



RESEARCH PAPER

LINGO-1 promotes lysosomal degradation of amyloid- β protein precursor

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Sequential proteolytic cleavages of amyloid- β protein precursor (A β PP) by β -secretase and γ -secretase generate amyloid β (A β) peptides, which are thought to contribute to Alzheimer's disease (AD). Much of this processing occurs in endosomes following endocytosis of A β PP from the plasma membrane. However, this pathogenic mode of processing A β PP may occur in competition with lysosomal degradation of A β PP, a common fate of membrane proteins trafficking through the endosomal system. Following up on published reports that LINGO-1 binds and promotes the amyloidogenic processing of A β PP we have examined the consequences of LINGO-1/A β PP interactions. We report that LINGO-1 and its paralogs, LINGO-2 and LINGO-3, decrease processing of A β PP in the amyloidogenic pathway by promoting lysosomal degradation of A β PP. We also report that LINGO-1 levels are reduced in AD brain, representing a possible pathogenic mechanism stimulating the generation of A β peptides in AD.

Keywords: APP; A β PP proteolysis; endosome; LINGO; trafficking; Alzheimer's disease

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Received: 21 August 2014; Revised: 4 February 2015; Accepted: 10 February 2015; Published: 9 March 2015

Alzheimer's disease (AD) is associated with aberrant accumulation of β -amyloid (A β), a peptide produced by proteolytic processing of the β -amyloid precursor protein (A β PP) (1–3). A β PP, a membrane protein, is subject to cleavage within its extracellular domain by α - or β -secretases, triggering cleavage within its transmembrane domain by γ -secretase. The β/γ cleavage sequence produces A β while the α/γ sequence produces the p3 peptide, commonly believed to be innocuous. Processing may occur during trafficking through the ER/Golgi compartments of the secretory pathway, or following trafficking to the cell surface (4–7). Most nascent A β PP molecules are cleaved by α -secretase in either the Golgi or a post-Golgi/plasma membrane compartment to produce soluble extracellular domain sA β PP α and an intracellular carboxy-terminal fragment (CTF α). A β PP is subject to rapid clathrin-mediated endocytosis, minimizing the life-time of the intact protein at the cell surface (8). Internalized A β PP then enters late endocytic compartments where cleavage by β -secretase produces sA β PP β and CTF β ,

and cleavage of CTF β by γ -secretase produces A β peptides (7–9). A β production may also occur via direct Golgi to endosome trafficking (7,10). Several membrane proteins act as sorting receptors that bind A β PP controlling its endocytosis and endosomal trafficking, thereby influencing production of A β peptides, such as LRP1, LRP1b (11), ApoER2 (9) and SorLA (12).

Using crosslinking to identify the *in vivo* interactions of A β PP, Bai and colleagues demonstrate that LINGO-1, a membrane protein, physically associates with A β PP in cortical neurons and that this interaction stimulated A β generation from A β PP, by increasing the production of CTF β while decreasing production of CTF α (13). Partially contradicting these findings, a subsequent study lead to the conclusion that LINGO-1 over-expression decreased levels of both CTF β and CTF α (14). Neither study elucidated the mechanism of the reported effects of LINGO-1.

LINGO-1 is evolutionarily related to LRIG-1, a membrane protein that promotes the endocytosis and

lysosomal degradation of a variety of membrane proteins, including receptor tyrosine kinases such as the EGF, HGF and GDNF receptors (15). This observation led us to initiate experiments to examine whether effects of LINGO-1 on A β PP processing occurs due to altered trafficking of A β PP through the endosome/lysosome pathway. Bai and coworkers employed C-terminally tagged LINGO-1 in their experiments examining the influence of LINGO-1 on A β PP processing. Our studies have revealed that C-terminal tags functionally disrupt a C-terminal domain that controls the trafficking of LINGO-1 between plasma membrane and intracellular membrane compartments (unpublished observations). Thus we employed LINGO-1 lacking C-terminal tags for our studies.

Our studies confirm that LINGO-1 does interact physically and functionally with A β PP. We find this association promotes the lysosomal degradation of A β PP by a non-amyloidogenic pathway suggesting that LINGO-1 deficiency might increase the proportion of A β PP processed by the amyloidogenic pathway. Indeed, we report that LINGO-1 expression in cortical grey matter is substantially reduced in individuals with AD, as compared to cognitively normal subjects.

Materials and methods

Vector construction

pRRL-cPPT-CMV-hLINGO-1-IRES-hrGFPII-PRE-SIN. We thank Dr. William Osborne for providing the lentiviral transfer vector pRRL-cPPT-CMV-X-PRE-SIN. Full length LINGO-1 was isolated from IMAGE clone 4214 343 using HindIII restriction enzyme. The digest was heat inactivated at 65°C for 20 min and the ends blunted with T4 DNA polymerase. This second reaction was heat inactivated at 75°C for 20 min and then further digested with EcoR1. The cut product (2,350 bp) was run on a 1% agarose gel and band purified. The fragment was ligated into an EcoR1 and EcoR5 linearized pRRL-cPPT-CMV-X-PRE-SIN. Confirmation of gene insertion was obtained by direct sequencing of the final product.

Cell culture, transfection and treatments

HEK293 and N2A cells were cultured in pH 7.2 DMEM (MediaTech) containing 10% fetal bovine serum (FBS) (Hyclone) at 37°C, 5% CO₂. Both HEK293 cells and N2A cells were plated at 30–50% confluency in 6-well plates 24 h prior to transfection. Cells were transfected using Lipofectamine 2000 (2.5 μ l/ μ g LIPO:DNA; Invitrogen) and 1 μ g (unless otherwise indicated) of plasmid DNA (per construct transfected) in DMEM containing 10% FBS without antibiotics. Cells transfected with expression vectors were harvested 24 h post-transfection while cells transfected with siRNA were harvested 48 h post-transfection. Cells plated on cover slips were plated

at 20–30% confluency. Cells transfected with siRNA were transfected with 2 μ l/well of Lipofectamine 2000 and 50 nM siRNAs. Where indicated, cultures were subjected to the following treatments in fresh media: DMSO (Sigma, 1:1000, times vary), DAPT (Sigma, 10 μ M, 20 h), bafilomycin a1 [Baf] (Calbiochem, 0.1 μ M, 12 h), epoxomicin [Epo] [EMD, 1 μ M, 6 h, a concentration found to be non-toxic in previous studies (16)].

Brain tissue sample preparation

Post-mortem human brain samples were obtained from the University of Washington Neuropathology Core and Alzheimer Disease Research Center (ADRC). The University of Washington Human Subjects Division determined that our use of these coded specimens did not meet the federal definition of research with human subjects under 45 CFR 46. Sections of frozen cortical grey matter were isolated, weighed, and added to a microcentrifuge tube. A matching volume of lysis buffer [20 mM Tris, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 250 mM sucrose, and protease inhibitor mixture (Sigma)] was added to each tube and the sample was gently prepared using a microcentrifuge tube pestle. The samples were triturated by passing them through a 20 gauge needle to shear DNA. The processed lysate was then sonicated for 10 pulses at 20% duty/output #2 before being clarified by centrifugation (16,000 g, 15 min at 4°C). The final clarified supernatant was collected and protein concentrations for each sample were quantified by Bradford assay for use in SDS-PAGE. SDS loading buffer (62 mM Tris pH 6.8, 2% SDS, 10% Glycerol, 2% BME, 0.02% BPB) was added to the lysates and they were boiled for five minutes.

Dissociated mouse primary neuronal culture

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington and conform to the National Research Council's guide for the care and use of laboratory animals. C57Bl6 mice of both sexes, were housed in the AALAC approved vivarium of the University of Washington. Neonatal mice were sacrificed by decapitation for harvest of brain tissue. Primary cortical neurons were prepared from postnatal day zero mouse pup brains. Pup brains were removed in Hanks' medium without Ca²⁺ and Mg²⁺, and then further dissected in dissection media (Neurobasal-A™ (Invitrogen) medium supplemented with 10 mM HEPES, and 500 μ M L-glutamine). After the dissection, cortices were digested with 3.75 mg/ml trypsin for 25 min at 37°C. The trypsin digestion was halted with trypsin inhibitors and the tissue was treated with 1 mg/ml DNase (Sigma). The tissue was dissociated by multiple rounds of pipetting and collected by centrifugation at 800 rpm for 3 min. The neurons were resuspended in dissection media and the process repeated three times to wash the neurons. On the final wash the

neurons were resuspended in plating media (Neurobasal-A™ with B27 supplement and 500 μ M L-glutamine). The neurons were plated on poly-d-lysine (Sigma, 0.1 mg/ml in dH₂O) coated 12 well plates. The neurons were allowed to recover for 2–3 days before transduction with virus or transfection with siRNA. Transduced neurons were cultured for 5 days without changing the media, while siRNA transfected neurons were harvested after 48 h. Neuronal cultures were transduced using 3 MOI of virus in serum free media. For experiments using the γ -secretase inhibitor DAPT, cells were treated with 10 μ M DAPT starting approximately 24 h after transfection and remained in the inhibitors for 16–20 h prior to lysis.

Immunocytochemistry

Cover slips were rinsed twice with phosphate-buffered saline (PBS, pH 7.4) and fixed with 4% formaldehyde in PBS for 10 min. Cover slips were then rinsed with PBS and permeabilized (0.25% v/v Triton-X100 in PBS for 15 min) before blocking in 10% w/v bovine serum albumin in PBS (BSA/PBS) for 10 min. The cover slips were then incubated overnight at 4°C with primary antibodies. Primary antibody dilutions used the following: rabbit anti-LINGO-1 (Upstate, 1:1000) and mouse anti-LINGO-1 (R&D, 1:500). Primary antibodies were removed and cover slips were rinsed four times with PBS. The cover slips were incubated in secondary reagents for 45 min (Molecular Probes; Alexafluor 594, 1:1000 and Alexafluor 488, 1:1000) at room temperature or 3 h at 4°C. The cover slips were then washed twice with PBS and once with deionized water before mounting in Vectashield with DAPI (Vector Labs).

Confocal microscopy

Confocal images of cultured mouse neurons or human cortex were acquired on a Nikon Eclipse Ti confocal microscope using 405 nm, 488 nm and 543 nm excitation lasers. Images were obtained at 40 \times 0.65 NA. Emission channels were sequentially acquired in frame-scan mode with line averaging (set to 4). The final multi-channel image was reconstructed using the microscope's resident imaging software package, NIS-Elements.

γ -secretase activity assay

APP-Gal4-VP16 expression construct was employed to measure γ -secretase activity in a variety of cell types. This construct reports the A β PP-cleavage dependent expression of luciferase and the independent constitutive expression of β -galactosidase, thereby allowing for luciferase activity levels to be normalized to transfection efficiency. HEK 293 or N2A cells were plated at 50,000 cells/well in a 24 well plate the day prior to transfection with APP-Gal4-VP16 and LINGO constructs. The cells were then grown for 72 h and then rinsed twice with ice-cold PBS and lysed in 100 μ l of cold lysis buffer (0.1%

Triton X-100, 1 mM DTT, 6 mM MgSO₄, 4 mM ATP, 100 mM potassium phosphate, pH 7.8) and the plates were frozen at –80°C to promote lysis. Each well of a 96-well microtiter white bottomed plate was filled with 200 μ l (100 mM potassium phosphate, 6 mM MgSO₄/4 mM ATP, pH 7.8) and 25 μ l lysates added to each well. Luciferase activity was then determined by employing a Berthold EG&G MicroLumat LB 96 V luminometer. The raw output from the luminometric assays were normalized to the constitutive and transfection-dependent β Gal value for each sample. Cells were transfected using Lipofectamine 2000 and 1 μ g of appropriate expression construct.

Immunoblot analysis

Cells were washed twice with cold PBS on ice, lysed in 20 mM Tris, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 250 mM sucrose, and protease inhibitor mixture (Sigma), and centrifuged 15 min at 16,000 \times g. The resulting clarified supernatants were quantified by Bradford assay for use in SDS-PAGE. SDS-PAGE, semi-dry transfer using 0.45 μ m PDVF (Millipore) were performed as previously described (17). The resulting membranes were blocked with 0.01% Tween-20, phosphate-buffered saline (PBS-T) containing 5% non-fat dried milk for 1 h at room temperature and then probed with primary antibody

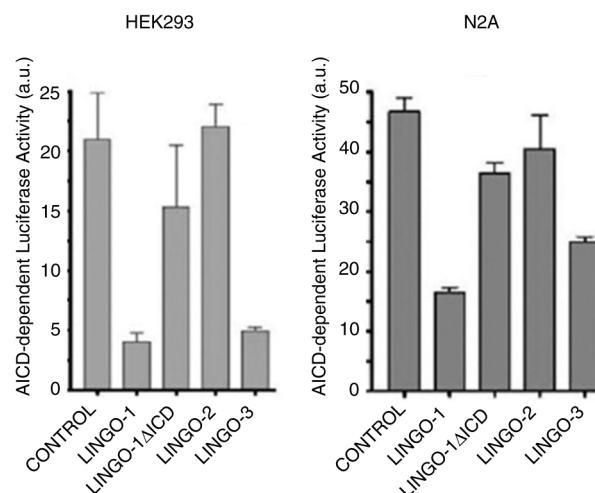


Fig. 1. LINGO-1 down-regulates full length A β PP and AICD. HEK293 and N2A cells were transfected with the APGV16 luciferase transcriptional reporter construct or APGV16 plus a construct encoding LINGO-1, LINGO-2, LINGO-3 or LINGO-1 with the C-terminal portion of the intracellular domain deleted (LINGO-1 Δ ICD). AICD – dependent luciferase activity is reduced by expression of LINGO-1 or LINGO-3, while LINGO-2 or LINGO-1 Δ ICD had little effect. Release of A β PP AICD was measured as transcriptionally coupled luciferase expression. Error bars represent s.e.m.; $N = 4$ experimental replicates. Luciferase activity is expressed as arbitrary luminometer units (a.u.).

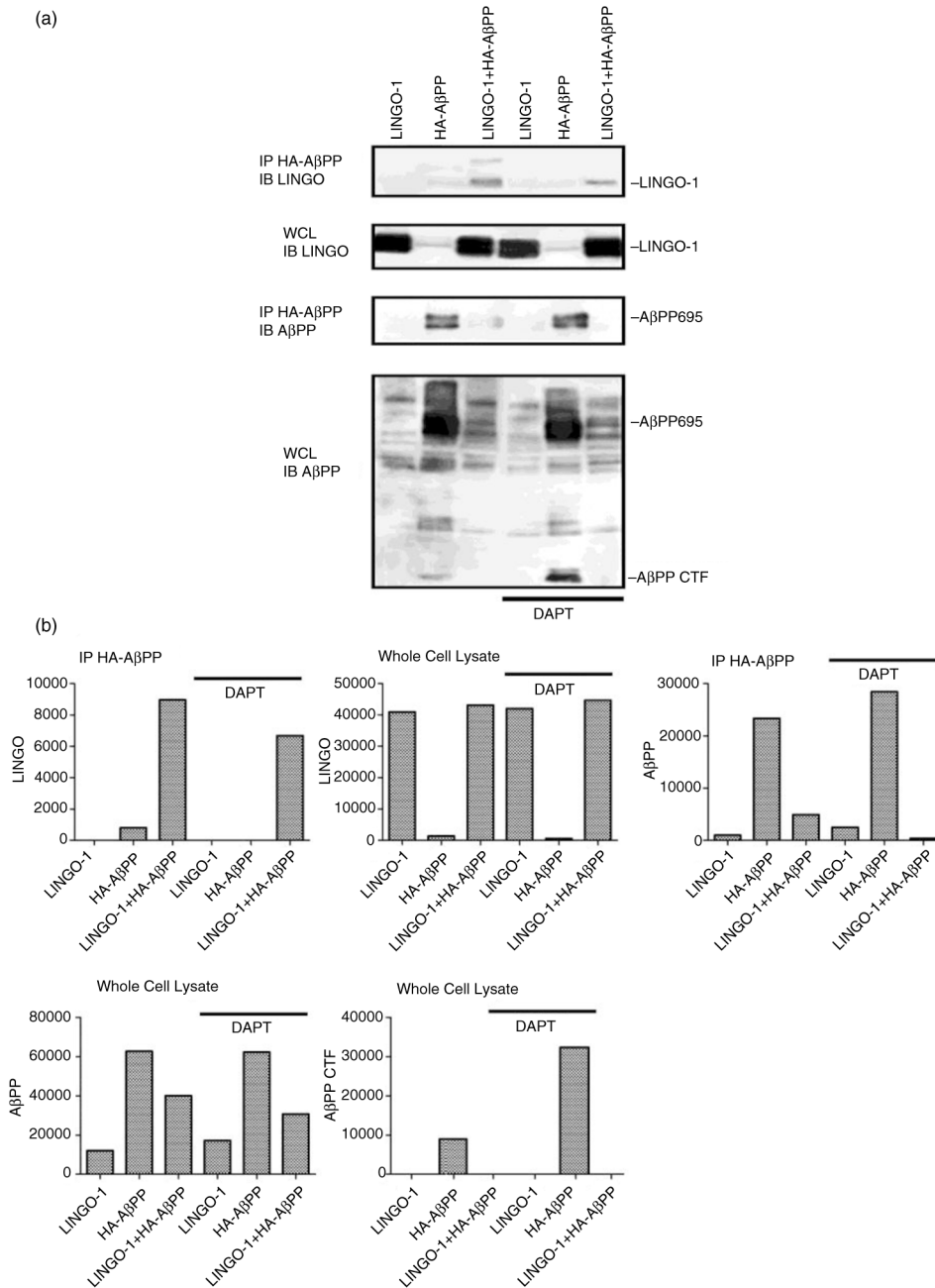


Fig. 2. LINGO-1 down-regulates full length AβPP as well as CTFα/CTFβ fragments. HEK293 cells were transfected with LINGO-1 (LINGO), hemagglutinin-tagged AβPP (HA-AβPP) or both, and cultured with a γ-secretase inhibitor (DAPT) or vehicle (DMSO). Harvested whole cell detergent lysates (WCL) or lysates immunoprecipitated with anti-HA to bring down AβPP were probed on immunoblots with anti-LINGO-1 and anti-AβPP. (a) Immunoblot image. (b) Densitometric quantification of bands on immunoblot image. Quantity of immunoreactive protein, in arbitrary units, is plotted. For LINGO-1 and AβPP, which are expressed as two bands, the sum of the quantities of the two species is plotted. The lower molecular weight band of LINGO-1 was co-precipitated with HA-AβPP. LINGO-1 over-expression dramatically reduced levels of both AβPP and its CTF fragments in cell lysates. This effect was observed even in the presence of DAPT.

overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary IgG (Jackson Immuno, 1:20,000) in 5% milk, PBS-T for 1 h at room temperature. After exposure to the secondary antibody, membranes were further washed and treated with ECL (0.2 mM coumaric acid, 1.25 mM Luminol, 1 mM Tris activated with 0.12% hydrogen peroxide in

water). Densitometry of resulting target bands was performed using NIH ImageJ.

Statistics

Unless otherwise indicated, statistical significance was evaluated using Student's *t*-test. Error bars for luciferase activity represent standard error of the mean.

Results

LINGO-1 diminishes γ -secretase-mediated processing of A β PP

Our initial experiments sought to confirm that LINGO-1 influences processing of A β PP. Quantitation of A β peptide production from A β PP is fraught with difficulty. Multiple A β PP-derived peptides are produced with varying length thereby affecting the reactivity with antibodies employed for immunoassay, and likely, varying the extractability from lysed cells. Consequently, we have employed a procedure that monitors γ -secretase mediated release of the intracellular domain (AICD) of A β PP, since release of A β peptides from A β PP by γ -secretase is accompanied by release of AICD (16,18). The methodology involves expression of A β PP modified by C-terminal fusion to a transcription factor, so that AICD release drives expression of a luciferase reporter gene. We examined the effect of LINGO-1 on AICD release using this reporter system. We have observed that mutation of residues at the C-terminus of the intracellular domain of LINGO-1 causes LINGO1 to traffic to the cell surface, in contrast to native LINGO-1 which is restricted to intracellular membrane compartments (unpublished results). Thus we used LINGO-1 mutated in this manner as a negative control. We found that LINGO-1 dramatically *decreased* release of AICD both in a non-neuronal cell line (HEK293) and in a neuronal cell line (N2A) while LINGO-1 bearing a C-terminal deletion mutation had little effect (Fig. 1).

LINGO-1 has three paralogs, LINGO-2, LINGO-3, and LINGO-4, which are similar in amino acid sequence (44–60% identical) and domain structure to LINGO-1. LINGO-2 and LINGO-3, like LINGO-1, are expressed in cortical neurons. This begs the question whether LINGO-1 paralogs also diminish release of AICD from A β PP. The data in Fig. 1 indicate that LINGO-3 affects production of AICD almost as strongly as LINGO-1 while LINGO-2 is substantially less effective.

LINGO-1 binds A β PP

The apparent inconsistency of our finding that LINGO-1 diminishes γ -secretase mediated processing of A β PP with previously reported enhancement of A β production by LINGO-1 caused us to assess whether published reports that LINGO-1 binds A β PP are correct. In order to assess whether LINGO-1 associates with A β PP, we expressed the two proteins by transient transfection of HEK 293 cells, and assessed whether LINGO-1 was present in A β PP immunoprecipitates. This experiment revealed an avid association of LINGO-1 with A β PP (Fig. 2). Similarly, A β PP was detected in LINGO-1 immunoprecipitates (data not shown).

LINGO-1 down-regulates A β PP

In the experiment illustrated in Fig. 2, we noted that expression of LINGO-1 substantially reduced the amount

of A β PP present in cell lysates. Furthermore, LINGO-1 diminished the amount of CTF, the C-terminal product of cleavage of A β PP by α - and β -secretases. (The similarly sized products of α and β cleavage were not well-resolved in this experiment). Reduced CTF accumulation could result from increased cleavage of CTF by γ -secretase. However, in the presence of the γ -secretase inhibitor, DAPT, LINGO-1 still strongly down-regulated the A β PP CTF. The results indicate that LINGO-1 promotes degradation of A β PP and CTF by a γ -secretase-independent pathway.

To examine this effect more systematically, we employed transient transfection with increasing quantities of LINGO-1 plasmid to assess whether the effect of LINGO-1 was dose-dependent. We observed that the amount of A β PP present in cells varied in inverse proportion to the amount of LINGO-1 expressed (Fig. 3). As a control to verify that A β PP down-regulation resulted from increased LINGO-1 expression rather than a

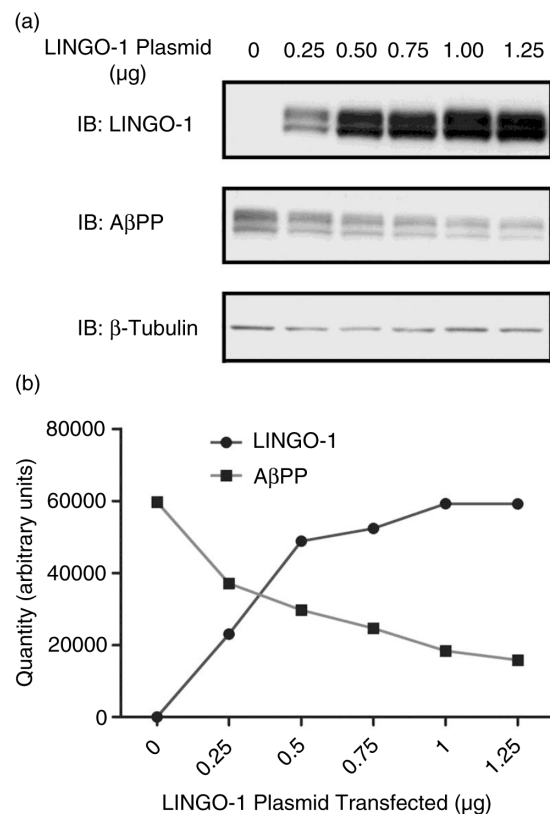


Fig. 3. A β PP is down-regulated by LINGO-1. HEK293 cells were transfected with 1 μ g A β PP plasmid and varying amounts of LINGO-1 plasmid ranging from 0 to 1.25 μ g (plus control plasmid to maintain constant total plasmid quantity). A β PP and LINGO-1 expression was assessed by probing immunoblots with anti-LINGO-1 and anti-A β PP. (a) Immunoblot image. (b) Densitometric quantification of bands on immunoblot image. Quantity of immunoreactive protein, in arbitrary units, is plotted. A β PP was down-regulated in proportion to the amount of LINGO-1 expressed.

non-specific effect of LINGO-1 plasmid, we verified that shRNA-mediated knockdown of LINGO-1 expression blocked the effect of LINGO-1 plasmid transfection on AβPP expression (Fig. 4).

LINGO-1 paralogs down-regulate AβPP

In light of our observation that the various LINGO-1 paralogs differed in their ability to reduce release of the AICD fragment of AβPP, we compared the effects of LINGO-1, LINGO-2 and LINGO-3 on AβPP down-regulation. We found that LINGO-1, LINGO-2 and LINGO-3 each down-regulated AβPP (Fig. 5). Although LINGO-2 was slightly less effective than the other paralogs, the differential effect of LINGO-2 was not as great as observed in the prior experiment assessing effects on production of AICD. However, this difference might simply reflect differing levels of LINGO-2 expression achieved in the two experiments.

LINGO-1 down-regulates AβPP in cortical neurons

The prior experiments all employed AβPP artificially expressed in cell lines. In order to determine whether LINGO-1 has a similar effect on endogenous AβPP in authentic neurons, we exposed cultured cortical neurons to a lentiviral vector expressing LINGO-1. The results

demonstrated that LINGO-1 overexpression down-regulates endogenous AβPP in cortical neurons (Fig. 6).

Since LINGO-1 down-regulates AβPP both when it is expressed from its native promoter and when it is expressed artificially from a constitutively active CMV promoter, it is unlikely that LINGO-1 acts by transcriptional regulation. Instead, it seems likely that LINGO-1 promotes AβPP degradation. Major cell pathways for protein turnover include degradation by lysosomes and degradation by proteasomes. Because of the structural similarity of LINGO-1 to LRIG1, which promotes lysosomal degradation of several membrane proteins, we hypothesized that LINGO-1 promotes lysosomal degradation of AβPP. We tested this hypothesis employing a pharmacological inhibitor of lysosome (bafilomycin a1) and compared the effect to the effect of a pharmacological inhibitor of proteasomes (epoxomicin), which represents another major cellular pathway for protein degradation (Fig. 7). The down-regulation of AβPP mediated by transduced LINGO-1 overexpression in neurons was not rescued by treatment with the proteasome inhibitor epoxomicin. However, AβPP expression was strongly elevated above control levels when transduced cultures were treated with the lysosome inhibitor bafilomycin a1, indicating that lysosomes mediate LINGO-1 dependent degradation of AβPP.

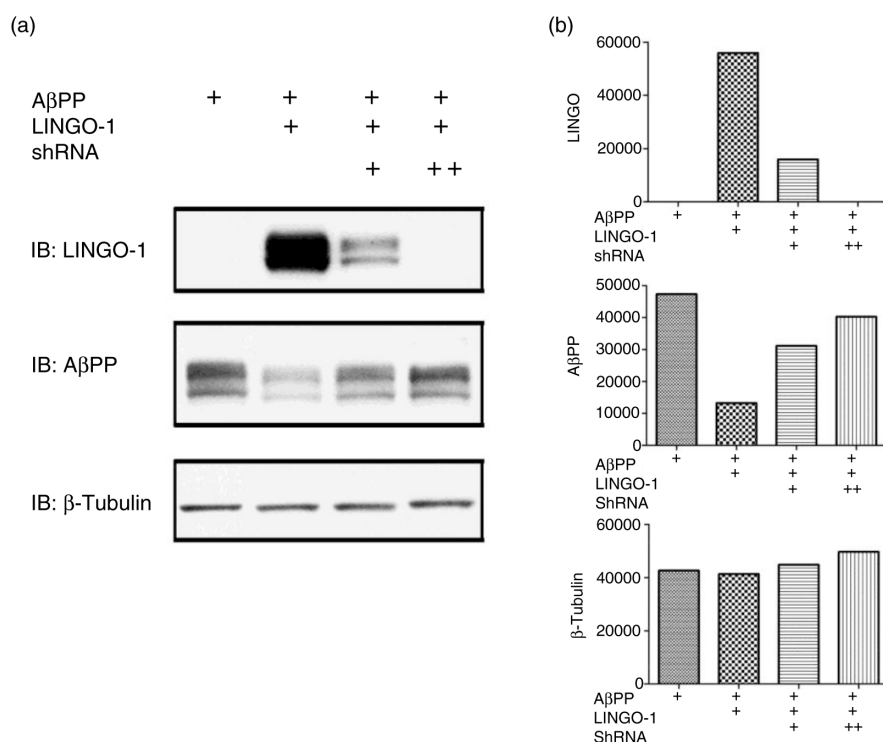


Fig. 4. shRNA knock down of LINGO-1 blocks effect of LINGO-1 expression on AβPP down-regulation. HEK293 cells were transfected with AβPP and LINGO-1 plasmids with or without plasmid encoding a short-hairpin RNA (shRNA) targeting LINGO-1 transcript. (a) Immunoblot image. (b) Densitometric quantification of bands on immunoblot image. Quantity of immunoreactive protein, in arbitrary units, is plotted.

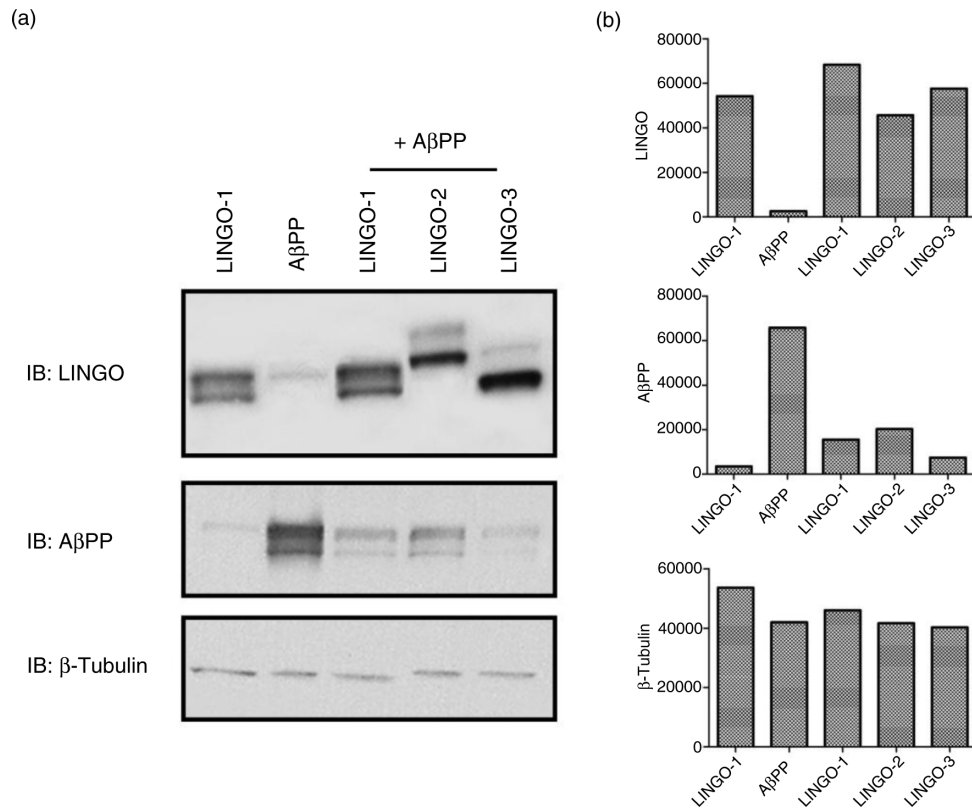


Fig. 5. LINGO paralogs down-regulate AβPP with varying effectiveness. HEK293 cells were transfected with AβPP plasmid, and LINGO-1, LINGO-2, or LINGO-3 plasmids [or empty vector as a negative control (1 μg each)]. Expression of LINGO and AβPP was assessed by immunoblot. (a) Immunoblot image. (b) Densitometric quantification of bands on immunoblot image. Quantity of immunoreactive protein, in arbitrary units, is plotted.

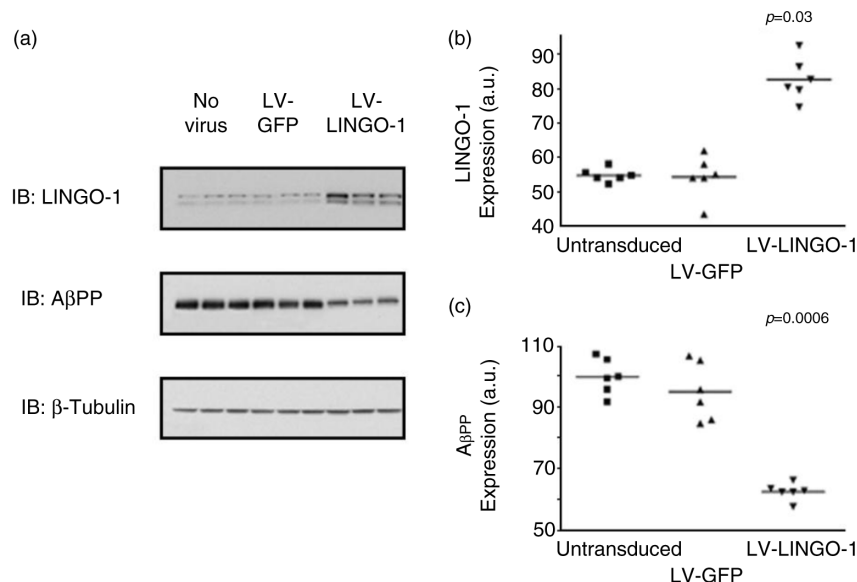


Fig. 6. LINGO-1 over-expression down-regulates AβPP in cultured cortical neurons. Neonatal mouse cortical cultures ($N=6$ for each condition) were infected at 3 MOI with lentiviral vectors encoding LINGO-1 or GFP and grown for 5 days (respectively, LV-LINGO-1 and LV-GFP). (a) A representative immunoblot image for three of the six replicates (20 μg protein loaded per lane). LINGO-1 and AβPP expression was quantified using densitometry and normalized with respect to the quantity of β-tubulin immunoreactivity. Normalized values for LINGO-1 (b) and AβPP expression (c) show AβPP expression is reduced by LINGO-1 over-expression. The lentiviral vector elevated LINGO-1 expression only modestly above endogenous levels in cortical neurons but this was sufficient to down-regulate AβPP. Densitometrically quantified quantity of immunoreactive protein is expressed as arbitrary units (a.u.).

Lysosomal inhibition also blocked LINGO-1-mediated down-regulation of endogenous A β PP expressed in HEK 293 and N2A cells (data not shown).

These results suggest that eliminating expression of endogenous LINGO-1 in cultured cortical neurons would up-regulate A β PP. Unfortunately, we were unable to test this hypothesis because our efforts to knock down expression of LINGO-1 with siRNAs were confounded by the fact that non-specific control siRNAs massively up-regulated LINGO-1 (data not shown). It is well known that siRNAs act non-specifically to activate toll-like receptors (19,20) so we suspect that the effect of siRNAs on LINGO-1 expression reflects transcriptional regulation by effectors such as NF- κ B that function down-stream of toll-like receptors.

LINGO-1 localization in cortical neurons

To assess the potential of LINGO-1 to act as a physiological regulator of A β PP turnover in brain cortex, we asked whether LINGO-1 and A β PP are co-expressed in the same neuronal cell types and in similar subcellular compartments. *In situ* hybridization data localizing LINGO-1 and A β PP mRNA in the developing mouse brain (21) indicate that LINGO-1 and A β PP are expressed in similar populations of brain neurons, particularly in the cortex. We employed immunofluorescence microscopy to compare the distribution of LINGO-1 and A β PP in cerebral cortex. To ensure that the immunostaining observed was specific, we compared results with two different LINGO-1 antibodies, directed against the N-terminus or C-terminus of LINGO-1. In preliminary experiments immunostaining HEK293 cells transfected with LINGO-1, LINGO-2 or LINGO-3 we observed that the antibody against LINGO-1 C-terminus recognized LINGO-2 and LINGO-3 equally well, while the antibody against LINGO-1 N-terminus did not recognize LINGO-2 or LINGO-3. We observed that

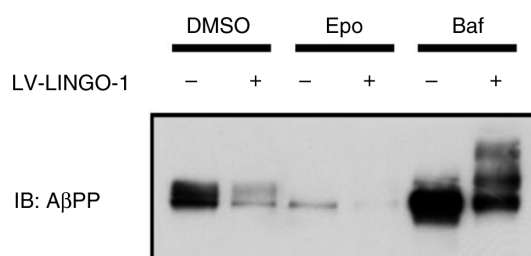


Fig. 7. Inhibition of lysosomes blocks LINGO-1-mediated down-regulation of A β PP. Mouse cortical neurons or neurons infected with LINGO-1 lentivirus were cultured in the presence of a proteasome inhibitor (epoxomicin; Epo), a lysosomal inhibitor (bafilomycin a1; Baf) or vehicle (DMSO). Detergent cell extracts were examined by immunoblot analysis with anti-A β PP. LINGO-1 mediated down-regulation of A β PP was prevented by bafilomycin a1. Epoxomicin reduced expression of A β PP, possibly because of toxicity, but did not prevent further reduction of A β PP levels by LINGO-1.

the two antibodies immunostained identical intracellular membrane compartments, and in the same cortical neurons in histological sections of human cortex and in neonatal mouse cortical neuronal cultures (Fig. 8). These results support the conclusion that both antibodies are specific. The results also suggest that LINGO-1 is the predominant LINGO isoform in cortex. This conclusion was confirmed by immunoprecipitating a mouse cortical homogenate with the pan-specific LINGO-1 antibody, followed by analysis of the immunoprecipitated proteins by mass spectrometry. Peptide fragments identified derived almost exclusively from LINGO-1 (data not shown).

We employed the antibody against the C-terminus (ICD) of LINGO-1 to compare the distribution of A β PP and LINGO-1 in cultured mouse cortical neurons (Fig. 9). The images revealed that most cortical neurons express both and A β PP and LINGO-1.

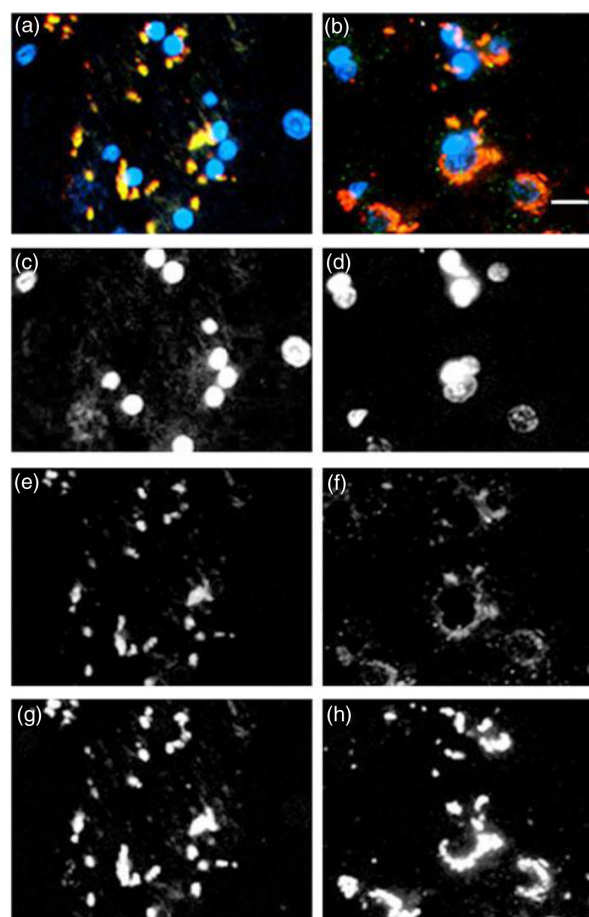


Fig. 8. LINGO-1 ICD and ECD antibodies label identical intracellular membrane compartments in cortical neurons. Using confocal microscopy fluorescently labeled antibodies against LINGO-1 ICD (green, e, f) and LINGO-1 ECD (red, g, h) were imaged in stained mouse cortical neurons (left panels a, c, e, g) and sections of human cortex (right panels b, d, f, h). Co-localization of the LINGO-1 ICD (green) and LINGO-1 ECD (red) appears as yellow-orange. Blue (c, d) is DAPI nuclear stain. Scale bar equals 10 μ m.

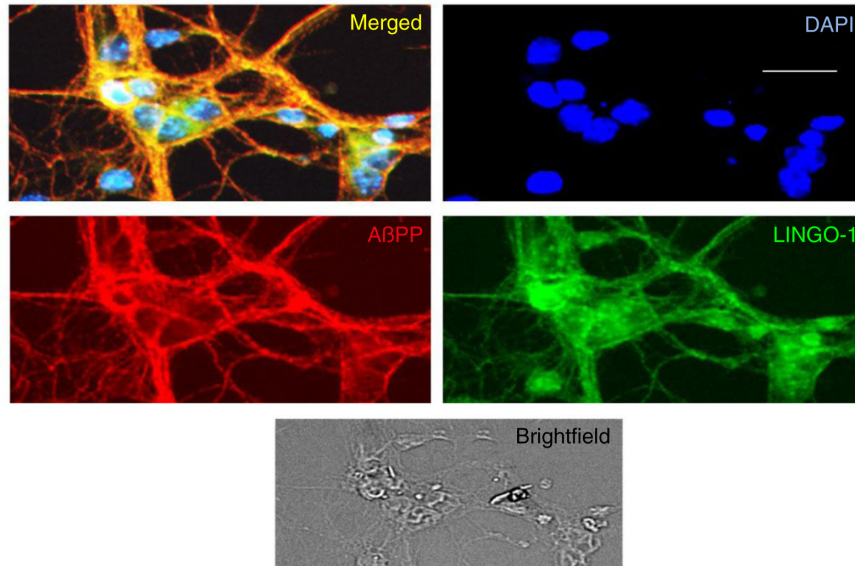


Fig. 9. LINGO and A β PP are co-expressed in mouse cortical neurons. Confocal immunofluorescence microscopy was employed to localize LINGO-1 (red) and A β PP (green) in cultured neonatal mouse cortical neurons. Blue is DAPI nuclear stain. Scale bar equals 50 μ m.

LINGO-1 levels are reduced in AD brain cortex

Our results indicating that LINGO-1 down-regulates A β PP by a non-amyloidogenic pathway suggest that the level of LINGO-1 expression in the brain might be a determinant of risk to develop AD. For this reason, we performed immunoblot experiments to assess whether LINGO-1 expression in parietal, frontal and temporal cortical grey matter from AD brains differs from age-matched normal subjects. Sample volumes of detergent brain extracts were adjusted to ensure that equivalent amounts of protein were loaded for each brain sample, and gel immunoblots were quantified densitometrically for LINGO-1 and β -tubulin. LINGO-1 expression was normalized to β -tubulin levels. Relative to controls, LINGO-1 levels were reduced by almost 50% in AD cortical samples (Fig. 10). This effect was observed in all three cortical regions examined.

Discussion

Our results indicate that LINGO-1 interaction with A β PP promotes lysosomal degradation of A β PP. Degradation in this manner reduces the opportunity for A β PP to undergo processing by the amyloidogenic pathway. LINGO-3 and, to a lesser extent, LINGO-2 appears to have the capacity to function redundantly with LINGO-1 in promoting degradation of A β PP but LINGO-1 mediated A β PP degradation probably predominates in cortex where LINGO-1 is the most abundant member of the LINGO family. However, the extent to which the various LINGO paralogs may function redundantly in cortical regulation of A β PP remains to be determined more fully.

Analysis of the interaction of deletion mutants of A β PP has indicated that neither the leucine rich repeat domain, the transmembrane domain, or the cytoplasmic domain of LINGO-1 are required for association with A β PP,

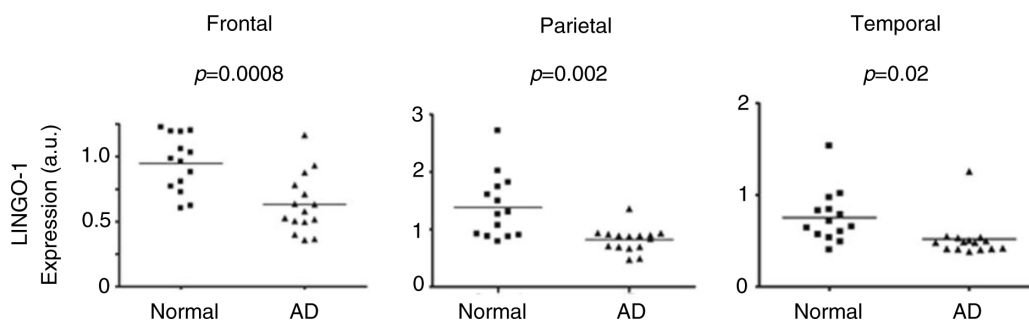


Fig. 10. LINGO-1 expression is diminished in multiple cortical areas in Alzheimer's disease. Immunoblots of frontal, parietal and temporal cortical grey matter tissues from age-matched normal ($N = 15$) and AD subjects ($N = 15$) were analyzed. LINGO-1 expression was normalized to the load control protein β -tubulin. Each data point represents the resulting ratio for a single unique subject. p -values are Student's t -test between Normal and AD for each tissue.

implying that the binding domain lies in the C-terminal portion of the extracellular domain (22). Our results indicate that the intracellular domain of LINGO-1 is required for effective stimulation of A β PP degradation. We speculate that the cytoplasmic domain of LINGO-1 interacts with cytoplasmic proteins that control trafficking through the secretory and/or endolysosomal pathway.

Following endocytosis, A β PP is apparently degraded by two alternative pathways, one involving lysosomal degradation, and the other involving sequential cleavage by α - or β - and γ -secretase. The consistently lower LINGO-1 levels we observed in post-mortem samples of cortex from individuals with AD compared to controls would be predicted to increase the proportion of A β PP processed by the amyloidogenic pathway and thereby promote the progression of neurodegeneration. The mechanism responsible for the reduction of LINGO-1 levels in AD brains remains to be determined. However, it is likely that the mechanism concerns altered transcriptional regulation. It has been reported that the glutamate agonist kainic acid transcriptionally up-regulates expression of LINGO-1 (23). Consequently we speculate that the decreased cortical activity that characterizes AD may be responsible for the decrease in LINGO-1 expression.

LINGO-1 has not been identified directly as a genetic risk factor for AD. However, genetic linkage studies have identified specific alleles of both LINGO-1 and LINGO-2 as risk factors for another common neurodegenerative disease, essential tremor and more controversially, Parkinson's disease (24–30). Individuals with essential tremor and Parkinson's disease have increased risk for AD (31). The role of LINGO-1 in regulating A β PP processing by shunting it from a pathogenic to a non-pathogenic proteolytic pathway may point the way to novel means of therapeutically reducing production of A β peptides in AD.

Acknowledgements

This work was supported by grants from the National Institute of Aging (1R01 AG21127; R21 AG02647) and the Alzheimer's Disease Association (IIRG-00-1935). Autopsy materials used in this study were obtained from the University of Washington Neuropathology Core, which is supported by the Alzheimer's Disease Research Center (AG05136), the Adult Changes in Thought Study (AG006781), and Morris K Udall Center of Excellence for Parkinson's Disease Research (NS062684).

Conflict of interest and funding

The authors have not received any funding or benefits from industry or elsewhere to conduct this study.

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