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Dynein Dysfunction Reproduces Age-Dependent Retromer Deficiency

Concomitant Disruption of Retrograde Trafficking Is Required for Alteration in β -Amyloid Precursor Protein Metabolism

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It is widely accepted that β -amyloid (A β) protein plays a pivotal role in Alzheimer disease pathogenesis, and accumulating evidence suggests that endocytic dysfunction is involved in A β pathology. Retromer, a conserved multisubunit complex, mediates the retrograde transport of numerous kinds of cargo from endosomes to the trans-Golgi network. Several studies have found that retromer deficiency enhances A β pathology both *in vitro* and *in vivo*. Cytoplasmic dynein, a microtubule-based motor protein, mediates minus-end-directed vesicle transport via interactions with dynactin, another microtubule-associated protein that also interacts with retromer. Aging attenuates the dynein-dynactin interaction, and dynein dysfunction reproduces age-dependent endocytic disturbance, resulting in the intracellular accumulation of beta-amyloid precursor protein (APP) and its β -cleavage products, including A β . Here, we report that aging itself affects retromer trafficking in cynomolgus monkey brains. In addition, dynein dysfunction reproduces this type of age-dependent retromer deficiency (ie, the endosomal accumulation of retromer-related proteins and APP). Moreover, we found that knockdown of Rab7, Rab9, or Rab11 did not alter endogenous APP metabolism, such as that observed in aged monkey brains and in dynein-depleted cells. These findings suggest that dynein dysfunction can cause retromer deficiency and that concomitant disruption of retrograde trafficking may be the key factor underlying age-dependent A β pathology. (*Am J Pathol* 2016, 186: 1952–1966; <http://dx.doi.org/10.1016/j.ajpath.2016.03.006>)

Alzheimer disease (AD) is a progressive neurodegenerative disorder that is histopathologically characterized by the formation of senile plaques (SPs) and neurofibrillary tangles (NFTs).¹ Although it is widely accepted that β -amyloid protein (A β), the major component of SPs, is the key molecule underlying AD pathogenesis,^{2,3} it remains unclear how aging processes contribute to sporadic AD, the major form of the disorder. The amyloidogenic cleavage of the beta-amyloid precursor protein (APP) (ie, A β production) is considered to occur through the endocytic pathway.^{4–6} In the brains of patients with early-stage AD, neuronal endocytic pathology, such as intracellular accumulation of abnormally enlarged

endosomes, is frequently observed,⁷ and recent genome-wide association studies identified several AD-associated variants in endocytosis-related genes, suggesting that perturbed endocytosis could be involved in AD pathogenesis.^{8–12}

The retromer complex is a key element of the endosomal protein sorting machinery, and it mediates the retrograde trafficking of numerous kinds of cargo from endosomes to the trans-Golgi network (TGN), the so-called retromer

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trafficking.¹³ Retromer complex comprises five proteins encoded by *VPS* genes and is functionally divided into two subcomplexes: a trimer consisting of VPS26/29/35 that mediates cargo selection and a dimer consisting of VPS5/17 (members of the sorting nexin family) that mediates tubule/vesicle formation for cargo incorporation.^{14–19} There are several prominent retromer cargoes, such as the cation-independent mannose 6-phosphate receptor (MPR)^{20,21} and the sortilin-related receptor (SORL1), also known as SorLA, that bind APP.^{22,23} AD-associated variants were identified in retromer complex–related genes, including SORL1,^{10,24} whose expression is also reduced in sporadic AD.²⁵ Thus, the alteration in retromer-mediated retrograde trafficking of APP via SORL1 could be involved in A β pathology.

Cytoplasmic dynein is a microtubule-based motor protein required for minus-end-directed axonal transport.^{26,27} Dynactin, another microtubule-associated protein, binds to dynein intermediate chain (DIC) to form a functional complex that enables motor activity.^{28–33} We previously found that aging attenuates the interaction between DIC and dynactin in monkey brain, suggesting that aging impairs dynein-mediated transport.³⁴ We also found that dynein dysfunction reproduced age-dependent endocytic pathology, resulting in the significant accumulation of APP and A β .³⁵ Specifically, dynein dysfunction reproduced a significant accumulation of APP in enlarged early endosomes, similar to that observed in AD patient neurons.³⁵ However, how dynein dysfunction causes such alteration in APP trafficking and metabolism remains unclear.³⁵

Recent findings indicate that retromer complex associates with dynactin, suggesting that the dynein-dynactin complex may also be involved in retromer trafficking.³⁶ Thus, we hypothesized that dynein dysfunction may affect retromer trafficking in a way that induces the endosomal accumulation of APP. We reveal that aging itself causes retromer deficiency in nonhuman primate brains. We also found that dynein dysfunction reproduces various features of age-dependent retromer deficiency, such as the accumulation of retromer-related proteins and APP in endosomes. Moreover, our RNA-mediated interference (RNAi) experiments reveal that knockdown of Rab7, Rab9, or Rab11 does not reproduce the alteration in APP metabolism, such as that observed in aged monkey brains and dynein-depleted cells. These findings suggest that dynein dysfunction can cause retromer deficiency and that the concomitant disruption of retrograde trafficking is important for age-dependent A β pathology.

Materials and Methods

Antibodies

We used the following antibodies: mouse monoclonal anti- β -actin antibody (Sigma, St. Louis, MO); mouse monoclonal anti-DIC antibody (Millipore, Temecula, CA); mouse monoclonal anti-golgin97 antibody (Santa Cruz

Biotechnology, Santa Cruz, CA); mouse monoclonal anti-MPR (Abcam, Cambridge, UK); mouse monoclonal anti-Rab5 antibody (Rab5; Santa Cruz Biotechnology); mouse monoclonal anti-Rab7 antibody (Rab7m; Abcam); mouse monoclonal anti-Rab9 antibody (Rab9m; Calbiochem, Darmstadt, Germany); mouse monoclonal anti-TGN38 (TGN) antibody (Abcam); goat polyclonal anti-cathepsin D (CatD) antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-dynein heavy chain (DHC) antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-ERp72 antibody (Cell Signaling Technology, Danvers, MA); rabbit polyclonal anti-full-length APP antibody (IBL, Gunma, Japan); rabbit polyclonal anti-Furin antibody (Santa Cruz Biotechnology); rabbit polyclonal anti-LAMP1 antibody (Abcam); rabbit polyclonal anti-Rab7 antibody (Rab7r; Sigma); rabbit polyclonal anti-Rab9 antibody (Rab9r; Sigma); rabbit polyclonal anti-SORL1 antibody (GeneTex, Irvine, CA); and rabbit polyclonal anti-VPS35 antibody (Proteintech, Chicago, IL).

Animals

Twenty-four cynomolgus monkey (*Macaca fascicularis*) brains were used in this study (Table 1). We defined monkeys younger than 10 years as young monkeys, monkeys between the ages of 10 and 20 years as adult monkeys, and monkeys older than 20 years as aged monkeys (Table 1). Among the aged monkeys, those with both SP and NFT lesions were defined as advanced aged monkeys (Table 1). Eight brains were from young monkeys [age: 6 years ($n = 4$) and 7 years ($n = 4$)]; four were from adult monkeys [age: 14 years ($n = 2$) and 15 years ($n = 2$)]; eight were from aged monkeys [age: 24 years ($n = 2$), 25 years ($n = 2$), 26 years ($n = 2$), 27 years ($n = 1$), and 30 years ($n = 1$)]; and four were from advanced aged monkeys [age: 32 years ($n = 2$) and 36 years ($n = 2$)] (Table 1). The temporal lobes of 16 cases [age: 6 years ($n = 4$), 7 years ($n = 4$), 24 years ($n = 2$), 25 years ($n = 2$), 26 years ($n = 2$), 27 years ($n = 1$), and 30 years ($n = 1$)] were used for immunohistochemical studies (Table 1). The temporal lobes of 16 cases [age: 7 years ($n = 4$), 14 years ($n = 2$), 15 years ($n = 2$), 24 years ($n = 2$), 25 years ($n = 2$), 32 years ($n = 2$), and 36 years ($n = 2$)] were used for Western blot analyses (Table 1). The temporal lobes of eight cases [age: 7 years ($n = 4$), 24 years ($n = 2$), and 25 years ($n = 2$)] were used for the subcellular fractionation study (Table 1). Other brain regions and other tissues were used for unrelated studies.

All brains were obtained from the Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation, Health, and Nutrition (NIBIOHN), Japan. All animals were bred and maintained in an air-conditioned room at the TPRC with controlled illumination (12 hours light/12 hours dark), temperature ($25^{\circ}\text{C} \pm 2^{\circ}\text{C}$), humidity ($60\% \pm 5\%$), and ventilation (10 cycles per hour). Each day they were given 70 g of commercially available pellet monkey chow (CMK-2; CLEA Japan, Inc., Tokyo, Japan),

Table 1 Cynomolgus Monkeys Used in the Present Study

Case No.	Age, years	Sex	SP	NFT	Experimental purpose
Young					
1	6	F	ND	ND	IH
2	6	F	ND	ND	IH
3	6	F	ND	ND	IH
4	6	M	ND	ND	IH
5	7	F	ND	ND	IH, WB, and SF
6	7	F	ND	ND	IH, WB, and SF
7	7	F	ND	ND	IH, WB, and SF
8	7	F	ND	ND	IH, WB, and SF
Adult					
9	14	F	ND	ND	WB
10	14	M	ND	ND	WB
11	15	F	ND	ND	WB
12	15	F	ND	ND	WB
Aged					
13	24	F	+	ND	IH, WB, and SF
14	24	F	+	ND	IH, WB, and SF
15	25	F	+	ND	IH, WB, and SF
16	25	M	+	ND	IH, WB, and SF
17	26	F	+	ND	IH
18	26	M	+	ND	IH
19	27	F	+	ND	IH
20	30	F	+	ND	IH
Advanced age					
21	32	M	+	+	WB
22	32	M	+	+	WB
23	36	F	+	+	WB
24	36	M	+	+	WB

F, female; M, male; +, detected; IH, immunohistochemistry; ND, not detected; NFT, neurofibrillary tangles; SF, subcellular fractionation; SP, senile plaques; WB, Western blot.

100 g of apples, and unlimited access to tap water.³⁷ Every morning their health status (eg, viability, appetite, coat appearance) was monitored. The maintenance and care of animals were according to the rules for animal care of the TPRC at NIBIOHN for the care, use, and biohazard countermeasures of laboratory animals.

This study was performed in strict accordance with the recommendations of the Animal Care and Use Committee of the NIBIOHN, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of the NIBIOHN. When a monkey presented with clinical symptoms characteristic of an injury or illness and if recovery from pain or morbidity was not expected, the prognosis was judged to be poor. In such cases, the monkeys were euthanized with an overdose of pentobarbital and used in the study. Animals that died of natural causes were also used in this study.

For primary cortical cultures, pregnant Institute for Cancer Research mice were purchased from SLC Japan (Shizuoka, Japan). The animals were maintained under controlled conditions (temperature, 24°C ± 1°C; humidity, 55% ± 5%) in plastic cages with sterilized wood shavings for bedding.

All animal experiments were conducted according to the guidelines of the Animal Care and Use Committee of National Center for Geriatrics and Gerontology (25-38) and NIBIOHN (DS17-001R1).

Biochemical Analysis of Monkey Brains

Frozen monkey brain tissue (0.2 g) was homogenized in a glass homogenizer with 4 mL of homogenate buffer solution (0.32 M sucrose, 10 mmol/L Tris-HCl [pH 7.6], 1 mmol/L EDTA, and Complete Mini proteinase inhibitor cocktail [Roche Molecular Biochemicals, Penzberg, Germany]) and then centrifuged at 1000 × *g* for 10 minutes to obtain the supernatant fraction. The supernatant (S1) was centrifuged at 13,000 × *g* for 15 minutes to yield the P2 fraction. The supernatant (S2) from the P2 fraction was centrifuged at 105,000 × *g* for 60 minutes to obtain the microsomal (pellet) fraction. The proteins in the fraction were adjusted to 5 µg and then subjected to SDS-PAGE by using 6% (DHC) or 12.5% acrylamide gels (other proteins). Separated proteins were blotted onto polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dried milk in phosphate-buffered saline (pH 7.0) and 0.1% Tween-20 for 1 hour at room temperature and then incubated with primary antibodies overnight at 4°C. We used the following primary antibodies: anti-APP (1:1000), anti-ERp72 (1:4000), anti-CatD (1:2000), anti-Furin (1:2000), anti-Rab7r (1:8000), anti-Rab9r (1:1000), anti-LAMP1 (1:2000), anti-MPR (1:1000), anti-SORL1 (1:1000), and anti-VPS35 (1:2000). After primary antibody incubation and thorough washing, the membranes were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG (Cell Signaling Technology), or chicken anti-goat IgG (Bethyl Laboratories, Montgomery, TX) for 1 hour at room temperature. Immunoreactive elements were visualized using enhanced chemiluminescence (Immobilon Western Detection Reagents; Millipore).

For the subcellular fractionation study, frozen monkey brain tissue (0.2 g) was homogenized in a glass homogenizer with 2 mL of homogenate buffer solution [0.25 M sucrose, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, and Complete Mini proteinase inhibitor cocktail (Roche Molecular Biochemicals)] and then centrifuged at 1000 × *g* for 10 minutes to obtain the supernatant fraction. The supernatant fraction was centrifuged at 100,000 × *g* for 60 minutes to obtain the pellet. The pellet was resuspended in 400 µL of homogenization buffer and sonicated. This sonicated fraction was adjusted with 40% sucrose buffer to give a final volume of 1.6 mL. The sample was overlaid with 1.6 mL of a 35% sucrose solution, followed by 1.6 mL of 8% sucrose, and then subjected to discontinuous equilibrium sucrose density gradient centrifugation at 150,000 × *g* for 2 hours using a swing-out MLS-50 Beckman rotor. Then, 400-µL gradient fractions were harvested beginning from

the top of the tube, and 20 μ L of each fraction was subjected to Western blot analyses. We used the following primary antibodies: anti-APP, anti-ERp72, anti-Furin, anti-Rab5 (1:1000), anti-Rab7r, anti-MPR, anti-SORL1, and anti-VPS35.

Double Immunohistochemistry

The brain samples were immersion-fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4- μ m-thick sections. The sections were deparaffinized and then prestained with 1% Sudan Black B to reduce the natural autofluorescence often associated with tissues from older animals. Sections were incubated overnight at 4°C in solutions that contained the following primary antibodies: anti-golgin97 (1:50), anti-LAMP1 (1:200), anti-MPR (1:200), anti-SORL1 (1:200), anti-TGN (1:200), anti-Rab5 (1:100), anti-Rab7m (1:100), anti-Rab9m (1:100), and anti-VPS35 (1:200). After thorough washing, the sections were then incubated with AlexaFluor 488–conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA), AlexaFluor 568–conjugated goat anti-rabbit IgG (Invitrogen), and DAPI nuclear stain (Santa Cruz Biotechnology) for 1 hour at room temperature. All sections were examined with a Digital Eclipse C1 confocal microscope (Nikon, Kanagawa, Japan).

Cell Cultures

COS-7 cells were cultured in culture medium (Dulbecco's modified Eagle's medium with 10% fetal calf serum). Cells were plated at 2.0×10^4 cells/cm² onto 6-well plates for Western blot analyses and at 1.0×10^4 cells/cm² onto 2-well LAB-TEK chamber slides (Nalge Nunc, Rochester, NY) coated with 0.01% poly-L-lysine (Wako, Osaka, Japan) for immunocytochemistry. A neuronal cell line, mouse neuroblastoma Neuro2a cells, was also cultured in culture medium. Cells were plated at 1.5×10^4 cells/cm² onto 6-well plates for Western blot analyses, at 1.5×10^4 cells/cm² onto 100-mm dishes for the subcellular fractionation study, and at 1.0×10^4 cells/cm² onto 2-well LAB-TEK chamber slides coated with 0.01% poly-L-lysine for immunocytochemistry.

RNA Interference

For double-stranded RNAi experiments, we used the following short double-stranded siRNA against primate-specific DHC (siDHC) for COS-7 cells and against mouse-specific DHC (siDHCm) for Neuro2a cells³⁸: siDHC, 5'-GGUGGGUGUA-CAUUACGAAUU-3' (sense) and 5'-UUCGUAAUGUACACCCACCUG-3' (antisense); siDHCm, 5'-GUAUCAGCACGGAGUUUUUGG-3' (sense) and 5'-AAAAACUCCGUGCUGAUACUC-3' (antisense).

To avoid off-target effects, all siRNAs were carefully designed by Enhanced siDirect (Tokyo, Japan). We also

examined other siRNAs: siDHC-2, 5'-CGAGAGCGUUCGUACCUAUGA-3' (sense) and 5'-AUAGGUACGAA-CGCUCUCGAU-3' (antisense); siDHCm-2, 5'-GCCAUUCGUGAAUACCAGACC-3' (sense) and 5'-UCUGGUAUUCACGAAUGGCCC-3' (antisense). Then, we confirmed their knockdown-specific effects, as previously reported.³⁶ The control siRNA had a random sequence. RNAi experiments were performed by using ScreenFect A lipid reagent (Wako), according to the manufacturer's protocol.

Seventy-two hours after siRNA transfection, cells plated on 6-well plates were lysed in a sample buffer solution that contained 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% sodium deoxycholate, 0.5% Triton X-100, 0.1% SDS, and Complete Mini proteinase inhibitor cocktail (Roche Molecular Biochemicals) to extract total cellular proteins. Total proteins were adjusted to 5 μ g and then subjected to Western blot analyses as described above. We used the following primary antibodies: anti- β -actin (1:50,000), anti-APP, anti-CatD, anti-DHC (1:500), anti-DIC (1:20,000), anti-Rab7r (1:8000), anti-Rab9r (1:1000), anti-MPR, anti-SORL1, and anti-VPS35. We performed three independent experiments ($n = 6$ for each experimental group), duplicating each experiment.

Cells plated on 100-mm dishes were subjected to subcellular fractionation and tissue samples from monkey brains. We performed three independent experiments, duplicating each experiment.

Cells plated on chamber slides were fixed with 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 5 minutes. After blocking with 3% bovine serum albumin, cells were incubated with primary antibodies overnight at 4°C. After thorough washing, cells were then incubated with AlexaFluor 488–conjugated anti-mouse IgG and AlexaFluor 555–conjugated anti-rabbit IgG (Invitrogen) for 1 hour at room temperature. All cells were examined with a Digital Eclipse C1 confocal microscope (Nikon). For immunocytochemistry, we used the following primary antibodies: anti-DIC (1:10,000), anti-TGN (1:1000), anti-Rab5 (1:2000), anti-Rab7m (1:2000), anti-Rab7r (1:2000), anti-Rab9m (1:2000), anti-Rab9r (1:2000), anti-VPS35 (1:2000), and anti-SORL1 (1:1000). We performed three independent experiments.

A β ELISA

A β level was determined using a sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA kit for human/rodent A β 1-40 was obtained from Wako. Seventy-two hours after siRNA transfection, cells plated on 6-well plates were lysed in 5 M guanidine-HCl buffer (pH 8.0). After incubation at room temperature for 3 hours, the homogenates were further diluted with 0.1% bovine serum albumin and phosphate-buffered saline and centrifuged at $16,000 \times g$ for 20 minutes. The resulting supernatants were then applied to ELISA. We performed two independent

experiments ($n = 6$ for each experimental group), and all samples were measured in duplicate.

Mouse Primary Cortical Cultures and Dynein Inhibitor Treatment

Mouse fetuses were removed at gestational day 17, and isolated fetal cerebral cortices were cut into small pieces in isolation medium (equal volumes of phosphate-buffered saline and Dulbecco's modified Eagle's medium). Small pieces of cerebral cortices were digested with 1.5 U/mL of papain, and cells were plated at 1.5×10^5 cells/cm² onto 12-well plates coated with 0.01% poly-L-lysine for Western blot analyses and at 5.0×10^4 cells/cm² onto 2-well LAB-TEK chamber slides coated with 0.01% poly-L-lysine for immunocytochemistry. Cells were maintained in neurobasal medium that contained B27 supplement (Invitrogen). A half volume of culture medium was replaced with fresh culture medium every 2 days.

After 6 days *in vitro*, primary cortical neurons were treated with the dynein ATPase inhibitor erythro-9-[3-(2-hydroxy-nonyl)]adenine (EHNA; Calbiochem) at final concentrations of 2 μ mol/L or 5 μ mol/L. After 24 hours of EHNA treatment, cells were subjected to Western blot analyses and immunocytochemistry as described above. For Western blot analyses, we used the following primary antibodies at the same dilutions listed above: anti-APP, anti- β -actin, anti-Rab5, anti-Rab7m, anti-Rab9m, anti-SORL1, and anti-VPS35. We performed three independent experiments ($n = 6$ for each experimental group), duplicating each experiment. For immunocytochemistry, we used the following primary antibodies: anti-Rab5, anti-SORL1, and anti-TGN. We performed three independent experiments.

Colocalization Analysis

To quantitatively assess the colocalization of AlexaFluor 488 and AlexaFluor 568 signals obtained from immunohistochemical and immunocytochemical experiments, we used Mander's overlap coefficient and the JACoP ImageJ plugin, which measures the direct overlap of green and red pixels in a confocal section.³⁹ For each double immunostained monkey brain section, we examined three different visual fields per

section and then calculated the mean overlap coefficient for young and aged monkeys ($n = 8$ each). For double-immunostained cells, we analyzed a mean of 30 cells in each experiment (three different visual fields per experiment) and then calculated the Mander's overlap coefficient.

Data Analysis

To confirm reproducibility, we quantified the immunoreactive bands obtained from the Western blots using commercially available software (Quantity One version 4.5; PDI Inc., Upper Saddle River, NJ). Data are expressed as means $\pm$ SD. For statistical analyses, one-way analysis of variances were performed, followed by the Bonferroni-Dunn *post hoc* test.

Results

Retromer Trafficking Is Disturbed in Aged Monkey Brains

In cynomolgus monkeys, aging itself causes endocytic pathology in the brain, including intraneuronal accumulation of abnormally enlarged endosomes, similar to that observed in the early stage of AD.³⁵ Moreover, both SPs and NFTs occur spontaneously in old cynomolgus monkeys,^{40,41} and the amino acid sequence of monkey A β is completely homologous with that of human A β .³⁸ Thus, cynomolgus monkeys are a useful and appropriate animal model to investigate age-dependent A β pathology. However, there are also limitations to using these animals because their availability, especially aged cynomolgus monkeys, is relatively meager. With smaller sample sizes, any observed differences may be less reliable and require replication.

We analyzed the brains of various aged monkeys to clarify whether aging affects retromer trafficking. Endosome trafficking is coordinated by small molecular weight G proteins called Rabs.^{42,43} In retromer trafficking, Rab7 is required to recruit retromer complexes consisting of VPS26/29/35 toward endosomes, whereas Rab9 mediates retrograde endosome trafficking toward the TGN.^{44,45} In cynomolgus monkey brains, both Rab7 and Rab9 levels increased in an age-dependent manner. With these Rab increases, APP levels subsequently were significantly increased in the brains of

Figure 1 Aging disrupts retromer-mediated trafficking in cynomolgus monkey brains. **A:** Representative Western blots showing the amounts of Rab7, Rab9, A β precursor protein (APP), VPS35, sortilin-related receptor (SORL1), Furin, and Erp72 in microsome fractions derived from the brains of young (Y, 7-year-old; case 5), adult (Ad, 14-year-old; case 9), aged (Ag, 24-year-old; case 13), and advanced aged (AA, 32-year-old; case 21) monkeys. **B:** Quantitation of Western blots. Histograms showing age-related changes in the amounts of Rab7, Rab9, APP, VPS35, SORL1, and Furin in Y, Ad, Ag, and AA monkey brains. Both Rab7 and Rab9 levels are significantly increased, even in adult monkey brains. The amount of APP is clearly elevated in Ag and AA monkey brains. On the other hand, retromer-related proteins, such as VPS35 and SORL1, and the trans-Golgi network (TGN) marker Furin, do not reveal prominent or reliable age-related changes. All data were normalized according to the mean value in young monkey brains. **C:** Representative photomicrographs of temporal lobe sections from Y (7-year-old; case 6) and Ag (27-year-old; case 19) cynomolgus monkeys. Sections were immunostained with antibodies against TGN, Rab5, Rab7, and VPS35. In Ag monkey brains, the colocalization of TGN and VPS35 was apparently altered, with VPS35 specifically accumulating in endosomes. **D:** Western blots showing the distribution of Erp72, Furin, Rab5, and Rab7 in sucrose-gradient fractions derived from Y (7-year-old; case 7) cynomolgus monkey brains. Fraction 4 (F4) was enriched with Rab5 and Rab7, suggesting that this was an endosome-rich fraction. **E:** Western blots showing the amounts of VPS35, SORL1, APP, and mannose 6-phosphate receptor (MPR) in endosome-rich fractions (F4) derived from Y (7-year-old; case 7) and Ag (25-year-old; case 15) monkey brains. Data are expressed as means $\pm$ SD ($n = 4$ for each group (B)). * $P < 0.05$, ** $P < 0.01$. Scale bars = 10 μ m.

aged and advanced aged monkeys (Figure 1, A and B). However, the amounts of retromer-related proteins, such as VPS35 and SORL1, did not have prominent age-related changes (Figure 1, A and B). The amounts of TGN protein, Furin, and ERp72 also remained unchanged regardless of the age of the animal (Figure 1, A and B).

VPS35 is the core protein of the retromer complex that is required for retromer trafficking.⁴⁶ In young monkey brains, VPS35 immunostaining had a granular pattern, which colocalized with the TGN marker (Mander's overlap coefficient, 0.41 ± 0.09) (Figure 1C). In young monkey brains, VPS35 colocalized less with endosome markers (Mander's overlap coefficient, Rab7-VPS35, 0.28 ± 0.08 ; Rab9-VPS35, 0.22 ± 0.09 ; Rab5-VPS35, 0.24 ± 0.08) (Figure 1C). By contrast, in aged monkey brains, both TGN- and VPS35-positive granules appeared in clusters, and their colocalization pattern was different (Mander's overlap coefficient, 0.20 ± 0.04) (Figure 1C). Instead, VPS35 accumulated in enlarged Rab7- and Rab9-positive endosomes (Mander's overlap coefficient, Rab7-VPS35, 0.49 ± 0.04 ; Rab9-VPS35, 0.46 ± 0.09) (Figure 1C). Indeed, in aged monkey brains, VPS35 accumulated even in enlarged Rab5-positive early endosomes (Mander's overlap coefficient, 0.48 ± 0.12) (Figure 1C). We also found that SORL1, the retromer-binding adaptor protein for APP, also accumulated in enlarged Rab5-positive early endosomes (Supplemental Figure S1).

To assess whether retromer-related proteins truly accumulate in the endosomes of aged monkey brains, we analyzed young and aged monkey brains using subcellular fractionation. Immunoblotting revealed that subcellular fractionation successfully separated brain samples into endosome-rich fractions (Figure 1D). In the endosome-rich fraction, the levels of retromer-related proteins, such as VPS35, SORL1, and MPR, were apparently increased in aged monkey brain samples (Figure 1E). Concomitantly, the amount of APP was also clearly increased in endosome-rich fractions derived from aged monkey brains (Figure 1E).

Aging Causes Mislocalization of MPR in LAMP1-Positive Late Endosomes/Lysosomes

The retromer trafficking of MPR is important for the transport of CatD, a lysosomal aspartyl protease, toward endosomes.^{20,21} In young monkey brains, MPR partially colocalized with the late endosome/lysosome marker LAMP1 (Mander's overlap coefficient, 0.22 ± 0.08) (Figure 2A). By contrast, in aged monkey brains, MPR significantly accumulated in enlarged LAMP1-positive late endosomes/lysosomes (Mander's overlap coefficient, 0.65 ± 0.10) (Figure 2A). Western blot analyses failed to identify any age-related changes in MPR and LAMP1 levels (Figure 2, B and C).

As the lumen of endosomes/lysosomes becomes acidic, CatD is activated and cleaved to produce CatD heavy chain (CatD-HC).⁴⁷ In cynomolgus monkey brains, CatD-HC levels tended to decrease with aging. This decrease,

however, was not statistically significant (Figure 2, B and C). Intriguingly, CatD-HC levels returned to normal levels in the brains of very old monkeys (Figure 2, B and C).

Dynein Depletion Reproduces the Endosomal Accumulation of Retromer-Related Proteins in Neuronal Cells

We previously found that aging attenuates the dynein-mediated transport system and that dynein dysfunction reproduces AD-related endocytic pathology, resulting in the intracellular accumulation of APP and A β .^{34,35} Because retromer complex associates with dynactin,³⁶ we hypothesized that dynein dysfunction may be involved in the age-dependent retromer deficiency observed in aged monkey brains (Figure 1). Although processes associated with brain aging cannot be fully reproduced *in vitro*, we tested our hypothesis in a well-established cell line used to study cellular mechanisms.

We performed RNAi experiments using COS-7 cells, which have large somas and are thus useful for studying endosome trafficking. As we previously reported,³⁵ both DHC and DIC expression levels clearly decreased in siDHC-transfected COS-7 cells, indicating that dynein was effectively depleted (Supplemental Figure S2). In siDHC-transfected COS-7 cells, both Rab7 and Rab9 levels significantly increased, as we observed in aged monkey brains (Supplemental Figure S2). However, the depletion of dynein did not affect VPS35 levels, even 72 hours after transfection (Supplemental Figure S2). It is noteworthy that immunocytochemistry revealed significant alterations in the localization of retromer-related proteins. In control siRNA-transfected COS-7 cells, both Rab7- and Rab9-positive endosomes partially colocalized with the TGN marker (Mander's overlap coefficient, TGN-Rab7, 0.50 ± 0.10 ; TGN-Rab9, 0.43 ± 0.09) (Supplemental Figure S2). VPS35 also colocalized with TGN (Mander's overlap coefficient, 0.48 ± 0.10) (Supplemental Figure S2). By contrast, in siDHC-transfected COS-7 cells, both Rab7- and Rab9-positive endosomes were significantly enlarged, and their colocalization with the TGN marker was apparently disrupted (Mander's overlap coefficient, TGN-Rab7, 0.25 ± 0.11 ; TGN-Rab9, 0.21 ± 0.05) (Supplemental Figure S2). In siDHC-transfected cells, the TGN localization of VPS35 was also perturbed (Mander's overlap coefficient, 0.20 ± 0.05), with VPS35 primarily accumulating in enlarged Rab7-, Rab9-, and Rab5-positive endosomes (Mander's overlap coefficient, Rab7-VPS35, 0.61 ± 0.17 ; Rab9-VPS35, 0.64 ± 0.18 ; Rab5-VPS35, 0.60 ± 0.16) (Supplemental Figure S2). This finding is consistent with our immunohistochemical data obtained from aged monkey brains (Figure 1C). Intriguingly, dynein depletion caused a change in the perinuclear localization of the TGN (Supplemental Figure S2).

We previously found that dynein dysfunction induces the endosomal accumulation of APP; however, the precise

Figure 2 Mannose 6-phosphate receptor (MPR) is mislocalized subcellularly, and cathepsin D heavy chain (CatD-HC) levels are altered in aged monkey brains. **A:** Fluorescent micrographs of temporal lobe sections from young (7-year-old; case 5) and aged (30-year-old; case 20) cynomolgus monkeys. Sections were immunostained with anti-MPR and anti-LAMP1 antibodies. In aged monkey brains, MPR accumulates abundantly in enlarged LAMP1-positive late endosomes/lysosomes. **B:** Western blots showing the amounts of MPR, LAMP1, CatD-HC, and Erp72 in microsome fractions derived from the brains of young (Y, 7-year-old; case 5), adult (Ad, 14-year-old; case 9), aged (Ag, 24-year-old; case 13), and advanced aged (AA, 32-year-old; case 21) monkeys. **C:** Histograms showing age-related changes in the amounts of MPR, LAMP1, and CatD-HC in young, adult, aged, and advanced aged monkey brains. Both MPR and LAMP1 do not show prominent age-related changes. CatD-HC levels tend to decrease with aging. However, CatD-HC levels appear to return to normal levels in the brains of advanced aged monkeys. All data were normalized according to the mean value in young monkey brains. Data are expressed as means $\pm$ SD. $n = 4$ for each group (B). Scale bars = 10 μ m.

mechanism for this remains unclear.³⁵ To test our hypothesis that dynein dysfunction may disrupt retromer trafficking of APP, we conducted RNAi studies using Neuro2a cells, a neuroblastoma cell line that fully expresses endogenous APP. In siDHCm-transfected Neuro2a cells, we confirmed the large increases in Rab7 and Rab9 levels, as well as the significant increase in APP levels, as previously reported (Figure 3, A and B).³⁵ In Neuro2a cells, dynein depletion did not affect the amount of VPS35 or SORL1 (Figure 3, A and B). Because SORL1 is the retromer-binding receptor for APP,^{22,23} we investigated whether dynein dysfunction altered the distribution of SORL1 in Neuro2a cells.

In control siRNA-transfected Neuro2a cells, SORL1 mainly localized to the cell soma and colocalized well with the TGN marker rather than Rab5-positive early endosomes (Mander's overlap coefficient, TGN-SORL1, 0.56 ± 0.09 ; Rab5-SORL1, 0.31 ± 0.04) (Figure 3C). In siDHCm-transfected Neuro2a cells, however, TGN localization of SORL1 was significantly disrupted, and SORL1 clearly accumulated in enlarged early endosomes (Mander's overlap coefficient, TGN-SORL1, 0.29 ± 0.07 ; Rab5-SORL1, 0.61 ± 0.14) (Figure 3C). As observed in COS-7 cells (Supplemental Figure S2), the perinuclear localization of TGN was also disrupted in siDHCm-transfected Neuro2a cells (Figure 3C). Notably, subcellular fractionation experiments confirmed that APP accumulates in endosome-rich fractions and that this is accompanied by the accumulation of SORL1 and VPS35 in dynein-knockdown cells (Figure 3, D and E).

Dynein ATPase Inhibitor Reproduces the Mislocalization of Retromer-Related Proteins in Primary Cortical Neurons

Although our RNAi study revealed that dynein dysfunction disrupts retromer trafficking in cell lines, it was questionable about whether this actually occurs in neurons. Because it is difficult to perform RNAi in neurons without using viral vectors, which may independently affect intracellular transport, we treated mouse primary cortical neurons with EHNA, a well-known dynein ATPase inhibitor. EHNA treatment increased both Rab7 and Rab9 levels in a dose-dependent manner, indicating that EHNA induced the same endocytic disturbances observed in our RNAi study using cell lines (Figure 4, A and B). As in the RNAi study with Neuro2a cells, EHNA treatment did not affect VPS35 or SORL1 levels, regardless of dose (Figure 4, A and B).

Immunocytochemistry revealed that VPS35 localized well to the TGN in control rat cortical neurons (Mander's overlap coefficient, 0.51 ± 0.16) (Figure 4C). Evidently, EHNA treatment disturbed the intracellular localization of VPS35 (Mander's overlap coefficient, 0.25 ± 0.10), and both VPS35 and SORL1 accumulated in enlarged endosomes (Mander's overlap coefficient, Rab7-VPS35, 0.57 ± 0.05 ; Rab9-VPS35, 0.58 ± 0.07 ; Rab5-VPS35, 0.58 ± 0.15 ; Rab5-SORL1, 0.59 ± 0.15) (Figure 4C). We observed that EHNA treatment disrupted the normal perinuclear localization of the TGN (Figure 4C).

Figure 3 siRNA-induced dynein dysfunction reproduces the endosomal accumulation of retromer-related proteins in Neuro2a cells. **A:** Western blots showing the amounts of dynein heavy chain (DHC), dynein intermediate chain (DIC), Rab7, Rab9, Aβ precursor protein (APP), VPS35, sortilin-related receptor (SORL1), and β-actin in extracts derived from Neuro2a cells 72 hours after siRNA transfection. **B:** Histograms showing that dynein depletion affect the amounts of Rab7, Rab9, APP, VPS35, and SORL1 in Neuro2a cells. **C:** Fluorescent micrographs of Neuro2a cells immunostained for DIC, trans-Golgi network (TGN), Rab5, and SORL1 72 hours after siRNA transfection. The depletion of dynein alters the colocalization of TGN and SORL1 and also induces the endosomal accumulation of SORL1. **D:** Western blots showing the distribution of ERp72, Furin, Rab5, and Rab7 in sucrose-gradient fractions derived from Neuro2a cells. Fraction 4 (F4) was enriched with Rab5 and Rab7, indicating that it is an endosome-rich fraction. **E:** Western blots showing the amounts of APP, SORL1, and VPS35 in an endosome-rich fraction (F4) derived from siRNA-transfected Neuro2a cells. Data are expressed as means ± SD. **P* < 0.05. Scale bars = 20 μm. CT, cells transfected with control siRNA; siDHC, cells transfected with mouse-specific dynein heavy chain.

Disturbance in a Single Endosome-Trafficking Pathway Cannot Induce Alterations in APP Metabolism

Our previous study revealed that dynein dysfunction causes the intracellular accumulation of APP and its β-site cleavage

products, such as soluble peptide APPβ (sAPPβ), β-secretase cleavage, and Aβ (Supplemental Figure S3).³⁵ We also found that dynein dysfunction primarily disrupts Rab7- and Rab11-mediated endosome trafficking, causing APP to accumulate in enlarged Rab5-positive early endosomes.³⁵

Figure 4 Erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA) treatment reproduces the endosomal accumulation of VPS35 and sortilin-related receptor (SORL1) in rat primary cortical neurons. **A:** Western blots showing the amounts of Rab7, Rab9, VPS35, SORL1, and β -actin in rat primary cortical neurons treated with the dynein ATPase inhibitor EHNA for 24 hours. Drug treatments increase the levels of Rab GTPases but not those of VPS35 or SORL1. **B:** Histograms showing the effect of EHNA treatment on Rab7, Rab9, VPS35, and SORL1. All data were normalized according to β -actin levels. **C:** Photomicrographs of primary cortical neurons immunostained for trans-Golgi network (TGN), Rab5, Rab7, Rab9, VPS35, and SORL1 24 hours after EHNA treatment. VPS35 and SORL1 accumulate in enlarged endosomes due to EHNA treatment. Data are expressed as means $\pm$ SD. * $P < 0.05$. Scale bars = 20 μ m. CT, control; EH2, cells treated with 2 μ mol/L EHNA; EH5, cells treated with 5 μ mol/L EHNA; EH5 μ mol/L, cells treated with 5 μ mol/L EHNA.

We observed that dynein dysfunction induces a concomitant increase in Rab9 and Rab7 levels, indicating that Rab9-mediated endosome trafficking is also affected at an early stage of dynein dysfunction—related endocytic disturbance (Figures 3 and 4 and Supplemental Figure S2). To assess which endosome trafficking pathway is primarily responsible for the alteration in APP metabolism, we performed additional RNAi studies using siRNAs specific for Rab7, Rab9, and Rab11 in Neuro2a cells. Western blot analyses confirmed that these siRNAs effectively knocked down Rab7, Rab9, and Rab11 (Figure 5, A and B). Seventy-two hours after siRNA transfection, the Rab5 level was increased in Rab7-, Rab9-, and Rab11-depleted cells, respectively (Figure 5, A and B). However, neither the level of APP nor the level of sAPP β increased after the knockdown of Rab7, Rab9, or Rab11 (Figure 5, A and B). ELISA analyses also revealed that knockdown of Rab7, Rab9, or Rab11 did not change intracellular A β levels,

which is in contrast to what occurs during dynein depletion (Figure 5C).

Discussion

We found that aging itself disturbs retromer trafficking. We also found that dynein dysfunction reproduces this age-dependent retromer deficiency and that it is characterized by the endosomal accumulation of retromer-related proteins and APP. Furthermore, our RNAi studies revealed that the knockdown of Rab7, Rab9, or Rab11 did not alter APP metabolism, as it does in dynein-depleted cells. We acknowledge that our current study has some limitations. For example, out of necessity, we used a small number of aged monkeys, and some of our findings are based on *in vitro* cell models. Thus, parts of our study face the same limitations as similar published studies using the same experimental approaches.^{48,49} Nonetheless, our findings

Figure 5 Single knockdown of Rab GTPases does not alter A β precursor protein (APP) metabolism in Neuro2a cells. **A:** Western blots showing the amounts of APP, soluble peptide APP β (sAPP β), Rab5, Rab7, Rab9, Rab11, and β -actin in extracts derived from Neuro2a cells 72 hours after siRNA transfection. Each siRNA sufficiently down-regulates the target Rab GTPase. Although Rab5 level slightly increases, neither APP nor sAPP β levels are affected by the depletion of Rab7, Rab9, or Rab11. **B:** Histograms showing the effect of Rab7, Rab9, or Rab11 knockdown on the amounts of APP, sAPP β , and Rab5 in Neuro2a cells. **C:** Histograms showing the effect of dynein, Rab7, Rab9, or Rab11 knockdown on intracellular A β levels in Neuro2a cells 72 hours after siRNA transfection. A β levels were assessed with enzyme-linked immunosorbent assay (ELISA). All data were normalized according to A β level in control siRNA-transfected cells. Data are expressed as means $\pm$ SD. * $P < 0.05$. CT, cells transfected with control siRNA; siDHC, cells transfected with mouse-specific dynein heavy chain; siR7, cells transfected with siRab7; siR9, cells transfected with siRab9; siR11, cells transfected with siRab11.

support the hypothesis that endosomal disturbances contribute to age-dependent A β pathology. Our findings suggest that dynein dysfunction itself can cause retromer deficiency and that the concomitant disruption of retrograde trafficking is required to alter APP metabolism. This finding may explain why axonal transport deficits are considered to be involved in AD pathogenesis⁵⁰ and why a system-wide breakdown in endocytosis, not disruption of a single endocytic pathway, is important for age-dependent A β pathology to be expressed.

We previously found that dynein-dynactin interactions are attenuated in aged monkey brains³⁴ and that dynein dysfunction reproduces age-dependent endocytic disturbance, resulting in the intracellular accumulation of APP and A β .^{35,51,52} Accumulating evidence suggests that endocytic disturbance is involved in AD pathogenesis, with recent findings from genome-wide association studies in patients with AD supporting this idea.^{8–12} Indeed, several studies indicate that a deficiency in retromer trafficking of APP enhances A β generation.^{53–55} Thus, in the present

study, we first analyzed the effect of aging on retromer trafficking using various aged monkey brains.

In cynomolgus monkey brains, Rab9 levels significantly increased with advancing age, indicating that aging alone affects the retromer trafficking pathway (Figure 1, A and B). Because Rab9 levels increased concomitantly with Rab7 levels, both Rab9- and Rab7-mediated endosome trafficking would be disturbed at an early stage of aging.³⁵ Moreover, the increase in both Rab7 and Rab9 levels precedes APP accumulation in monkey brains, suggesting that the alteration in Rab7- and Rab9-mediated endosome trafficking also occurs earlier, before APP metabolism is altered (Figure 1, A and B). Immunohistochemistry results also revealed that aging affects the localization of VPS35, which clearly accumulates in endosomes of aged monkey brains (Figure 1C). Subcellular fractionation experiments also confirm that retromer-related proteins accumulate in endosome-rich fractions, suggesting that retromer trafficking is truly disrupted in aged brains (Figure 1, D and E).

It is widely known that lysosomal degradation deteriorates in AD brains and that the malfunction of lysosomes can also induce endocytic disturbance.^{51,52,56,57} MPR mediates the transport of CatD to lysosomes, and then MPR is recycled back to the TGN by retromer trafficking.^{20,21} In cynomolgus monkey brains, both MPR and LAMP1 levels did not reveal prominent age-related changes; however, MPR significantly accumulated in LAMP1-positive late endosome/lysosomes in aged monkey brains (Figure 2). Western blot analyses confirmed that CatD-HC levels tended to decrease in an age-dependent manner, suggesting that the transport and/or activation of CatD may be disturbed in aged monkey brains (Figure 2, B and C). Intriguingly, CatD-HC levels recovered in brains of advanced aged monkeys—the oldest old of the monkeys in our study (Figure 2, B and C). A previous study found that CatD expression is much greater in AD brains and correlates well with the severity of AD pathology.⁵⁷ We previously found that NFTs are observed only in monkeys older than 32 years (ie, advanced aged monkey brains).³⁹ Thus, it would be reasonable that NFT formation may cause the compensatory up-regulation of CatD, leading to the increase of CatD-HC in the brains of advanced aged monkeys compared with that of younger aged monkeys.

It is noteworthy that our present study indicates that dynein dysfunction closely reproduces age-dependent retromer deficiency characterized by the accumulation of retromer-related proteins in endosomes and APP (Figures 3 and 4 and Supplemental Figure S2). In dynein knockdown COS-7 cells, both Rab7- and Rab9-positive endosomes were significantly enlarged, and their colocalization with the TGN marker was apparently disrupted (Supplemental Figure S2). This finding suggests that dynein dysfunction really disrupts retrograde endosome trafficking toward the TGN. In dynein-depleted cells, we confirmed that VPS35 accumulated in both enlarged Rab7- and Rab9-positive endosomes, suggesting that neither the recruitment of

Figure 6 Hypothetical model of intracellular APP trafficking and Aβ precursor protein (APP) metabolism during normal conditions (A) and during endocytic disturbance induced by dynein dysfunction (B). **B:** Taken together with our previous findings, dynein dysfunction concomitantly disrupts multiple endosomal trafficking pathways, including endosomal/lysosomal trafficking, recycling trafficking, and retromer trafficking. This disruption creates a significant traffic jam, leading to an alteration in APP metabolism. Moreover, dynein dysfunction might deteriorate lysosomal degradation by disrupting the retromer trafficking of cation-independent mannose 6-phosphate receptor (MPR), which can also contribute to endocytic dysfunction.

retromer complex to Rab7 nor the transport of retromer complex to Rab9-positive endosomes were disrupted by dynein dysfunction (Supplemental Figure S2). Because both VPS35 and SORL1 accumulated in enlarged Rab5-positive endosomes in aged monkey brains (Figure 1C and Supplemental Figure S1), some VPS35 may exist in early endosomes, even without the recruitment to Rab7, and/or dynein dysfunction may perturb the transport of retromer complex from early endosomes to late endosomes (Supplemental Figure S2).

Our previous study found that dynein dysfunction induces a significant endosomal accumulation of APP.³⁵ In the present study, we observed that dynein dysfunction altered the distribution of SORL1, the retromer-binding

receptor for APP, and that SORL1 significantly accumulated in endosomes, as observed in aged monkey brains (Figure 3C and Figure 4C and Supplemental Figure S1). Subcellular fractionation experiments also confirmed that dynein dysfunction reproduces the accumulation of both retromer-related proteins and APP in the endosome-rich fraction, as observed in aged monkey brains (Figure 1D and Figure 3D). These findings suggest that dynein dysfunction disrupts the retromer trafficking of SORL1 toward TGN, leading to the endosomal accumulation of APP.

We found that dynein dysfunction disrupts the perinuclear localization of TGN not only in dynein-depleted cell lines but also in EHNA-treated primary neurons (Figures 3 and 4 and Supplemental Figure S2). We observed that Golgi

fragmentation evidently occurs in aged monkey brains (Supplemental Figure S4), although we could not detect obvious fragmentation in brain sections immunostained for the TGN marker we used (Figure 1C). Several studies found that adequate dynein motor activity is required for the perinuclear localization of Golgi compartments.⁵⁸ Thus, dynein dysfunction can lead to the dismantling of the Golgi compartment, which may also contribute to intracellular trafficking deficiency accompanied by perturbation of endosome trafficking. This finding also suggests that dynein dysfunction might be responsible for Golgi fragmentation, one of the pathological features of cells in AD brains.⁵⁹

We previously found that dynein dysfunction induces the intracellular accumulation of APP and β -site cleavage products and that such alteration in APP metabolism correlates with the perturbation of Rab5-mediated endosome trafficking.³⁵ Our previous study also found that the disruption of Rab7- and Rab11-mediated trafficking pathways precedes the disruption of the Rab5-mediated trafficking pathway.³⁵ In the present study, we found that Rab9-mediated trafficking is affected at the same time as Rab7-mediated trafficking (Figure 1, A and B). This observation prompted us to conduct additional RNAi experiments to clarify which Rab-mediated trafficking pathway is responsible for the alteration in Rab5-mediated trafficking and the alteration in APP metabolism. Surprisingly, the knockdown of Rab7, Rab9, or Rab11 increased Rab5 levels but not APP or sAPP β levels, even 72 hours after siRNA transfection in Neuro2a cells (Figure 5, A and B). ELISA analyses revealed that intracellular A β levels are unchanged by the knockdown of Rab7, Rab9, or Rab11 (Figure 5C). These findings suggest that the disruption of Rab7-, Rab9-, or Rab11-mediated endosome trafficking can alter Rab5-mediated trafficking to some extent; however, it alone may not alter APP metabolism. This finding is consistent with a previous report that the knockdown of Rab7, Rab9, or Rab11 did not induce A β secretion into the culture medium.⁶⁰ Although they reported that the knockdown of Rab11 strongly decreased A β secretion, they mainly examined HeLa cells that stably express mutant APP.⁵⁷ In the present study, we investigated endogenous APP metabolism, which may explain why there is some discrepancy about the effect of Rab11 knockdown.

The dynein-dynactin complex is required for the retrograde trafficking of Rab5-, Rab7-, and Rab11-positive endosomes.^{26,27,35} The recent finding that dynactin associates with retromer complex³⁶ suggests that dynein-dynactin is also involved in Rab9-positive endosome trafficking. Thus, dynein dysfunction can alter Rab5-, Rab7-, Rab9-, and Rab11-mediated endosome trafficking concomitantly, and the concomitant disruption of retrograde endosome trafficking might be responsible for the alteration of APP metabolism, as seen in aged monkey brains (Figure 6). It remains unclear why aging causes dynein dysfunction. Previous studies found that the phosphorylation of DHC down-regulates its activity as an ATPase for transport^{61,62} and that the phosphorylation of DIC causes its dissociation from the dynein-dynactin

complex.⁶² Several studies also found that aging affects the activity of protein phosphatases in the brain^{62–64} and that the activity of certain protein phosphatases, such as PP2A, is clearly decreased in AD brains.^{65–68} Our previous study found that the phosphatase inhibitor okadaic acid can also reproduce endocytic pathology in Neuro2a cells in a dose- and time-dependent manner.³⁵ These findings suggest that the alteration in the activity of protein phosphatases may be one of the causative factors for age-dependent dynein dysfunction. It is widely known that dynactin regulates dynein-mediated retrograde transport and that it consists of several subunits, such as dynactin-P150glued and dynactin-P50.^{28–33} Recently, Aboud et al⁶⁹ found that dynactin-P50 levels were significantly reduced in AD brains and that this reduction was much more severe in patients carrying the ApoE ϵ 4/4 allele. This finding strongly suggests that dynein dysfunction is indeed involved in age-dependent AD pathology and that it may be one of the key factors contributing to AD pathogenesis.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.03.006>.

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