Dynein Dysfunction Induces Endocytic Pathology Accompanied by an Increase in Rab GTPases

A POTENTIAL MECHANISM UNDERLYING AGE-DEPENDENT ENDOCYTIC DYSFUNCTION

Nobuyuki Kimura†1, Makoto Inoue5, Sachi Okabayashi§8, Fumiko Ono§8, and Takayuki Negishi∥

From the 1Laboratory of Disease Control, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba-shi, Ibaraki 305-0843, 5DNAVEC Research Inc., 1-25-11 Kannondai, Tsukuba-shi, Ibaraki 305-8421
The Corporation for Production and Research of Laboratory Primates, 1 Hachimandai, Tsukuba-shi, Ibaraki 305-0843, and the 8Department of Chemistry and Biological Science, School of Science and Engineering, Aoyama Gakuin University, 5-10-1 Fuchinobe, Sagamihara-shi, Kanagawa 229-8558, Japan

The appearance of neuronal endocytic pathology before senile plaque deposition suggests that endocytic dysfunction is involved in early stage Alzheimer disease (AD) pathology, such as the accumulation of β-amyloid precursor protein in enlarged early endosomes. However, it remains unclear how endocytic dysfunction is induced in an age-dependent manner. Cytoplasmic dynein, a microtubule-based motor protein, interacts with another microtubule-associated protein, dynactin. The resulting dynein-dynactin complex mediates endosome trafficking, including endosome trafficking. We have previously shown that the interaction between dynein-dynactin complexes is clearly attenuated in aged monkey brains, suggesting that dynein-mediated transport dysfunction exists in aged brains. Our immunohistochemical analyses revealed that age-dependent endocytic pathology was accompanied by an increase in Rab GTPases in aged monkey brains. Here, we demonstrated that siRNA-induced dynein dysfunction reproduced the endocytic pathology accompanied by increased Rab GTPases seen in aged monkey brains and significantly disrupted exosome release. Moreover, it also resulted in endosomal β-amyloid precursor protein accumulation characterized by increased β-site cleavage. These findings suggest that dynein dysfunction may underlie age-dependent endocytic dysfunction via the up-regulation of Rab GTPases. In addition, this vicious circle may worsen endocytic dysfunction, ultimately leading to Alzheimer disease pathology.

Growing evidence suggests that endocytic dysfunction is intimately involved in early stage Alzheimer disease pathology, such as the accumulation of β-amyloid precursor protein in enlarged early endosomes. However, it remains unclear how endocytic dysfunction is induced in an age-dependent manner. Cytoplasmic dynein, a microtubule-based motor protein, interacts with another microtubule-associated protein, dynactin. The resulting dynein-dynactin complex mediates endosome trafficking, including endosome trafficking. We have previously shown that the interaction between dynein-dynactin complexes is clearly attenuated in aged monkey brains, suggesting that dynein-mediated transport dysfunction exists in aged brains. Our immunohistochemical analyses revealed that age-dependent endocytic pathology was accompanied by an increase in Rab GTPases in aged monkey brains. Here, we demonstrated that siRNA-induced dynein dysfunction reproduced the endocytic pathology accompanied by increased Rab GTPases seen in aged monkey brains and significantly disrupted exosome release. Moreover, it also resulted in endosomal β-amyloid precursor protein accumulation characterized by increased β-site cleavage. These findings suggest that dynein dysfunction may underlie age-dependent endocytic dysfunction via the up-regulation of Rab GTPases. In addition, this vicious circle may worsen endocytic dysfunction, ultimately leading to Alzheimer disease pathology.

The appearance of neuronal endocytic pathology before senile plaque deposition suggests that endocytic dysfunction is involved in early stage Alzheimer disease (AD) pathology, such as the accumulation of β-amyloid precursor protein (APP) in enlarged early endosomes (1–4). β-Amyloid protein (Aβ), the major component of senile plaques, is produced from APP through sequential proteolytic cleavages by β- and γ-secretases, and such amyloidogenic cleavage, so-called β-site cleavage, of APP can occur through the endocytic pathway (1, 3). These findings suggest that endocytosis is involved in APP metabolism, and its dysfunction may lead to AD pathology. However, it remains unclear how endocytic dysfunction is induced in an age-dependent manner.

Cytoplasmic dynein is a microtubule-based motor protein required for minus end-directed axonal transport (5, 6). Dynactin, another microtubule-associated protein, binds to dynein intermediate chain (DIC) via its subunit, P150glued/dynactin (DYN) to form dynein-dynactin complexes that mediate minus end-directed vesicle transport, which includes endosome trafficking (7–12). We have previously shown that the interaction between DIC and DYN is clearly attenuated in aged monkey brains, suggesting that aging may impair dynein-mediated transport (13). Other studies also support this idea (14–17). Thus, in this study we investigated age-dependent endocytic pathology in cynomologus monkey brains and tested our hypothesis of whether dysfunction of dynein causes endocytic dysfunction leading to AD pathology. Here, we demonstrated that siRNA-induced dysfunction of dynein reproduced the endocytic pathology and increased Rab GTPase levels observed in aged monkey brains. Moreover, dynein dysfunction also resulted in endosomal APP accumulation with a significant increase in β-site cleavage, representing early stage AD pathology.

EXPERIMENTAL PROCEDURES

Animals—Fourteen cynomolgus monkey (Macaca fascicularis) brains were used in this study. Of these, 6 brains were from young monkeys (ages 4 and 6 years), and 8 were from aged monkeys (ages 25, 26, 32, and 36 years). The frontal and temporal lobes of all the brains were used for immunohistochemical studies. The temporal lobes of eight cases (ages 4 years (n = 4), 25 years (n = 2), 26 years (n = 2)) were used for Western blot analyses.

All brains were obtained from the Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Japan. All animals were housed in individual cages and maintained according to the National Institute of Biomedical Innovation...
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rules and guidelines for experimental animal welfare. Three monkeys (ages 26 and 36 years) died naturally. The remaining animals were deeply anesthetized with pentobarbital.

**Antibodies**—In this study we used the following antibodies: mouse monoclonal anti-β-actin antibody (Sigma), mouse monoclonal anti-α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-DIC antibody (Chemicon, Temecula, CA), mouse monoclonal anti-secreted fragment of APP by α-secretase (sAPPα) antibody (IBL, Gunma, Japan), mouse monoclonal anti-Aβ40 C-terminal 35–40 residue antibody (1A10; IBL), mouse monoclonal anti-rodonet αβ N-terminal antibody (14F1; IBL), mouse monoclonal anti-Rab5 antibody (Rab5m; Santa Cruz Biotechnology), mouse monoclonal anti-Rab11 antibody (Rab11m; Abcam, Cambridge, UK), mouse monoclonal anti-trans-Golgi network (TGN) antibody (Abcam), mouse monoclonal anti-Alix antibody (Santa Cruz Biotechnology), mouse monoclonal antitransferrin receptor (TfR) antibody (Zymed Laboratories Inc., Carlsbad, CA), rabbit polyclonal anti-dynein heavy chain (DHC) antibody (Santa Cruz Biotechnology), rabbit polyclonal anti-DYN antibody (Santa Cruz Biotechnology), rabbit polyclonal anti-Rab5 antibody (Rab5r; Santa Cruz Biotechnology), rabbit polyclonal anti-Rab7 antibody (Rab7r; Sigma), rabbit polyclonal anti-Rab11 antibody (Rab11r; Abcam), rabbit polyclonal anti-flotillin-1 (Flo-1) antibody (Santa Cruz Biotechnology), rabbit polyclonal anti-APP C-terminal antibody (APPc; Zymed Laboratories Inc.), and rabbit polyclonal anti-secreted fragment of APP by β-secretase (sAPPβ) antibody (IBL). APPn predominantly recognizes full-length APP in Neuro2a cells (data not shown), and then it was used for immunocytochemistry. Because APPc can recognize both full-length APP and predominantly recognizes full-length APP in Neuro2a cells (data not shown), and then it was used for immunocytochemistry.

**Immunohistochemistry**—Immuno-staining of monkey brains was performed as described elsewhere (18). Briefly, the brain samples were immersion-fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. The sections were deparaffinized by pretreating with 0.5% periodic acid followed by autoclaving for 5 min at 121 °C, then incubated with a primary antibody solution overnight at 4 °C. In this study we used the following primary antibodies: anti-Rab5r (1:100), anti-Rab7r (1:200), and anti-Rab11r (1:200). After brief washes with buffer, the sections were sequentially incubated with biotinylated goat anti-mouse or anti-rabbit IgG then with a streptavidin-biotin-horseradish peroxidase complex (sABC kit; DAKO, Denmark). Immunoreactive elements were visualized by treating the sections with 3–3′ diaminobenzidine tetroxide (Dojin Kagaku, Japan). The sections were then counterstained with hematoxylin.

**Subcellular Fractionation of Monkey Brains**—Subcellular fractions from the temporal lobes of 8 monkeys were prepared at 0–4 °C as described by Tamai et al. (19). Briefly, sucrose solutions were prepared in a buffer containing 10 mM Tris-HCl (pH 7.6), 0.25 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. Brain tissue (~1.0 g) was homogenized in a glass homogenizer with 10 ml of 0.32 M sucrose solution and then centrifuged at 1000 × g for 10 min. The pellet (P1) was resuspended in 0.32 M sucrose, and this solution was layered over a discontinuous density gradient consisting of 1.2 and 0.85 M sucrose solutions. The suspension was centrifuged at 75,000 × g for 30 min in a Hitachi RPS-27 swing rotor to separate out P1 myelin and nuclei fractions. The supernatant (S1) from the P1 fraction was centrifuged at 13,000 × g for 15 min to yield the P2 fraction. The supernatant (S2) from the P2 fraction was centrifuged at 105000 × g for 60 min to obtain the microsomal (pellet) and cytosol (supernatant) fractions.

In this study we used the microsomal fraction for Western blot analyses. To normalize loading differences and confirm the purity of the fraction, we immunoblotted the fractions as described previously (20). Endoplasmic reticula and vesicles in the microsomal fraction were examined with mouse monoclonal anti-calnexin antibody (Santa Cruz Biotechnology; 1:500).

**Western Blot Analyses for Age-related Changes in Rab GTPases in Monkey Brains**—The proteins in the microsomal fractions were adjusted to 10 μg, and then each fraction was analyzed using SDS-PAGE and 12.5% acrylamide gels. Separated proteins were blotted onto polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dried milk in 20 mM PBS (pH 7.0) and 0.1% Tween 20 for 1 h at room temperature and then incubated with primary antibodies overnight at 4 °C. In this study we used the following primary antibodies: anti-Rab5 (1:1000), anti-Rab7 (1:5000), and anti-Rab11 (1:5000). They were then incubated with either horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. Immunoreactive elements were visualized using enhanced chemiluminescence (Immobilon Western Detection Reagents; Millipore).

**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/cm^2 onto 6-well plates coated with 0.01% poly-l-lysine (Wako, Osaka, Japan) for Western blot analyses and at 1.0 × 10^4 cells/cm^2 onto 2-well LAB-TEK chamber slides (Nalge Nunc, Rochester, NY) coated with 0.01% poly-l-lysine 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/cm^2 onto 6-well plates coated with 0.01% poly-l-lysine for Western blot analyses at 1.5 × 10^4 cells/cm^2 onto 60-mm dishes coated with 0.01% poly-l-lysine for immunonoprecipitation and at 1.0 × 10^4 cells/cm^2 onto 2-well LAB-TEK chamber slides (Nalge Nunc) coated with 0.01% poly-l-lysine for immunocytochemistry.

**RNA Interference**—For double-stranded RNA-mediated interference (RNAi) experiments we used the following short double-stranded RNA (siRNA) against primate-specific DHC (siDHC) and DYN (siDYN) for COS-7 cells and against mouse-specific DHC (siDHCM) for Neuro2a cells: siDHC, 5'-GGUG-GGUCCAGUAAUACGGAU-3' (sense) and 5'-UUCGUAAU- GUACCCACCCUG-3' (antisense); siDYN, 5'-GGACCGCG- UGAUCUGAGCAC-3' (sense) and 5'-UCUUCGCAUAC- AGGCGUCAC-3' (antisense); siDHCM, 5'-GUACACGA- CGGAGUUCGUG-3' (sense) and 5'-AAAAACUCGGUCG-
UGAUACUC-3' (antisense). To avoid off-target effects, all siRNAs were carefully designed by Enhanced siDirect®. We also examined two different siRNAs for each target (see the supplemental table) and confirmed their knockdown-specific effects (data not shown). The control siRNA had a random sequence. RNAi experiments were performed by using siLentFect™ lipid reagent (Bio-Rad) according to the manufacturer's protocol.

Twenty-four or seventy-two hours after siRNA transfection, cells plated on 6-well plates were harvested in PBS and centrifugated to obtain cell pellets. The pellets were lysed in a sample buffer solution containing 62.5 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.5% Triton X-100, 2% SDS, and Complete Mini™ protease inhibitor mixture (Roche Applied Science) to extract total cellular proteins. Total proteins were adjusted to 10 μg and then subjected to SDS-PAGE by using 6% (DHC), 12.5% (other proteins), or 16% acrylamide gels (α-CTF, β-CTF, and Aβ). Separated proteins were blotted onto nitrocellulose membranes (Pall Life Sciences, Ann Arbor, MI) (Aβ) or polyvinylidene fluoride membranes (other proteins). Blotted nitrocellulose membranes were heated in boiling PBS for 5 min to enhance the signal (21) and then subjected to Western blot analyses as described above. We used the following primary antibodies: anti-β-actin (1:50000), anti-DHC (1:500), anti-DIC (1:20000), anti-DYN (1:2000), anti-Rab5r, anti-Rab7, anti-Rab11r, anti-APPn (1:1000), anti-sAPPα (1:1000), anti-sAPPβ (1:5000), and anti-Aβ40 C-terminal (1:500). We performed three independent experiments (n = 6 for each experimental group), duplicating each experiment.

Cells plated on chamber slides were fixed with 4% paraformaldehyde and then permeabilized with 0.01% (COS-7) or 0.1% (Neuro2a) Triton X-100 for 5 min at room temperature. All cells were examined with a Digital Eclipse C1 confocal microscope (NIKON, Kanagawa, Japan). For immunocytochemistry, we used the following primary antibodies: anti-β-actin, anti-DIC, anti-DYN, anti-Rab5r, anti-Rab7, and anti-Rab11r. We performed three independent experiments. We used the following primary antibodies: anti-β-actin, anti-α-tubulin (1:1000), anti-DYN, anti-Rab5r, anti-Rab7, and anti-Rab11r. We performed three independent experiments.

Semiquantitative Reverse Transcription-PCR—Primer sets were designed to recognize and amplify nucleotide sequences encoding mouse DHC (NM_030238), APP (NM_007471), and β-actin (NM_007393). cDNA sequences and/or the homologue(s) was identified using the BLAST (Basic Local Alignment Search Tool) computer program (National Center for Biotechnology Information, Bethesda, MD). Primers were designed using the Primer3 computer program (Whitehead Institute, Cambridge, MA).

Seventy-two hours after siRNA transfection, total cellular RNAs from Neuro2A cells were isolated using TRizol reagent (Invitrogen). Single-stranded cDNAs were prepared via reverse transcription of a reaction mixture of total RNA (1 μg in 20 μl of reaction mixture), oligo(dT)12–18 primers, and Superscript™ II (Invitrogen). PCR amplification was performed using Thermo-Start™ (Thermo Fisher Scientific, Epsom, UK) and the following primers: DHC (−), 5'-CTCGGAAGCTTCTGACAAAC-3'; DHC (+), 5'-CATCAGCTGT-TTCCAGTG3'; APP (+), 5'-GGTCTGGGCTGACAAAC- CAT-3'; APP (−), 5'-GTGATGACAATCACGGTTG3'-3' β-actin (+), 5'-ATGGATGACGATATCCTG-3'; β-actin (−), 5'-ATGGAGGATGTCTGTAGG-3'.

PCR products were electrophoresed in 1.5% agarose gels, and gels were subsequently stained with ethidium bromide. Densitometric analyses of gels were carried out with Quantity One Software (PDI Inc.). We performed three independent experiments (n = 6 for each experimental group), duplicating each experiment.

Immunoprecipitation—All steps were performed at 4 °C unless otherwise noted. For immunoprecipitation, we used rec-protein G-Sepharose™ 4B beads (Zymed Laboratories Inc.). Seventy-two hours after siRNA treatment, Neuro2a cell cultures (60 mm dish) were lysed in immunoprecipitation buffer solution containing 25 mM Tris-HCl (pH 8.0), 0.5% Triton X-100, 0.5% Nonidet P-40, and Complete Mini™ protease inhibitor mixture and then centrifuged at 105,000 × g for 15 min. The supernatant was recovered and then precleaned with Sepharose beads for 1 h. The precleared supernatant containing 10 μg of proteins was incubated with anti-Aβ40 C-terminal antibody (1A10; 1 μg) or mouse IgG (Rockland Immunochemicals, Gilbertsville, PA) for 2 h. After immunoreactions, antibody-linked supernatant was incubated with precleared beads for 1 h. The beads were washed with lysis buffer twice and then finally with Tris-buffered saline (50 mM Tris-HCl, pH 6.8). Protein was eluted from the beads with electrophoresis sample buffer and then analyzed using SDS-PAGE (16% acrylamide gels). Separated proteins were blotted onto nitrocellulose membranes. Blotted nitrocellulose membranes were heated in boiling PBS for 5 min to enhance the signal (21) and then subjected to Western blot analyses with anti-rodent Aβ N-terminal antibody (14F1; 1:2000). We performed five independent experiments.

Preparation of the Extracellular Membrane Fraction—Seventy-two hours after siRNA treatment, culture media from...
each well of Neuro2a cell cultures (6-well plate) were collected and centrifuged for 10 min at 1000 × g to exclude cell debris. The supernatant was subjected to ultracentrifugation at 10,000 × g for 30 min and at 100,000 × g for 1 h. The resulting pellets were resuspended in sample buffer and then subjected to Western blot analyses. We used the following primary antibodies: anti-Alix (1:1000), anti-Flo-1 (1:10000), anti-TfR (1:10000), and anti-APPc. We examined 12 independent samples for each group.

Data Analyses—To confirm reproducibility, immunoreactive bands obtained from the Western blots were quantified using commercially available software (Quantity One; PDI, Inc., Upper Saddle River, NJ). Data are shown as the means ± S.D. For statistical analyses, one-way analysis of variance was performed followed by the Bonferroni Dunn post hoc test.

RESULTS

Age-dependent Endocytic Pathology Is Observed in Aged Monkey Brains, and Rab GTPases Are Concurrently Increased—The cynomolgus monkey is a good animal model for studying age-dependent AB pathology without transgenic manipulations (18, 22, 23). Thus, we used this model first to investigate whether aging itself causes endocytic pathology in monkey brains. Immunohistochemical analyses revealed that early (Rab5-positive), late (Rab7-positive), and recycling (Rab11-positive) endosomes were apparently enlarged mainly in large pyramidal neurons of aged monkey brains (Fig. 1A). Endosome trafficking is regulated by small Rab GTPases such as Rab5, Rab7, and Rab11 (24). Therefore, to investigate the relationship between age-dependent endocytic pathology and Rab GTPase levels, we performed Western blot analyses on tissue from the same monkey brains used for immunohistochemistry. In aged monkey brains, the amounts of Rab GTPases were significantly increased respectively (Fig. 1, B and C).

siRNA-induced Dysfunction of Dynein Causes Endocytic Pathology with a Concomitant Increase in Rab GTPases—We previously showed that dynein-dynactin interactions are attenuated in aged monkey brains, suggesting that dynein-mediated transport in aged brains is dysfunctional (13). To test our hypothesis that dysfunction of dynein-mediated transport may cause the age-dependent endocytic pathology observed in aged monkey brains, we performed RNAi experiments using COS-7 cells. COS-7 cells have large somas and are, thus, useful for studying endosomal trafficking. We chose DHC as a main target to knock down because it is well known that siRNAs targeting DHC also induce significant down-regulation of DIC (25). DHC has an ATPase activity for dynein-mediated transport, and DIC interacts with DYN to assemble dynein-dynactin complexes responsible for minus end-directed vesicle transport (5–12, 26). Hence, the depletion of both DHC and DIC should cause severe dysfunction of dynein-mediated transport.

In siDHC-transfected cells, both DHC and DIC expression levels clearly dropped 24 h after transfection, indicating effective depletion of dynein (Fig. 2A). Dynein depletion noticeably lasted up to 72 h after transfection (Fig. 2A). In contrast to control siRNA-transfected cells, in siDHC-transfected cells dynein depletion resulted in an increase in Rab7 24 h after transfection; this increase became significant 72 h after transfection (Fig. 2, A and B). Unlike Rab7, Rab5 expression levels remained more or less the same 24 h after transfection before increasing at the 72-h point (Fig. 2, A and B). Interestingly, Rab11 expression clearly increased 24 h after transfection, but it increased even more 72 h after transfection (Fig. 2, A and B). Dynein depletion also resulted in a significant increase in DYN (Fig. 2, A and B).

To confirm the relationship between elevated Rab GTPase levels and endocytic pathology, we immunostained cells with antibodies against various Rab GTPases 72 h after siRNA transfection. DIC-negative cells (i.e. cells depleted of dynein) possessed enlarged endosomes that showed variable localization patterns (Fig. 2C). By contrast, Rab7-positive endosomes mainly localized around nuclei in control siRNA-transfected cells and enlarged Rab7-positive endosomes distributed around nuclei as well as in peripheral parts of the cytoplasm (Fig. 2C). The localization of Rab11-positive endosomes showed the most drastic change. In control siRNA-transfected cells, most Rab11-positive endosomes gathered and localized to the juxtanuclear region, also known as the juxtanuclear endocytic recycling compartment (Fig. 2C). The depletion of dynein disrupted the juxtanuclear assembly of Rab11-positive endosomes such that enlarged Rab11-positive endosomes distributed throughout the cytoplasm (Fig. 2C). Although Rab5 expression only increased slightly, even 72 h after transfection both Rab7 and Rab11 expression increased significantly due to the depletion of DYN (supplemental Fig. S1). Interestingly, the depletion of DYN resulted in the significant increase in DIC (supplemental Fig. S1).

Drug-induced Perturbation of Endosome Trafficking to lysosomes Can Also Cause Recycling Endocytic Pathology—Recruitment of recycling endosomes to endocytic recycling compartment is mediated by dynein (27, 28). To assess whether recycling endocytic pathology is directly caused by disturbance of its recruitment or whether it represents a secondary consequence of disrupting endosome trafficking to the lysosomal degradation pathway, we examined how chloroquine and ammonium chloride, two drugs well known to perturb endosome trafficking to lysosomes independent of dynein function (29), affect the levels of endosome-related GTPases.

FIGURE 1. Endocytic pathology accompanied by increase in Rab GTPases in aged monkey brains. A, photomicrographs of temporal lobe sections from a 4-year-old (Young) monkey and a 26-year-old (Aged) cynomolgus monkey are shown. Sections were immunostained with anti-Rab5r, anti-Rab7, and anti-Rab11r antibodies and then counterstained with hematoxylin. In aged monkey brains, early (Rab5-positive), late (Rab7-positive), and recycling (Rab11-positive) endosomes were enlarged mainly in large pyramidal neurons. The high magnification image (inset) shows enlarged endosomes (arrowheads) accumulated in an aged monkey brain. Scale bars, 10 μm. B, Western blots show the amounts of Rab5, Rab7, Rab11, and calnexin in microsomal fractions derived from 4- and 26-year-old monkey brains. The amounts of Rab GTPases were clearly increased in aged monkey brains. C, histograms show age-related changes in the amounts of Rab GTPases in young (n = 4) and aged (n = 4) monkey brains. All data were normalized according to calnexin levels. Values are the means ± S.D. *, p < 0.01; **, p < 0.001. y axes show the mean values of the quantified data.
As expected, both chloroquine and ammonium chloride significantly increased Rab5 and Rab7 expression, respectively. Moreover, enlarged Rab5- and Rab7-positive endosomes were clearly observed in treated cells, indicating that endocytic dysfunction was successfully induced (Fig. 3). Noteworthy, Rab11 significantly increased after the drug treatments, and enlarged Rab11-positive endosomes were also observed (Fig. 3).

siRNA-induced Dysfunction of Dynein Results in Endosomal APP Accumulation Leading to Enhancement of β-Site Cleavage and Also Disturbs Exosome Release—Finally, to test our hypothesis that dysfunction of dynein may cause endocytic dysfunction leading to early-stage AD pathology, we performed RNAi experiments using a neuronal cell line, Neuro2a. As with COS-7 cells, Neuro2a cells depleted of...
Dynein also showed a significant increase in Rab GTPases (supplemental Fig. S2). Because dynein-dynactin complexes associate with vesicle cargo via dynactin (30–35), DYN can be used as a marker for minus end-directed vesicle cargo. Immunocytochemical analyses demonstrated that DYN accumulated in the distal ends of neurite-like processes due to depletion of dynein (supplemental Fig. S2), indicating that dynein depletion disturbed minus end-directed vesicle transport.

In this study we aimed to examine endogenous APP metabolism. Noteworthy, dynein depletion significantly increased endogenous APP and its β-site cleavage products such as sAPPβ, βCTF, and Aβ, respectively (Fig. 4, A and B). Immunoprecipitation analyses also confirmed that the amount of Aβ was increased in siDHCm-transfected cells (Fig. 4C). APP can be alternately cleaved by α-secretase within the Aβ domain, resulting in productions of sAPPα and αCTF (36, 37). In contrast to β-site cleavage products, the amount of sAPPα and αCTF seemed unchanged by dynein depletion (Fig. 4, A and B). Immunocytochemical analyses confirmed that APP clearly accumulated in dynein-depleted cells and mainly localized to enlarged Rab5-positive endosomes, i.e. early endosomes (Fig. 4F). Moreover, dynein depletion also caused APP to accumulate in the distal ends of neurite-like processes (Fig. 4F).

To determine whether exosome secretion pathway is also affected by dynein dysfunction, we prepared extracellular membrane fractions from siRNA-transfected Neuro2a cells 72 h after transfection. In contrast to control siRNA-transfected cells, the amounts of both Alix and Flo-1, two exosome markers, were significantly decreased in extracellular membrane fractions derived from dynein-depleted cells (Fig. 4G).
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A

DHC
DIC
APP
sAPPα
sAPPβ
βCTF
αCTF
Aβ
β-actin

B

APP
sAPPα
sAPPβ

αCTF
βCTF
Aβ

C

IP : 1A10
WB : 14F1

IP : Mouse IgG
WB : 14F1

CT siRNA

CT siRNA

D

CT siRNA

E

APP mRNA

F

CT

DIC
APP
Merge

siRNA

DIC
APP
Merge

CT

Rab5
APP
Merge

siRNA

Rab5
APP
Merge

siRNA

TGN
APP
Merge

G

Cell

EM

CT siRNA

CT siRNA

Alix
Flo-1
TfR
APP

CT siRNA

CT siRNA
Furthermore, TFR and APP levels were also considerably decreased in extracellular membrane fractions (Fig. 4G).

**DISCUSSION**

Here, we demonstrated that dysfunction of dynein causes multi-endocytic pathology with an increase of Rab GTPases, and it may underlie age-dependent endocytic dysfunction leading to early stage of AD pathology such as endosomal accumulation of APP.

In this study immunohistochemical analyses of monkey brains indicated that age-dependent endocytic pathology was accompanied by an increase in Rab GTPases (Fig. 1). A recent study showed that over-activation of Rab GTPase causes aberrant up-regulation of endosome trafficking and endocytosis, resulting in endocytic pathology (38). Taken together, these findings suggest that aging can cause an increase in Rab GTPases and induce endocytic pathology.

We previously showed that dynein-dynactin interactions are attenuated in aged monkey brains (13). Because the dynein-dynactin complex mediates endosome trafficking (39–43), we hypothesized that age-dependent dysfunction of dynein-mediated transport disrupts endosome trafficking, resulting in the compensatory up-regulation of Rab GTPases. Our RNAi experiments demonstrated that the depletion of dynein induced a significant increase in Rab GTPases. Immunocytochemical analyses confirmed endocytic pathology in the dynein-depleted cells (Fig. 2). This finding suggests that dynein dysfunction itself can cause aberrant up-regulation of endosome trafficking, leading to endocytic pathology. Importantly, the aberrant up-regulation of endosome trafficking also perturbs dynein-mediated transport (38). Thus, the dysfunction of dynein and the up-regulation of endosome trafficking may represent a “vicious circle” that leads to endocytic dysfunction.

In the present study the increase of Rab7 preceded that of Rab5 (Fig. 2), suggesting that dynein dysfunction primarily or strongly affects the trafficking of late endosomes before subsequently affecting the trafficking of early endosomes retrogradely. Because dynein mediates the fusion of late endosomes with lysosomes (39, 41, 43, 44), dynein dysfunction would perturb fusion, resulting in impairment of lysosomal degradation (Fig. 5). Thus, it is reasonable that the compensatory up-regulation of endosome trafficking may represent a “vicious circle” that leads to endocytic dysfunction.

Intriguingly, the depletion of dynein caused a significant increase in DYN, whereas the depletion of DYN caused a significant increase in DIC and vice versa (Fig. 1, supplemental Fig. S1). This finding suggests that dysfunction of one component of the dynein-dynactin complex may induce compensatory up-regulation of the other component. Because we have previously shown that DYN levels were significantly increased in aged monkey brains (13), age-dependent dysfunction of minus-end directed vesicle transport may be caused by a dysfunction in dynein rather than dynactin.

Most noteworthy, our RNAi experiments with Neuro2a cells demonstrated that dysfunction of dynein caused intracellular accumulation of APP, which was accompanied by a significant increase in β-site cleavage products, resulting in intracellular accumulation of Aβ (Fig. 4). Because neither sAPPα nor AβCTF showed significant changes in dynein-depleted cells, dynein dysfunction would not affect α-site cleavage. Immunocytochemistry also confirmed early-stage AD pathology, such as APP accumulation in enlarged early endosomes. Although semiquantitative reverse transcription-PCR analyses showed that the APP mRNA level seemed rather increased in dynein-depleted cells, APP mainly accumulates in enlarged early endosomes but not recycling endosome or TGN, and the APP level in extracellular membrane fraction was significantly decreased (Fig. 4). These findings suggest that APP endocytosis is really induced by the dysfunction of dynein, and intracellular accumulation of APP would not be the simple consequence of enhanced APP expression but may be attributed to endosomal retention and subsequent avoidance of lysosomal degradation. Although additional studies are needed, this finding suggests that dysfunction of dynein-mediated transport is involved at least partly in age-dependent AD pathology via endocytic dysfunction.

Moreover, the dysfunction of dynein also resulted in an increase in Rab11, the recycling endosome-associated Rab GTPase (Fig. 2). Our drug treatment experiments confirmed that drug-induced perturbation of endosome trafficking to lysosomes increases Rab11 and causes recycling endocytic pathology (Fig. 3). Hence, although we cannot fully exclude the possibility that dynein dysfunction directly disturbs the recruitment of recycling endosomes to endocytic recycling compartment, impaired trafficking of late endosomes to lysosomes may shift early endosomes to the recycling pathway to avoid intracellular vesicle accumulations.
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We hypothesize that age-dependent dysfunction of dynein-mediated transport may cause compensatory up-regulation of Rab GTPase, resulting in up-regulation of endosome trafficking (black solid arrows). Consequently, the up-regulation of Rab GTPases causes endosomes to enlarge (thick circle), which in turn perturbs endosome trafficking (dotted arrows) and leads to the deterioration of dynein-mediated transport. This vicious circle may cause the unwanted uptake and/or accumulation of APP in early endosomes, and the retardation of endosome trafficking may result in enhanced endosomal β-site cleavage. Moreover, the disruption of recycling endosome trafficking and the decrease of exosome release may also cause intracellular protein/vesicle accumulation. Additional investigations are needed, however, to clarify whether multivesicular body (MVB) formation is up-regulated or down-regulated. EE, early endosome; LE, late endosome; RE, recycling endosome; LS, lysosome; EX, exosome.

FIGURE 5. Hypothetical scenario illustrating age-dependent endocytic dysfunction; a vicious circle leading to AD pathology. We hypothesize that age-dependent dysfunction of dynein-mediated transport may cause compensatory up-regulation of Rab GTPase, resulting in up-regulation of endosome trafficking (black solid arrows). Consequently, the up-regulation of Rab GTPases causes endosomes to enlarge (thick circle), which in turn perturbs endosome trafficking (dotted arrows) and leads to the deterioration of dynein-mediated transport. This vicious circle may cause the unwanted uptake and/or accumulation of APP in early endosomes, and the retardation of endosome trafficking may result in enhanced endosomal β-site cleavage. Moreover, the disruption of recycling endosome trafficking and the decrease of exosome release may also cause intracellular protein/vesicle accumulation. Additional investigations are needed, however, to clarify whether multivesicular body (MVB) formation is up-regulated or down-regulated. EE, early endosome; LE, late endosome; RE, recycling endosome; LS, lysosome; EX, exosome.

Our immunocytochemistry analyses revealed that APP accumulated within the distal ends of processes partly localized to Rab5-positive endosomes (Fig. 4). This suggests that age-dependent synaptic accumulation of APP may be caused not solely by age-dependent dysfunction of minus-end transport but also by local up-regulation of APP endocytosis. This, in turn, may lead to the concurrent accumulation of synaptic Aβ with the age-dependent down-regulation of neprilysin, an Aβ-degrading enzyme that mainly localizes to synapses (45).

We also demonstrated that dynein dysfunction significantly decreased exosome release (Fig. 4). Recent findings suggest that β-site cleavage may occur in multivesicular bodies (46) and that Aβ-containing exosomes are released into the extracellular space (47, 48). Although it remains unclear whether the release of Aβ represents a method of eliminating intracellular Aβ or simply achieves some other biological function, dynein dysfunction clearly decreased exosome release (Fig. 4) and may also lead to the accumulation of intracellular Aβ.

TIR levels in extracellular membrane fractions were also significantly decreased by the dysfunction of dynein even though TIR levels in whole-cell lysates did not significantly change (Fig. 4). This finding supports the premise that dynein dysfunction definitely disturbs recycling endosome trafficking. These findings suggest that dynein dysfunction may cause proteins to accumulate intracellularly by disturbing endosome trafficking at multiple levels, such as perturbations in lysosomal degradation, recycling endosome trafficking, and exosome release (Fig. 5). The intracellular accumulation of causative proteins is a common pathological feature of age-dependent neurodegenerative diseases (49–51). Although additional studies are needed, we propose that the age-dependent dysfunction of dynein may be a risk factor not only for AD but also for other age-dependent neurodegenerative diseases.

How aging causes dynein dysfunction remains unclear. One possible way is through the abnormal up-regulation of protein phosphorylation. Phosphorylation of DHC down-regulates DHC activity as an ATPase for transport (52, 53). On the other hand, phosphorylation of DIC causes it to detach from DYN, resulting in the dissociation of the dynein-dynactin complex (53). Several studies have shown that aging affects the activity of protein phosphatases in the brain (54–56) and that the activity of certain protein phosphatases, such as PP2A, is clearly decreased in AD brains (57–61). Moreover, neurofibrillary tangles, a late-stage neuropathological hallmark of AD, evidently result from the hyperphosphorylation of Tau protein (62). To assess the hypothesis that abnormal phosphorylation is responsible for age-related dynein dysfunction, we treated Neuro2a cells with the phosphatase inhibitor OA. Even at very low concentrations, OA treatment increased Rab7 levels in a dose- and time-dependent manner (supplemental Fig. S3). One study showed that OA treatment induces calpain activity leading to DIC degradation (63); however, we did not find evidence to support this observation at the dose used in the present study. Instead, OA treatment increased DYN levels, which is indicative of dynein dysfunction (supplemental Fig. S3). Immunocytochemistry confirmed that in OA-treated cells Rab7-positive

caused APP accumulation in the distal ends of neurite-like processes (Fig. 4).

We previously showed that APP significantly accumulates in the nerve-ending fraction, which includes synaptic vesicles and membranes, from aged monkey brains (22). Intracellular Aβ content is also significantly increased in the nerve-ending fraction with aging (18). Our immunocytochemistry analyses revealed that APP accumulated within the distal ends of processes partly localized to Rab5-positive endosomes (Fig. 4). This suggests that age-dependent synaptic accumulation of APP may be caused not solely by age-dependent dysfunction of minus-end transport but also by local up-regulation of APP endocytosis. This, in turn, may lead to the concurrent accumulation of synaptic Aβ with the age-dependent down-regulation of neprilysin, an Aβ-degrading enzyme that mainly localizes to synapses (45).
endosomes were enlarged, and their localization was affected even though microtubule assembly was not significantly changed (supplemental Fig. S3). These findings suggest that the increase in Rab7, reflecting dynein dysfunction, induced by OA treatment resulted neither from DIC depletion nor microtubule disruption. Because the colocalization of DIC and DYN was not appreciably affected by OA treatment at the dose we used (supplemental Fig. S3), dynein dysfunction may be caused by phosphorylation of DHC.

In conclusion, we demonstrated that dysfunction of dynein induces endocytic pathology accompanied by an increase in Rab GTPases, which may underlie age-dependent endocytic dysfunction. Although additional investigations are needed, we believe that this vicious circle continues to worsen endocytic dysfunction, ultimately leading to AD pathology such as the accumulation of intracellular APP and Aβ (Fig. 5). Moreover, because dynein also mediates the transport and fusion of autophagosomes with lysosomes (64–66), the intracellular transport system may represent a prime target for the development of new therapeutics used to treat not only AD but also other neurodegenerative disorders characterized by the abnormal intracellular accumulation of causative proteins.

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