The role of PTB domain containing adaptor proteins on PICALM-mediated APP endocytosis and localization

Lisa Merthan^{*}, Amelie Haller^{*}, Dietmar R. Thal^{**}, Bjoern von Einem^{*} and Christine A.F. von Arnim^{*,***1}

^{*} Department of Neurology, Ulm University, Helmholtzstraße 8/1, 89081 Ulm

^{**} Department of Neuroscience, KU Leuven and Department of Pathology, UZ Leuven, Belgium

^{***} Clinic for Neurogeriatrics and neurological rehabilitation, Oberer Eselsberg 45, 89081 Ulm

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¹ To whom correspondence should be addressed: Prof. Christine A.F. von Arnim, MD, Clinic for Neurogeriatrics and neurological rehabilitation, Oberer Eselsberg 45, 89081 Ulm; E-mail: christine.arnim@uni-ulm.de; phone: +49-731-177-5201; fax: +49-731-500-63011

Abstract

One hallmark of Alzheimer's disease (AD) is the presence of amyloid plaques, which mainly consist of the amyloid precursor protein (APP) cleavage product amyloid β ($A\beta$). For cleavage to occur, APP must be endocytosed from the cell surface. The phosphatidylinositol binding clathrin assembly protein (PICALM) is involved in clathrin-mediated endocytosis and polymorphisms in and near the gene locus were identified as genetic risk factors for AD. PICALM overexpression enhances APP internalization and $A\beta$ production. Furthermore, PICALM shuttles into the nucleus, but its function within the nucleus is still unknown. Using co-immunoprecipitation (CoIP), we demonstrated an interaction between PICALM and APP, which is abrogated by mutation of the APP NPXY-motif. Since the NPXY-motif is an internalization signal that binds to phosphotyrosine-binding domain-containing adaptor proteins (PTB-APs), we hypothesized that PTB-APs can modulate the APP-PICALM interaction. We found that interaction between PICALM and the PTB-APs (Numb, JIP1b and GULP1) enhances the APP-PICALM interaction. FACS analysis and internalization assays revealed differentially altered APP cell surface levels and endocytosis rates that depended upon the presence of PICALM and co-expression of distinct PTB-APs. Additionally, we were able to show an impact of PICALM nuclear shuttling upon co-expression of PTB-APs and PICALM, with the magnitude of the effect depending on which PTB-AP was co-expressed. Taken together, our results indicate a modulating effect of PTB-APs on PICALM-mediated APP endocytosis and localization.

Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease, affecting approximately 10% of the population over 65 years old and 50% over 85 years old (1). AD can be broadly classified into early onset AD (EOAD; <65 age of onset; rare) and late onset AD (LOAD; >65 age of onset; more common). Mutations in amyloid precursor protein (APP), presenilin 1, and presenilin 2 cause EOAD and are rare, but highly penetrant (2-4). LOAD is the more common form of AD. Apolipoprotein allele epsilon 4 (APOE4) is the best-known genetic risk factor for LOAD. Roughly, 20% of LOAD is attributed to APOE4 (5, 6). Therefore, large genome-wide association studies were performed to identify further AD risk factors. Amongst others, certain SNPs near the gene locus of the phosphatidylinositol binding clathrin assembly protein (PICALM) appeared to be highly associated with the risk of developing AD (7, 8). We and others found that the PICALM rs541458 polymorphism is associated with decreased CSF $A\beta_{42}$ levels (9, 10). The protein product of the PICALM gene was first identified in acute myeloid leukemia (11) and it was shown that it is involved in clathrin-mediated endocytosis, in which it binds to phosphatidylinositol 4,5-bisphosphate

(PIP₂) and recruits AP2 and clathrin to initiate endocytosis (12, 13). It is well known that APP endocytosis plays a key role in A β production (14, 15), as APP has to be internalized to endosomal compartments for the initial β -secretase cleavage. Subsequent γ -secretase cleavage releases the amyloid β (A β) protein (16-18).

Several studies have focused on PICALM and its effect on APP. One study reported that PICALM is involved in APP processing and enhances A β production (19, 20). Furthermore, it was shown that PICALM enhances APP internalization and thereby influences the generation of A β (21). These findings have been confirmed by Kanatsu and colleagues. They found that PICALM influences γ -secretase activity, leading to altered A β 42 level (22). In addition, they were able to detect decreased A β level in APP transgenic mice upon reduced PICALM expression (20). However, in previous studies it was not possible to verify a direct interaction between PICALM and APP.

In addition to PICALM, several proteins were found to modulate APP internalization, transport and processing. Among these is a group of adaptor proteins (PTB-AP) that possess a phosphotyrosine-binding (PTB) domain which binds directly to a c-terminal sorting motif, called NPXY. This NPXY motif was identified in a variety of transmembrane proteins, including LRP1 and 2, VLDL receptor, insulin receptor, integrin beta 3, NOTCH, and APP (23-25). It has even been shown, by Zhao and colleagues, that PICALM is able to interact with LRP1 and thereby accelerates A β clearance (26). The PTB domain displays a consensus sequence, which is present in several PTB-APs including Numb, JIP1b and GULP1 (17, 27). Numb is a protein that is involved in neurogenesis and plays a role as an inhibitor of the NOTCH1 pathway. It binds with its PTB domain to the APP NPXY motif and influences trafficking and processing of APP (28, 29). JIP1b was described as a JNK signal cascade regulator. It has been reported that JIP1b stabilizes immature APP and suppresses production of A β 40 and 42 (30). GULP1 was first described as a protein involved in the engulfment of apoptotic cells in *C.elegans* (31, 32). We and others identified GULP1 as an interacting partner of APP (33, 34). However, there is conflicting data as to whether GULP1 increases or decreases A β production (35).

PICALM recruits various PTB-APs and clathrin to the plasma membrane to initiate endocytosis. In addition, it was shown that PICALM is able to shuttle into the nucleus. PICALM shares this ability with PTB-APs such as Fe65 (36, 37). The APP cleavage product APP intracellular domain (AICD) can likewise translocate to the nucleus, where AICD can act as a transactivator (38). In addition, various PTB-APs have been shown to differentially regulate APP internalization as well as its nuclear shuttling. Since modulation of APP endocytosis may present a new therapeutic strategy for decreasing A β production, we here addressed the question of whether the PTB-APs Numb, JIP1b, and GULP1 can interact with PICALM and thereby modulate APP transport and processing.

Material and methods

Antibodies

Antibodies directed against the tags hemagglutinin (HA, #H6908) (1:1000) or enhanced green fluorescence protein (eGFP, N-terminal, #G1544) (1:2500) were purchased from Sigma-Aldrich (St. Louis, USA). The following antibodies were used for protein specific detection: rabbit anti-APP (C-Terminal, #A8717, Sigma-Aldrich) (1:1000), mouse anti APP (6E10, C-Terminal, #SIG-39320, Convance, Emeryville, USA) (Western blot: 1:1000) and (FACS: 1:500), mouse anti APP (4G8, 17-24, # SIG-39220, BioLegend, San Diego, USA) (CoIP, 2 μ g), rabbit anti-MEK1/2 (47E6, #9122, Cell Signaling, Danvers, USA) (1:1000), mouse anti c-myc (9E10, # M4439, Sigma-Aldrich), goat anti-PICALM (CALM (C-18), C-Terminal, #sc-6433, Santa Cruz Inc., Dallas, USA) (1:1000), mouse anti-p84 (5E10, #ab487, Abcam, Cambridge, USA) (1:1000). Secondary antibodies coupled to the fluorescent dye Alexa-488 (1:500), horseradish peroxidase (1:10.000) were obtained from Molecular Probes/Invitrogen (Carlsbad, USA), horseradish peroxidase TrueBlot® (Rockland, Pottstown, USA (1:5000) or horseradish peroxidase Clean-Blot™ IP Detection Reagent (#21230, Thermo Fisher).

Generation of expression constructs

An overview of all protein constructs used in this study are presented in Fig. 1.

GFP-PICALM was generated by PCR-amplification of the PICALM cDNA (human, isoform 5; Origene, Rockville, USA) with primers containing XhoI and BamHI restriction sites and subsequent cloning into the multiple cloning site of the pEGFP-C1 vector (Clontech, Mountain View, USA). For generation of PICALM-myc, PCR-amplification of PICALM cDNA was performed with primers containing NdeI and EcoRV and cloning into pcDNA3.1(-) Myc/His A (Thermo Fisher).

HA-tagged Numb or JIP1b were also generated by PCR amplification and subsequent insertion into the pHCMV3 vector (Genlantis, San Diego, USA) using XhoI and EcoRI for Numb (mouse, isoform 4) and EcoRI and KpnI for JIP1b (mouse, isoform 2), both kind gifts of Dr. Uwe Konietzko, ETH Zürich, CH.

N-terminus (aa 1-489) and c-terminus (aa 490-778) of mTurquoise2 were generated by PCR amplification using mTurquoise2 C1 (Addgene, Cambridge, USA) as template and primers containing the restriction sites of AgeI and EcoRI for n-term and AfeI and XhoI for c-term (39). Constructs for the BiFC assay containing c-terminus of mTurquoise2 were generated by replacing the GFP of GFP-PICALM with the PCR-product. For generation of other constructs containing the mTurquoise2 c-terminus, PICALM was replaced by the corresponding cDNAs using the restriction enzymes XhoI and BamHI. For the generation of PICALM mTurquoise2_n-term, PICALM mTurquoise2_c-term was replaced by using AgeI and EcoRI restriction sites. Subsequently, cDNAs of interest were inserted as described before for PICALM mTurquoise2_c-term.

Generation of human APP695-GFP, APP Y682/687A-mutant and GULP1-HA were described previously (34).

Cell culture and transfection

Human embryonic kidney cells (HEK293) (DSMZ no.: ACC 305) were cultured under standard cell culture conditions (37°C, 5% CO₂) in DMEM (Gibco, #41966) supplemented with 10% fetal bovine serum (FBS, Biochrom GmbH, Berlin, Germany) and 1% penicillin/streptomycin (PAN - Biotech, Aidenbach, Germany).

Primary hippocampal neurons (PHN) from embryonic brains (C57BL/6; E18) were cultured as described before (40).

Transient transfections were performed using the calcium phosphate transfection method according to Flemington lab (Tulane, USA).

Human tissue

Human postmortem cortex samples from non-demented cases with vascular pathologies (Table 1) were provided by the University Hospital Ulm (Germany) in accordance with the law and permission of the local ethical committees. The samples for this analysis were taken from unaffected parts of the cortex and represent normal cortex. All experiments were performed in accordance with the Declaration of Helsinki. Informed consent to participate in this study was provided. For Western blot analysis, brain tissue was homogenized in Brain extraction lysis buffer (BEX, 25 mM Tris pH 8.0, 20 mM NaCl, 0.6% desoxycholate, 0.6 % Igepal CA-630) (41) containing phosphatase/protease inhibitor cocktail (HALT, # 78430, Thermo Fisher) and 0.2% SDS.

Co-immunoprecipitation (CoIP)

Co-immunoprecipitation (CoIP) was performed by using µMACS epitope tag protein isolation kits (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to manufacturer's instructions. Briefly, 24 h after transfection, HEK293 cells were lysed in 1 ml lysis buffer (Miltenyi Biotec) containing 1x proteinase and phosphatase inhibitor (HALT, Thermo Fisher). Cellular debris were pelleted and 1 ml of supernatants were incubated with anti-tag MicroBeads for 1 h on ice. Afterwards, cell lysates were applied onto equilibrated µmacs columns and washed stringently to remove non-specific interacting molecules. Proteins were eluted with 1x pre-heated 95°C NuPage lithium dodecyl sulfate (LDS) sample buffer

(Thermo Fisher) supplemented with 100 mM Dithiothreitol (DTT, AppliChem, Darmstadt, Germany). Raw lysates and eluates were analyzed by Western blot.

For endogenous CoIP, PHNs were lysed in lysis buffer (Miltenyi Biotec) supplemented with 1x HALT. Lysates were centrifuged and supernatant was transferred to fresh Eppendorf cups. Lysate of PHNs or human tissue was adjusted to 1 ml with lysis buffer. Afterwards, lysates were incubated with 2 µg of the required antibodies for 2 h at 4°C. Afterwards, cell lysates were incubated with anti protein G MicroBeads (Miltenyi Biotec) for 1 h on ice. Immunoprecipitations were performed as described above.

Bimolecular fluorescence complementation assay (BiFC)

Protein interaction in living cells was tested by performing a bimolecular fluorescence complementation assay (BiFC). The fluorescent protein mTurquoise2 was split into two parts. One part was fused to PICALM and the other part to one of the adaptor proteins. Upon interaction between PICALM and PTB-APs the N-term and C-term fragments are able to reconstitute and complement mTurquoise2. BACE1 and PICALM were used as negative control. Construct generation was performed as described above. HEK293 cells were transiently co-transfected with the desired constructs at a density of 60%. Cells were analyzed 24 h after transfection by fluorescence microscopy with a Zeiss Axiovert 200 (Carl Zeiss, Jena, Germany). The transfection efficient was around 30-50% depending on the construct.

Cell surface APP quantification

To measure the amount of endogenous APP on the cell surface, a fluorescence activated cell sorting (FACS) analysis was performed (42). 24 h after transfection, HEK293 cells were washed off in PBS containing 20 mM ethylenediaminetetraacetic acid (EDTA, AppliChem). Cells were fixed by 4% paraformaldehyde (PFA)/sucrose. After fixation, cells were incubated with 100 µl anti-APP antibody (6E10; 1:500 in staining buffer, PBS containing 2% FCS and 2 mM EDTA) for 45 min. Afterwards, APP was labeled with 100 µl Alexa488 conjugated secondary antibody for 45 min protected from light. Subsequently, the cells were analyzed using a FACSCalibur™ (BD Bioscience, Franklin Lakes, USA).

The AlexaFluor488 labelled endogenous APP was detected using the FL1 channel equipped with a 525nm band pass filter. 10 000 cells were counted per sample. They were gated with a life gate drawn in an SSC/FSC density plot in order to remove cell fragments (very low in the FSC). Representative dot plots of SSC/FSC as well as a histogram plot of the FL1 channel is shown in Supplementary Figure 1.

Biotinylated internalization assay

Biotinylated internalization assay was performed according to (21, 43-46) with minor modifications. All steps, except the internalization step, were performed at 4°C on ice. Briefly, 24 h after transfection, HEK293 cells were incubated and shaken for 45 min with 1 mg/ml EZ-Link™ Sulfo-NHS-SS-Biotin (Thermo Fisher) in PBS. Subsequently, biotin were quenched by PBS containing 100 mM glycine (Sigma-Aldrich). Next, endocytosis was started by incubation cells in preheated DMEM containing 10% FCS and 1% penicillin/streptomycin for 5 min at 37°C. Endocytosis was stopped by rapid cooling down on ice. Biotin labeled, and not internalized APP was cleaved by incubation in a cleavage buffer (50 mM 2-mercaptoethanesulfonic acid, 50 mM TRIS pH 8.6, 100 mM NaCl, 2.5 mM CaCl₂) 3 times for 7 minutes. To control for this, cells were lysed immediately after quenching-step without an endocytosis step. Cells treated with cleavage buffer, were washed in PBS containing 20 mM HEPES (AppliChem) to neutralize the cleavage buffer. Subsequently, cells were lysed by addition of 200 µl biotin buffer (25 mM TRIS pH 7.6, 150 mM NaCl, 1% TRITON X-100, 0.5% NaDeoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM NaFluoride). 40 µl of each lysate was stored at -20°C for Western blot analysis. The remaining lysates were incubated with 100 µl Pierce™ NeutrAvidin™ Agarose beads (Thermo Fisher) for 2 h. Elution of biotinylated APP was performed by adding 30 µl of elution buffer (4x LDS +DTT) to the beads and boiling at 95°C for 5 min. The eluted APP was analyzed by Western blot. Densitometric quantification was performed of the 5 min internalization samples.

Nuclear extraction assay

HEK293 cells were transiently transfected with PICALM and HA or PICALM and PTB-APs. For collection of the cytosolic fraction, cells were harvested and centrifuged for 5 min at 510 g and 4°C. The cell pellet was resuspended in 250 µl Sucrose Buffer (0.32 M Sucrose, 10 mM Tris HCl pH 8.0, 3 mM CaCl₂, 2 mM MgAc, 0.1 mM EDTA and 0.5% Igepal CA-630) 1 mM DTT and 1x HALT were added freshly and centrifuged at 14.000 g for 15 min at 4°C. The supernatant (cytosol) was transferred to a fresh tube.

To collect the nuclear fraction, the pellet was resuspended in 500 µl Sucrose buffer without Igepal CA-630 and centrifuged 510 g for 5 min at 4°C. Cell pellet was resuspended in 60 µl low salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT) containing freshly added 1x HALT. Subsequently, 60 µl of a high salt buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 800 mM KCl, 0.2 mM EDTA, 25% glycerol, 1% Igepal CA-630, 0.5 mM DTT) containing freshly added 1x HALT was added to extract the nucleoplasm. Afterwards, the mixture was shaken for 45 min at 4°C and 600 rpm. The suspension was centrifuged for 15 min at 14.000 g and 4°C and supernatant (nucleolus) was transferred to a fresh tube. The cytosolic as well as the nuclear fraction was analyzed by Western blot.

Transactivation assay

The transactivation assay was performed as described previously (34, 47, 48). Briefly, HEK293 cells were transfected with expression plasmids coding for firefly luciferase (pG5E1B-luc) (0.25 µg), Gal4 binding domain (pMST-Gal4) (0.25 µg) and Gal4-PICALM + PTB-APs and/or empty controls (0.3 µg each). Transfected HEK293 cells with pG5e1B-luc and pMST-Gal4 were used as negative control. 24 hours after transfection, cells were lysed in 2x Reporter Lysis Buffer (Promega, Madison, USA). Expression was controlled by Western blot analysis. Luciferase activity was measured in duplicate using the VICTOR X3 multilabel plate reader (Perkin-Elmer, Waltham, USA) according to manufacturer's instructions. Briefly, samples were diluted 1:2 in 1x Reporter Lysis Buffer and 100 µl of luciferase substrate (Beetle Juice, PJK GmbH, Kleinbittersdorf, Germany) was added by an automated dispenser. Luciferase activity was measured after 2 sec incubation time and with 5 sec integration time.

Western Blot Analysis

After lysis, proteins (20-25 µg total protein concentration, determined by BCA assay) were separated by lithium dodecyl sulphate (LDS)-polyacrylamide gel electrophoresis (NuPage Novex Bis-Tris, Thermo Fisher). For endogenous CoIP 400 µl of NuPage Novex antioxidant (Thermo Fisher) was added to maintain proteins on a reduced state. Afterwards electrophoretic transfer onto 0.2 µm pore size PVDF membrane (BioRad, Munich, Germany) was performed by tank blot. After blocking with 5% non-fat dry milk (Sigma-Aldrich) the membrane was incubated with primary antibody for 2 h at room temperature or overnight at 4°C. Afterwards, blot was incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected by ECL Luminata Forte (Millipore, Darmstadt, Germany) and LAS-4000 (GE, Healthcare, Chicago, USA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0. Data were tested for normality with the D'Agostino-Pearson omnibus test and the data were analyzed by t test or One-way ANOVA Bonferroni test. When normality test failed, the groups were compared by Kruskal-Wallis test. $P < 0.05$, $p < 0.01$, $p < 0.001$, was considered statistically significant and indicated by an asterisk.

Results

PICALM interacts with APP and PTB-APs

Previously, it has been shown that PICALM and APP co-localize within the cell and that PICALM overexpression leads to an enhanced internalization of APP and increased A β production (21). However, the interaction of these two proteins has not yet been demonstrated. Therefore, we performed co-immunoprecipitations (CoIP) of PICALM and APP. HEK293 cells were transiently transfected with APP-GFP and PICALM-myc. PICALM-myc plus GFP empty vector was used as a negative control. To test the interaction between PICALM and APP, APP was immunoprecipitated and co-precipitation of PICALM

was detected by Western blot analysis (Fig. 2A, lower panel, lane 2). We were thus able to show an interaction of PICALM and APP. Since it is known that the NPXY motif of APP is important for the regulation of APP internalization, we hypothesized that the interaction between PICALM and APP is abrogated upon mutation of the NPXY motif. We co-transfected PICALM-myc and the NPXY-mutated APP Y682/687A-GFP (APP_{yy}-GFP). As expected, co-immunoprecipitation of PICALM was not possible when APP_{yy}-GFP was immunoprecipitated (Fig. 2A, lower panel, lane 3). The protein expression was controlled by Western blot (Fig. 2A, upper panel). Densitometric analysis revealed a significant ($p < 0.05$) difference in the interaction of PICALM and APP_{wt} compared to PICALM and APP_{yy} (Fig. 2, statistical analysis). To show the functional relevance of the interaction between PICALM and APP, we performed CoIPs in PHNs. PHN lysate incubated with anti-myc (9E10) antibody was used as negative control. Immunoprecipitated endogenous APP as well as co-precipitated endogenous PICALM was detected by Western blot (Fig. 2B, eluate, upper and lower panel). Furthermore, we tested interaction between endogenous PICALM and APP in human post mortem cortex samples. As described before endogenous APP was able to co-precipitate endogenous PICALM (Fig. 2C).

Because the NPXY motif is required for binding and transport regulation by PTB-APs and because Xiao and colleagues found only a co-localization of APP and PICALM, we asked whether the interaction between PICALM and APP could be mediated or modulated by PTB-APs. To test this hypothesis, we performed CoIP with HEK293 cells that were co-transfected with GFP-PICALM and HA-tagged PTB-APs (Numb, JIP1b, and GULP1). As a negative control GFP-PICALM plus HA empty vector was used. To control for protein expression, lysates were analyzed by Western blot (Fig. 2D, upper panel). For the CoIP, the PTB-APs were immunoprecipitated and co-immunoprecipitation of PICALM was detected by Western blot analysis (Fig. 2D, lower panel) and vice versa (data not shown). We were able to show an interaction between PICALM and all three investigated PTB-APs. Noticeably, JIP1b combined with PICALM exhibited lower expression levels compared with the other PTB-APs, but upon co-precipitated the signal was as strong as for the other constructs. This could be an indicator of a stronger affinity of JIP1b to PICALM compared with the other PTB-APs. To confirm the CoIP results we used a complementary method, a bimolecular fluorescence complementation (BiFC) assay. One part of split mTurquoise2 was fused to the N- or C-terminus of PICALM, whereas the second part was fused to the N- or C-terminus of PTB-APs. The strongest fluorescent signals were observed from the combination of PICALM mTurquoise2_{n-term} and Numb mTurquoise2_{c-term}, mTurquoise2_{n-term} GULP1 and PICALM mTurquoise2_{c-term}, and JIP1b mTurquoise2_{n-term} and PICALM mTurquoise2_{c-term}. These three combinations were used for all further experiments. HEK293 cells were co-transfected with PICALM split mTurquoise2 and corresponding PTB-APs split mTurquoise2 constructs and analyzed by fluorescence microscopy (Fig. 2E). As a negative control, mTurquoise2_{n-term} was fused to BACE1 and mTurquoise2_{c-term} was fused to PICALM (Fig. 2E, left image). To confirm the negative control, we performed CoIPs with GFP-tagged PICALM and HA-tagged BACE1. Expression of the proteins was controlled by Western blotting (Fig. 2D, left panel, lysate). Immunoprecipitated BACE1 was detected by HA antibody (Fig. 2D, left panel, eluate). Co-precipitation of PICALM was not detectable (Fig. 2D, left panel, eluate). Taken together, these experiments confirm a specific interaction of PICALM with all three investigated PTB-APs (Numb, JIP1b, and GULP1) in living cells.

PTB-APs enhancement of PICALM and APP interaction is PTB domain dependent

Since we have found that PICALM interacts with both APP and PTB-APs we hypothesized that PTB-APs influence or mediate the interaction between APP and PICALM. To test this hypothesis, we performed CoIPs with lysates of cells co-expressing PICALM-myc and APP-GFP and either HA empty vector, PTB-APs or PTB deletion mutants of these APs. As the PTB domain mediates the interaction between APP and the APs, deletion of this domain should impair APP-PICALM interaction.

To control for protein expression, lysates were analyzed by Western blot (Fig. 3, upper panel). To investigate whether PTB-APs or delta PTB-APs influence the interaction between PICALM and APP, APP-GFP was immunoprecipitated (Fig. 3, lower panel). The presence of PTB-APs exhibited increased levels of PICALM after co-precipitation indicating an enhanced interaction between PICALM and APP (Fig. 3, lower panel). Furthermore, deletion of the PTB domain abrogated the enhanced binding between PICALM

and APP (Fig. 3A, eluate lane 5, 6 and 7). We quantified the Western blots by densitometry, normalized co-precipitated PICALM levels to immunoprecipitated APP-GFP levels and performed a statistical analysis using a one-way ANOVA (Fig. 3B). The presence of JIP1b resulted in a significant ($p<0.001$) increase in the PICALM/APP interaction compared to control. Although not significant, the presence of GULP1 and to a lesser extent Numb also showed a trend towards an enhanced PICALM/APP interaction. Taken together, our data indicate that the PTB-APs and especially the PTB domain bridge between PICALM and APP.

Co-expression of PICALM and JIP1b decreased APP internalization

Because we detected an impact of PTB-APs on PICALM/APP interaction by CoIP, we hypothesized that these PTB-APs could influence PICALM-mediated APP transport and APP localization. Therefore, we performed a FACS analysis to measure the amount of APP on the cell surface. HEK293 cells were co-transfected with PICALM-myc and one of the PTB-APs (Numb, JIP1b or GULP1). The endogenous APP was labeled at the cell surface and mean green fluorescence intensity was measured. All conditions were compared to cells treated with transfection reagent only (mock control). The combination of PICALM and HA empty vector led to a significant ($p<0.001$) reduction in APP cell surface level compared to mock control (upper panel, lane 1). When co-expressing Numb-HA or GULP1-HA (upper panel, lane 2, lane 4) APP cell surface levels were significantly (Numb: $p<0.001$; GULP1: $p<0.05$) reduced at the same level. In contrast, co-expression of JIP1b-HA restored the amount of APP on the cell surface (upper panel, lane 3). Additionally, we transfected HEK293 cells with PICALM-myc or PTB-APs alone. As observed previously, PICALM expression significantly ($p<0.05$) reduced APP cell surface levels compared to mock control (Fig. 4B, upper panel, lane 1). Besides Numb and GULP1, also JIP1b alone significantly (Numb: $p<0.001$; GULP1: $p<0.01$; JIP1b: $p<0.05$) decreased APP cell surface levels (Fig. 4B, upper panel, lane 2, 3 and 4), indicating that the blocking of APP internalization requires the presence of both JIP1b and PICALM. The expression of transfected construct was controlled by Western blot (Fig. 4B, lower panel). To control for altered endocytosis of APP, we performed a biotinylated internalization assay. Therefore, HEK293 cells were co-transfected with PICALM-myc and HA empty vector or combined with one of the PTB-APs (Fig. 4C). The endogenous APP on the cell surface was labeled by biotin and the levels were analyzed by Western blot. Densitometric analysis revealed a trend towards an increase of APP endocytosis upon co-transfection of PICALM-myc and HA empty vector compared to mock control. Likewise, PICALM-myc and Numb-HA co-expression showed an increase of APP endocytosis without reaching significance (Fig. 4C, upper panel, lane 2 and 3). In line with our previous experiment, the presence of JIP1b-HA did not increase APP endocytosis and GULP1 had only a minor impact on APP internalization (Fig. 4C, upper panel, lane 4 and 5). To investigate the expression of all transfected constructs a Western blot was performed. To correct for differences in protein expression, the amount of biotinylated APP was normalized to total concentration of APP (Fig. 4C). Subsequently, we transfected HEK293 cells with PICALM-myc or PTB-APs alone. Although not significant ($p=0.24$), PICALM-myc showed a 1.81-fold increase of APP endocytosis compared with the mock control, whereas the PTB-APs alone had no impact on APP endocytosis (Fig. 4D, upper panel). To control for expression of different constructs we performed Western blot analysis and normalized biotinylated APP to total level of APP (Fig. 4D). Taken together, we were able to show that PICALM co-expressed with JIP1b caused the greatest increase in the amount of APP at the cell surface. Therefore, we speculate that JIP1b stabilizes APP cell surface presents and prevents the PICALM-mediated endocytosis of APP.

Numb enhances nuclear shuttling of PICALM and its transcriptional activity

Because Numb showed no additive effect on APP cell surface level or endocytosis compared with PICALM alone, we asked whether the Numb-PICALM interaction is involved in processes in addition to endocytosis. As described before, it is known that PICALM and PTB-APs shuttle into the nucleus. We therefore investigated whether nuclear translocation of PICALM is altered by the PTB-APs. We performed a nuclear extraction assay to test for altered nuclear translocation of PICALM upon co-expression with the PTB-APs. The cytoplasm and the nucleus were separated 24 h after co-transfection with PICALM and HA empty vector or PICALM and PTB-APs. PICALM and HA empty vector were used as control. The results of the nuclear extraction assay revealed a significantly increased ($p<0.05$) nuclear translocation of PICALM upon

Numb co-expression. However, JIP1b and GULP1 had no significant impact on the nuclear shuttling of PICALM (Fig. 5A). The proper separation of cytoplasm and nucleus was controlled by Western blot using specific antibodies against the nuclear protein (p84) and the cytoplasmic protein (MEK1/2) (Fig. 5A). Furthermore, we tested the nuclear shuttling of endogenous PICALM. Therefore, we transfected HEK293 cells either with HA empty vector or PTB-APs only. We found a slight increase in the nuclear shuttling of endogenous PICALM upon expression of the PTB-APs (Fig. 5B), with Numb showing the strongest effect (Fig. 5B, lane 2). Proper separation of cytoplasm and nucleus as well as the expression of the single proteins was controlled by Western blot (Fig. 5B). To correct for differences in protein expression, the amount of the PICALM was normalized to p84. Both experiments support the idea that Numb enhances the nuclear shuttling of PICALM.

To confirm these findings with a more quantitative method, a transactivation assay according to Cao and Südhof was performed (47). HEK293 cells were transfected with pG5E1B-luc, Gal4-construct and Numb/JIP1/GULP1 or empty vector. Protein expression was controlled by Western blot (Fig. 5C). Luciferase activity as measured in relative luminescence units (RLU) revealed a significant increase ($p < 0.05$) in the nuclear presence of PICALM compared to control (Fig. 5C, lane 2). The co-expression of Numb further enhanced the luciferase activity ($p < 0.01$) (Fig. 5C, lane 3). JIP1 co-expression, on the other hand, led to a significantly ($p < 0.05$) decreased luciferase activity compared to PICALM and HA empty vector (Fig. 5C, lane 4). The combination of PICALM and GULP1 had no significant effect on luciferase activity, indicating that GULP1 has only minor influence on the nuclear transport of PICALM (Fig. 5C, lane 5).

Discussion

Previous studies have demonstrated co-localization of PICALM and APP in endocytic vesicles and an increase in A β production upon PICALM expression (21). However, they did not show an interaction between PICALM and APP. We demonstrate here for the first time an interaction between PICALM and APP in transfected HEK293 cells as well as in PHNs and in post mortem tissue (Fig. 2A, B and C). Furthermore, we were able to disrupt the interaction by mutation in the highly conserved NPXY motif of APP, which is known to be an interaction site for PTB-APs (49). We therefore speculated that PTB-APs might influence or mediate the PICALM-APP interaction. To test this hypothesis, we examined the interaction between PICALM and the PTB-APs Numb, JIP1b and GULP1. CoIP experiments indicated that PICALM interacts with APP and with the PTB-APs (Fig. 2D). Using a BiFC approach, we confirmed the interaction between PICALM and the PTB-APs (Fig. 2E).

Our CoIP approach, where PICALM as well as APP and one of the PTB-APs are co-expressed, revealed that all PTB-APs increased the interaction of PICALM and APP, but there was variation between the different PTB-APs (Fig. 3), with Numb exhibiting the smallest effect. However, it is possible that the interaction could be a dynamic process, mediated by various factors (e.g., phosphorylation). Previous studies have shown that phosphorylation of Tyr682 in APP influences the interaction between APP and PTB-APs (50). Therefore, it is possible that posttranslational modifications of APP or PICALM modulated the interaction with the different PTB-APs. On the other hand, it is also known that different splice isoforms of the PTB-APs exist, and these might exhibit different binding affinities towards PICALM and APP (28, 30). The hypothesis that the PTB-APs facilitate the interaction between PICALM and APP was confirmed by CoIP, as co-expression of PTB-APs increased the binding between PICALM and APP. This result is in line with other publications, showing that PTB-APs act as mediators between proteins, which do not interact directly (51).

It has been described previously that different PTB-APs are involved in distinct pathways. Dab1 for example is involved in clathrin- and caveolin dependent internalization (52). Therefore, it is reasonable to speculate that the PTB-APs investigated in this study might also operate in several transport pathways. This idea supports our observation that the PTB-APs had a distinct impact on PICALM mediated APP endocytosis and APP cell surface levels as well as on nuclear translocation of PICALM. Co-expression of JIP1b with PICALM resulted in marked APP redistribution and internalization in biotinylated internalization assays and FACS, in contrast to co-expression of Numb or GULP1, which showed no effect

on PICALM-mediated APP endocytosis (Fig. 4). In nuclear extraction assays, however, we found a significantly increased shuttling of PICALM into the nucleus in the presence of Numb and JIP1b (Fig. 5) but no significant change for GULP1 co-expression.

Numb is known as an endocytic protein and was described in previous studies as a regulator of APP processing, in which it was found to enhance A β production (28, 53). However, based on our data, it seems that PICALM-mediated APP endocytosis is independent of Numb's effect on APP transport (Fig. 4). However, we detected a significant increase of nuclear translocation of PICALM in the presence of Numb (Fig. 5), what could be a hint, that PTB-APs mediate next to PICALM-mediated endocytosis also other pathways.

For GULP1, we and others identified this PTB-AP as an interaction partner and modulator of APP endocytosis (33, 34). Furthermore, it was found that GULP1 decreases the amount of APP on the cell surface (34). However, in this study, the co-expression of GULP1 with PICALM had no effect on PICALM-mediated APP endocytosis (Fig. 4). Also, the nuclear translocation of PICALM was not altered upon GULP1 expression (Fig. 5).

In contrast, JIP1b, which has been shown to stabilize immature APP and to transport APP along axons (54), enhanced the amount of APP on the cell surface when it was co-expressed with PICALM (Fig. 4). Based on the findings of Taru and colleagues, which observed a stabilization of immature APP by JIP1b, we speculate that JIP1b might also act as a stabilizer for APP on the cell surface (30). It might be speculated that PICALM-JIP1b is involved in highly dynamic cell surface recycling of APP. Due to its limitation regarding time resolution, the performed endocytosis assay is most likely unsuitable in measuring this highly dynamic trafficking. Furthermore, JIP1b blocked the nuclear localization of PICALM (Fig. 5). This data indicates that JIP1b-modifying effects on APP might be mediated by PICALM.

These results confirm the cargo-dependent functions of the individual PTB-APs in the cellular transport system. More importantly, our study shows that interaction of PICALM with different PTB-APs can modify PICALM's impact on APP internalization and processing. Regulatory functions of PTB-APs on other proteins have been described previously. For example, Numb is known to be a key player in the inhibition of the Notch pathway. McGill and McGlade found that Numb, which acts in endocytosis (53), promotes the ubiquitination of Notch and thereby its enhanced degradation. So, it might be speculated that the effects observed here may be due to varying functions and cargo specificity of PTB-APs in PICALM mediated endocytosis (55). However, the underlying molecular mechanisms of the distinct roles of the various PTB-APs have to be revealed in further studies.

Taken together, our current findings suggest that expression of PTB-APs, especially JIP1b, can be a possible new therapeutic strategy in the attempt to modulate altered APP transport and processing to find an effective treatment against AD (Fig. 6).

Abbreviations

The abbreviations used are: AD, Alzheimer's Disease; APOE4, apolipoprotein allele epsilon 4; APP, amyloid precursor protein; A β , amyloid beta; BiFC, bimolecular fluorescence complementation; CoIP, co-immunoprecipitation; EOAD, early-onset Alzheimer's disease; FACS, fluorescence activated cell sorting; FAD, familial Alzheimer's disease; GFP, green fluorescence protein; GULP1, engulfment adaptor PTB domain containing 1; GWAS, genome wide association study; HA, Human influenza hemagglutinin; LOAD, late-onset Alzheimer's disease; Numb, endocytic adaptor protein; JIP1b, mitogen-activated protein kinase 8 interacting protein 1; PICALM, phosphatidylinositol binding clathrin assembly protein; PIP2, phosphatidylinositol 4,5-bisphosphate; PTB, phosphotyrosine binding domain; PTB-AP, PTB containing adaptor proteins; SNP, single nucleotide polymorphism.

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Author Contribution:

Study concept and design: CAFvA, LM, BvE; acquisition of data: LM, AH, DRT; analysis and interpretation of data: LM, BvE, CAFvA; drafting of the manuscript: LM, BvE, CAFvA; critical revision of the manuscript for important intellectual content: CAFvA, BvE, DRT

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FIGURE LEGENDS

Figure 1: Overview of constructs used in this study

All proteins used for BiFC were tagged n- or c-terminal with the N- or C-term of mTurquoise2. For other applications, proteins were tagged n- or c-terminally with GFP, HA or myc, respectively. Antibody detection is indicated.

Figure 2: PICALM interacts with APP_{WT} as well as with PTB-APs but not with APP_{YY}

(A) HEK293 cells were transfected with PICALM-myc and APP_{WT}-GFP or APP_{YY}-GFP. Co-transfection of PICALM-myc and GFP empty vector was used as negative control. Expression of proteins was analyzed by Western blot (lysate, upper panel). APP-GFP was immunoprecipitated by anti-tag magnetic beads and analyzed by Western blot (eluate, lower panel). Co-precipitated PICALM-myc was detected by PICALM antibody (eluate, lane 2), which was abrogated by a mutation of NPXY motif of APP (eluate, lane 3). Shown is a representative blot of N=3 independent experiments. Densitometric analysis of CoIP with PICALM and APP_{WT}-GFP or APP_{YY}-GFP revealed a significant ($p < 0.05$) decrease of PICALM/APP_{YY} interaction compared to PICALM/APP_{WT}. Shown are the mean values $\pm$ SEM of N=3 independent experiments. Statistical analysis was performed using t-test.; * $p < 0.05$.

(B) PHN lysates were incubated with either 2 μ g of an anti-APP monoclonal antibody (4G8) or an IgG negative control (anti myc /9E10). Subsequently, both preparations were incubated with protein G beads (Miltenyi Biotec) and immunoprecipitation was performed according to the manufacturer's instructions. APP immunoprecipitation was confirmed by Western blot analysis using an anti APP-c-term specific antibody (eluate, upper panel). Co-precipitated endogenous PICALM was detected by PICALM antibody (eluate, lower panel). Shown is a representative blot of N=4 independent experiments.

(C) Human post mortem cortex lysates were incubated with either 2 μ g of an anti-APP monoclonal antibody (4G8) or an IgG negative control (anti myc /9E10). Subsequently, both preparations were incubated with protein G beads (Miltenyi Biotec) and immunoprecipitation was performed according to the manufacturer's instructions. APP immunoprecipitation was confirmed by Western blot analysis using an anti APP-c-term specific antibody (eluate, upper panel). Co-precipitated endogenous PICALM was detected by PICALM antibody (eluate, lower panel). Shown is a representative blot of N=3 independent experiments; unspecific bands were indicated by asterisks.

(D) HEK293 cells were co-transfected with GFP-PICALM and either HA-tagged PTB-APs or HA-tagged BACE1 as negative control. Equivalent expression of proteins was confirmed by Western blot (lysate). The PTB-APs were immunoprecipitated by magnetic anti-HA beads (eluate, upper panel). The co-precipitated GFP-PICALM was detected by GFP antibody (eluate, lower panel). Shown is a representative blot of N=4 independent experiments.

(E) HEK293 cells were co-transfected with PICALM mTurquoise2_n- or c-term and PTB-APs n- or c-terminally tagged with mTurquoise2_n- or c-term. Fluorescence microscopy analysis confirmed an interaction of PICALM and the various PTB-APs by reconstitution of mTurquoise2 (the three images on the right). Co-expression of mTurquoise2_n-term fused to BACE1 and c-term fused to PICALM alone was used as negative control and showed no unspecific reconstitution of mTurquoise2 (left image). N=5. Bar: 50 μ m

Figure 3: PTB-APs facilitation of PICALM-APP interaction is PTB-domain dependent

(A) HEK293 cells were co-transfected with APP-GFP, PICALM-myc and either HA-tagged PTB-APs or HA-tagged delta PTB-APs. 24 h after transfection cells were lysed and the expression of the proteins was analyzed by Western blot (lysate). APP was immunoprecipitated by anti-GFP magnetic beads and detected by an anti APP c-term antibody (eluate, upper panel). Co-precipitated PICALM was analyzed by PICALM antibody (eluate, middle panel). All full-length PTB-APs enhance the interaction between PICALM and APP (eluate, middle panel, lane 1, 2 and 3), whereas in the presence of delta PTB-APs the interaction between PICALM and APP is unaltered (eluate, middle panel, lane 5, 6 and 7). Co-precipitated full-length and delta PTB-APs were detected using anti HA antibody (eluate, lower panel). (B) Densitometric analysis

revealed a significant ($p<0.001$) increase in APP-PICALM interaction only for the combination of PICALM, APP and JIP1b compared to control. Shown are the main values $\pm$ SEM of N=4 independent experiments. Statistical analysis: One way-ANOVA; Bonferroni test; *** $p<0.001$.

Figure 4: Co-expression of PICALM and JIP1b decreased APP internalization

(A) HEK293 cells were transfected with either PICALM-myc alone or in combination with PTB-APs. 24 hours after transfection, cells were fixed and surface APP was stained with Alexa488. The mean green fluorescence intensity of stained cells was determined by FACS analysis. All conditions were compared to cells treated with transfection reagent only (mock control). The combination of PICALM and HA empty vector, led to a significant ($p<0.001$) reduction in APP cell surface level compared to mock control (upper panel, lane 1). Likewise, PICALM with Numb-HA or GULP1-HA (upper panel, lane 2, lane 4) significantly (Numb: $p<0.001$; GULP1: $p<0.05$) reduced APP cell surface level, without further increasing this effect. In contrast, co-expression of PICALM-myc and JIP1b-HA restored the amount of APP on the cell surface without any significance (upper panel, lane 3). Protein expression was controlled by Western blot (lower panel). (B) FACS analysis was performed with PTB-APs and PICALM alone. As observed previously, PICALM expression significantly ($p<0.05$) reduced APP cell surface levels compared to mock control (upper panel). Besides Numb and GULP1, also JIP1b alone significantly (Numb: $p<0.001$; GULP1: $p<0.01$; JIP1b: $p<0.05$) decreased APP cell surface levels, indicating that the blocking of APP internalization requires the presence of both JIP1b and PICALM. Protein expression was controlled by Western blot (lower panel). Shown are the medians of N=5 independent experiments with boxes representing the 25th and 75th percentiles and whiskers representing the 10th and 90th percentiles; outliers are indicated by black dots. One way-ANOVA; Bonferroni test * $p<0.05$; ** $p<0.01$; *** $p<0.001$. (C) Cell surface APP was labeled with biotin and detected by an APP antibody (upper panel). Internalization time points of 0 min or 5 min are indicated with 0 and 5. The total amount of biotinylated APP on cell surface is indicated with T. Quantification was performed using the 5 min internalization samples. Densitometric analysis revealed an enhanced internalization of APP upon PICALM-myc and HA or Numb-HA co-expression, without reaching significance ($p=0.85$) (upper panel, lane 2 and 3). Protein expression was controlled by Western blot (lower panel). (D) A 1.81-fold higher but not significant ($p=0.24$) internalization of APP upon PICALM expression was detected, whereas the PTB-APs alone had no impact on the APP internalization. Protein expression was controlled by Western blot (lower panel). Shown are the mean values $\pm$ SEM of N=5 independent experiments. One way-ANOVA and Kruskal-Wallis test; Dunn's test.

Figure 5: Co-expression of PICALM and Numb enhances and JIP1b blocks the shuttling of PICALM into the nucleus

(A) HEK293 cells were co-transfected with PICALM and either HA empty vector or Numb-/ JIP1b-/GULP1-HA. PICALM and HA empty vector were used as control. Cytosol and nucleus were separated from each other. To control for protein expression and a proper separation of the cytosolic and the nuclear fractions, Western blot was performed (left panel). Densitometric analysis revealed a significant ($p<0.05$) increase of PICALMs nuclear shuttling upon co-expression of PICALM-myc and Numb-HA compared to control. In contrast, PICALM and JIP1b or GULP1 had no significant impact on PICALM levels in the nucleus. Shown are the mean values $\pm$ SEM of N=5 independent experiments. One way-ANOVA; Bonferroni test; * $p<0.05$.

(B) Nuclear shuttling of endogenous PICALM was performed by transfection of HEK293 cells with either HA empty vector or the single PTB-APs. To control for protein expression and proper separation of the cytosolic and nuclear fractions, Western blot was performed (middle panel). Densitometric analysis revealed no significant differences in nuclear translocation of endogenous PICALM. However, in presence of Numb a trend towards enhanced nuclear translocation of PICALM was detectable. Shown are the mean values $\pm$ SEM of N=3 independent experiments. One way-ANOVA; Bonferroni test.

(C) HEK293 cells were transfected with pG5E1B-luc, Gal4-construct, Numb/JIP1/GULP1 or empty vector. Expression of constructs was confirmed by Western blot (right panel). Gal4 and pG5e1B-luc empty vector was used as control. Luciferase activity was measured and we found significantly ($p<0.05$) increased transcriptional activity upon co-expression of PICALM and HA empty vector compared with control. Numb co-expression further enhanced this effect ($p<0.01$), whereas co-expression with JIP1b reduced the transcriptional activity significantly ($p<0.05$). GULP1 had no significant influence compared with control. Shown are the mean values $\pm$ SEM of N=5 independent experiments. One way-ANOVA; Bonferroni test; * $p<0.05$; ** $p<0.01$.

Figure 6: Influence of co-expressed PICALM and PTB-APs on PICALM translocation and APP trafficking

(A) PICALM alone enhanced the APP endocytosis as well as nuclear localization of PICALM. (B) Co-expressed PICALM and JIP1b stabilized APP on cell surface and has no effect on APP endocytosis as well as nuclear localization of PICALM. (C) Co-expressed PICALM and GULP1 no effect on APP endocytosis, APP on the cell surface and nuclear translocation of PICALM. (D) Co-expression of PICALM and Numb revealed significant increase of nuclear localization of PICALM but no significant impact on PICALM mediated APP endocytosis.

Supplementary Figure 1: FACS analysis of endogenous labeled APP on cell surface of HEK293 cells

FACS analysis (population mean) of endogenous APP labeled with Alexa488. Dot plot of autofluorescence HEK293 cells (upper panel, left) and its associated FL1 histogram plot (upper panel, right). Dot plot of labeled endogenous APP on cell surface (lower panel, left) and its associated FL1 histogram plot (lower panel, right).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

#	Age	Sex	Diagnosis
1	66	M	Fresh intracerebral hemorrhage
2	71	F	Old frontal microinfarct
3	60	M	Traumatic brain defect, old lacunar infarct (basal ganglia), hemorrhagic infarcts

Supplementary Figure 1