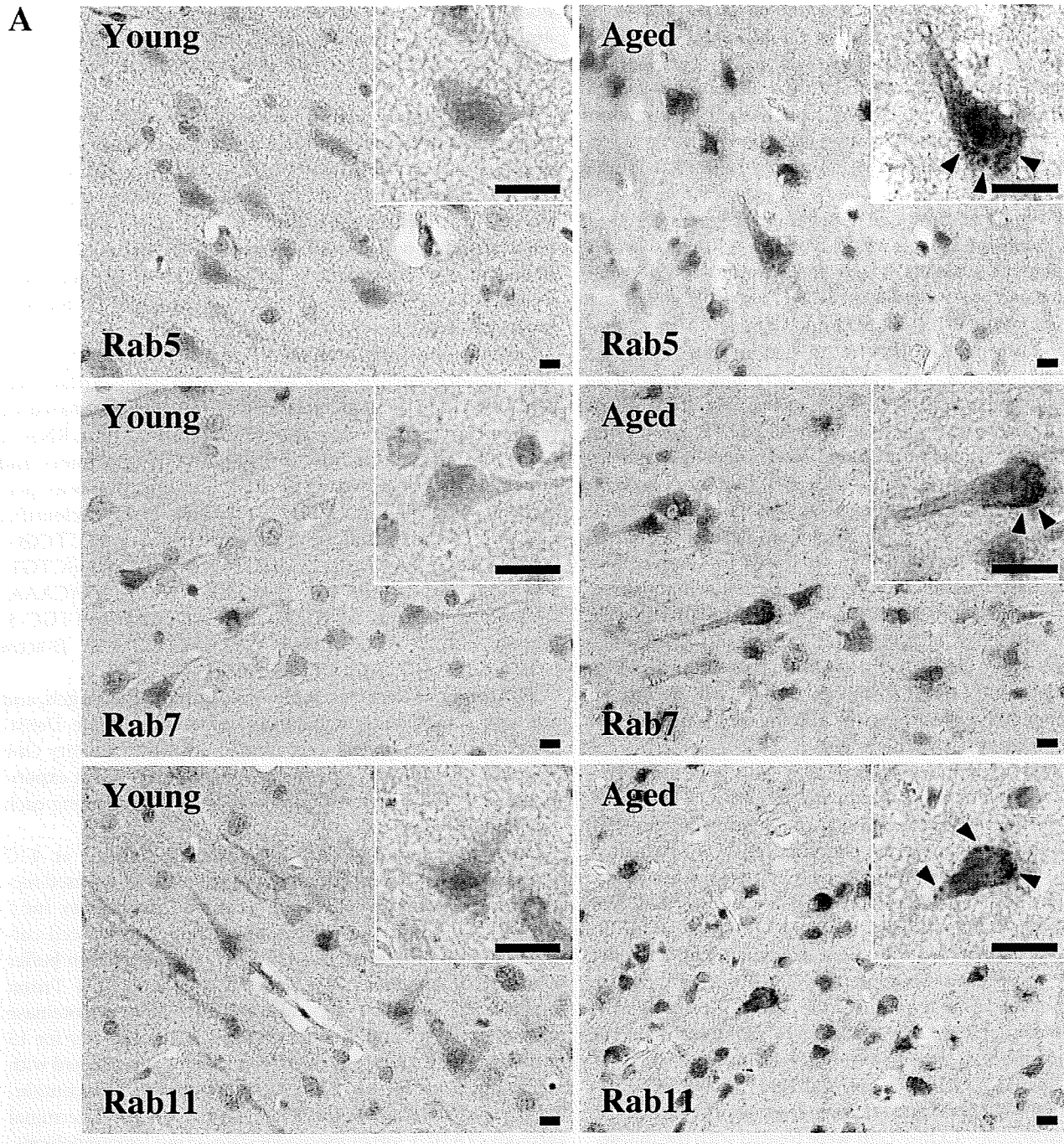
A
Young
Rab5
Aged
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Rab7
Rab11
Calnexin
C
Rab5
*
Young
Aged
Rab7
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Aged
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Young
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each well of Neuro2a 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 Aβ 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. 1*A*). 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. 2*A*). Dynein depletion noticeably lasted up to 72 h after transfection (Fig. 2*A*). 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. 2*C*). 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. 2*C*). 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. 2*C*). The depletion of dynein disrupted the juxtanuclear assembly of Rab11-postive endosomes such that enlarged Rab11-positive endosomes distributed throughout the cytoplasm (Fig. 2*C*). 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.

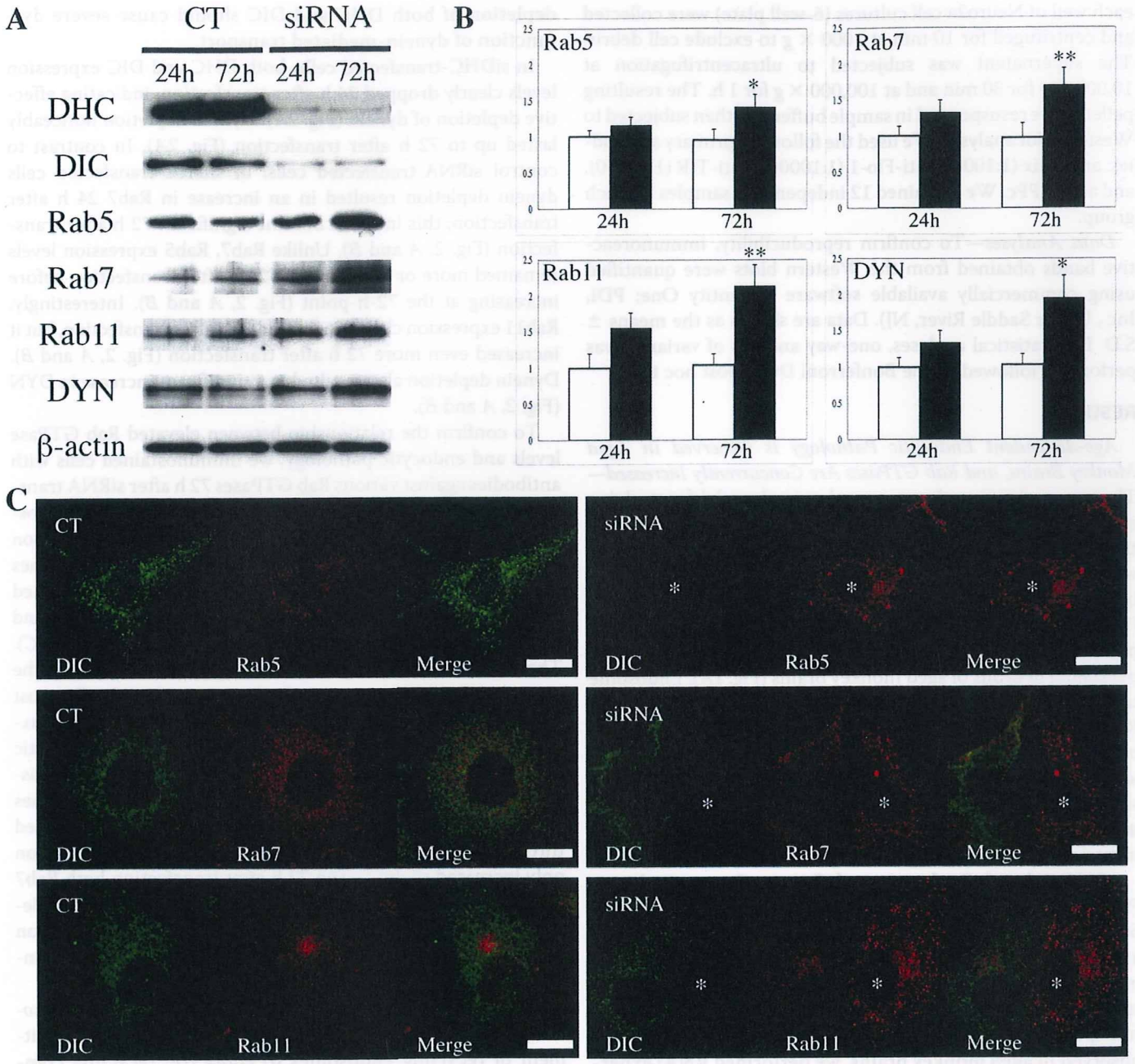


FIGURE 2. **siRNA-induced dynein dysfunction reproduces endocytic pathology accompanied by an increase in Rab GTPases.** *A*, Western blots show the amounts of DHC, DIC, Rab5, Rab7, Rab11, DYN, and β-actin in extracts derived from COS-7 cells 24 and 72 h after siRNA transfection. The amounts of DHC and DIC clearly dropped even as soon as 24 h after transfection. The knockdown effectively lasted up to 72 h after transfection. The depletion of dynein resulted in the time-dependent increase in Rab GTPases and DYN in COS-7 cells. *B*, histograms show the effect of dynein depletion on the amounts of Rab GTPases and DYN in COS-7 cells. The increase in Rab7 preceded that of Rab5 and became significant 72 h after transfection. The amount of Rab11 began to increase as early as 24 h after transfection and became quite significant 72 h after transfection. The amount of DYN also significantly increased 72 h after transfection. All data were normalized according to β-actin levels. Values are the means ± S.D. $*p < 0.05$; $**p < 0.001$. *y* axes show the mean values of the quantified data. *C*, photomicrographs of COS-7 cells immunostained for DIC, Rab5, Rab7, and Rab11 72 h after siRNA transfection. The depletion of dynein resulted in the accumulation of enlarged endosomes. *Scale bars*, 10 μm. *CT*, cells transfected with control si-RNA; *siRNA*, cells transfected with siDHC. *Asterisks*, DIC-immunonegative cells (dynein-depleted cells).

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

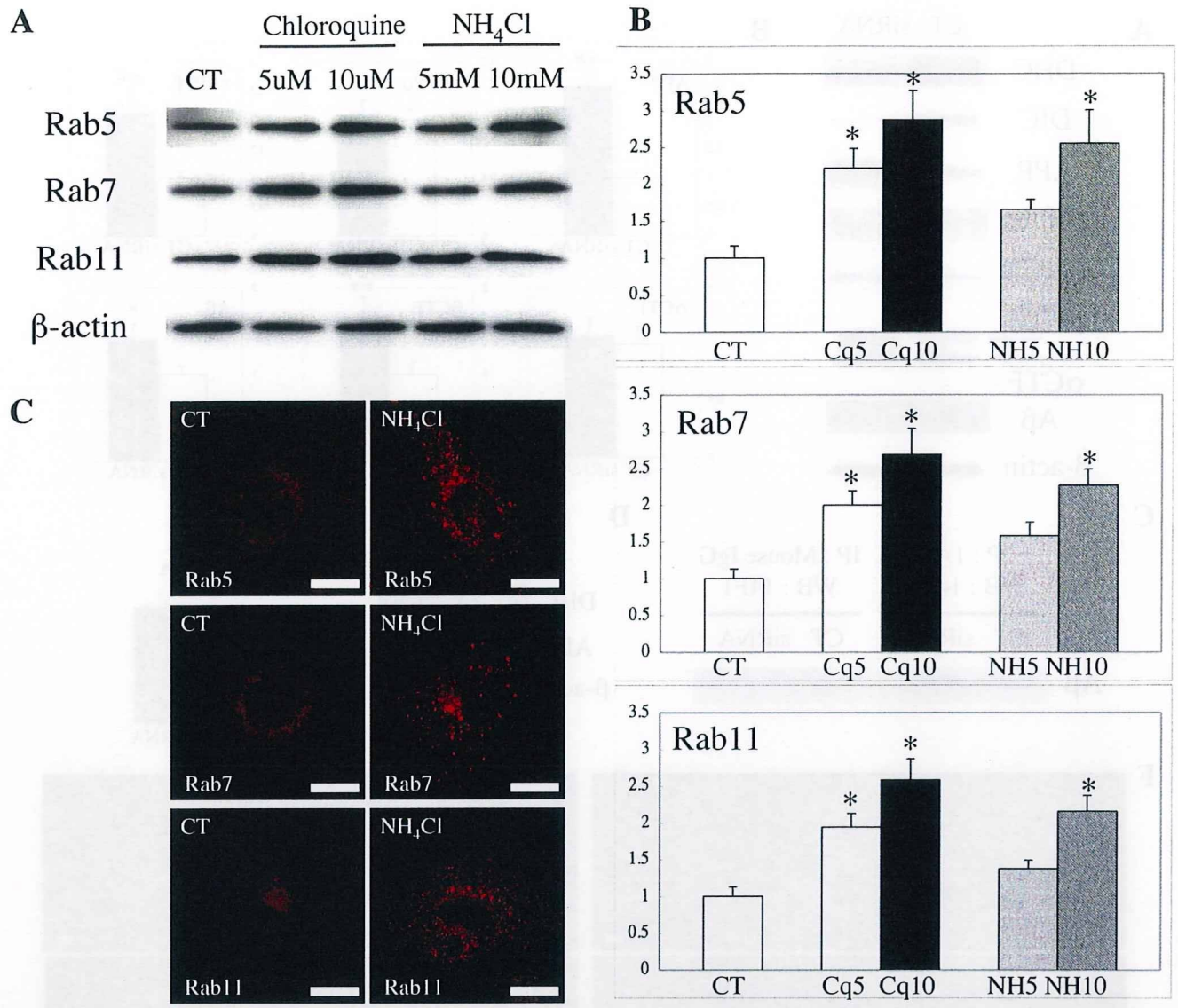
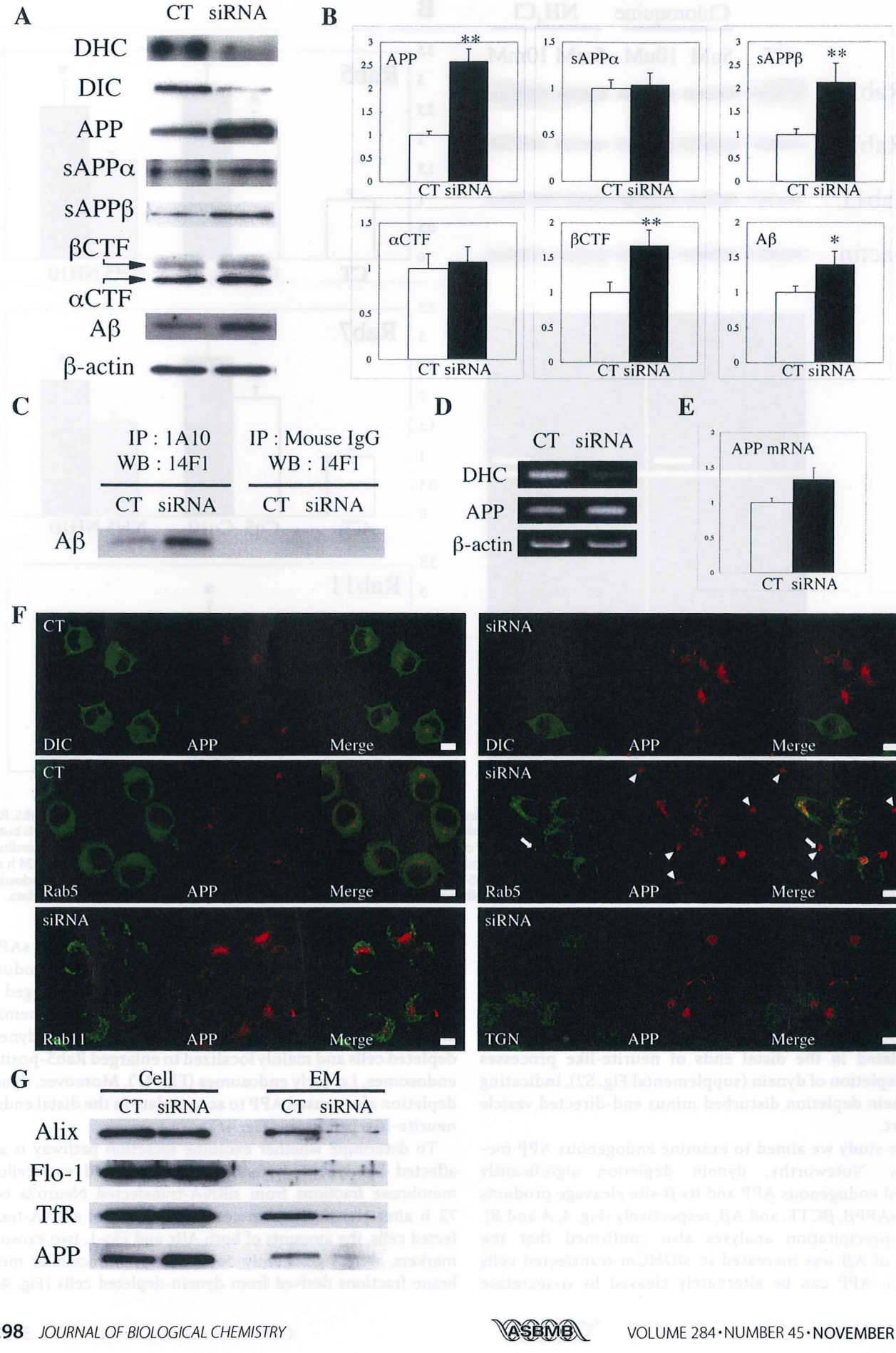


FIGURE 3. **Drug treatment reproduced recycling endocytic pathology independent of dynein function.** *A*, Western blots show the amounts of Rab5, Rab7, Rab11, and β-actin in COS-7 cells treated with chloroquine and ammonium chloride (NH_4Cl). Drug treatments induced increases in Rab GTPases levels but not β-actin levels, which remained unchanged. *B*, histograms show the effect of drug treatments on Rab GTPases levels. All data were normalized according to β-actin levels (*CT*, control). Values are the means ± S.D. *, $p < 0.05$. *C*, photomicrographs of COS-7 cells immunostained for Rab5, Rab7, and Rab11 24 h after drug treatment. NH_4Cl treatment induced enlargement not only of early (Rab5-positive) and late (Rab7-positive) endosomes but also of recycling endosomes (Rab11-positive). *Scale bars*, 10 μm. *Cq*, cells treated with chloroquine; *NH*, cells treated with NH_4Cl. *y* axes show the mean values of the quantified data.

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. 4*C*). 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. 4*F*). Moreover, dynein depletion also caused APP to accumulate in the distal ends of neurite-like processes (Fig. 4*F*).

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. 4*G*).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CT siRNA
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βCTF
Aβ
CT siRNA
C
IP : 1A10
WB : 14F1
IP : Mouse IgG
WB : 14F1
CT siRNA
Aβ
D
CT siRNA
DHC
APP
β-actin
E
APP mRNA
CT siRNA
F
CT
siRNA
DIC
APP
Merge
Rab5
Rab11
TGN
G
Cell
EM
CT siRNA
Alix
Flo-1
TfR
APP

Furthermore, TfR and APP levels were also considerably decreased in extracellular membrane fractions (Fig. 4*G*).

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 Rab7 would primarily be necessary to ensure cell viability, as Rab7 up-regulation enhances trafficking of late endosomes to fuse with lysosomes. Indeed, dynein dysfunction 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.

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 αCTF 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

FIGURE 4. **siRNA-induced dysfunction of dynein caused endosomal APP accumulation with enhanced β-site cleavage and perturbed recycling endosome trafficking and exosome release in Neuro2a neuronal cells.** *A*, Western blots show the amounts of DHC, DIC, APP, sAPPα, sAPPβ, αCTF, βCTF, Aβ, and β-actin in extracts from Neuro2a cells 72 h after siRNA transfection. The amount of APP was clearly increased, and the amount of β-site cleavage products such as sAPPβ, βCTF, and Aβ also increased concurrently. *B*, histograms show the effect of dynein depletion on the amounts of APP, sAPPα, sAPPβ, αCTF, βCTF, and Aβ in Neuro2a cells. The depletion of dynein significantly increased APP, sAPPβ, βCTF, and Aβ 72 h after siRNA transfection. All data were normalized according to β-actin levels. Values are the means ± S.D. *, $p < 0.05$; **, $p < 0.001$. *y* axes show the mean values of the quantified data. *C*, shown are immunoprecipitation (*IP*) analyses of endogenous Aβ in Neuro2a cells 72 h after siRNA transfection. The amount of Aβ was clearly increased in siDHCm-transfected cells. *WB*, Western blot. *D*, expression patterns of DHC, APP, and β-actin mRNA in Neuro2a cells 72 h after siRNA transfection. Expression was assessed with semiquantitative reverse transcription-PCR. The expression of DHC mRNA clearly dropped in siDHCm-transfected cells, indicating successful siRNA-induced down-regulation of DHC. *E*, the histogram shows the effect of dynein depletion on the expression of APP mRNA in Neuro2a cells. Data were normalized according to β-actin levels. Values are the means ± S.D. *y* axes show the mean values of the quantified data. *F*, photomicrographs are shown of Neuro2a cells immunostained for DIC, Rab5, Rab11, TGN, and APP 72 h after siRNA transfection. The depletion of dynein resulted in the accumulation of APP, which mainly localized to enlarged early endosomes. *Scale bars*, 10 μm. *Arrows*, enlarged early endosomes in the distal ends of neurite-like processes; *arrowheads*, accumulations of APP in the distal ends of neurite-like processes. *G*, Western blots showing the amounts of Alix, Flo-1, TfR, and APP in whole-cell extracts and extracellular membrane (*EM*) fractions from Neuro2a cells 72 h after siRNA transfection. Dynein depletion significantly decreased the levels of Alix and Flo-1, two exosome markers, in extracellular membrane fractions but not in whole-cell extracts (*Cell*). The depletion of dynein also significantly decreased TfR and APP levels in EM 72 h after siRNA transfection. *CT*, cells transfected with control siRNA; *siRNA*, cells transfected with siDHCm.

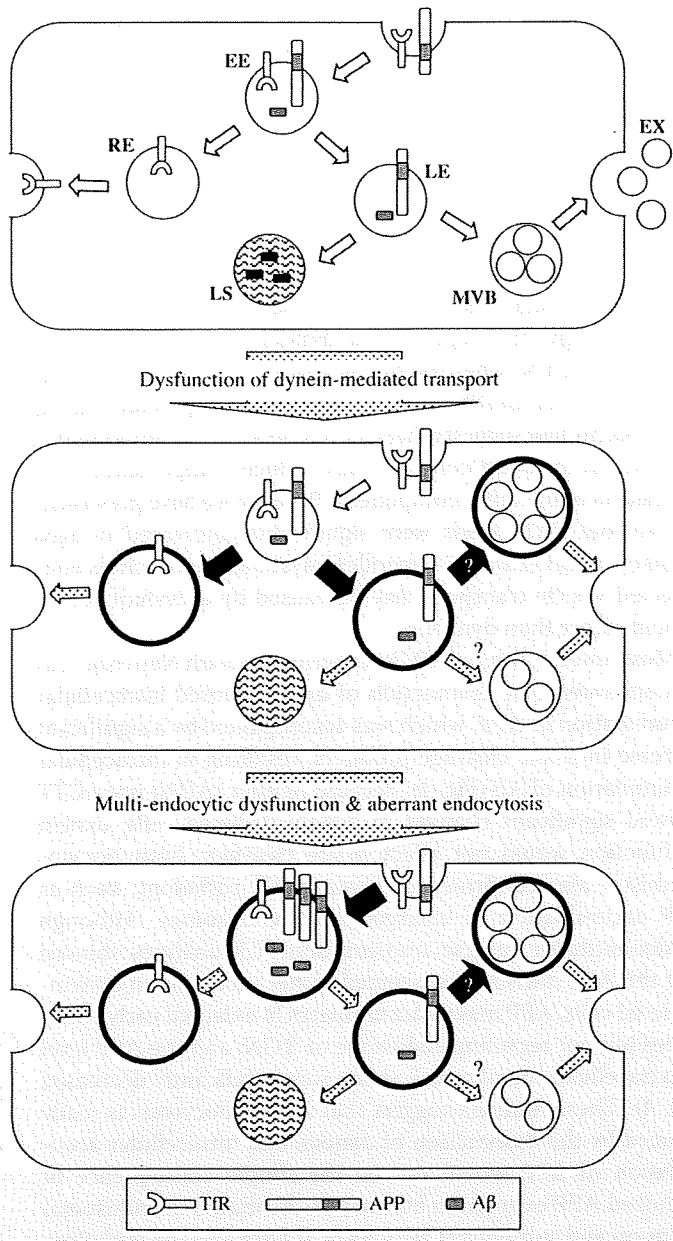

FIGURE 5. **Hypothetical scenario illustrating age-dependent endocytic dysfunction; a vicious circle leading to AD pathology.** We hypothesize that age-dependent dysfunction of dynein-mediated transport may cause compensatory up-regulation of Rab GTPase, resulting in up-regulation of endosome trafficking (*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.

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).

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.

TfR levels in extracellular membrane fractions were also significantly decreased by the dysfunction of dynein even though TfR 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

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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