Evidence that the Rab5 effector APPL1 mediates APP-βCTF induced dysfunction of endosomes in Down syndrome and Alzheimer’s disease

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Abstract

β-amyloid precursor protein (APP) and its cleaved products are strongly implicated in Alzheimer’s Disease (AD). Endosomes are highly active APP processing sites and endosome anomalies associated with upregulated expression of early endosomal regulator, rab5, are the earliest known disease-specific neuronal response in AD. Here, we show that the rab5 effector APPL1 mediates rab5 over-activation in Down Syndrome (DS) and AD, which is caused by elevated levels of the β-cleaved carboxy-terminal fragment of APP (βCTF). βCTF recruits APPL1 to rab5 endosomes, where it stabilizes active GTP-rab5, leading to pathologically accelerated endocytosis, endosome swelling, and selectively impaired axonal transport of rab5 endosomes. In DS fibroblasts, APPL1 knockdown corrects these endosomal anomalies. βCTF levels are also elevated in Alzheimer brain, which is accompanied by abnormally high recruitment of APPL1 to rab5 endosomes as seen in DS fibroblasts. These studies indicate that persistent rab5 over-activation through βCTF-APPL1 interactions constitutes a novel APP-dependent pathogenic pathway in AD.
Introduction

β-amylloid precursor protein (APP) and its cleaved product, amyloid beta peptide (Aβ), are strongly implicated in Alzheimer’s Disease (AD) via β-amloid toxicity although disease pathogenesis is increasingly considered multifactorial possibly involving additional APP metabolites. Endosomes are highly active APP processing sites and genes that influence endocytosis are over-represented as AD risk factors. Endosome anomalies associated with upregulated expression of rab5 and other endocytosis-related genes are the earliest known disease-specific neuronal response in AD. They develop in early in Down syndrome (DS, Trisomy 21), a cause of early-onset AD linked to an extra copy of APP, wherein APP-dependent endosome abnormalities are associated with late endosome anomalies and defective endosomal signaling leading to cholinergic neurodegeneration in DS brains. Similar endosome dysfunction is seen in neurons generated from induced pluripotent stem cells from individuals with familial and sporadic AD patients and DS fibroblasts. In particular, endosomal abnormality found in DS cells are caused by the β-cleaved carboxy-terminal fragment of APP (βCTF). Endocytosis is particularly important in neurons for receptor trafficking, neurotrophin signaling, and neurotransmission. It is also critical for regulating nuclear signaling via endosome-mediated interactions of APPL1 (adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain, and leucine zipper motif), a binding partner and effector of rab5. APPL1 is localized mainly in early endosomal membrane. There are also populations of APPL1 vesicles distinct from rab5 positive endosomes although its identity has not been determined. APPL1 on rab5-positive endosomes translocates from endosomal membranes to the nucleus where it regulates chromatin structure and gene expression. It also mediates several signaling processes, including the NF-κB and insulin pathways, the Akt pathway via phosphoinositides, and EGF receptor signaling. The small GTPase, rab5, regulates these processes and controls diverse signaling and cell functions of early endosomes. Abnormal activation of rab5 is implicated in AD and DS-related endosome dysfunction, however, the mechanism underlying pathological rab5 activation in AD is unknown.

Although it has been shown that βCTF can promote rab5-mediated endosomal pathology in DS fibroblasts, it is not known to interact directly with rab5. Although rab5 activation-induced clathrin-dependent APP endocytosis has been suggested to participate in βCTF and amyloid beta production through a rab5 dependent pathway, it is not clear how rab5 over-activation contributes to disease onset and progression. Here, we show that APPL1 mediates rab5 activation caused by elevated levels of βCTF in DS and AD. By binding the PTB domain of APPL1, βCTF recruits APPL1 to endosomes, where it stabilizes active GTP-rab5 and increases rab5 activity on endosomes, leading to pathologically accelerated endocytosis, followed by AD-like endosome swelling and selectively impaired axonal transport of endosomes in neurons. In fibroblasts from individuals with DS, siRNA silencing of APPL1 corrects known endocytic anomalies and reverses elevated nuclear translocation of p65/RelA, an indication of activated NF-κB signaling, which is known to be mediated by APPL1/rab5-endosomes. Finally, we show, for the first time, that βCTF levels are elevated in Alzheimer brain despite normal APP levels, which is accompanied by abnormally high

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recruitment of APPL1 to rab5-endosomes in AD brain, similar to that seen in cells from individuals with DS. These studies indicate that persistent rab5 over-activation through βCTF-APPL1 interactions constitutes a novel βCTF-dependent, and Aβ-independent, pathogenic pathway contributing to the development of AD and DS.

Materials and Methods

Cell culture and transfection

Embryonic mouse cortical neurons from E17-18 pregnant C57BL/6J females were cultured as described previously. All animal studies were performed with an approved protocol from the Nathan Kline Institute Institutional Animal Care and Use Committee.

Statistics

Results are presented as mean±S.E.M. Unless otherwise noted, statistical significance was determined by unpaired two-tailed Student’s t-test for two samples and one-way analysis of variance (ANOVA) followed by Tukey’s test to determine statistical significance for multiple comparisons. A p value of p<0.05 was considered statistically significant.

Details of the materials and methods are given in supplementary information materials and methods.

Results

βCTF activates rab5 and induces endosomal enlargement

To characterize APP influences on endocytosis, we first analyzed N2a mouse neuroblastoma cells overexpressing wild-type human APP stably (N2aAPP). N2aAPP cells internalized horseradish peroxidase (HRP) 2-fold more rapidly than N2a cells (Supplementary Fig. 1a), consistent with evidences of accelerated endocytosis in DS fibroblasts measured by transferrin, HRP, and EGF uptake. We documented rab5 activation by three assays. First, we detected 50% higher levels of membrane-bound rab5 (GTP-loaded active form) in N2aAPP cells compared to N2a cells using crude membrane fractionation analysis (Supplementary Fig. 1b). Second, we measured rates of fluorescence recovery after photobleaching (FRAP) on GFP-rab5 endosomes to reveal rab5 flux through the GDP-GTP cycle, which requires dissociation of bleached GFP-rab5 from endosomal membranes and insertion of unbleached GFP-rab5 from the surrounding cytosol. As expected, endosomes in N2a cells expressing GFP-rab5 Q79L, a dominant active mutant unable to hydrolyze GTP, showed a very diminished rate of recovery of fluorescence, indicating the constitutively activated state of the rab5 mutant (Fig. 1a). Overexpression of wild-type human APP (APPwt) in N2a cells also significantly decreased the rate of FRAP on endosomes when compared to that in control N2a cells, indicating greater rab5 activation (Fig. 1a). In a third approach, we directly measured levels of active rab5 by selectively immunoprecipitating this form with an antibody specific for GTP-rab5. This immunoblot analysis confirmed increased GTP-rab5 levels in HEK293 cells expressing APPwt compared to control cells, indicating that APPwt overexpression is sufficient to increase active rab5 levels (Fig. 1b). Consistent with actions
of activated rab5 in promoting endosome fusion\textsuperscript{25}, GFP-rab5 positive endosomes in N2a cells overexpressing APP\textsubscript{wt}, on average, were significantly larger than those in control N2a cells based on morphometric analyses, providing an independent functional evidence of rab5 activation (Fig. 1c). Taken together, these data show that APP\textsubscript{wt} overexpression activates rab5 on endosomes, upregulating endocytosis and endosomal fusion\textsuperscript{25}, which promotes endosome enlargement, as seen in early AD and DS\textsuperscript{8}.

Because one particular APP cleavage product, $\beta$CTF, is known to produce endosomal pathology in DS cells\textsuperscript{3, 4}, we tested if $\beta$CTF generation was sufficient to induce rab5 activation by using a mutant APP M596V (APP\textsubscript{mv}) that is unable to generate $\beta$CTF\textsuperscript{26}. Expressing APP\textsubscript{mv} had no effect on endosome FRAP in N2a cells (Fig. 1a), active rab5 levels in HEK293 cells (Fig. 1b), or endosome size in N2a cells (Fig. 1c), whereas a $\gamma$-secretase inhibitor (L685,458) significantly increased levels of $\beta$CTF by blocking its $\gamma$-cleavage to $\alpha$3 (Fig. 1c), slowed GTP-rab5 fluorescence recovery (Fig. 1a) and enlarged endosomes (Fig. 1c). Consistent with previous findings in DS fibroblasts\textsuperscript{3}, endosomal enlargement was not dependent on $\alpha$CTF levels that were higher in APP\textsubscript{mv}-overexpressed cells than in control N2a cells (Fig. 1c), confirming that rab5 activation is mediated specifically by $\beta$CTF. Similar to the pattern in AD brain\textsuperscript{27}, endosomal enlargement was disproportionately greater in the endosome population larger than 0.5$\mu$m$^2$ (Supplementary Fig. 1c). Thus, these studies show that APP-dependent rab5 activation requires $\beta$-cleavage of APP, but not $\alpha$- or $\gamma$-cleavages, as we previously showed in DS fibroblasts\textsuperscript{3}.

### $\beta$CTF selectively binds via its YENPTY domain to the PTB domain of APPL1

Since APP is not known to interact directly with rab5, we sought a protein mediator of $\beta$CTF-dependent rab5 activation, capitalizing on knowledge that APP interacts with proteins containing the PTB domain\textsuperscript{28}. We considered APPL1 a good candidate because it contains the PTB domain but also has other domains mediating selective binding to active GTP-bound rab5, which then stabilizes rab5 in its activated GTP state on endosomal membranes and recruits additional rab5\textsuperscript{13, 29}. Moreover, perisomatic APPL1 distribution is altered in AD brain\textsuperscript{30}. Based on double-immunolabeling analyses of APPL1 and GFP-rab5, APP\textsubscript{wt} overexpression in N2a cells indeed increased APPL1 recruitment to rab5-positive endosomes (Supplementary Fig. 2a). To identify the interaction between APP and APPL1, we carried out a series of co-immunoprecipitation (co-IP) analyses in transfected HEK293 cells. In lysates from cells overexpressing APP\textsubscript{wt} and APPL1\textsubscript{wt}, an antibody (C1/6.1) against the extreme C-terminal end of APP pulled down APPL1 along with full-length APP (flAPP), $\beta$CTF, and $\alpha$CTF, while a specific APPL1 antibody precipitated APPL1 only with $\beta$CTF and not flAPP and $\alpha$CTF (Fig. 2a). Similar analysis using cells overexpressing the APP\textsubscript{mv} mutant and APPL1 yielded no APPL1-$\beta$CTF co-IP (Fig. 2b), indicating that APPL1 selectively interacts with $\beta$CTF. Similarly, an overexpressed mutant APPL1 (APPL1\textsubscript{ΔPTB}) lacking the PTB domain did not co-IP with $\beta$CTF using either antibody (Fig. 2c), suggesting that the PTB domain of APPL1 is required for $\beta$CTF binding.

Because some proteins that contain the PTB domain have been shown to interact with the YENPTY motif of APP\textsuperscript{28, 31}, we tested a mutant APP (APP\textsubscript{AENATA}) that contains AENATA instead of YENPTY\textsuperscript{32} to see if this domain is important for the interaction between APPL1
and βCTF. Because C1/6.1 does not recognize this construct due to the mutation, we used 6E10 antibody against amino acids 1–17 of human Aβ, which recognizes APP\textsubscript{AENATA}. In cells overexpressing both mutant APP and APPL1, the APP\textsubscript{AENATA} mutant did not co-IP APPL1 using 6E10, and APPL1 antibody no longer co-immunoprecipitated βCTF (Fig. 2d), suggesting that the YENPTY motif of βCTF mediated its binding to APPL1. FE65, an adaptor protein containing the PTB domain and known to interact with APP via the YENPTY motif\textsuperscript{33}, was also unable to bind to APP\textsubscript{AENATA} (Fig. 2d–e), confirming that the mutation disrupts the interaction with the PTB domain.

APP-BP1, a NEDD8-activating enzyme E1 regulatory subunit, is known to interact with the carboxy-terminal of APP and also with rab5 to promote endosomal enlargement\textsuperscript{10, 34}. It is possible, therefore, that APP-BP1 could mediate the interaction between APPL1 and βCTF. Using APPL1 and 6E10 antibodies, we found that, while APP-BP1 was able to bind to APP, it did not co-IP with APPL1 in lysates of cells overexpressing both proteins (Fig. 2e), suggesting that APP-BP1 does not mediate APPL1-βCTF interaction.

\(\text{flAPP}\) and αCTF are mainly localized on the plasma membrane whereas βCTF is mainly generated on endosomes\textsuperscript{35}. Blocking endocytosis should therefore diminish the association of βCTF with an endosomal adaptor protein, such as APPL1. To test this idea, we treated N2a cells overexpressing APP\textsubscript{wt}/APPL1\textsubscript{wt} with MiTMAB\textsuperscript{™}, a cell-permeable inhibitor for dynamin I and II to block endocytosis, or Pro-Myristic Acid, an inactive form of MiTMAB\textsuperscript{™}, as a negative control and performed co-IP using C1/6.1 or APPL1 antibody (Fig. 2f). MiTMAB\textsuperscript{™} reduced βCTF levels (Fig. 2f) as shown previously\textsuperscript{36} and increased αCTFs (Fig. 2f) as expected given that this cleavage is considered to take place at the plasma membrane\textsuperscript{36, 37}. MiTMAB\textsuperscript{™} significantly decreased the interaction of APPL1 with βCTF (Fig. 2f and supplementary Fig. 2b), while FE65, which is known to interact with APP in the plasma membrane\textsuperscript{38}, was still able to bind to APP (Fig. 2f). Taken together, these analyses show that APPL1 specifically binds to internalized βCTF through the interaction between PTB and YENPTY domains.

**APPL1 knockdown reverses βCTF-induced rab5 activation and endosomal enlargement**

APPL1 siRNA treatment, which significantly lowered APPL1 levels (Supplementary Fig. 2c–d), reversed GTP-rab5 levels (Fig. 3a), endosomal size (Fig. 3b), and FRAP rate (Fig. 3c) induced by APP\textsubscript{wt} overexpression, suggesting that APPL1 mediates APP-induced rab5 activation. Notably, APPL1 knockdown did not alter endosome size or rab5 activation in the absence of APP\textsubscript{wt} overexpression, implying that APPL1 plays no major role in normal constitutive rab5 function (Fig. 3b–c), which is consistent with previous findings\textsuperscript{29, 39}. Furthermore, APPL1 knockdown by using siRNA did not alter Aβ formation (Supplementary Fig. 3a–b). Combined overexpression of APP\textsubscript{wt} and APPL1\textsubscript{wt} in N2a cells also induced endosomal enlargement and decreased FRAP rate compared with non-transfected control N2a cells (Fig. 3d–e). However, co-expression of APP\textsubscript{mv} with APPL1\textsubscript{wt} or APP\textsubscript{wt} with APPL1\textsubscript{ΔPTB} had no effect on these endosomal functions (Fig. 3d–e). These data indicate that βCTF-mediated rab5 activation is dependent on APPL1. Although APPL1 shares high sequence homology with its isoform APPL2, proteins have been identified that selectively bind to APPL1 but not to APPL2\textsuperscript{40, 41}. This differential binding is possible...
because APPL1 has additional regions that are absent in APPL2. Substantial reduction of APPL2 levels had no effect on endosomal enlargement in N2a cells overexpressing APPwt (Supplementary Fig. 4), suggesting that βCTF-induced endosomal changes are selectively mediated by APPL1.

**APPL1 mediates βCTF-induced impairment of endosomal transport**

Because neurotrophic endosome signaling is dependent on microtubule-based retrograde axonal transport and is impaired in some neurodegenerative diseases, including mouse models of DS, we analyzed how axonal transport of endosomes is affected by overexpression of APPwt and/or rab5 in cultured mouse cortical neurons. To monitor endosome behavior directly, we transfected neurons with GFP-rhoB, a small GTPase located on both early and late endosomes. Expression of rab5 or APPwt significantly increased the average size of GFP-rhoB endosomes in an AD-like pattern (Fig. 4a, Supplementary Table 1). Dynamic behaviors of GFP-rab5 endosomes captured from time-lapse images and displayed on kymographs (Supplementary Fig. 5) revealed a pattern of predominantly short-range bidirectional movements contrasting with the long-range retrograde movements of rab7-positive late endosomes. Unlike rab5 endosomes, GFP-rhoB endosomes remained fluorescent after acquiring rab7 and displayed both transport patterns as expected (Supplementary Fig. 5). Overexpression of either rab5 or APPwt markedly slowed the average velocity of GFP-rhoB endosomes (Fig. 4b and Supplementary Table 1) but did not induce the extended pausing (stationary behavior during a 250 second observation) compared to neurons not overexpressing rab5 or APPwt (Fig 4c and Supplementary Table 1). By comparison, combined overexpression of APPwt and GFP-rab5 induced much greater endosome enlargement and endosome slowing and also increased proportions of endosomes that remained paused for longer than 250 seconds (stationary behavior) (Fig. 4d–f). These effects approached the magnitude induced by expressing the constitutively active Q79L rab5 mutant (Supplementary Table 1). Further supporting the specificity of these APP effects, overexpression of transferrin receptor (TFR), a single pass membrane protein that undergoes endocytosis, exerted no effects on endosome size or transport (Supplementary Table 1). Notably, overexpression of the β-cleavage incompetent APPmv mutant in neurons altered neither endosome size nor transport whereas elevating βCTF by inhibiting γ-secretase with L685,458 induced endosomal enlargement and disrupted endosome transport (Fig. 4d–f, Supplementary Table 1), implicating βCTF as the critical factor driving these APP-related pathological effects. We can also exclude general transport failure as a basis for endosome transport slowing after APPwt overexpression because mitochondrial transport, monitored by DsRed-Mito, was unaltered (Supplementary Table 2).

APPL1 depletion using APPL1 siRNA reversed endosome enlargement and completely rescued endosome transport defects in neurons transfected with APPwt and GFP-rab5, whereas a control scrambled siRNA had no effect (Fig. 4g–i and Supplementary table 1), indicating that APPL1 mediated APP-induced endosomal trafficking defects. Co-transfection of APPwt and APPL1wt significantly increased endosomal sizes and impaired transport, but APPwt/APPL1ΔPTB did not (Fig. 4j–l and Supplementary table 1), suggesting that the PTB domain of APPL1 is required for APP-induced impairment of endosomal transport. Notably, we found that, under experimental conditions, stationary endosomes were
consistently larger (>0.5μm²) than moving endosomes which were, on average, smaller than 0.5μm² (Supplementary Fig. 6), consistent with considerable evidence that axonal transport rate is negatively influenced by increasing vesicle volume beyond a normal limit due to steric hindrance and cargo drag.\(^{43-45}\) Taken together, these data show that βCTF/APPL1-mediated rab5 activation impairs endosomal transport and can be considered a significant pathogenic factor in multiple neurodegenerative diseases, including AD and DS\(^ {11,46}\).

**APPL1 siRNA rescues endosomal pathobiology in DS fibroblasts**

We next investigated the pathogenic importance of APPL1 alterations in fibroblasts from individuals with DS, where elevated βCTF induces rab5 activation and diverse endosome anomalies\(^ 3,8,9\). APPL1 co-localized with rab5-positive endosomes to a significantly greater degree in DS fibroblasts than in control cells (Fig. 5a), consistent with APPwt overexpression in N2a cells (Supplementary Fig. 2a) and suggesting that APP overexpression in DS cells recruits more APPL1 to rab5 endosomes. In addition, APPL1 siRNA knockdown in DS fibroblasts reduced APPL1 levels by about 50% (Fig 5b and supplementary Fig. 7) and restored normal endosomal size (Fig. 5b) and reversed the abnormally high endocytic HRP uptake compared with control fibroblasts (Fig. 5c and supplementary Fig. 8). We also found that APPL1 siRNA had no effect on endosomal size and HRP uptake in control cells (Fig. 5b–c and supplementary Fig. 7). By contrast, siRNA knockdown of APPL2 did not reverse endosomal enlargement in DS cells (Supplementary Fig. 9). This suggests that, as we observed in N2a cells (Supplementary Fig. 4), βCTF-induced rab5 activation in DS fibroblasts is mediated specifically by APPL1.

To establish an impact of APPL1-mediated rab5 activation on downstream endosomal signaling, we investigated a key aspect of NF-κB signaling, the nuclear translocation of the transcriptional activator p65/RelA, which was recently shown to require rab5-dependent endosomal recruitment of APPL1\(^ {16}\). NF-κB pathway activation is reported to occur in various neurological diseases, including AD and DS\(^ {21,47}\). Immunoblot analysis revealed that p65/RelA levels were elevated 75% in the nuclear fraction of DS fibroblasts (Fig. 5d), as seen previously\(^ {21}\). Knockdown of APPL1 using siRNA, however, significantly reversed this elevation. Collectively, these data establish that APPL1 is essential for APP/βCTF-induced rab5 activation and pathological endosome-related NF-κB signaling in DS fibroblasts.

**βCTF and endosomal APPL1 levels are elevated in Alzheimer brains**

We further investigated the relevance of APPL1 to neuronal endosome anomalies in late-onset AD\(^ 8\). APP levels have been found to be normal in human AD brain\(^ {48}\), but β-secretase activity is reported to be increased\(^ {49}\), suggesting a higher rate of βCTF generation, although comparative βCTF levels in AD and matched controls have not been reported. We observed that βCTF levels, assayed as a ratio with flAPP, were significantly elevated in the AD cerebral cortex (Fig. 5e), while levels of flAPP and αCTF were not significantly altered (Fig. 5e), which results were also observed in brains of both Ts2 DS model mice and older adult DS individuals (unpublished data). In addition, the membrane-association of APPL1 was significantly increased in AD brains (Fig. 5f) and in older adult DS brains (unpublished data). No influences of postmortem variables and demographics (eg. age and gender) were detected (Supplementary Table 3). Furthermore, a quantitative double-immunofluorescence
labeling analysis of AD and control neocortex with rab5 and APPL1 antibodies revealed a significantly higher co-localization of APPL1 with rab5-endosomes in neurons of cortical layers III and V (Fig. 5g). Notably, APPL1 colocalization with rab5 endosomes was increased with greater endosome size in AD brains. APPL1 recruitment to rab5 endosomes was significantly higher in AD brains and was most abnormal (greater than 2-fold higher than control) on the abnormally large rab5 endosomes (>0.5μm²) (Fig. 5h). Thus, as in DS, elevated βCTF levels in Alzheimer brain are linked to the abnormal endosomal recruitment of APPL1, rab5 upregulation, and characteristic endosome anomalies of early AD.

Discussion

Our studies define a novel βCTF-dependent pathogenic pathway that accounts for the signature endosome pathology in AD appearing at early stages of the disease and accord with growing evidence implicating the genes regulating endocytosis as frequent negative risk factors for AD. βCTF has neurotoxic properties not dependent on cleavage to Aβ. βCTF overexpression in transgenic mice and CTF elevations in a mouse model of Danish Dementia induce age-dependent neurodegeneration and cognitive impairment. BACE1 overexpression in mice, which raises brain βCTF levels and lowers Aβ, has similar effects. Importantly, our finding that βCTF levels are elevated in sporadic AD brain reveals how a βCTF-driven mechanism may apply to late-onset AD. In addition, rab5-endosome dysfunction can be potentially promoted further by multiple AD-related factors, including increased BACE1 expression and activity, inheritance of the ApoE E4 allele, lowered Vps35, and altered handling of cholesterol, which increase BACE1 activity and βCTF levels.

Endosome anomalies are among the earliest disease specific neuronal responses in AD and DS. In addition to βCTF of APP, other genetic factors have been reported to induce endosomal dysfunction in AD and DS, including synaptojanin1 and Vps34, a class III phosphoinositide 3-kinase and possible their relationships to rab5-dependent or -independent mechanisms deserve further investigation. Rab5-mediated acceleration of endocytosis and endosome fusion causes endosomes to enlarge and selectively disrupts their transport in neurons, possibly mediated by steric hindrance or rab5-mediated activation of Vps34. Substantial transport slowing of enlarged vesicles is seen under various experimental conditions and the tipping point for axonal transport slowing of organelles occurs when vesicular expansion reaches sizes above 0.5μm² (unpublished data). In addition, Vps34 activity, which is modulated by rab5, has been implicated in endosomal transport and endosome-related disease pathogenesis. Although the exact mechanism of trafficking failure of enlarged endosomes needs further investigation, rab5 overactivation promoted by a βCTF-APPL1 interaction provides a plausible basis for the APP-dependent failure of retrograde neurotrophin signaling by endosomes previously implicated in the neurodegeneration of cholinergic neurons in DS mouse model. Disrupted transport of endosomes and their focal accumulation in axons mainly with autophagic vacuoles, which likely reflects attempted endosome clearance by autophagy, leads to neuritic dystrophy resembling that observed at early stages of AD. Furthermore, accelerated endocytosis causes endocytic cargos to accumulate within enlarged late endosomes and impairs lysosome functions in a βCTF-dependent manner (unpublished data). Therefore, the
pathological consequences of βCTF-induced pathological rab5 activation may be diverse and include, as shown here, selective impairment of endosome transport in neurons - a possible basis for impaired endosomal signaling linked to neurodegeneration

and alteration of NF-κB pathway signaling via endosomes - an effect known to promote neuronal apoptosis in some contexts.

We present multiple lines of evidence from neuronal APP models and DS patient fibroblasts and additional support from analyses of Alzheimer brains, indicating that APPL1 is the mediator of βCTF-induced rab5 activation, which underlies the very early-appearing endocytic dysfunction in AD. This mechanism is consistent with the reported ability of APPL1 to increase rab5 localization on enlarged endosomes. Our data suggest that APPL1 recruited by βCTF could stabilize rab5 in its active GTP state and slow its loss from endosomes. We have previously shown that levels of αCTF do not influence rab5 upregulation in DS fibroblasts and, consistent with this finding, we observed that APPL1 antibodies did not pull down appreciable αCTF even though it contains the same APPL1-interacting domain as βCTF. In this regard, αCTF is mainly generated at the cell surface and APPL1 is excluded from the plasma membrane. By contrast, βCTF is more abundantly generated on endosomes where a distinctive set of signaling ligands may contribute to its selective interaction with APPL1. In this regard, APP intracellular domain (AICD) generated from βCTF by γ-secretase on endosomes can be stabilized by binding to other proteins, such as JIP-1, and translocated to the nucleus, whereas AICD produced from cell surface αCTF is degraded into smaller fragments. To further support these earlier data, we showed that inhibition of endocytosis decreases APPL1-βCTF interaction and increases levels of αCTF, but not its interaction with APPL1 (Fig. 2f), consistent with its relative inaccessibility to endosomal proteins, like APPL1. Finally, we show here that αCTFs levels do not influence endosomal pathology in cells (Fig. 1c), as previously seen in DS fibroblasts. Thus, we suggest that selective APPL1 with βCTF may partly reflect to the differential compartmentalization of full-length, α-cleaved, and β-cleaved APP although it is possible that unidentified proteins interacting with βCTF and APPL1 on endosomes may enhance specificity of this interaction.

Notably, APPL1 on endosomes is normally replaced by phosphatidylinositol 3-triphosphate (PI3P) binding proteins, such as EEA1 (early endosome antigen 1). The reduced PI3P levels seen in AD brain could contribute to APPL1 elevation on endosomes. APPL1 overexpression mimicked pathological effects of βCTF on endosome morphology and function, while APPL1 knockdown prevented these effects and reversed them in DS cells. Furthermore, APPL1 positively regulates TNFα-independent NF-κB activation via APPL1/rab5-endosomes. We used this phenomenon as additional functional evidence of increased APPL1-dependent activity on endosomes in DS beyond its effect on stabilizing active rab5 on endosomes. Increased NF-κB activation in several major neurological diseases, including AD, is implicated in neurodegenerative mechanisms, although its range of effects on neuronal vulnerability are complex. Importantly, we observed that APPL1 knockdown minimally affected endocytosis by cells that express endogenous APP at normal levels, implying that βCTF/APPL1-dependent rab5 activation is not essential for normal constitutive endocytosis but is instead a superimposed pathological phenomenon. Moreover, APPL1 overexpression did not affect βCTF levels (Supplementary Fig. 10), suggesting that...
βCTF, not APPL1, is the more upstream initiator of endosomal pathology in both AD and DS. These observations raise the possibility that the pathogenic endocytosis mediated by elevated βCTF-APPL1 could be modulated therapeutically at multiple possible targets without altering vital functions of basal endocytosis.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Figure 1. βCTF activates rab5 on endosomes and increases endosome size**

(a) Rab5 activation, reflected by the rate of **Fluorescence Recovery After Photobleaching** (FRAP), is measured as the rate at which photobleached GTP-bound-rab5 (activated rab5) on individual endosomes is replaced by fluorescent cytosolic GDP-rab5 (inactive rab5). The rate of FRAP for endosomal GFP-rab5 is significantly decreased when βCTF levels are increased by overexpressing wild-type APP (APPwt) in N2a cells (N2a APPwt) or blocking βCTF cleavage with 10μM γ-secretase inhibitor L685,458 (N2a+L685), as compared to control cells expressing βCTF at endogenous levels (N2a) or transfected with an APP mutant construct unable to be cleaved to βCTF (N2a APPmv). The FRAP rate after transfecting a dominant active mutant GFP-rab5 Q79L as a positive control is extremely reduced, indicating persistent rab5 activation. The summary graph reflects by the percent of average fluorescence recovery at 270 s after photobleaching in each condition (n=20 endosomes, one endosome per cell, total 20 cells, mean ± S.E.M., one-way ANOVA, Tukey’s test, ***p<0.001).

(b) βCTF activates rab5 in HEK293 cells. APPwt overexpression increases levels of active rab5 (GTP-bound rab5) detected with a GTP-rab5-specific antibody (1st lane) while APPmv mutant has no effect (2nd lane), as compared with untransfected control (ctrl) cells (3rd lane). A bar graph presents mean immunoreactive GTP-rab5 signal ± S.E.M. for 3 separate immunoblot experiments (representative blot shown) (**p<0.01, one-way ANOVA, Tukey’s test). 10% of cell lysate is used as total rab5. (c) βCTF induces endosomal enlargement in N2a cells. Cross-sectional area of rab5-positive endosomes is increased by APPwt overexpression or 10μM L685,458 (lanes 2 and 3), which raise αCTF and βCTF levels compared to those in control (ctrl) cells (lane 1). APPmv mutant expression elevates αCTF levels but not βCTF levels (lane 4) and does not enlarge endosomes. Each graph bar indicates number of measured endosomes from 20 cells (*p<0.05 and **p<0.01, respectively, mean ± S.E.M., one-way ANOVA, Tukey’s test).
Figure 2. βCTF generated on endosomes binds via its YENPTY domain to the PTB domain of APPL1

(a) βCTF interacts with APPL1: immunoblot analysis shows co-immunoprecipitation (co-IP) of βCTF and APPL1 from cell lysates of HEK293 cells overexpressing APPwt and APPL1 using antibodies against APPL1 or against APP (APP C1/6.1 antibody is raised against amino acid residues 676–695 of human APP695 and recognizes full-length APP (flAPP), αCTF, and βCTF). (b) APPL1 interaction with APP requires β-secretase cleavage: APPL1 does not co-IP with a β-secretase-cleavage incompetent mutant APP (APPmv) from lysates of HEK293 cells overexpressing both proteins. (c) βCTF-APPL1 interaction requires the PTB domain of APPL1: βCTF does not co-IP with a mutant APPL1 (APPL1ΔPTB) lacking the PTB domain when this mutant and APPwt are overexpressed in HEK293 cells. (d) APPL1 interacts via the YENPTY domain of βCTF: APPL1 does not co-IP with an APP mutant containing a YENPTY domain variant (APP\textsubscript{AENATA}) from lysates of HEK293 cells overexpressing both proteins. (e) FE65 and APP-BP1 are immunoprecipitated with APP using 6E10 antibody, raised against the amino acid 1–17 of human Aβ, detects human flAPP, βCTF, and Aβ, but not with APPL1 from lysates of HEK293 cells overexpressing APPwt and APPL1. (f) APPL1 selectively interacts with endocytosed βCTF: In N2a cells overexpressing APPwt and APPL1, APPL1 does not co-IP with βCTF when βCTF generation on endosomes is prevented by blocking endocytosis with MiTMAB\textsuperscript{TM} (M), a dynamin inhibitor. Treatment with Pro-myristic acid (P), an inactive form of MiTMAB\textsuperscript{TM}, a negative control for endocytic blockade, does not prevent APPL1 wt co-IP with βCTF. In all co-IP experiments, 10% of total cell lysate is used as an input standard. (+) and (−) indicates the presence and absence of the antibody used in IP, respectively.
Figure 3. APPL1 mediates βCTF-induced endosomal enlargement and Rab5 activation

(a) APPL1 siRNA knockdown blocks APPwt-mediated Rab5 activation: APPwt overexpression in HEK293 cells significantly elevates the ratio of activated (GTP-bound) Rab5 to total Rab5 relative to the ratio in cells lacking APPwt overexpression (lane 2 vs lane 1) (**p<0.01, one-way ANOVA, Tukey’s test). siRNA knockdown of APPL1 prevents Rab5 activation in the presence of APPwt over-expression (4th lane) but has no effect on Rab5 activation when APP is expressed at endogenous levels (3rd lane). The bar graph depicts mean ± S.E.M. for four separate experiments (one representative immunoblot shown).

(b) APPL1 mediates APPwt-induced Rab5 enlargement. Increases in average size (cross-sectional area) of GFP-Rab5 endosomes induced by APPwt overexpression in N2a cells are reversed with APPL1 siRNA treatment but not with scrambled control siRNA (ctrl) (***p<0.001, one-way ANOVA, Tukey’s test). APPL1 siRNA does not affect endosomal size in cells expressing APP at endogenous levels. Each graph bar indicates number of measured endosomes from 20 cells and shows values as mean ± S.E.M. 10% of total cell lysate is used as a standard.

(c) APPL1 mediates APPwt-induced Rab5 activation on endosomes: APPL1 siRNA, but not control siRNA, blocks the slowed FRAP of GFP-Rab5 in N2a cells overexpressing APPwt but does not alter endosomal Rab5 activation (FRAP) in cells expressing APP at normal endogenous levels endosomes. The summary graph reflects by the percent of average fluorescence recovery at 270 s after photobleaching in each condition (n=20 endosomes, one endosome per cell, total 20 cells, mean ± S.E.M., one-way ANOVA, Tukey’s test, *p<0.05 and *p<0.01).

(d) The PTB domain of APPL1 is required for βCTF-induced Rab5 enlargement. The increased GFP-Rab5 endosome size induced by APPwt and APPL1wt overexpression in N2a cells is prevented when either the APPmv mutant or the APPL1ΔPTB (PTB domain deleted APPL1) mutant is substituted for APPL1wt as the corresponding overexpressed construct. Each graph bar indicates number of measured endosomes from 20 cells (*p<0.05, mean ± S.E.M., one-way ANOVA, Tukey’s test).

(e) The PTB domain of APPL1 is required for βCTF-induced GFP-Rab5 activation on endosomes: FRAP in N2a cells. Overexpression of APPwt and APPL1wt in N2a cells reduces the FRAP on endosomes, indicating Rab5 activation, while APPmv mutant or APPL1ΔPTB overexpression do not alter the FRAP relative to that in control cells. The summary graph reflects the percent of average fluorescence recovery at 270 s after photobleaching in each condition (n=20 endosomes, one endosome per cell, total 20 cells, mean ± S.E.M., one-way ANOVA, Tukey’s test, ***p<0.001).
Figure 4. APPL1 mediates βCTF-induced enlargement and impaired transport of endosomes in neurons
(a) Overexpression of APPwt or rab5 increases average cross-sectional areas of individual rhoB-positive endosomes in primary cultures of mouse cortical neurons (one-way ANOVA, Tukey’s test, *p<0.05 and **p<0.01, respectively). (b) Rab5 or APPwt overexpression reduces average transport velocities of rhoB-positive endosomes in cultured neurons (one-way ANOVA, Tukey’s test, ***p<0.001). (c) Rab5 or APPwt overexpression has no effect on transport interruption of rhoB-positive endosomes in cultured neurons. (d–f) Raising βCTF levels by APPwt overexpression or exposure to the γ-secretase inhibitor L685,458 (L685) in cultured neurons increases average cross-sectional area of rab5-positive endosomes (d), decreases average speeds of rab5-positive endosomes (e), and increases numbers of stationary rab5 endosomes (f) (one-way ANOVA, Tukey’s test, **p<0.01 and ***p<0.001). Overexpressing the β-secretase cleavage-incompetent APPmv mutant does not alter these parameters (d–f). (g–i) Treatment with APPL1 siRNA but not a control scrambled siRNA prevents from the increased cross-sectional area of rab5 endosomes (g), the reduced rab5 endosome transport velocity (h), and the transport interruption of rab5 endosomes (i.e. increased stationary endosome number) (i) that are induced in primary cultures of mouse cortical neurons by APPwt overexpression (unpaired two-tailed t-test, **p<0.01 and
***p<0.001. (j-l) The PTB domain of APPL1 is required for βCTF-induced endosome alterations in primary cortical neurons. Increased endosomal cross-sectional area (j), lowered transport rate of endosomes (k), and transport interruption of rab5 endosomes (elevated stationary endosomes) (l) are induced by overexpressing APPwt and APPL1wt (one-way ANOVA, Tukey’s test, **p<0.01 and ***p<0.001), but are not induced by overexpression of either APPL1wt with APPmv or APPwt with APPL1ΔPTB (PTB domain deleted APPL1 mutant). Results are presented as mean ± S.E.M.
Figure 5. APPL1 and βCTF-dependent endosomal abnormalities in DS fibroblasts and AD brain
(a) Greater APPL1 colocalization with rab5 in DS fibroblasts compared with control (ctrl) fibroblasts is seen by immunocytochemistry as shown in representative images of cells double-immunolabeled with antibodies to APPL1 (green) and rab5 (red). The graph shows a higher APPL1 and rab5 colocalization coefficient (R) in DS fibroblasts, as calculated by Pearson’s correlation coefficient in 30 DS and 30 control cells (mean ± S.E.M., unpaired two-tailed t-test *p<0.05). (b) APPL1 mediates endosomal enlargement in DS cells. Treatment with APPL1 siRNA, but not scrambled control siRNA, blocks the increase in cross-sectional area of endosomes in DS fibroblasts but has no effect on normal endosomes in age-matched 2N control (ctrl) fibroblasts (n=30 cells, one-way ANOVA, Tukey’s test, mean ± S.E.M., *p<0.05 and ***p<0.001). A representative blot with a GAPDH loading control and graphic quantitation from three separate experiments are shown. (c) APPL1 mediates the abnormally elevated endocytosis in DS fibroblasts. Fluorescent HRP-positive puncta are higher in DS fibroblasts at 30 min after addition of HRP to the medium compared with control cells and are reduced in DS cells treated with APPL1 siRNA compared to cells treated with scrambled (ctrl) siRNA while APPL1 siRNA has no effect on HRP uptake in control fibroblasts. Quantitative analysis of fluorescence at 0, 5, 15, and 30 min after addition of HRP to the medium shows significantly reduced HRP uptake after APPL1 siRNA compared to ctrl-ctrl siRNA; 15, 15, 20, and 19 cells, Ctrl-siRNA; 16, 28, 17, and 25 cells, DS-ctrl siRNA; n=26, 10, 18, and 36 cells, DS-APPL1 siRNA; n=12, 16, 39, and 42 cells in 0, 5, 15 and 30 min from two experiments, mean ± S.E.M., one-way ANOVA, Tukey’s test, mean ± S.E.M., ***p<0.001). (d) APPL1 mediates abnormally increased NF-κB pathway activation in DS fibroblasts. Functional evidence for APPL1-mediated rab5 activation on endosomes is reflected in greater APPL1/rab5-endosomal-mediated nuclear localization of the NF-κB p65 subunit (p65/RelA). Levels of nuclear p65/RelA normalized to nuclear histone H3 as a loading control (3rd lane) are elevated in DS fibroblasts compared to those in control (ctrl) cells (1st lane). This elevation is reversed by APPL1 siRNA (4th lane) but not scrambled (ctrl) siRNA as shown on an immunoblot representative of three experiments quantified in the graph (mean ± S.E.M.)
S.E.M., two-way ANOVA with interaction, *p<0.05). (e) βCTF levels, but not flAPP or αCTF levels, are significantly elevated in AD brain compared with age-matched control (ctrl) brain, as shown on an immunoblot representative of three experiments quantified in the graph. C1/6.1 antibody was used to measure βCTF and αCTF, which are normalized to the level of APP (n=13 control and 13 AD brains, mean ± S.E.M., unpaired two-tailed t-test *p<0.05). Identities of βCTF (C1–C4) and αCTF (C5) were confirmed using 6E10 (data not shown). Actin is used as a loading control. (f) Membrane-associated APPL1 levels are increased in AD brains compared with control (ctrl) brains as shown on an immunoblot representative of three experiments quantified in the graph as a ratio of membrane-associated APPL1 in a 50μg pellet (P) of brain homogenate to the APPL1 level in the 50μg supernatant (S) (n=13 control and 13 AD brains, mean ± S.E.M., unpaired two-tailed t-test *p<0.05). (g) APPL1 association with rab5-positive endosomes is increased in AD brain. Double immunofluorescence labeling confirms rab5-positive endosome enlargement in neocortical pyramidal neurons of AD brain and demonstrates greater APPL1 co-localization (arrowheads) in these enlarged endosomes as compared to neuropathologically normal control brains. The graph shows a higher APPL1 and rab5 colocalization coefficient (R) in AD brains, as calculated by Pearson’s correlation coefficient (n=90 cells, mean ± S.E.M., unpaired two-tailed t-test *p<0.05). (h) Recruitment of APPL1 to rab5-endosomes is higher in neuronal endosomes of AD brain than in control (ctrl) brain, as reflected by an increased percent of rab5-endosome surface area occupied by APPL1 immunoreactive signal quantified from images similar to those in panel g. Comparison of rab5 endosomes of different size ranges demonstrates increased APPL1 colocalization on endosomes of greater size in AD brains (n=90 cells; 3824, 408, and 224 endosomes for control, 3194, 269, and 156 endosomes for AD in each size bin, mean ± S.E.M., unpaired two-tailed t-test, ***p<0.001).