Dynamin 1Regulates Amyloid Generation through Modulation of BACE-1

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Abstract

**Background:** Several lines of investigation support the notion that endocytosis is crucial for Alzheimer’s disease (AD) pathogenesis. Substantial evidence have already been reported regarding the mechanisms underlying amyloid precursor protein (APP) traffic, but the regulation of beta-site APP-Cleaving Enzyme 1 (BACE-1) distribution among endosomes, TGN and plasma membrane remains unclear. Dynamin, an important adaptor protein that controls sorting of many molecules, has recently been associated with AD but its functions remain controversial. Here we studied possible roles for dynamin 1 (dyn1) in Aβ biogenesis.

**Principal Findings:** We found that genetic perturbation of dyn1 reduces both secreted and intracellular Aβ levels in cell culture. There is a dramatic reduction in BACE-1 cleavage products of APP (sAPPβ and βCTF). Moreover, dyn1 knockdown (KD) leads to BACE-1 redistribution from the Golgi-TGN/endosome to the cell surface. There is an increase in the amount of surface holoAPP upon dyn1 KD, with resultant elevation of α-secretase cleavage products sAPPαs and sCTF. But no changes are seen in the amount of nicastrin (NCT) or PS1 N-terminal fragment (NTF) at cell surface with dyn1 KD. Furthermore, treatment with a selective dynamin inhibitor Dynasore leads to similar reduction in βCTF and Aβ levels, comparable to changes with BACE inhibitor treatment. But combined inhibition of BACE-1 and dyn1 does not lead to further reduction in Aβ, suggesting that the Aβ-lowering effects of dynamin inhibition are mainly mediated through regulation of BACE-1 internalization. Aβ levels in dyn1−/− primary neurons, as well as in 3-month old dyn1 haploinsufficient animals with AD transgenic background are consistently reduced when compared to their wildtype counterparts.

**Conclusions:** In summary, these data suggest a previously unknown mechanism by which dyn1 affects amyloid generation through regulation of BACE-1 subcellular localization and therefore its enzymatic activities.

Introduction

Late-onset Alzheimer’s disease (LOAD) typically manifests after the sixth decade, accounting for over 95% of all AD cases. Genetic studies of LOAD point to a number of risk factor genes, such as apolipoprotein E epsilon4 (ApoEε4) allele [1], and several endocytic proteins. For example, single nucleotide polymorphism (SNP) studies from two research groups studying Japanese and Belgian populations have independently identified an association of dynamin binding protein gene (DNMBP) on chromosome 10 to LOAD, particularly in individuals lacking the APOE ε4 allele [2,3]. Furthermore, a significant association of LOAD with the dynamin 2 (DNM2) gene was detected by SNP analysis, especially in non-carriers of the ApoEε4 allele [4,5].

Dynamin is a GTPase that plays a critical role in endocytic vesicle fission [6]. It is encoded by three different genes (DNM1, DNM2, and DNM3) in mammals [7]. Dynamin 1 (dyn1) is highly and selectively expressed in the nervous system and represents the major dynamin isoform expressed in this tissue [8]. Dyn1 has been linked to the biology of AD. For example, dominant-negative dyn1 (K44A mutant), which blocks endocytosis, reduces Aβ levels in interstitial spinal fluid (ISF) and prevents activity-dependent increases in Aβ [9]. Dyn1 K44A mutant also reduces oligomer Aβ1-42-induced neuronal death [10] and increases APP ectodomain shedding [11]. Others showed an increase in BACE-1 cleavage of APP and Aβ generation at the cell surface in dyn1 K44A expressing HeLa cells [12]. Taken together, a role for dyn1 in AD is implicated but precise molecular mechanism(s) remains elusive.

Herein, we report that using gene silencing techniques to knockdown dyn1 levels reduces both secreted and intracellular Aβ levels in cell culture. There is a dramatic reduction in beta-site APP-Cleaving Enzyme 1 (BACE-1) cleavage products of APP.

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(sAPPβ and βCTF). Moreover, dyn1 knockdown (KD) leads to BACE-1 redistribution from the Golgi-TGN/endosome to the cell surface. There is an increase in the amount of surface holoAPP upon dyn1 KD, with resultant elevation of α-secretase cleavage products sAPPβ2 and βCTF. But no changes are seen in the amount of nicastrin (NCT) or PS1 N-terminal fragment (NTF) at cell surface with dyn1 KD. Furthermore, treatment with a selective dynamin inhibitor Dynasore leads to similar reduction in βCTF and Aβ levels, comparable to changes with BACE inhibitor treatment. But combined inhibition of BACE-1 and dyn1 does not lead to further reduction in Aβ, suggesting that the Aβ-lowering effects of dynamin inhibition are mainly mediated through regulation of BACE internalization. Aβ Levels in dyn1−/− primary neurons, as well as in 3-month old dyn1 haptopoinsufficient animals with AD transgenic background are consistently reduced when compared to their wildtype counterparts.

In summary, these data suggest a previously unknown modulatory mechanism by which dyn1 affects amyloid generation through regulation of BACE-1 subcellular localization and therefore its enzymatic activities. Together, our findings provide mechanistic evidence that inhibition of dyn1 functions may prevent certain pathologic changes associated with AD.

Materials and Methods

Ethics Statements

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal studies were approved by the Institutional Animal Care and Use Committee of James J Peters VA Medical Center ( Permit Number: CAI-10-044a). All efforts were made to minimize suffering.

Antibodies

4G8, 6E10 and sAPPβ (Covance), anti-APP N-terminus MAB348 (clone 22C11, Millipore) and anti-BACE C-terminus MAB309 (clone 61, Millipore), anti-dynamin clone 41, anti-EA1, anti-γ-adaptin and anti-nicastrin (NCT) (BD Transduction), anti-β actin (Santa Cruz), anti-Pen-2 antibody NE1008 (Calbiochem), anti-mouse and rabbit HRP conjugates (Vector Laboratories), Texas-Red conjugated anti-mouse IgG (Vector Laboratories Inc.) were purchased. pAb369 (C-terminal APP antibody) [13], RU690 (C-terminal BACE1 antibody) [14], RUWN-b1 (C-terminal sAPPβ antibody), RU717 (N-terminal nicastrin antibody) [15] and Ab14 (N-terminal PSI antibody) [16] generously provided by Dr. Greengard at The Rockefeller University, were also used for APP/CTFs, BACE1, sAPPβ, NCT and PSI detection. Synthetic sAPPβ peptide (Covance) and BACE-1 lysates from 293 cells (Santa Cruz Biotechnology) were used as positive controls.

Cell Lines

N2a cells stably transfected with cDNAs encoding human APP695 provided by Dr. Greengard at The Rockefeller University [17] were transfected with dyn1siRNA and maintained for 4–5 days to achieve about 50–80% knockdown of dyn1 protein levels. Fibroblast cell lines from dynamin 1, dynamin 2 conditional knockout mice expressing tamoxifen-inducible Cre recombinase (dyn KD) provided by Dr. Pietro De Camilli at Yale School of Medicine [18] lose dynamin expression upon treatment with 1μM 4-hydroxy-tamoxifen. Wild type fibroblast cells were treated by tamoxifen as a control. Alternatively, cells were treated with a dyn inhibitor Dynasore [19] at 10 μM (Sigma), or a BACE inhibitor at 15 μM (Inhibitor IV, EMD) overnight before subjected for further analysis.

Neuronal Cultures

Postnatal day 0–1 cortical neurons from dyn1+/+ or dyn1−/− mouse pups [8] were cultured on poly-D-lysine (Sigma) pre-coated dishes for 14–21 days.

siRNA Oligonucleotides

The siRNA duplexes specifically targeting dyn1 (IDT Inc.) were synthesized. Sequences are as follows: forward 5′-rGrGCr rUrUrA rCrArU rGrArA rUrArC rGrArC rGrGA A-3′; reverse, 5′-rUrUrC rGrUrG rGrUrU rGrUrG rArUrU rCrArU rGrUrA rArGrCrGrArG-3′. Stealth siRNA Control GC Duplex was used as a negative control (IDT Inc.). Sequences are as follows: forward 5′-rCrGrU rUrArA rUrCrG rCrGrU rArUrA rCrGrC rGrUA T-3′; reverse, 5′-rArUrA rCrGrC rGrUrA rUrArA rArGrG rArGrC rGrArC-3′.

Cell Transfection

For siRNA analysis, N2a 695 cells were seeded at 50% to 60% confluence and transfected with 200 pmol siRNA dyn1 versus control duplex using Lipofectamine RNAimax (Invitrogen). For transient transfection of cDNA constructs, dyn KD fibroblasts were seeded at 70% confluence and transfected with 0.5 μg of BACE1-EGFP cDNA provided by Dr. Bradley Hyman at Harvard Medical Center, [20] using Lipofectamine 2000 (Invitrogen).

Cell Lysate Analysis

After transfection, cells were harvested in lysis buffer [21]. Equal amounts of total protein were loaded for Western blot using 6E10 to detect holoAPP (full-length APP), Aβ40 and Aβ42 levels in media were determined by human ELISA kits (WAKO), according to the manufacturer’s instructions. Alternatively, mouse Aβ40 levels in media of Dyn KD fibroblasts after various treatments were determined by mouse ELISA kits (WAKO).

Immunoprecipitation

Media or lysates were diluted with immunoprecipitation buffer [22] and immunoprecipitated using 4G8 followed by immunoblotting with 6E10 for Aβ and βCTF detection. Media were immunoprecipitated using MAB348 (Millipore) followed by immunoblotted with sAPPβ antibody (for sAPPβ) or 6E10 antibody (for sAPPβ).

Biotinylation/Cell Surface Assay

Dyn KD fibroblasts were incubated at 4°C with 0.5 mg/ml sulfo N-hydroxysuccinimide biotin (Pierce) to label surface proteins as described previously [17]. Samples of both biotinylated and non-biotinylated BACE-1 were analyzed by Western blot using MAB309 or RU690. Purified lysates from BACE-1 expressing 293 cells were used as positive controls. Alternatively, samples of both biotinylated and non-biotinylated holoAPP were analyzed by Western blot using 6E10.

Immunofluorescence Confocal Microscopy

Dyn KD fibroblasts transfected with EGF-P-BACE1 were treated with or without tamoxifen for dyn1 knockdown, followed by fixation and permeabilization. Cells were then incubated with primary antibody against an endosomal marker EEA1 (1:1000) or a Golgi/TGN marker γ-adaptin (1:1000) following incubation with Texas Red conjugated anti-mouse IgG (1:1000). Immuno-
fluorescence staining was examined by confocal microscopy (LSM510, Zeiss).

Measurement of Newly Synthesized Aβ Production

Primary neurons derived from dyn1+/+ or dyn1−/− animals [8] were labeled with [35S] methionine (500 μCi/ml) for 5 hours at 37°C. Media was immunoprecipitated with 4G8 and then subjected to autoradiography to determine levels of Aβ1–40/42 and Aβ11–40/42 [23,24].

Generation of Dyn1 Haploinsufficient Mice with AD TRANSGENIC Mouse Background

Human Swedish βAPP and FAD-linked PS1 ΔE9 mutant transgenic mice from Jackson Laboratory [25,26] were mated with heterozygous dyn1 null mice (dyn1+/−) provided by Dr. Pietro De Camilli from Yale School of Medicine [8]. Double heterozygous F1s were then bred with heterozygous dyn1 null mice only to generate offsprings that express human Swedish APP and FAD-linked PS1 ΔE9 in the dyn1+/+ or dyn1−/− background. Genotypes were determined by PCR amplification as described previously.

Brain Lysate Preparation and Analysis

Mouse brains from APP/PS1+/+/dyn1−/+ or APP/PS1−−/ dyn1−/+ at 2–3 months of age, were rapidly dissected and snap frozen for further analysis. Each frozen hemi-brain was then processed via step-wise solubilization [21,27]. Lysates were analyzed by Western blot using 6E10 to determine levels of holoAPP and βCTF. Levels of Aβ40 were determined by human Aβ40 ELISA kits (WAKO), according to the manufacturer’s instructions. Results were normalized to wet brain weight and expressed as picomoles per gram.

Statistical Analysis

Densitometric analysis of western blot bands (integrated density) was performed using Multigauge v3.1 software (Fujifilm). Levels of holoAPP, βCTF, sAPPβ, Aβ, and BACE-1 were normalized to actin and expressed as percentage of control. Absolute Aβ40 concentrations were quantitatively determined by sandwich ELISA (Wako). Independent-samples t tests (parametric design) were used to determine significant mean differences between groups. Significance for t tests are reported with a p < 0.05 using two-tailed tests with an α level of 0.05. All statistical analysis was performed using SPSS v18.0.

Results

1. Dynamin 1 (Dyn1) Knockdown in N2a 695 Cells Reduces Aβ and βCTF Levels

To determine whether dyn1 modulates APP metabolism, we first analyzed the effects of dyn1 knockdown on levels of APP and its metabolites in N2a cells stably expressing human APP695 (N2a 695 cells). Using siRNA transfection methods, 50–80% reduction in dyn1 protein levels was achieved after 4–5 days. Both secreted and intracellular Aβ levels were significantly decreased by 64.5% and 77.8% respectively, compared to control siRNA transfection.
(Fig.1A, p<0.001). There was 62.5% reduction in \( \Delta \beta \), and 44.7% reduction in \( \Delta \alpha \), levels measured by sandwich ELISA (Fig.1B, p<0.001). There was a dramatic reduction in other APP metabolites sAPP\( \beta \) and \( \beta \)-CTF levels (57.4%, p=0.021 and 45.7%, p<0.001 respectively), without significant changes in holoAPP levels (119.16% of controls, p=0.101). In addition, the levels of BACE-1 as well as \( \gamma \)-secretase components such as PS1 NTF and Pen-2 were unchanged (Figure S1A).

To confirm the specificity of effects of dyn1 siRNA on APP metabolism, three different dynamin 1 siRNA duplex constructs were synthesized (IDT Inc.) and transfected into N2a 695 cells to determine APP processing. Secreted \( \beta \)-APP levels were similarly decreased by all three siRNA constructs compared to control siRNA transfection (Figure S1B). The specificity of \( \beta \)-APP detection was determined by comparing to transfection of APP siRNA. Moreover, to confirm the specificity of \( \beta \)-CTF detection, lysates from N2a 695 cells with dyn1 or control siRNA transfection were immunoprecipitated with 4G8 followed by immunoblotting with 6E10 (Figure S1B, left panels) to determine levels of \( \beta \)-CTF and \( \beta \)-APP. Alternatively, lysates were directly blotted with 6E10 to detect holoAPP, \( \beta \)-CTF and \( \beta \)-APP, followed by reprobing with 369 for detection of \( \alpha \)/\( \beta \)-CTF (Figure S1C, right panels).

Based on our findings, the consistent changes in levels of \( \beta \)-CTF and sAPP\( \beta \) support the notion that dyn1 knockdown inhibits APP cleavage by BACE-1 rather than accelerating degradation of \( \beta \)-CTF.

2. Dyn1 Down-regulation Changes BACE-1 Intracellular Distribution

Next, we evaluated the effects of dyn1 knockdown on BACE1 intracellular traffic using dyn1 knockdown (KD) fibroblasts derived from dyn conditional knockout mice expressing tamoxifen-inducible Cre recombinase [18]. These experimental conditions allowed us to study dynamic changes in BACE-1 with inducible dyn1 down-regulation. After tamoxifen treatment at 1\( \mu \)M for 5–7 days, dyn1 protein levels were reduced by 56.6% (Figure S2A, p<0.03) in the KD fibroblasts. Alternatively, wildtype fibroblast cells were cultured with tamoxifen as a control and there was no change in dyn1 protein levels with tamoxifen treatment (Figure S2B, right panel). Similar to the results using dyn1 siRNA in the N2a 695 cell line, reduced \( \beta \)-CTF levels (Figure S2A; 66.7% of reduction, p=0.008) were observed in dyn1 KD fibroblasts with tamoxifen treatment. Tamoxifen treatment in wildtype cells did not cause any changes in \( \alpha \)/\( \beta \)-CTF levels, ruling out an effect of tamoxifen itself (Figure S2B, left panel).

BACE-1 intracellular distribution in dyn1 KD cells was studied after transfection with EGFP-BACE1 construct [20]. As shown in Fig. 2, amounts of EGFP-BACE-1 at the cell surface were significantly increased with dyn1 knockdown. Under control conditions, BACE-1 (green fluorescence) was mainly localized in the endosomes and TGN/Golgi, determined by an endosomal marker EEA1 (red fluorescence, Fig. 2A), as well as a TGN marker \( \gamma \)-adaptin (red fluorescence, Figure 2B). However, after dyn1 knockdown, amounts of green fluorescence (EGFP-BACE1) were dramatically increased at cell surface with little remaining within the TGN/Golgi and endosomes. Transfected EGFP-BACE-1 expression levels were comparable between control cells and dyn1 KD cells (Figure S3A).

3. Dyn1 Knockdown Increases Cell Surface BACE-1 and holoAPP Levels

Using surface biotinylation methods, we next analyzed the intracellular and surface distribution of endogenous BACE-1 in dyn1 KD fibroblasts without over-expression of EGFP-BACE-1. As shown in Fig.3A (top panel), surface BACE-1 levels were elevated by 251.8% with dyn1 knockdown when compared to controls (p=0.047). There was a modest elevation in total BACE-1 protein levels without achieving statistical significance (increase by 78.3%, p=0.00). The specificity of BACE-1 detection was confirmed by using cell lysates extracted from BACE-1 expressing 293 cells as positive controls.

Interestingly, cell surface holoAPP levels in dyn KD cells were also elevated by 131.1% (p=0.02) upon tamoxifen induction, without any significant changes in total APP protein levels (111.6% of controls, p=0.615) as shown in Figure 3B. In addition, the levels of sAPP\( \beta \) in N2a 695 cells determined by immunoprecipitation with MAB348 followed by immunoblotting with 6E10 antibody, was increased by 296.3% (p=0.042) upon dyn1 knockdown by siRNA transfection. Consistently, the levels of \( \alpha \)-CTF were also elevated with dyn1 KD in both N2a 695 cells (Figure S1C) and in dyn1 KD fibroblasts (Figure S2A; increase by 84.9%, p=0.011). The increased holoAPP at cell surface likely promotes \( \alpha \)-secretase cleavage to generate sAPP\( \beta \) and \( \alpha \)-CTF.

However, it should be noted that dyn1 knockdown does not have a general effect on the internalization of all proteins because no significant differences were found in the amounts of nicastrin (NCT) or PS1 NTF at cell surface upon dyn KD conditions (Figure S3B). In addition, the total protein levels of NCT and NCT were unchanged with dyn1 KD (Figure S3B, input panels). The changes in both BACE-1 and holoAPP at cell surface with dyn1 KD suggest that dyn1 regulates a common trafficking pathway for those two proteins between endosomes and plasma membrane.

Taken together, these data suggest that dyn1 knockdown inhibits endocytosis of BACE-1, thereby preventing delivery of BACE-1 to its active sites in endosomes [29]. The BACE-1 on the cell surface is likely to be inactive and unable to process holoAPP to generate \( \beta \)-CTF and \( \beta \)-APP. Therefore, increased holoAPP availability at surface for \( \alpha \)-secretase cleavage leads to elevation in sAPP\( \beta \) (Figure 3B) and \( \alpha \)-CTF (Figure S1C and S2A).

4. A Selective Dynamin Inhibitor Dynasore Reduces \( \beta \)-CTF and \( \beta \)-APP Levels

Next, to determine whether pharmacological inhibition of dyn1 could affect APP processing, N2a 695 cells were treated with a selective dynamin inhibitor dynasore at 10\( \mu \)M [19,29,30]. As shown in Figure 4A, levels of \( \beta \)-CTF and \( \beta \)-APP secreted in media were decreased by 47.4% (p=0.008) and 70.4% (p<0.001), comparable to that with a BACE inhibitor treatment [31] (reduction of \( \beta \)-CTF by 38% p=0.006, and \( \beta \)-APP by 50.4% p=0.011).

Furthermore, the effects of dyn1 knockdown on BACE-1 cleavage of endogenous APP were confirmed in dyn1 KD fibroblast cell lines. Similar to the results using siRNA and chemical inhibitors in N2a 695 cells, reduction in \( \beta \)-CTF levels (Figure S2A; 66.7% of reduction, p=0.008) was observed in these cells upon dyn1 knockdown. The total \( \beta \)-APP levels measured by sandwich ELISA were reduced by 39.4% (p<0.001), comparable to the reduction induced by a BACE inhibitor treatment (41.3% reduction, p<0.001; Fig. 4B). However, the combination of BACE inhibitor IV with tamoxifen-induced dyn1 KD did not lead to further reduction in \( \beta \)-APP levels than either treatment alone (41.3% reduction). Consistently, \( \beta \)-APP levels were reduced by 40% in dyn1 KD fibroblasts with dynasore treatment alone. The combination of dynasore and BACE inhibitor treatment did not lead to further reduction in \( \beta \)-APP levels as well (43.5% reduction). The lack of additive effects with combined treatments suggests that the \( \beta \)-
lowering effects of dyn1 KD, dynasore and BACE inhibitor IV all act through the same step: BACE1 inhibition.

5. Genetic Perturbation of Dyn1 in Animals Reduces Aβ Generation

Dyn1 has been implicated in regulating activity-dependent Aβ secretion through endocytosis [9]. To determine whether dyn1 has a direct effect on endogenous Aβ generation, we next analyzed levels of newly synthesized Aβ secreted from dyn1−/− neurons [8] using S35-methionine labeling approach. When compared to dyn1+/+ neurons, levels of S35-labeled Aβ (both Aβ1–40/42 and Aβ11–40/42 species as described previously [23,24]) secreted into the media were decreased by 55.9% in dyn1−/− neurons after 5 hours of incubation (Fig. 5A, p = 0.004). The dyn1 genotypes were confirmed by PCR results (Fig. 5A, middle panel) and the levels of

Figure 2. Dyn1 down-regulation changes BACE-1 intracellular distribution. A) Dyn1 fibroblast cells treated without (control; ctrl) or with tamoxifen (dyn1 KD) were transiently transfected with EGFP-BACE-1 followed by fixation. Localization of BACE-1 is shown as green fluorescence (labeled as EGFP-BACE). In addition, cells were double stained with an endosomal marker, EEA1 (red; labeled as EEA1). Overlay of both signals is shown as yellow (labeled as Overlay). Nuclear staining by DAPI is shown as blue (labeled as DAPI). B) Localization of BACE-1 is shown as green fluorescence (labeled as EGFP-BACE). In addition, cells were double stained with a TGN marker, γ-adaptin (red; labeled as γ-adaptin). Overlay of both signals is shown as yellow (labeled as Overlay). Nuclear staining by DAPI is shown as blue (labeled as DAPI). BACE-1 (green fluorescence) is mainly localized in the endosomes and TGN/Golgi in control cells. However, after dyn1 knockdown, the amounts of green fluorescence (EGFP-BACE1) are dramatically increased at cell surface with a little remaining within the TGN/Golgi and endosomes.

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Dyn1 knockout animals can only survive up to 2 weeks [8]. To determine whether genetic perturbation of dyn1 affects Aβ generation in adult animals in vivo, we next analyzed the levels of Aβ, holoAPP and BCTF in the brains of dyn1 haploinsufficient (dyn1+/−) mice with AD transgenic background expressing human Swedish APP and PS1ΔE9 mutations [25,26]. At 2–3 months of age, levels of Aβ1−40 determined by sandwich ELISA in total brain lysates from AD transgenic mice carrying dyn1 haploinsufficiency (n=8), were decreased by 35.5% when compared to controls (n=9; p=0.013; Fig. 5B). However, levels of Aβ1−42 at this age group were too low for detection of any statistically significant changes. The levels of BCTF in the brains of dyn1 haploinsufficient animals (dyn1+/−) were slightly decreased (13.7%) when compared to dyn1 wild-type (dyn1+/+) animals (Figure S4). There was a trend of reduction in BCTF in dyn1+/− mouse brains, but due to large variations in these transgenic animals (even within the same group), this effect did not achieve statistical significance (p=0.613). The levels of holoAPP were comparable between the two groups (Figure S4: 107.4% of controls in dyn1+/− mice; p=0.679).

β-actin were comparable between dyn1+/+ and dyn1−/− neurons (Fig. 5A, bottom panel).

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Discussion

It is well established that sorting mechanisms which cause APP, BACE-1 and \( \gamma \)-secretase to colocalize in the same subcellular compartment play important roles in the regulation of A\( \beta \) production [32]. Further, endocytic pathways are important for regulation of BACE-1 activity (Huse et al., 2000). Substantial data regarding the nature and mechanisms underlying APP endocytosis have been reported, but the role of endocytosis in regulating BACE-1 distribution among endosomes, TGN and plasma membrane remain unclear.

As an important endocytic adaptor protein, the involvement of dynamin in A\( \beta \) biogenesis has been described [9,11,12], raising the possibility that it may affect A\( \beta \) biogenesis as a step in the pathogenesis of AD. However, there are discrepancies between prior reports regarding its effects on A\( \beta \). One group showed increased cell surface A\( \beta \) production with dominant negative dyn1 K44A [12] but other groups reported reduced A\( \beta \) production by dyn1 K44A [9,11]. It was also suggested that impaired APP endocytosis by dyn1 K44A contributes to reduction in activity-dependent A\( \beta \) secretion [9]. A similar situation has been proposed for deficiencies of other sorting proteins (e.g., SorL1 and Vps35), both of which, like dynamin, will undoubtedly alter sorting of multiple substrates. Yet, deficiencies of either SorL1 or Vps35 can promote A\( \beta \) generation and accumulation as prominent phenotypes at the whole brain level [21,33].

Our data, using gene silencing in three different cell lines (including N2a cells overexpressing human APP695 cells, fibroblasts with endogenous APP expression, and primary neurons derived from dyn1 \( ^{-/-} \) animals), as well as in animal models, demonstrates a previously unknown mechanism by which dyn1 regulates A\( \beta \) generation; i.e., through modulation of BACE-1 traffic between the plasma membrane and endosomes. By reducing BACE-1 localization to the endosomal compartment, dyn1 inhibits A\( \beta \) generation and secretion. These data further support the idea that endosomes are the most optimal compartments for BACE-1 enzymatic activities and that prevention of BACE-1 distribution into endosomes can reduce APP processing into A\( \beta \). The reduction of both A\( \beta \) and \( \beta \)CTF/sAPP\( \beta \) through dyn1 knockdown may be a better therapeutic strategy compared to those targeting \( \gamma \)-secretase, given that recent data suggest \( \beta \)-but not \( \gamma \)-secretase-mediated proteolysis of APP causes synaptic and memory deficits in AD [34].

Figure 4. A selective dynamin inhibitor Dynasore reduces \( \beta \)CTF and A\( \beta \) levels. A) N2a 695 cells were treated with dynasore at 10 \( \mu \)M or BACE Inhibitor IV at 15\( \mu \)M before subjected for further analysis. Levels of \( \beta \)CTF and A\( \beta \) were determined and significant reductions were observed upon dynasore or BACE inhibitor treatment, as compared to control (*\( p < 0.05 \); **, \( p < 0.001 \)). Protein levels were normalized to \( \beta \)-actin and expressed as percentage of control. Data were collected in duplicate or triplicate from three independent experiments. B) Dyn1 KD fibroblast cells were treated with tamoxifen (to induce dyn1 knockdown), BACE inhibitor IV or combination of both (top panels). Levels of A\( \beta \)40 in the media are determined by sandwich ELISA analysis (**\( p < 0.001 \) comparing each treatment or combination to control). Alternatively cells were treated with dynasore, or BACE inhibitor IV or combination of both (bottom panels). Levels of A\( \beta \)40 in the media are determined by sandwich ELISA analysis (**\( p < 0.001 \) comparing each treatment or combination to control).

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We would suggest that the previously reported effects of dyn1 knockdown on Aβ generation [9,11,12] are more likely to be attributable to modulation of BACE-1 sorting and less likely to be attributable to the conventional wisdom that dyn1 is primarily acting via reduction of APP internalization. A combination of dynasore (or dyn1 knockdown) and BACE inhibitor treatment did not cause any further reduction in levels of Aβ generation when compared with the maximal effect of either treatment on its own (Figure 4B), consistent with the interpretation that they both act on the same pathway. If dynamin knockdown/blockade caused an inhibition of Aβ generation by acting via inhibition of APP internalization, then there would be further reduction of Aβ when the BACE inhibitor was added. Together, our data suggest that the Aβ-lowering effects of dynamin inhibition are mainly mediated through regulation of BACE-1 internalization.

Interestingly, a recent report demonstrated that an immediate early gene, Arc/Arg3.1 which recruits dynamin and endophilin to early/recycling endosomes at post-synaptic terminals, can mediate PS1/γ-secretase trafficking through the endosomal pathway, and thereby affect activity-dependent Aβ generation [35]. These data suggest that dyn1-regulated endocytosis may also regulate γ-secretase function. However, our data demonstrate that dyn1 knockdown affects only BACE-1 and holoAPP traffic to plasma membrane but not the γ-secretase components nicastrin and PS1 NTF (Figure S3B), suggesting a shared pathway for BACE-1/ holoAPP selectively regulated by dyn1.

Conclusions
In summary, here we report a previously unknown regulatory mechanism by which dyn1 modulates APP proteolysis and Aβ homeostasis through regulation of BACE-1 subcellular distribution. Without directly targeting BACE-1 enzymatic activities, modulation of BACE-1 subcellular localization by targeting dyn1 suggests new directions for developing therapeutic strategies for AD.

Supporting Information
Figure S1 Genetic silencing of dyn1 in N2a cells reduces Aβ levels. A) Western blot analysis of PS1 NTF by Ab14, Pen-2 by rabbit polyclonal anti Pen-2 antibody NE1008 (Calbiochem), and BACE-1 by clone 61 and RU690, were
performed in N2a 695 cells with transfection of dyn1 siRNA or control duplex. There were no significant differences in total protein levels of BACE-1 or \( \gamma \)-secretase components with dyn1 KD conditions. B) Three different dyn1 siRNA duplex were synthesized (IDT Inc.) and transfected into N2a 695 cells to determine the specificity of observed effects of dyn1 KD on APP processing. The secreted \( \beta \) levels were similarly decreased in three different dyn1 siRNA treatment, compared to control siRNA transfection. The specificity of \( \beta \) detection was determined by comparing to transfection of APP siRNA. Sequences of the dyn1 siRNAs are as follows: Duplex 1 forward 5'-rGrGrUC rUrUrU rGrArA rUrArm rGrArG rCrGAg A-3'; reverse, 5'-rUrUrU rGrGrU rGrUrG rGrUrU rGrUrU rGrUrU rGrUrA rUrGrU rGrArG rCrGArG-3'. Duplex 2 forward 5'-rGrGrA rCrArU rCrGrA rCrGrG rCrGrA rGrArGATC-3'; reverse, 5'-rGrArU rGrUrU rCrUrU rGrCrC rGrUrC rUrArU rGrUrC rGrUrU rCrUrU rGrUrC rUrUrA rGrArG rCrGrA rCrGrG rCrArG rGrArC rCrArG rCrArG-3'. Stealth siRNA Control Medium GC Duplex was used as a negative control (IDT Inc.). Sequences are described as before. APP siRNA sequences are as follows: forward 5'-rGrGrU rUrGrC rGrUrG rCrUrG rUrArU rUrUrU rGrUrG rCrArG rCrArC rGrArA rCrArA rUrArU rGrGrA rCrGrA rCrGrG rGrAgA-3'. Media and lysates from N2a 695 cells with dyn1 or control siRNA transfection were immunoprecipitated with 4G8 followed by immunoblotted with 6E10 to determine levels of \( \beta \)CTF and \( \alpha \)CTF. Alternatively, lysates were directly blotted with 6E10 to detect holoAPP, \( \beta \)CTF and \( \alpha \)CTF, followed by reprobed with 369 for detection of \( \alpha / \beta \)CTF.

Figure S2 Dyn1 knockdown in fibroblast cells changes endogenous APP processing. A) Dyn1 KD cells were treated with tamoxifen to induce dyn1 knockdown. After tamoxifen treatment at 1 \( \mu \)M for 5–7 days, dyn1 protein levels were reduced by 56.6% \( (p = 0.03) \) in the dyn1 KD cells. Similar to the results in N2a 695 cells, changes in metabolism of endogenous APP including reduction in \( \beta \)CTF levels \( (66.7\% \) of reduction; \( p = 0.008 \)) were observed in these cells upon dyn1 knockdown. Elevation of \( \alpha \)CTF levels was also observed \( (84.9\% \) of increase; \( p = 0.011 \)). B) Wild type fibroblast cells were treated with tamoxifen at 1 \( \mu \)M for 5–7 days as a control. The levels of \( \beta \)CTF and \( \alpha \)CTF, as well as dyn1 protein levels were unchanged or with tamoxifen treatment (ctrl versus Tamoxifen).

Figure S3 Dyn1 knockdown increases cell surface BACE-1 levels. A) Dyn KD fibroblast cells were treated with tamoxifen to induce dyn1 knockdown followed by transiently transfected with EGFP-BACE-1. The levels of EGFP-BACE1 in dyn DKO cells with or without tamoxifen induction (three independent transfection experiments in both control and DKO cells) were comparable as shown in top panel (determined by Invitrogen anti-GFP antibody 3E6). The levels of dyn1 and actin were also determined (bottom panels). B) The amounts of nicastrin (NCT) or PS1 NTF at cell surface were also determined by biotinylation followed by streptavidin pull down. Western blot analysis of surface and total (input) NCT by anti-nicastrin antibody (BD Transduction) and RU717, as well as surface and total (input) PS1 NTF by Ab14 was performed. No significant changes were seen in the amounts of NCT and PS1NTF at cell surface or total cell lysates upon dyn KD conditions.

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