EphA4 regulates Aβ production via BACE1 expression in neurons

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Abstract

Background

Several lines of evidence suggest that the aggregation and deposition of amyloid-β peptide (Aβ) initiate the pathology of Alzheimer disease (AD). Recently, a genome- wide association study demonstrated that a single-nucleotide polymorphism proximal to the *EPHA4* gene, which encodes a receptor tyrosine kinase, is associated with AD risk. However, the molecular mechanism of EphA4 in the pathogenesis of AD, particularly in Aβ production, remains unknown.

Methods

To clarify the molecular regulatory mechanism of EphA4 in detail, we performed several pharmacological and biological experiments both *in vitro* and *in vivo*. In addition, we referred to two public RNAseq datasets to confirm the changes in *EPHA4* mRNA expression levels in the brains of AD patients.

Results

We demonstrated that EphA4 is responsible for the regulation of Aβ production. Pharmacological inhibition of EphA4 signaling and knockdown of *Epha4* led to increased Aβ levels accompanied by increased expression of β-site APP cleaving enzyme 1 (BACE1), which is an enzyme responsible for Aβ production. On the other hand, EPHA4 overexpression and activation of EphA4 signaling via ephrin ligands decreased Aβ levels. In particular, the sterile-alpha motif domain of EphA4 was necessary for the regulation of Aβ production. Finally, *EPHA4* mRNA levels were significantly reduced in the brains of AD patients, and negatively correlated with *BACE1* mRNA levels.

Conclusions

Our results indicate a novel mechanism of Aβ regulation by EphA4, which is involved in AD pathogenesis.

Background

Alzheimer disease (AD) is the most common neurodegenerative disease involving dementia, in which patients demonstrate neuronal loss in the brain(1–3). A characteristic pathological hallmark of AD are senile plaques, which are aggregate depositions composed of amyloid-β peptide (Aβ). Several lines of evidence have demonstrated that the increased production and aggregation of Aβ in the brain of patients induce severe synaptic dysfunction and neuronal loss, contributing to the pathogenesis of AD(4–6).

Aβ is produced by the sequential cleavage of amyloid precursor protein (APP) by β-site APP cleaving enzyme 1 (BACE1) and γ-secretase. The initial proteolysis by BACE1 occurs at a position located 99 amino acids from the C-terminus of APP, which releases soluble form of APP (sAPPβ) into the extracellular space, and generates the stub of APP. Subsequent cleavage of the stub, which is mediated by γ-secretase, occurs at various positions, leading to the production of Aβ with various C-terminal

lengths(7,8). Whereas the major product is Aβ40, which is composed of 40 amino acids, a small portion of the products is Aβ42, which is composed of 42 amino acids, and is much more aggregation-prone and hence accumulates as senile plaque(9).

Eph receptors are synaptic adhesion molecules, and are large receptors with tyrosine kinase activity. Eph receptors are classified into two subclasses, EphA and EphB, depending on their structural similarities and binding affinities to their ligands, the ephrins. In cell-to-cell trans-interactions, binding *in trans* between Eph receptors and membrane- associated ephrin ligands triggers the clustering of each molecule, followed by the promotion of autophosphorylation, leading to bidirectional activation of intracellular canonical signaling in both cells(10,11). This contact-dependent bidirectional signaling of Eph receptors and ephrin ligands has been reported to regulate a wide variety of biological functions, not only including cell adhesion and cell proliferation in peripheral tissues, but also the development, stabilization, and plasticity of synapses in the central nervous system(12,13).

In recent years, genome-wide association studies have demonstrated that single- nucleotide polymorphisms proximal to *EPHA1* and *EPHA4* are associated with the genetic risk of AD, and copy number variations in *EPHA5* and *EPHA6* have been identified in families with early-onset familial AD(14–16), indicating that EphA receptors and/or their signaling pathways are involved in AD. In particular, EphA4 has been reported to be associated with the progression of AD(17–21). For example, a recent study has reported the decreased expression of EPHA4 in the hippocampus of AD patients and AD model mice before the development of cognitive impairment(17). In addition, it has been reported that EphA4 functions as a receptor for Aβ oligomers and triggers synaptic impairment in the hippocampus(18,20). These studies indicated that EphA4 plays an important role in the pathogenesis of AD; however, the detailed molecular regulatory mechanisms of EphA4, particularly underlying Aβ production, remain unclear.

In this study, we demonstrated the role of EphA4 in the regulation of Aβ production via BACE1 expression through both *in vitro* and *in vivo* analyses. Pharmacological inhibition of EphA4 signaling and knockdown of *Epha4* led to an increase in Aβ levels, accompanied by the increased expression of BACE1. On the other hand, EPHA4 overexpression and the activation of EphA4 signaling via ephrin ligands decreased Aβ levels. Notably, we confirmed that the sterile-alpha motif (SAM) domain of EPHA4 contributes substantially to the regulation of Aβ production. Finally, we confirmed that *EPHA4* mRNA levels were reduced in the brains of AD patients, showing a negative correlation with *BACE1* mRNA levels. Taken together, our results suggest a novel regulatory mechanism of Aβ production by EphA4, which is involved in the pathogenesis of AD.

Methods

Antibodies

The following antibodies were purchased from commercial suppliers: anti-C- terminal domain of APP (#18961, Immuno-Biological Laboratories, Gunma, Japan, 1:1000 dilution), anti-C terminus of sAPPβ (#18957, Immuno-Biological Laboratories, Gunma, Japan, 1:1000 dilution), anti-C-terminal domain of BACE1 (#18711, Immuno- Biological Laboratories, Gunma, Japan, 1:1000 dilution), anti-Nct C terminus (N1660, Sigma, MO, USA, 1:1000 dilution), anti-α-tubulin DM1A (T6199, Sigma, MO, USA, 1:2000 dilution), anti-V5 Tag antibody (Thermo Fisher Scientific, MA, USA, 1:1000 dilution), TUJ1 anti-human βIII tubulin (R&D systems, MN, USA, 1:2000 dilution), Clone N103/39 anti-ALDH1L1 antibody (NeuroMab, CA, USA, 1:1000 dilution), 6H7 anti-EPHA4 antibody (Abnova, Taipei, Taiwan, 1:1000 dilution), 4G10 anti-Phosphotyramine-KLH antibody (Merck Millipore, MA, USA, 1:500 dilution).

Peptides and reagents

KYL (H2N-KYLPYWPVLSSL-COOH), biotinylated KYL (H2N- KYLPYWPVLSSLGSGSK-(biotin)-COOH), WDC (H2N-WDCNGPYCHWLG-COOH), and biotinylated WDC (H2N-WDCNGPYCHWLGGSGSK-(biotin)-COOH) were synthesized by BEX CO., LTD. (Tokyo, Japan). Recombinant ephrin-Fc were obtained from the companies as following: mouse ephrin-A1/Fc Chimera and mouse ephrin-B1/Fc Chimera from R&D Systems (MN, USA), ChromPure Human IgG Fc Fragment and AffiniPure goat anti-human IgG Fc fragment specific from Jackson Immuno Research Laboratories (PA, USA). 100 µL of ephrin-Fc (100 µg/mL) was mixed with 19 µL anti-human IgG and added to cells after 1 hr of incubation at 37°C.

Plasmids preparation and transfection

Epha4-WT plasmid was kindly provided by Dr. Atsuko Sehara at Kyoto University. V5-His was tagged at the C terminus of *Epha4*-WT. *Epha5*-WT was cloned using the same vector. *Epha4*-KD (kinase-dead) has a K653M mutation, leading to the loss of kinase activity as previously reported(22). *Epha4*-ΔSAM has a deletion range from 908 to 964 amino acid residues, only remains 12 amino acids of SAM domain at the upstream of the PDZ binding motif.

For overexpression of EPHA4 and EPHA4 mutants, Neuro2a (N2a) cells were transfected with a mixture of the plasmid with polyethylenimine (PEI) or FuGENE6 (Promega, WI, USA) solution according to the manufacture's instruction.

Cell culture and generation of stable cell lines

N2a cells (#CCL–131, ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, UT, USA), and 50 unit/mL Penicillin/Streptomycin (Thermo Fisher Scientific, MA, USA). Primary neuron-glia mixed cells were cultured in Neurobasal medium (Thermo Fisher Scientific, MA, USA) supplemented with 2 mM L-Glutamine (Thermo Fisher Scientific, MA, USA), 50 unit/mL Penicillin/Streptomycin and 2% B–27 supplement (Thermo Fisher Scientific, MA, USA). All cell lines were maintained in a 5% CO2, 95% air

atmosphere incubator at 37°C. Contamination of mycoplasma is routinely tested by PCR and DNA agarose electrophoresis.

To generate stable cell lines, N2a cells were transiently transfected with plasmids coding murine *Epha4*-WT or *Epha4*-mutants using PEI solution and underwent neomycin G418 selection (Millipore Sigma, St. Louis, USA).

For neuron-glial mixed culture and primary neuron-enriched culture, the plate was coated using 250 μL of poly-L-ornithine solution (PLO; SIGMA, MO, USA) overnight. PLO was washed by FBS-free DMEM before collect primary cells. Primary cells were obtained from the fetuses of E18 or E19 pregnant Wistar rat (Japan SLC, Inc., Shizuoka, Japan). All procedure was carried out using cold Hanks' Balanced Salt Solution (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The collected brain tissue was incubated with 0.25% Trypsin (Thermo Fisher Scientific, MA, USA), 0.1 μL/mL DNase (Nippon Gene, Toyama, Japan), 0.8 mM MgSO4 (Kanto Chemical, Tokyo, Japan), and 1.85 mM CaCl2 (Kanto Chemical, Tokyo, Japan) at 37°C. After centrifugation, cells were counted for the appropriate amount and plated into the plate. For primary neuron-enriched culture, cells were cultured in Neurobasal medium containing 1 μM Ara-C (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) from DIV1. For primary glia-enriched culture, the plate without pre-coating with PLO was used and cells were cultured in DMEM supplemented with 10% FBS.

Aβ detection

For the measurement of the secreted Aβ, conditioned media were collected, and cell debris was removed by the centrifugation at 240 x g for 3 min. For secreted Aβ from primary cells, Aβ levels were analyzed by two-site enzyme-linked immunosorbent assay (ELISA) using Human/Rat β Amyloid (40) ELISA Kit (294–64701, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) and Human/Rat β -Amyloid (42) ELISA Kit, High Sensitivity (292–64501, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), as described(23,24). The secreted Aβ from N2a cells was analyzed by ELISA using a homemade Aβ detecting plate based on the same principle of manufacturer's ELISA Kit(9).

Aβ levels measured by ELISA were then standardized by protein concentrations of the cell lysates and further normalized to the control in each experiment as indicated.

Immunoblotting

All procedures of immunoblotting were performed as previously described(24). Briefly, for sample preparation, cells were lysed by Laemlli 1X sample buffer (2% sodium dodecyl sulfate (SDS; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 80 mM Tris-HCl with pH 6.8, 15% glycerol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 0.0025% Brilliant green (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 0.00625% Coomassie Brilliant Blue G–250 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan)) and sonicated (BRANSON, Danbury, CT, USA). Protein concentrations were

measured by using BCA protein assay (Takara Bio, CA, USA) following the manufacturer's instruction. The conditioned medium was collected and diluted using a 5X sample buffer. All samples were added with 1% 2- mercaptoethanol (Millipore Sigma, St. Louis, USA) and boiled at 100°C.

Samples and protein marker, Precision Plus Protein Dual Xtra Standards (BIORAD, CA, USA), were applied to SDS-polyacrylamide gel (7.5–15% Tris-Glycine or Tris-Tris gels) and transferred onto PVDF membrane (Millipore, MA, USA). The immunodetection was used ImmunoStar detection kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) or Supersignal West Femto (Thermo Fisher Scientific, MA, USA), and chemiluminescence was detected using Image Quant LAS4000 (GE Healthcare, IL, USA). The immunoreactive protein bands were digitally captured and quantified using ImageJ (NIH) software.

Biotinylated antagonist binding assay

N2a cells overexpressing EPHA4 or EPHA5 were harvested the day after transfection. Cells were washed with phosphate-buffered saline (PBS; 8 mM Na2HPO412H2O, 2 mM NaH2PO42H2O, 130 mM NaCl), mixed with the appropriate volume of HEPES lysis buffer (10 mM HEPES pH7.4 (DOJINDO, Kumamoto, Japan), 150 mM NaCl, 1% TritonX–100 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 1 mM EDTA, 10% glycerol) and sonicated. All samples were adjusted to contain an equal amount of total protein after quantification by BCA protein assay. Streptavidin Sepharose (Thermo Fisher Scientific, MA, USA) was then added, followed by the rotation at 4°C for 1 hr. After centrifugation at 15,000 rpm for 3 min, the supernatant was collected and a small portion of it was used as an input sample in immunoblotting. The remained supernatant was added with biotinylated KYL or biotinylated WDC with/without non-tagged KYL or WDC at the indicated concentration, and rotated at 4°C overnight. After centrifugation at 15,000 rpm for 3 min, the pellet was washed by lysis buffer and added with sample buffer as pulled down sample in immunoblotting.

Stereotaxic injection of KYL peptide

All experiments using animals in this study were performed according to the guidelines provided by the Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences at the University of Tokyo (protocol no.: P26–9). 10 mM KYL peptide/PBS and PBS were injected into the right hippocampus (Anteroposterior: –2.0, Mediolateral: –1.5, Dorsoventral: –1.6) and left hippocampus (Anteroposterior: –2.0, Mediolateral: +1.5, Dorsoventral: –1.6), respectively, of 8 weeks old C57BL/6J male mice (Japan SLC, Inc., Shizuoka, Japan). 8 hrs after injection, both hippocampi were collected, lysed using RIPA buffer (Thermo Scientific, Waltham, MA, USA) containing Complete protease inhibitor cocktail (Millipore Sigma, St. Louis, USA), homogenized, and ultra-centrifuged at 444,000 rpm at 4°C for 20 min. The supernatant was used for Aβ detection by ELISA.

Immunoprecipitation

Cells were harvested, washed by cold PBS, and lysed with 1% 3-[(3- cholamidopropyl) dimethylammonio]–2-hydroxypropanesulfonate (CHAPSO; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) / HEPES buffer (10 mM HEPES pH7.4, 150 mM NaCl, Complete protease inhibitor cocktail EDTA free (Millipore Sigma, St. Louis, USA)). After the centrifugation at 15,000 rpm at 4°C for 3 min, the appropriate amount of the supernatant was taken as an input sample. Aliquots of the supernatant were mixed with 30 µL of 50% Protein G agarose (Thermo Fisher Scientific, MA, USA) / Tris-buffered saline (TS) and rotated at 4°C for 1 hr. After the centrifugation at 3,000 rpm for 5 min, the supernatant was added with an anti-EPHA4 antibody, and rotated at 4°C overnight. 30 µL of 50% Protein G agarose/TS was added to all samples on the following day. After the additional rotation at 4°C overnight, samples were centrifuged at 3,000 rpm, 4°C for 5 min. The pellet was washed several times with lysis buffer, and added with sample buffer to each sample, followed by the immunoblotting using 4G10 anti-Phosphotyramine-KLH antibody.

Knockdown by shRNA treatment

For knockdown of *Epha4* in the primary neuron, shRNA targeting *Epha4* sequence (CCGGgatatgtccaatcaagatgtTTCAAGAGAacatcttgattggacatatccTTTTTG) was cloned into the pLKO.1 puro vector (#8453, Addgene). LentiX–293T cells were transiently co-transfected with the packaging plasmids (pCAG-KGP4.1R, pCAG4-RTR2, and pCAGS-VSVG) and the prepared plasmid using PEI solution. After the collection of the medium including lentivirus particles, it was concentrated using Lenti-X™ concentrator (Clontech, CA, USA). The lentiviral particles were resuspended in 500 µL Neurobasal medium and added at 30–60 µL/well into the primary neuron (DIV3). Medium change with fresh 250 µL Neurobasal medium was done at DIV7. The conditioned medium for detecting Aβ levels was collected after 24 hrs of incubation and the cells were collected by sample buffer for immunoblotting.

Data availability

Two public RNAseq datasets were obtained from AMP-AD Knowledge Portal (https://www.synapse.org/#!Synapse:syn2580853) as previously described(25): the Mayo sample set(26) and MSBB studies. The Mayo study comprises temporal cortex samples from 164 subjects with the following pathological diagnosis: 84 patients with AD and 80 controls. We assessed *EPHA4* expression of the temporal cortex between AD patients and controls by a simple model (syn6090804) adjusting for key covariates: age at death, gender, RIN, source, and flow cell. For the MSBB study, we obtained Clinical information of each subject, RNAseq covariates, and normalized *EPHA4* or *BACE1* normalized RNA read counts (syn7391833). We selected 201 samples of the parahippocampal gyrus (BM36) from subjects and excluded the samples without the information of the Braak NFT stage. These data were applied and analyzed using RStudio. The comparison of *EPHA4* and *BACE1* gene expression levels was performed under different set conditions. We divided samples into two categories, healthy control subjects (CT) and AD patients, depending on the NP.1 stage, neuropathology Category as measured by CERAD. As described in the figure legend, we also divided degrees of neuritic plaque density

(plaque levels) into five categories depending on the provided plaque mean which is the mean neocortical plaque density across 5 regions, the middle frontal gyrus, orbital frontal cortex, superior temporal gyrus, inferior parietal cortex and calcarine cortex (of plaques/mm2). Suitable statistical analysis, Mann-Whitney U test, Kruskal-Wallis test with Dunn's post hoc analysis, and Kendall rank correlation were performed to compare the *EPHA4* and *BACE1*gene expression levels under each condition.

Statistical analysis

Data analyses were carried out from independent cells and were not conducted in a blinded fashion. And we excluded samples only when there is evidence of contamination, cell peeling, or cell death prior to the experiments. Data are presented as mean values and error bars indicate the standard error of the mean (s.e.m.). Suitable statistical analysis, unpaired/paired two-tailed Student's *t*-test or ANOVA with Tukey's or Dunnett's post hoc test, was performed. A *p*-value of less than 0.05 was considered to have a statistically significant difference between groups.

Results

KYL peptide increases Aβ production accompanied by increased BACE1 expression

KYL peptide comprised of 12 amino acid residues, was previously identified as an EphA4-binding peptide by the phage-display technique, and was shown to inhibit endogenous EphA4 signaling(27). To confirm the binding specificity of the KYL peptide to EphA4, we performed the biotin-streptavidin pull-down assay using a biotinylated KYL peptide. The biotinylated KYL peptide successfully pulled down EPHA4 overexpressed in Neuro2a cells, which is a murine neuroblastoma cell line. This binding was competitively inhibited by the non-tagged KYL peptide in a dose-dependent manner (sFig. 1; upper panel). On the other hand, EPHA5, which is a member of the Eph receptor family, together with EphA4, was not precipitated (sFig. 1; the rightmost in the lower panel), suggesting specific binding between the KYL peptide and EPHA4. Likewise, the WDC peptide, which is an EphA5 antagonist that was reported previously(28), also showed specific binding to EPHA5, but not to EPHA4 (sFig. 1; lower panel and the rightmost in the upper panel).

To clarify the effects of the KYL peptide on Aβ production, primary neuron-glia mixed cultured cells were used. The KYL peptide substantially increased the secretion of both Aβ40 and Aβ42 in a dose-dependent manner (Fig. 1A). The expression levels of neither APP nor nicastrin (Nct), the latter being a γ-secretase component, were altered. However, BACE1 expression was increased in the presence of the KYL peptide (Fig. 1B, C). These results suggested the possibility that increased BACE1 expression promotes the cleavage of APP, resulting in the upregulation of Aβ production. Consistent with the results of the primary cultures, injection of the KYL peptide into the hippocampus of wild-type mice also induced an increase in

Aβ40 levels (Fig. 1D). Taken together, our results both *in vitro* and *in vivo* indicated that the KYL peptide upregulates Aβ production, accompanied by increased BACE1 expression.

EphA4 signaling negatively regulates Aβ production in neuronal cells

Consistent with a previous study demonstrating the expression of EPHA4 in both neurons and glia(29), we confirmed the endogenous expression of EPHA4 in both primary neuron-enriched and primary glia-enriched cultures (sFig. 2A). Whereas both neurons and glial cells have the ability to produce Aβ(30) (sFig. 2B), addition of the KYL peptide only increased Aβ production in primary neuron-enriched cultures (sFig. 2B; left graph), and not in the primary glia-enriched cultures (sFig. 2B; right graph). These results suggested that the KYL peptide upregulates Aβ production only in neurons. To confirm the role of EphA4 in the regulation of Aβ production, we knocked down *Epha4* mRNA in primary neural cultures. As expected from the results of KYL peptide treatment, the reduced expression of EPHA4 substantially increased Aβ levels (Fig. 2A, B), indicating that EphA4 negatively regulates Aβ production.

Among the ephrin ligands, EphA4 can be activated by both the ephrinA and ephrinB ligands that are expressed on the surface of neighboring cells. To induce EphA4 signaling in neurons, the Human IgG Fc Fragment (Fc)-fused recombinant ephrin ligands ephrinA1 and ephrinB1, clustered using anti-Fc antibodies(12), were used. Both clustered ephrinA1-Fc, as well as ephrinB1-Fc, caused a significant decrease in Aβ secretion (Fig. 2C). Therefore, these data indicated that EphA4 signaling is involved in the regulation of Aβ production, and correlates inversely with secreted Aβ levels.

Mouse neuroblastoma Neuro2a (N2a) cells endogenously produce a substantial amount of Aβ, similarly to primary neurons. The overexpression of EPHA4 in N2a cells decreased the levels of secreted Aβ40 and Aβ42 (Fig. 3A), indicating that activation of EphA4 signaling, which is induced by overexpressed EPHA4, also suppressed Aβ production in N2a cells. In addition, EPHA4 overexpression resulted in decreased BACE1 expression, leading to a decreased level of sAPPβ, which is a direct proteolytic product of BACE1 activity (Fig. 3B, C). These results suggested that the activation of EphA4 signaling reduced BACE1 expression, thereby resulting in decreased Aβ production from neuronal cells.

SAM domain is necessary for the regulation of Aβ production by EphA4

In EphA4 signaling, the SAM domain in the cytoplasmic region is involved in protein-protein interactions. On the other hand, the tyrosine kinase domain of EPHA4 undergoes autophosphorylation, and initiates canonical EphA4 signaling(22,31). To clarify which domain of EPHA4 plays a role in regulating Aβ production, the following EPHA4 mutants were analyzed: EPHA4-kinase dead (KD), which has the K653M mutation within the tyrosine kinase domain (23), and EPHA4-ΔSAM, with a deletion of amino acid residues 908–964 of the SAM domain (Fig. 4A). As expected, no phosphorylated EPHA4 was observed in N2a cells overexpressing EPHA4-KD, whereas it was observed in N2a cells overexpressing EPHA4-WT

(Fig. 4B), supporting that the K653M mutation abolished the kinase activity. However, the overexpression of EPHA4- KD reduced Aβ40 secretion, showing the same effect as EPHA4-WT on Aβ production (Fig. 4C). On the other hand, the overexpression of EPHA4-ΔSAM in N2a cells did not induce a significant decrease in Aβ40 levels, and did not alter BACE1 expression (Fig. 4D, E). These data indicated that the SAM domain of EPHA4 in the cytoplasmic region rather than kinase activity mediates the regulation of Aβ production.

Reduction of *EPHA4* mRNA levels in AD patients

To confirm the changes in *EPHA4* mRNA expression levels in the brains of AD patients, we referred to two public RNAseq datasets deposited in the accelerating medicines partnership-Alzheimer's disease (AMP-AD) knowledge portal, namely, the Mayo RNAseq (MayoRNAseq)(26) and Mount Sinai Brain Bank (MSBB) AD cohorts(25). In the Mayo sample set, a significant decrease in *EPHA4* mRNA levels was observed in the temporal cortex of AD patients (Table 1). Furthermore, analysis of the MSBB, which contains data of more specific brain regions, showed that the expression of *EPHA4* mRNA was significantly decreased in Brodmann area (BM) 36 of AD patients, including in the parahippocampal gyrus where *EPHA4* expression is high in normal subjects (Fig. 5A). Most importantly, the decrease in *EPHA4* mRNA levels clearly correlated with increased amyloid plaque burden in the MSBB sample set (Fig. 5B). In addition, we observed a slight negative correlation between *EPHA4* and *BACE1* mRNA levels (Fig. 5C). Collectively, these data strengthen our findings that the downregulation of EphA4 is associated with increased Aβ levels, and thus its involvement in AD pathology.

Discussion

Several lines of evidence have suggested that EphA4 is associated with the progression of AD; however, the details of the regulatory mechanism underlying Aβ production by EphA4 remained unclear. In the present study, our pharmacological and molecular biological experiments clearly demonstrated the EphA4 signaling-dependent regulation of Aβ production, accompanied by the modulation of BACE1 expression (Figs. 1–3). We also showed that the SAM domain of EPHA4 was responsible for this regulation (Fig. 4). These results suggested the possibility that EphA4 signaling via the SAM domain in neurons reduced BACE1 expression, resulting in a decrease in Aβ production by suppressing the β-site cleavage of APP (Fig. 6; left panel). On the other hand, the downregulation of this EphA4 signaling is involved in AD pathogenesis via an upregulation of Aβ production (Fig. 6; right panel). Consistent with this conclusion, we also successfully demonstrated a significant decrease in *EPHA4* mRNA levels in both the temporal cortex and parahippocampal gyrus of AD patients, demonstrating a negative correlation with Aβ plaque burden and *BACE1* mRNA levels (Table 1 and Fig. 5). Considering previous reports showing that BACE1 expression is upregulated in AD brains(32,33), our results suggest that the EphA4-dependent regulation of Aβ production is associated with AD pathogenesis.

We showed lines of evidence that the SAM domain of EPHA4, rather than its kinase activity, is required for the regulation of Aβ production (Fig. 4). The SAM domain is located C-terminal to the kinase domain, and

mediates the dimerization/oligomerization of Eph receptors(10). In addition, previous studies have shown that the phosphorylation of conserved tyrosine residues located within the SAM domain is able to recruit SH2 domain-containing proteins, leading to the regulation of various cellular processes(31,34–36). Considering the previously reported kinase activity-independent, particularly SAM-dependent EphA4 signaling(10), proteins recruited to the SAM domain are expected to be important and to be associated with the regulation of Aβ production. Another possible mechanism is via the proteolytically cleaved product of EphA4. EphA4 is known to be a substrate of γ-secretase, and to be sequentially cleaved by matrix metalloproteases and γ-secretase in the regulation processes of its signaling, generating the EphA4 intracellular domain (EICD), including the SAM domain within the cell(37). As the EICD has been reported to increase dendritic spine formation(37), the SAM domain of EICD may regulate Aβ production after the cleavage of EphA4. In addition, the SAM domain of Smaug, a major regulator of maternal mRNA destabilization in *Drosophila,* interacts with the 3′ untranslated region (3′ UTR) of the target mRNAs(38,39). These lines of evidence led to the hypothesis that the SAM domain of EICD directly regulates BACE1 expression. Further molecular and cellular studies are necessary to clarify the mechanistic role of EphA4.

In the present study, we identified regulation of BACE1 expression as a mechanism of Aβ regulation by EphA4. Although our results showing the negative correlation between *EPHA4* and *BACE1* mRNA levels (Fig. 5C) suggested that EphA4 signaling directly regulates BACE1 expression at the transcriptional level, we do not exclude other possibilities, such as the translation and degradation of BACE1. Recent studies have demonstrated that eukaryotic initiation factor–2α (eIF2α) kinase is activated in response to diverse cellular stress stimuli, resulting in the activation of gene-specific translation of BACE1(40,41). On the other hand, a neuron-specific F-box protein, Fbx2, has been demonstrated to directly interact with BACE1, to promote BACE1 ubiquitinationandproteasomaldegradation(42,43).Inaddition,thelysosomal degradation of BACE1 has also been reported to be modulated by both ubiquitin- dependent and ubiquitin-independent trafficking, which are regulated by golgi-localized γ-ear-containing ARF-binding protein 3 (GGA3) and bridging integrator 1 (BIN1)(44– 46), respectively. Therefore, it is also possible that these molecules involved in the regulation of BACE1 levels are modulated by SAM domain-dependent EphA4 signaling.

In the central nervous system, EPHA4 has been found to be expressed in both neurons and glia(29), which is consistent with our results (sFig. 2A). Given the fact that BACE1 is mainly expressed in neurons(47), the EphA4-dependent regulation of Aβ production is expected to occur primarily in neurons. On the other hand, we demonstrated that the ephrin ligands ephrinA1 and ephrinB1, whose mRNA expression are mainly in glia rather than in neurons(48,49), regulate Aβ production (Fig. 2C). Additionally, other ephrin ligands, namely, ephrinA3 and ephrinA5, are also expressed not only in neurons but also in glia. These EphA4/ephrin intercellular interactions between neurons and glia have been reported to regulate synaptic function(47). Thus, intercellular communication between neurons and glia, as well as between neurons, may also play an important role in regulating EphA4 signaling, and thus mediate Aβ production in neurons.

Conclusion

In summary, we demonstrated the regulation of Aβ production by EphA4 signaling via BACE1 expression in neurons. The disruption of this regulation is thought to play an essential role in the pathogenesis of AD.

List Of Abbreviations

Aβ, amyloid-β peptide; AD, Alzheimer disease; EphA4, Ephrin type-A receptor 4; APP, amyloid precursor protein; BACE1, β-site APP cleaving enzyme 1; SAM, sterile-alpha motif; Nct, nicastrin; N2a, Neuro2a; KD; kinase-dead; BM, Brodmann area; EICD, EphA4 intracellular domain; PEI, polyethylenimine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ELISA, enzyme-linked immunosorbent assay; CHAPSO, 3-[(3-cholamidopropyl) dimethylammonio]–2-hydroxypropanesulfonate; PLO, poly-L-ornithine

Declarations

Ethics approval and consent to participate

All experiments using animals in this study were performed according to the guidelines provided by the Institutional Animal Care Committee of the Graduate School of Pharmaceutical Sciences at the University of Tokyo (protocol no.: P26–9).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing financial interests associated with this study.

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Authors' contributions

K. T., YW. C., Y. H. and T. T. designed the research. K. T. and YW. C. performed the biological analysis. A. S. generated the material. K. T., YW. C., Y. H. and T. T. analyzed the data. K. T., YW. C., Y. H. and T. T. wrote the paper. All authors discussed the results and approved the final manuscript.

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Table

Table 1. Mayo RNAseq cohort shows the decreased *EPHA4* mRNA level in AD patients

Gene Name	Dx.Beta	Effect Direction	Dx.q Value
EPHA4	-0.477782278	Down In AD	0.034724205

Mayo RNAseq data of temporal cortex RNA was collected and analyzed as described in the previous study(26). This table demonstrated the comparison of the *EPHA4* mRNA level from 80 control and 84 Alzheimer Disease subjects. Normalized read counts of *EPHA4* was assessed by the simple model considering the factors including Age at Death, Gender, RIN (RNA integrity number), Source, FLOWCELL. Dx.Beta represents the regression coefficient. Effect Direction indicates the changes in target mRNA level between control and AD patients. Dx.q Value represents the adjusted *p* value with a significant difference when Dx.q < 0.05. This analysis showed a significant reduction of *EPHA4* mRNA expression in the temporal cortex of AD patients.

Figures

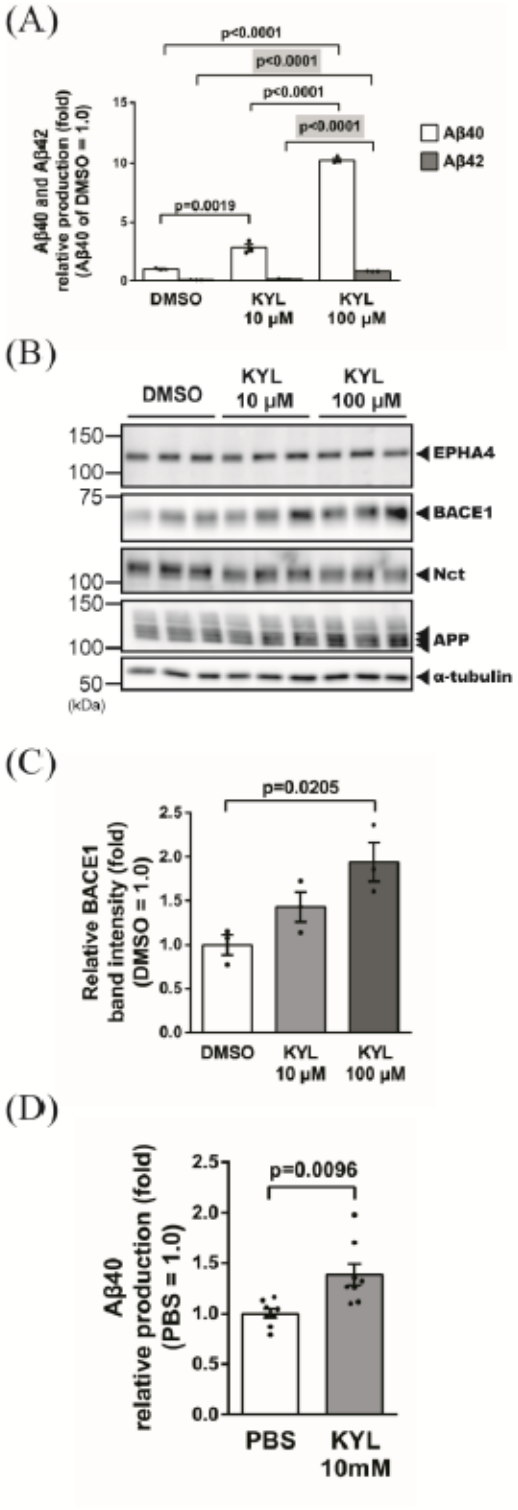

Figure 1

KYL peptide upregulates the secreted Aβ levels accompanied by increased BACE1 expression (A) Primary neuron-glia mixed cultured cells at DIV9 were treated with KYL peptide at the indicated concentration. After 24 hrs of incubation, the secreted Aβ levels were measured by two-side ELISA (n = 3, mean ± s.e.m., p value was assessed by one-way ANOVA with Tukey's HSD post hoc analysis, p value with a gray background indicates the comparison of Aβ42). (B) Immunoblotting of cell lysates in (A) using antibodies

against EPHA4, BACE1, Nct, APP, and α-tubulin. (C) Quantification of band intensities of BACE1 in (B). KYL peptide treatment induced an increased expression of BACE1 (n = 3, mean ± s.e.m., p value was assessed by one- way ANOVA with Tukey's HSD post hoc analysis). (D) Intrahippocampal KYL injection in 8 weeks old wild-type male mice. 8 hrs after injection, the hippocampus was collected, and soluble Aβ40 was measured by two-side ELISA (n = 8, mean ± s.e.m., p value was assessed by paired t-test).

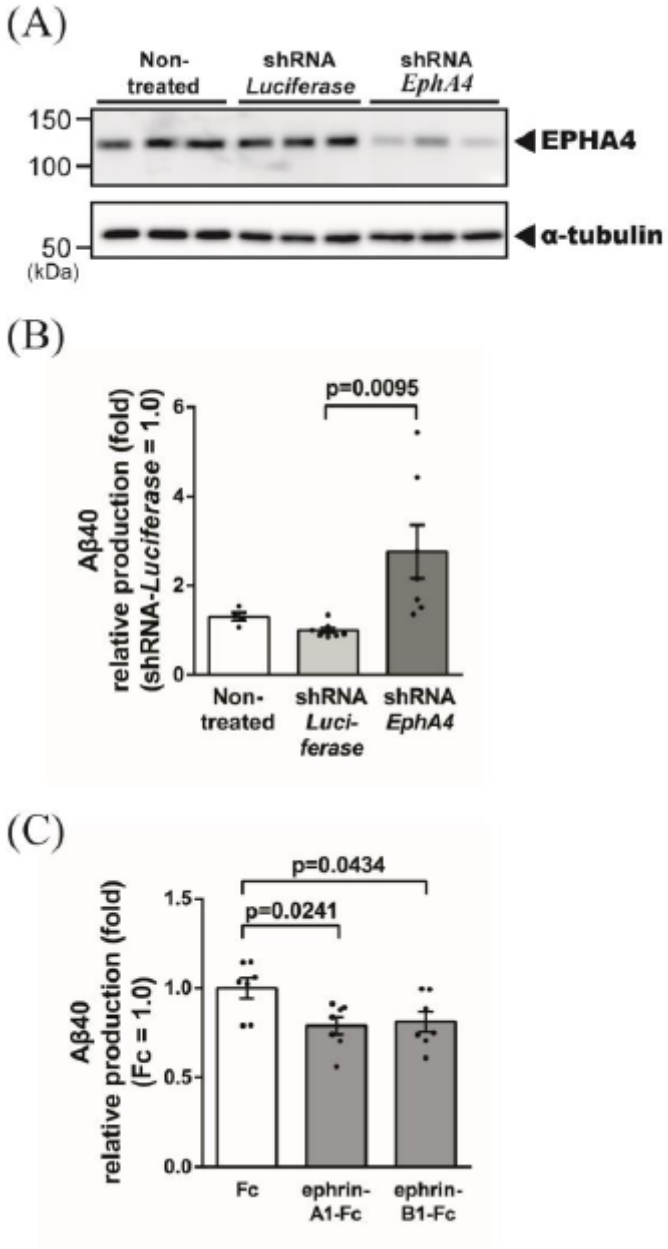

Figure 2

EphA4 signaling negatively regulates Aβ production (A) Immunoblotting of EPHA4 and α-tubulin. EPHA4 was successfully knocked down by Epha4-shRNA. (B) Secreted Aβ40 level in (A) was measured by two-side ELISA (n = 4-7, from at least 4 independent experiments, mean ± s.e.m., p value was assessed by one-way ANOVA with Dunnett's HSD post hoc analysis). (C) Clustered recombinant ephrinA1-Fc/ephrinB1-Fc was added into primary neuron- glia mixed culture at DIV9. After 8 hrs of incubation, secreted Aβ40

level was measured by two-side ELISA (n = 7, mean ± s.e.m., p value was assessed by one-way ANOVA with Dunnett's HSD post hoc analysis).

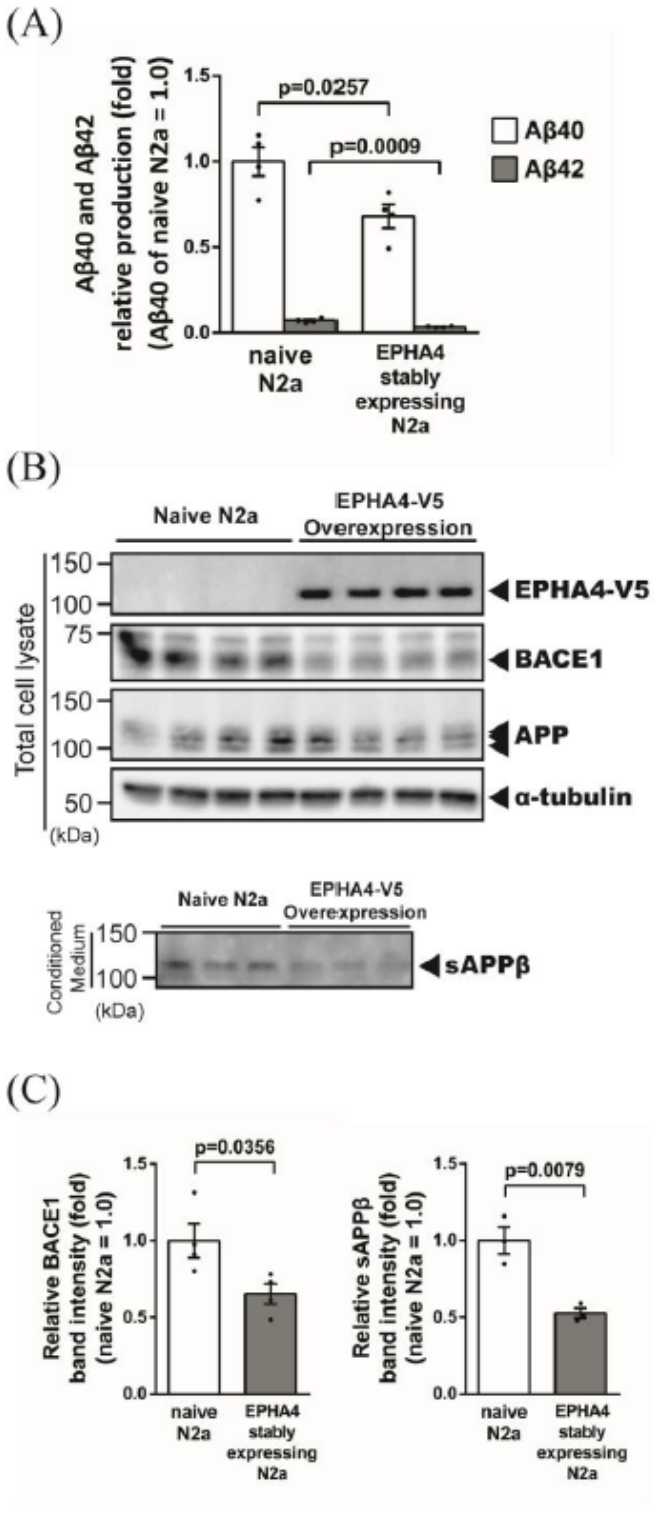

Figure 3

Overexpression of EPHA4 decreases Aβ production via BACE1 expression in N2a cells (A) Secreted Aβ levels of N2a cells stably overexpressing of EPHA4 were measured by two-side ELISA (n = 4, mean ± s.e.m., p value was assessed by Student's t-test). (B) Immunoblotting of cell lysates in (A) using

antibodies against V5, BACE1, APP, α- tubulin, and sAPPβ. (C) Quantification of band intensities of BACE1 and sAPPβ in (B) (n = 4 (BACE1), n = 3 (sAPPβ), mean ± s.e.m., p value was assessed by Student's t-test).

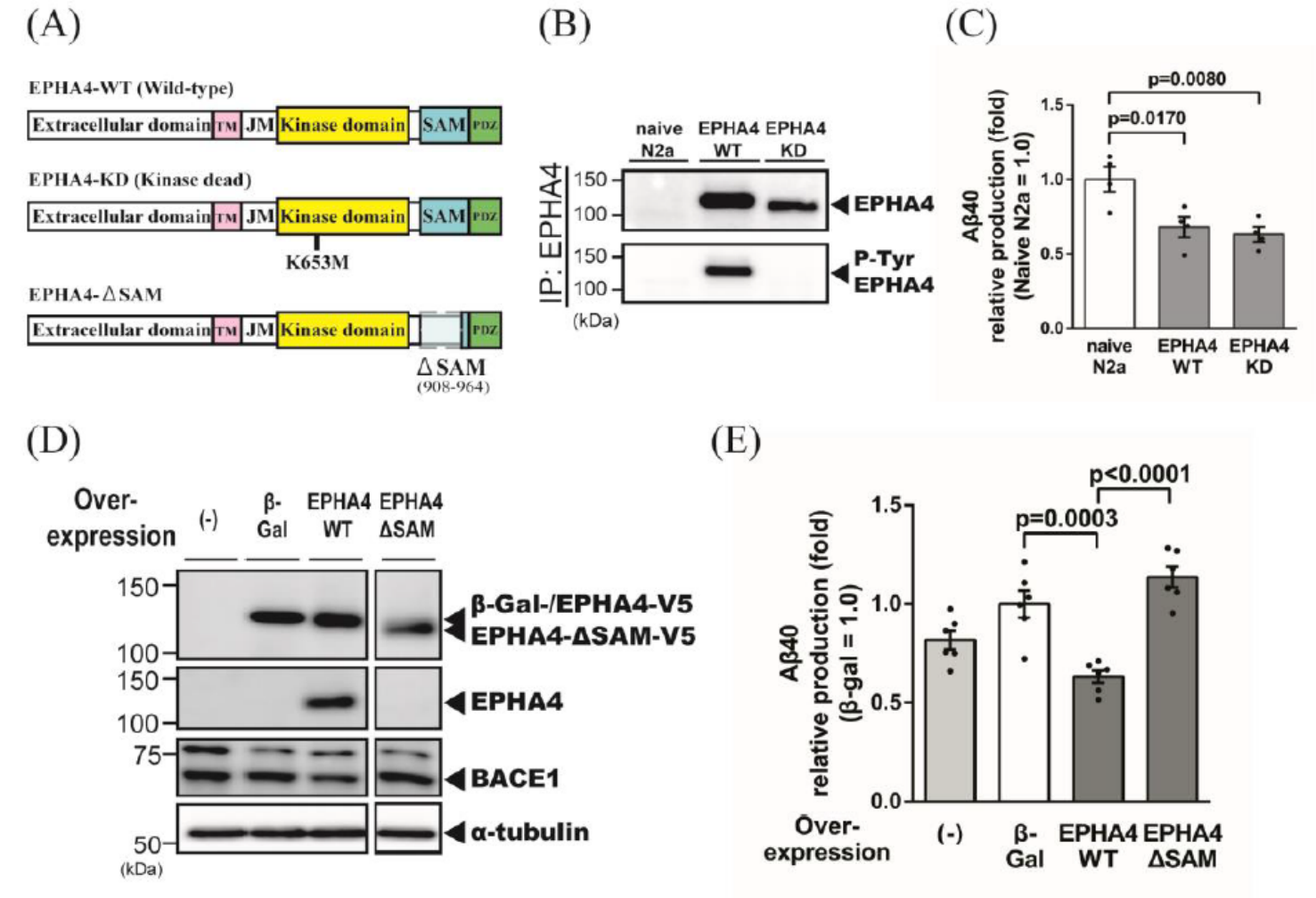

Figure 4

SAM domain is necessary for the regulation of Aβ production by EphA4 (A) Schemes of WT and cytoplasmic mutants of EPHA4. EPHA4-KD has a mutation of Lysin to Methionine at 653. EPHA4-ΔSAM has a deletion range from 908 to 964 amino acid residues of SAM domain. TM, transmembrane domain; JM, juxtamembrane domain. SAM, sterile-alpha motif. KD, kinase-dead. PDZ indicates the PDZ binding motif. The dotted blue rectangle indicates the deleted SAM domain. (B) Immunoblotting of phosphorylation in cells overexpressing EPHA4-WT or EPHA4- KD using antibodies against EPHA4 and phosphotyrosine-KLH. (C) Secreted Aβ40 levels of N2a cells stably overexpressing EPHA4 or EPHA4-KD were measured by two-side ELISA (n = 4, mean ± s.e.m., p value was assessed by one-way ANOVA with Dunnett's HSD post hoc analysis). (D) Immunoblotting of cells overexpressing EPHA4-WT or EPHA4-ΔSAM using antibodies against EPHA4, BACE1, V5, and α-tubulin. (E) Secreted Aβ40 levels of N2a cells stably overexpressing β-Gal, EPHA4-WT, or EPHA4-ΔSAM were measured by two-side ELISA (n = 3, mean ± s.e.m., p value was assessed by one-way ANOVA with Tukey's HSD post hoc analysis).

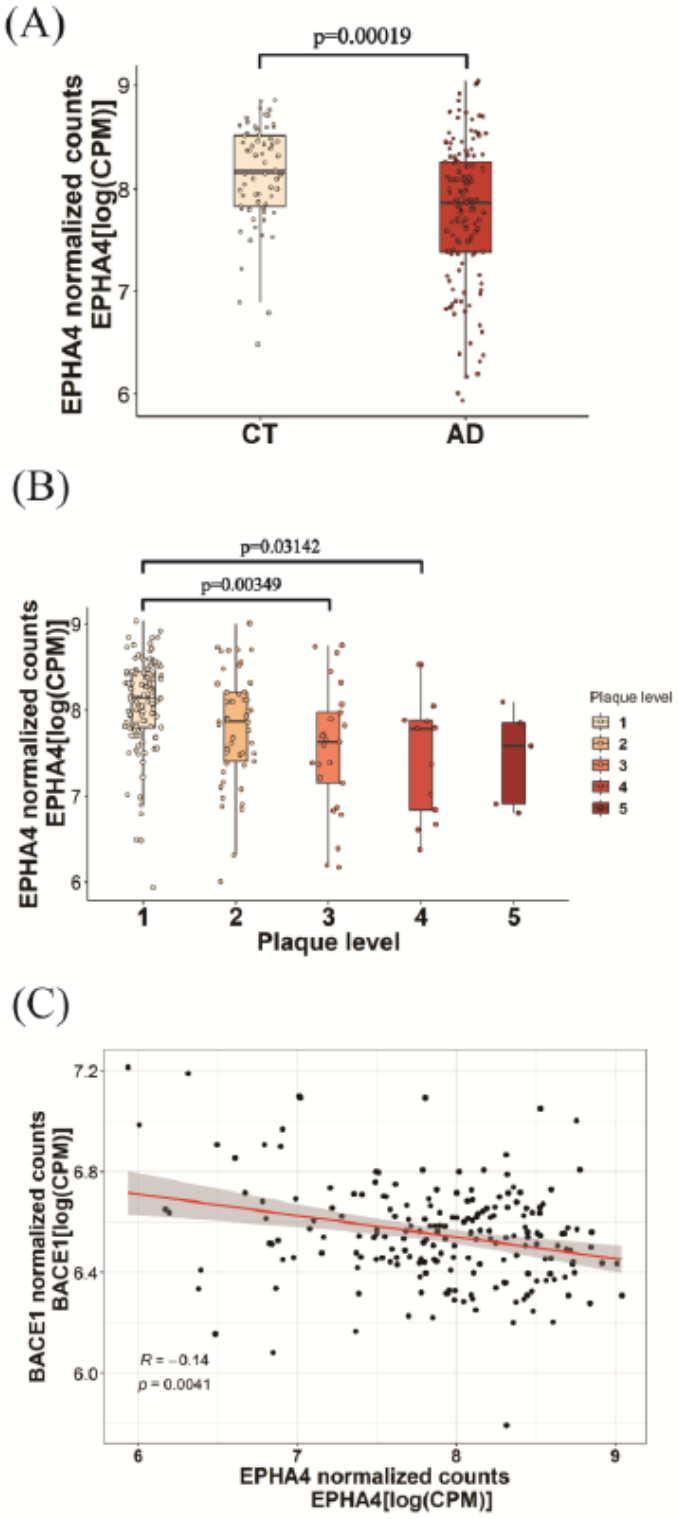

Figure 5

Reduction of EPHA4 mRNA levels in the brains of AD patients Normalized RNA read counts from the Brodmann area (BM36) including the parahippocampal gyrus of 201 subjects were provided as previously described(25). (A) Healthy control subjects (CT) was defined when NP.1, neuropathology Category as measured by CERAD, is equal to 1 (n = 64). AD patients were the other subjects when NP.1 range from 2 to 4 (n = 137). Compared to control, the EPHA4 mRNA level significantly decreased (p value

was assessed by the Mann–Whitney U test). (B) Plaque levels were defined according to the mean neocortical plaque density (of plaques/mm2) as following: 1 when plaque mean lower than 7 (n = 109), 2 when plaque mean range from 7 to 14 (n = 49), 3 when plaque mean range from 14 to 22 (n = 25), 4 when plaque mean rage from 22 to 30 (n = 13), 5 when plaque mean is greater than 30 (n = 5). EPHA4 mRNA level reduced when plaque level increased (p value was assessed by Kruskal-Wallis test with Dunn's post hoc analysis in which adjusted p value was assessed by Holm–Bonferroni method). (C) EPHA4 and BACE1 mRNA levels from 201 subjects showed a significantly weak negative correlation. The regression line is indicated as the red line. Gray background indicates the confidence interval. The correlation coefficient with the p-value was assessed from the Kendall rank correlation coefficient.

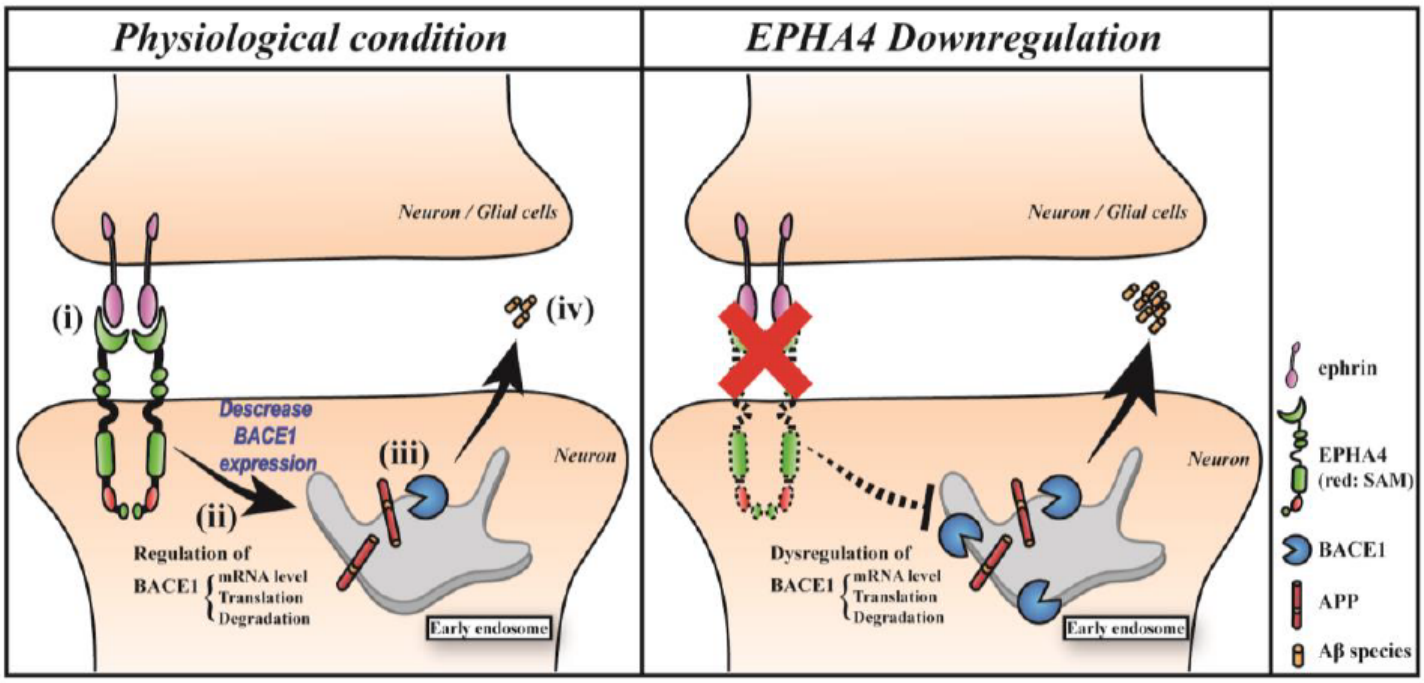

Figure 6

Scheme of EphA4 regulation in Aβ production The activation of EphA4 signaling (i) decreases the expression of BACE1 by regulation of the mRNA level, translation or degradation of BACE1 (ii), reducing the β-site cleavage of APP (iii), resulting in a decrease in Aβ production (iv) (left panel). On the other hand, the downregulation of EphA4 signaling induces an increase in BACE1 expression, upregulating Aβ production, which is involved in AD pathogenesis (right panel). The red part in EPHA4 indicates SAM domain of EPHA4, which is necessary for this regulation.

Supplementary Files

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- SupplementaryfiguresTamuraetal20200527molneurodegenerformfin.pdf