunotherapy against $A\beta$ (St-Amour et al., 2016) managed to slow down the cognitive decline in AD patients. Given these repetitive failures in efforts targeting extracellular $A\beta$, both the academic and industrial area of AD research should pay at-

Figure 7. Downregulation of RBO/Efr3a-PI4KIII α facilitates cellular A β_{42} release. Normalized ELISA quantification of A β_{42} concentrations in the media culturing dissected A β_{arc} -expressing larvae with and without PAO treatment (**A**), *rbo* mutation (**B**), *PI4KIII α* mutation (**C**), and culturing APP-HEK293T cells with PAO (**D**), A1 (**E**), and *Efr3a* RNAi/*PI4KA* RNAi (**F**) treatment. **A–C**, **E**, Minimum $n = 6$ replicates per bar. **D**, **F**, Minimum $n = 4$ replicates per bar, t test. **G**, Normalized activities of α -, β -, and γ -secretases with and without PAO treatment at different concentrations. $n = 5$ for each bar, one-way ANOVA with Dunnett's test. **H**, Representative immunoblot showing the APP level in HEK293T cells with and without PAO treatment at different concentrations, repeated at least 3 times. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Table 2. Composition of liposome preparations

	CAS no.	MW	CCM-lipid ^a (mol %)	Ctrl-L ^b (μ l)	PI-L ^b (μ l)	PI ₄ P-L ^b (μ l)	PI _{4,5} P-L ^b (μ l)
Phosphatidylcholine	63-89-8	734.04	15.4	113	113	113	113
Phosphatidylserine	145849-32-7	757.95	8.9	68	68	68	68
Phosphatidylethanolamine	1069-79-0	748.07	26.5	198	198	198	198
Sphingomyelin	85187-10-6	973.55	8.9	87	87	87	87
Cholesterol	57-88-5	386.65	14.8	57	57	57	57
Cerebroside	—	—	15.9	—	—	—	—
GM1	—	—	9.7	—	—	—	—
A β_{42}	—	4514.04	—	45	45	45	45
PI	119943-95-2	833.01	—	—	17	—	—
PI ₄ P	214332-61-3	956.96	—	—	—	19	—
PI _{4,5} P	120595-88-2	1080.9	—	—	—	—	22
Total volume (μ l)	—	—	—	568	585	587	589

^aThe liposome solution prepared in the study by Tashima et al. (2004), which was composed of a lipid mixture similar in composition to cerebral cortex membranes (CCM-lipid).

^bCtrl-L, PI-L, PI₄P-L, and PI_{4,5}P-L represent the liposome solution used in the present study, which contains 10 μ M A β_{42} , plus 20 μ M (~3% w/w of total lipids) PI, PI₄P, and PI_{4,5}P, respectively.

Synthetic A β applied to the extracellular space of cultured brain tissue and cells has been reported to produce toxicity (Lambert et al., 1998; Walsh et al., 2002; Lacor et al., 2004). One

PAO is a very potent inhibitor of PI4KIII α . It binds to vicinal thiols and may influence other targets or cellular events, such as the activities of tyrosine phosphatase, receptor endocytosis, and glucose transport, but only at concentrations much higher than those used in the present study (Le Cabec and Maridonneau-

Parini, 1995). Importantly, PAO's effect on cellular A β_{42} release via inhibiting PI4KIII α 's enzymatic activity was confirmed by using another structurally different PI4KIII α -specific inhibitor A1, and by genetic downregulation of PI4KIII α and Efr3a.

The less robust effect of RBO-PI4KIII α downregulation on HEK293 cells than fly CNS neurons could be because A β_{42} was mostly produced on the plasma membrane in fly neurons expressing A β_{42} , while A β_{42} was produced both on the plasma membrane and intracellular endosomes/lysosomes in HEK293 cells, and that the examination of A β_{42} release from fly CNS was a semi-*in vivo* assay whereas the former was a pure *in vitro* assay.

In control flies, reduction of RBO or PI4KIII α level, weakening of the interaction between RBO and PI4KIII α , or inhibition PI4KIII α 's enzymatic activity by PAO did not significantly change the synaptic transmission, mobility, and lifespan. These results are consistent with the discovery that a significant number of normal humans have only one copy of the PI4KA gene (Conrad et al., 2010). One possible explanation is that insufficiency of RBO/PI4KIII α may not significantly change the plasmalemmal level of PI $_{4,5}$ P, whose alternation may remarkably influence synaptic transmission and cellular function. This explanation is supported by the reports that complete deletion of PI4KA gene or inhibition of PI4KIII α with much higher concentration of PAO produces minor or moderate effect on the level of plasmalemma PI $_{4,5}$ P, despite that the plasmalemma PI $_{4,5}$ P is markedly reduced (Hammond et al., 2012; Nakatsu et al., 2012). Interestingly, in the presence of A β_{42} , downregulation of PI4KIII α may actually attenuate the depletion of plasmalemmal PI $_{4,5}$ P caused by oligomeric A β_{42} (Berman et al., 2008) via reducing PI $_{4,5}$ P-facilitated formation of oligomeric A β_{42} .

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