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Increased Expression of Reticulon 3 in Neurons Leads to Reduced Axonal Transport of β Site Amyloid Precursor Protein-cleaving Enzyme 1*

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Background: Axonal transport of BACE1 and the regulation of BACE1 synaptic localization remain to be fully characterized.
Results: Colocalization of BACE1 with synaptophysin was reduced by overexpression of RTN3. This reduction was due to reduced BACE1 axonal transport.
Conclusion: Increased interaction of RTN3 with BACE1 in the soma impacts axonal transport of BACE1.
Significance: Changes of BACE1 synaptic localization potentially alter synaptic Aβ generation and amyloid deposition.

BACE1 is the sole enzyme responsible for cleaving amyloid precursor protein at the β-secretase site, and this cleavage initiates the generation of β-amyloid peptide (Aβ). Because amyloid precursor protein is predominantly expressed by neurons and deposition of Aβ aggregates in the human brain is highly correlated with the Aβ released at axonal terminals, we focused our investigation of BACE1 localization on the neuritic region. We show that BACE1 was not only enriched in the late Golgi, trans-Golgi network, and early endosomes but also in both axons and dendrites. BACE1 was colocalized with the presynaptic vesicle marker synaptophysin, indicating the presence of BACE1 in synapses. Because the excessive release of Aβ from synapses is attributable to an increase in amyloid deposition, we further explored whether the presence of BACE1 in synapses was regulated by reticulon 3 (RTN3), a protein identified previously as a negative regulator of BACE1. We found that RTN3 is not only localized in the endoplasmic reticulum but also in neuritic regions where no endoplasmic reticulum-shaping proteins are detected, implicating additional functions of RTN3 in neurons. Coexpression of RTN3 with BACE1 in cultured neurons was sufficient to reduce colocalization of BACE1 with synaptophysin. This reduction correlated with decreased anterograde transport of BACE1 in axons in response to overexpressed RTN3. Our results in this study suggest that altered RTN3 levels can impact the axonal transport of BACE1 and demonstrate that reducing axonal transport of BACE1 in axons is a viable strategy for decreasing BACE1 in axonal terminals and, perhaps, reducing amyloid deposition.

One of the pathological hallmarks of Alzheimer disease is the presence of neuritic plaques in which amyloid deposits are surrounded by reactive astrocytes, activated microglia, and dystrophic neurites (1). The amyloid deposits contain mostly aggregates of amyloid peptides (Aβ)[3] that are excised from amyloid precursor protein (APP) by two endopeptidases: β- and γ-secretase (2, 3). BACE1 has been established as the β-secretase for initiating the generation of Aβ (4–8). Increased β-secretase activity is linked to the enhanced generation of Aβ and amyloid deposition in Alzheimer disease patients (9–12). Thus, how BACE1 activity is increased in Alzheimer disease patients has been the topic of intense research over the past several years.

As a type I transmembrane aspartyl protease, BACE1 is mainly localized in the late Golgi/trans-Golgi network (TGN) and early endosomes in cultured stable cell lines. The cellular trafficking of BACE1 is regulated by various proteins such as adaptor complexes and retromers (13–15). For example, increased levels of sortilin, a Vps10p domain-sorting receptor, enhances the retrograde transport of BACE1 from endosomes to the TGN (16). Decreased expression of vps35, another critical component of the retromer, correspondingly reduces retrograde transport of BACE1 from endosomes to the TGN, leading to increased BACE1 processing of APP (17). Decreased levels of Golgi-localized γ-ear-containing ADP-ribosylation factor (ARF)-binding proteins (GGAs) favor the colocalization of BACE1 and APP in the TGN and endosomes, resulting in increased Aβ production (18–21). Reduced GGA3 has also been observed in Alzheimer disease brains (18, 22).

We and others have demonstrated that BACE1 activity is negatively modulated by a protein family known as the reticu-

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[3] The abbreviations used are: Aβ, amyloid β; APP, amyloid precursor protein; TGN, trans-Golgi network; RTN, reticulon; ER, endoplasmic reticulum; eGFP, enhanced GFP; DIV, days *in vitro*.

lon/Nogo protein family (23–25). The reticulon (RTN) family of proteins consists of four members: RTN1 to RTN4. RTN4 is also known as Nogo, a protein first identified as an inhibitor of neurite outgrowth (26, 27). RTNs share the same structural organization in which the N-terminal domain is divergent, whereas the C-terminal domain, named the RTN-homologous domain, is evolutionarily conserved. The RTN-homologous domain is composed of two long stretches of hydrophobic amino acids separated by a loop of 66 amino acids (28, 29). This RTN-homologous domain anchors RTNs to the membrane and also governs RTN to adopt a ω-shape membrane topology with both the N- and C-terminal tails facing the cytoplasm (30–32).

BACE1 interacts with RTNs on the membrane in a region near their C-terminal sequences (33, 34). Increased expression of RTNs significantly reduces the production of Aβ in both cultured cells and transgenic mouse models (23, 35–38). This reduction is due to at least two effects. The tight interaction between BACE1 and RTN prevents BACE1 from accessing its APP substrate, and RTN can also increase the retention of BACE1 in the endoplasmic compartment (ER), which reduces Aβ production because BACE1 has a higher proteolytic activity in more acidic compartments, such as the endosomes (39).

Among the RTN members, RTN3 is the most extensively studied in regulating BACE1 activity because of its rich expression by neurons. Unlike most peripheral tissue cells, neurons are highly polarized. Because RTN proteins are largely viewed as ER-resident proteins, it is intriguing to know whether and how RTN3 is enriched in axons and dendrites. We have demonstrated previously that increased expression of RTN3 in hippocampal neurons promotes the formation of dystrophic neurites in transgenic mouse models or aging wild-type mice (35, 40). This finding also raises the question of how elevated expression of RTN3 in neurons facilitates the formation of dystrophic neurites. In addition, it is not clear whether expression of RTN3 will impact the neuronal localization of BACE1.

In this study, we investigated how RTN3 and BACE1 are localized in neurons and whether RTN3 alters the localization and/or activity of BACE1. To address these questions, we used cultured hippocampal neurons, which allowed us to monitor the localization of RTN3 and BACE1 in neurites. We expressed eGFP-tagged RTN3 or YFP-tagged BACE1 in cultured primary neurons via a lentivirus-expressing system. We showed that BACE1 was clearly localized in axons and dendrites in addition to the late Golgi and early endosomal compartments. RTN3, which interacts with BACE1, is largely expressed in the ER and neurites. Both RTN3 and BACE1 were also observed to be partially colocalized with the presynaptic marker synaptophysin, suggesting that these two proteins were present in synapses. Strikingly, increased expression of RTN3 reduced the localization of BACE1 in synapses. Our mechanistic study suggests that increased expression of RTN3 significantly reduces the anterograde transport of BACE1 in axons and influences Aβ generation. We also noted that the effect of overexpressed RTN3 on BACE1 in axonal transport was specific because it did not alter the axonal transport of APP. Hence, our results demonstrate that altered RTN3 expression can impact the neuritic localization and transport of BACE1, and increased RTN3 levels have been shown in elderly mouse brains.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Mouse primary neurons were dissected from embryonic cortices of Imprinting Control Region (ICR) mice at embryonic day 17.5. Culture dishes and plates were precoated with poly-D-lysine (catalog no. A-003-E, Millipore) overnight and rinsed twice with Hanks' balanced salt solution (catalog no. 14170, Invitrogen) before plating. Neurons were seeded in DMEM high-glucose (catalog no. SH30022.01B, Hyclone) with 10% FBS (catalog no. SH30084.03, Hyclone) for one night and then maintained in NeuroBasal (catalog no. 21103, Invitrogen) supplemented with 1% L-glutamine (catalog no. 25030, Invitrogen) and 2% B-27 (catalog no. 17504-044, Invitrogen). 2× PBS buffer (pH 6.95) used in phosphate calcium transfection was made from diluting the following chemicals in double distilled (dd)H_2O: 274 mM NaCl (catalog no. S7653, Sigma Aldrich), 10 mM KCl (catalog no. P9333, Sigma Aldrich), 1.4 mM $Na_2HPO_4{\cdot}7H_2O$ (catalog no. S7907, Sigma Aldrich), 15 mM glucose (catalog no. G7528, Sigma Aldrich), and 42 mM HEPES (catalog no. H7523, Sigma Aldrich).

Generation of BACE1 and RTN3 Constructs—RTN3-eGFP-N3 and BACE1-mRFP were generated by PCR amplification and standard subcloning. RTN3-myc-pcDNA 3.1 myc his A(-) was made by PCR-amplifying RTN3 from the previous construct and inserting it into the DNA in the EcoR1/BamH1 sites in pcDNA3.1 myc his A(-) (catalog no. V855-20, Invitrogen). RTN3 cDNA was then amplified and inserted between BamH1/Age1 of FUGW (catalog no. 14883, Addgene) to get RTN3-FUGW. BACE1-eGFP-N1 was made by subcloning BACE1 cDNA amplified from the previous construct in between the BamH1/Age1 sites of peGFP-N1 (catalog no. 6085-1, Clontech). BACE1-mRFP was obtained by replacing eGFP of the construct BACE1-eGFP with mRFP inserted between Age1/Not1. The DNA sequence encoding the BACE1-mRFP fusion protein was subcloned between BamH1/EcoR1 to make BACE1-mRFP-FUGW. The APP-eGFP fusion protein was generated in the same manner as BACE1-eGFP. The helper constructs pV-SVG, pREV, and pMDLG were purchased from Invitrogen.

Immunofluorescence and Confocal Microscopy—Neurons were cultured until DIV7 and then transfected with phosphate calcium. For one well of a 24-well plate, 2 μg of each construct was used. 48 h after transfection, cells were fixed and permeabilized. Antibodies against MAP2 (catalog no. M9942, Sigma Aldrich), calnexin (catalog no. C4731, Sigma Aldrich), Syntaxin6 (catalog no. 2869S, Cell Signaling Technology), EEA1 (catalog no. 3288S, Cell Signaling Technology), and synaptophysin (catalog no. MAB368, Millipore) were used to detect dendrites, the ER, the TGN, early endosomes, and presynaptic vesicles, respectively. Analysis of BACE1-eGFP puncta size in neuronal somata was performed by measuring the average size of the five biggest aggregates in one neuron, and 25 neurons were chosen for quantification.

For colocalization between BACE1 and synaptophysin, line profiles from the two fluorescent channels were analyzed using the ImageJ red-green-blue (RGB) Profiler plug-in (41). Briefly, neurons were transfected for BACE1-mRFP together with eGFP or RTN3-eGFP, and the fixed neurons on the coverslip were stained with an antibody against synaptophysin. BACE1-

mRFP fluorescent particles were randomly picked from the red channel, and multiple-channel line profiles were drawn for the same particle in the corresponding merged image. The line profile implies the distribution of the two fluorophores on the given line analyzed. The *x* axis represents the distance along the line, and the *y* axis represents the pixel intensity. Colocalization of BACE1 and synaptophysin was defined as overlapped red or blue peaks. Quantification analysis was performed by counting colocalized BACE1 and synaptophysin particles.

Live-cell Imaging—Neurons were cultured until DIV7 and transfected with BACE1-eGFP plus RTN3-myc or pcDNA 3.1. For one well of a 24-well plate, 2 μg of each construct was used. 48 h after transfection, BACE1-eGFP-positive neurons were recorded at 1 s/interval for 1 min under confocal microscopy (Leica) using a ×63 objective lens plus ×3.5 digital amplification by the software Leica Application Suite Advanced Fluorescence (LASAF). To minimize phototoxicity, only the eGFP channel was imaged. Kymographs were generated with the Multi Kymograph plug-in of the ImageJ software according to the instructions of J. Rietdorf, European Molecular Biology Laboratory, Heidelberg, Germany. BACE1-eGFP particle mobility was measured by quantifying the lines in the kymographs. Each line represents one vesicle. Vertical lines represent stationary BACE1 vesicles. Oblique lines or curves to the right represent anterograde movements, and lines to the left indicate retrograde transport.

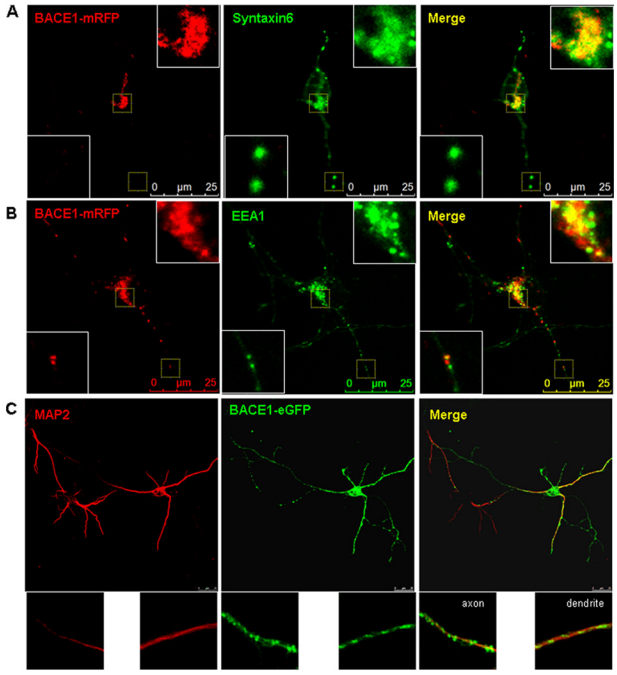

FIGURE 1. **Neuronal localization of BACE1.** Cultured mouse hippocampal neurons at DIV7 were transfected with either BACE1-mRFP (*A* and *B*) or BACE1-eGFP (*C*) for 48 h. The fixed cultured neurons were immunostained with an antibody specific to syntaxin 6 to label late Golgi and TGN compartments (*A*), EEA1 for early endosomal (*B*), or MAP2 for dendrites (*C*). The *boxed area* is enlarged and shown as an *inset*.

RESULTS

Localization of BACE1 in Cultured Neurons—The cellular localization of BACE1 in established cell lines has been investigated extensively (13). However, its localization in primary neurons is less well described because of limitations in the detection of endogenous BACE1 by commercially available antibodies. To determine BACE1 localization in cultured primary neurons, we generated the lentiviral expression constructs BACE1-eGFP and BACE1-mRFP, in which either eGFP or mRFP was fused to the C terminus of BACE1. Confocal examination of expressed BACE1-mRFP or BACE1-eGFP in hippocampal neurons showed localization of BACE1 in both somata and neurites. In the soma, BACE1-mRFP was colocalized with the TGN marker syntaxin 6 (Fig. 1*A*) and the early endosomal marker EEA1 (*B*). However, BACE1-eGFP puncta were rarely colocalized with either syntaxin 6 or EEA1 in neurites (see Fig. 1, *A* and *B*, *insets*), indicating that BACE1 puncta in neurites are not identical to these trafficking vesicles. Staining of transfected neurons with the dendritic marker MAP2 indicated that BACE1-eGFP was present in both dendrites and axons (Fig. 1*C*).

Localization of RTN3 in Cultured Neurons—Because BACE1 is negatively regulated by RTN/Nogo proteins and RTN3 is expressed by neurons (39), we asked whether neuronal localization of BACE1 is affected by altered expression of RTN3. To address this question, we generated RTN3-eGFP in a lentiviral expression construct and infected cultured neurons with this lentiviral expression vector. Expression of RTN3-eGFP in cultured neurons showed strong localization in the ER of soma. We compared the localization of RTN3-eGFP with that of the ER integral membrane protein calnexin (Fig. 2*A*, *center panel*), a calcium-binding protein embedded in the rough ER membrane, and an ER pore-forming protein, Sec61, located in the ER and ER-Golgi intermediate compartments (*B*, *center panel*). We observed that the expression pattern of RTN3-eGFP was similar to the localization of Sec61, indicating a more restricted localization.

In non-neuronal cells, it has been established that RTN/Nogo proteins function as tubular ER structural proteins (31, 42). To verify the localization of RTN3-eGFP in the tubular ER, we expressed RTN3-eGFP in endothelial cells as a control experiment. We showed a clear tubular ER localization of RTN3-eGFP (Fig. 2, *C* and *D*), confirming that this eGFP tag did not alter its localization in the tubular ER. A more enlarged view of RTN3-eGFP in cultured neurons showed that the tubular ER structure in cultured neurons is morphologically different from that in non-neuronal cells because RTN3-eGFP in cultured neurons appeared to be mostly discontinuing tubular structures captured by a confocal microscope (Fig. 2*E*). Hence, RTN3 in cultured neuronal soma is also localized in the tubular ER.

Noticeably, in the neuritic region, RTN3-eGFP was rarely colocalized with calnexin (Fig. 2*A*, *insets*) and only partially colocalized with Sec61 (*B*, *insets*). Although ER compartments are found in both dendrites and axons (41), the partial colocalization of RTN3-eGFP with ER marker proteins in neurites indicates the presence of RTN3 in different trafficking vesicles. RTN3 in axons was further confirmed by the staining of neurons with MAP2 antibody because MAP2, a dendritic protein, was not colocalized with RTN3-eGFP in axons (Fig. 2*C*).

Colocalization of BACE1 and RTN3 in Cultured Neurons—In cultured neurons cotransfected with BACE1-mRFP and RTN3-eGFP, BACE1 was mainly colocalized with RTN3 in the soma (Fig. 3*A*). This partial colocalization is likely related to the colo-

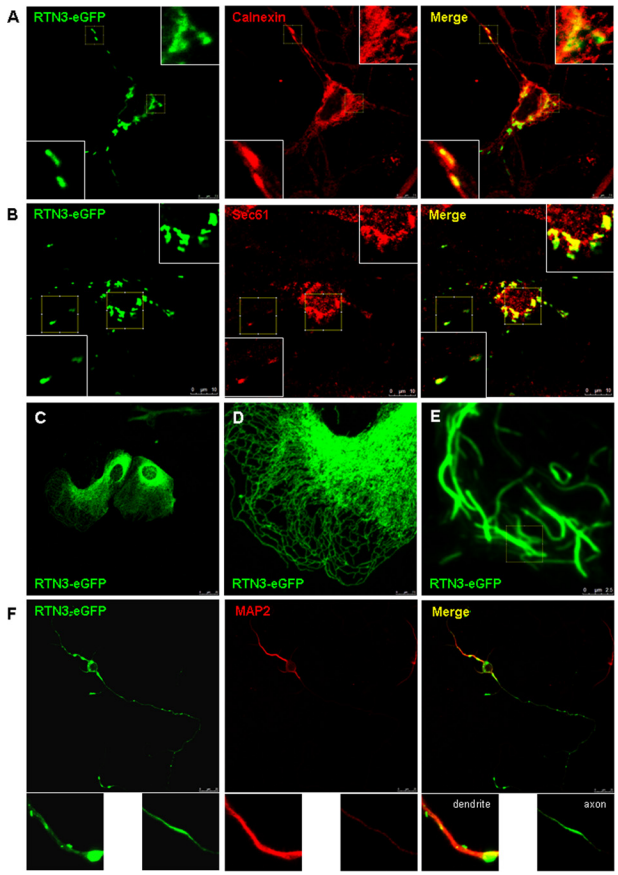

FIGURE 2. **Neuronal localization of RTN3.** The RTN3-eGFP expression construct was used to infect cultured mouse hippocampal neurons for 48 h, and the fixed neurons were reacted with anti-calnexin to mark the ER (*A*) or MAP2 to mark dendrites (*F*). The Sec61-myc expression construct (*B*) was coexpressed with RTN3 to determine their colocalization. *C* and *D*, the RTN3-eGFP expression construct was used to transfect endothelial cells, and fixed cells were visualized and imaged using a confocal microscope. The images showed clear localization of RTN3 in the tubular ER. The *boxed area* is enlarged and shown as an *inset*. *E*, higher magnification of the ER structure of cultured neurons transfected with the RTN3-eGFP expression construct was captured to observe tubular ER in neurons. *Scale bars* = 2.5 μm in *E* and 10 μm in the other panels.

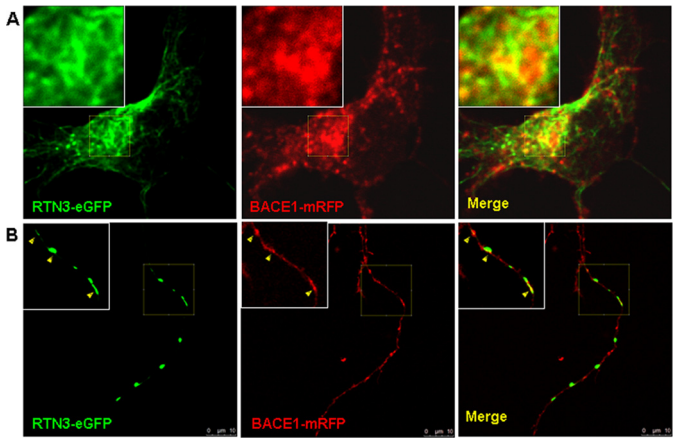

FIGURE 3. **RTN3 colocalizes with BACE1 in the soma.** Cultured mouse neurons were coinfected with BACE1-mRFP or RTN3-eGFP for 48 h, and fixed neurons were imaged by a confocal microscope. Colocalization of BACE1 with RTN3 was mainly in the soma (*A*). BACE1 in axons were significantly weaker than RTN3 (*A*), and partial colocalization of these two proteins is evident in *B*. The *boxed area* is enlarged and shown as an *inset*. *Scale bar* = 10 μm.

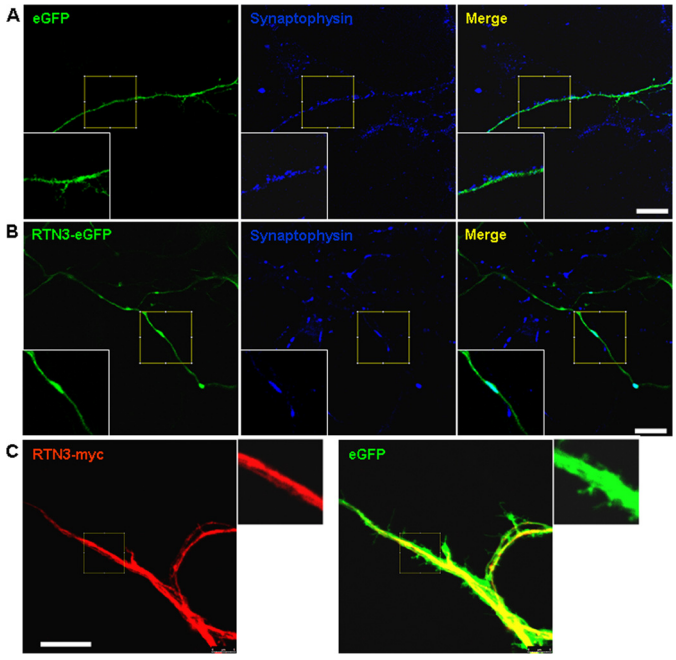

FIGURE 4. **Colocalization of RTN3 with synaptophysin.** *A* and *B*, cultured mouse hippocampal neurons were infected with a lentiviral vector expressing either eGFP or RTN3-eGFP for 48 h, and fixed neurons were then reacted with an antibody specific to synaptophysin, a protein abundantly enriched in presynaptic vesicles. *C*, RTN3-myc and eGFP were cotransfected in cultured neurons, and staining of dendritic spines was labeled by eGFP. The *boxed area* is enlarged and shown as an *inset*. *Scale bars* = 10 μm in *A* and *B*.

calization of RTN3 in the ER and ER-Golgi intermediate compartments because RTN3 is more enriched in these compartments. BACE1-mRFP in the endosomes was evidently not colocalized with RTN3 (see the merged picture in Fig. 3*A*). Interestingly, although both RTN3 and BACE1 are localized in the axon, RTN3 was only moderately colocalized with BACE1 (Fig. 3*B*). Morphologically, RTN3 in axons were enriched in bead-like puncta, whereas BACE1 was more diffusely spread along axons.

RTN3 Is Present in Presynaptic Vesicles—We further asked whether RTN3 or BACE1 is localized in synapses because both proteins are clearly present in neurites. To address this question, we infected cultured neurons with RTN3-eGFP, BACE1-mRFP, or both lentiviral expression constructs and marked infected neurons with an antibody specific to synaptophysin, an abundant presynaptic vesicle protein that regulates synapse formation. In cultured neurons, synaptophysin appeared in spots, mostly along neuritic regions (Fig. 4*A*, eGFP was transfected to nonspecifically labeled neurites). Overexpressed RTN3-eGFP present in axons appeared more similar to small beads on a string, and RTN3-eGFP beads were colocalized with synaptophysin spots in axons (Fig. 4*B*). In a separate experiment, we coexpressed myc-tagged RTN3 with eGFP in cultured neurons and examined RTN3 localization in the dendritic spines. Clearly, RTN3-eGFP was not readily detected in the dendritic spines (Fig. 4*C*, spines are labeled by eGFP), indicating that RTN3 is mostly in the presynaptic, but not postsynaptic, vesicles.

Synaptic Localization of BACE1 Is Altered by Overexpressed RTN3—Small beads marked by BACE1-mRFP were clearly visible along axons, although BACE1-mRFP mostly resided in neuronal soma (Fig. 5*A*). In BACE1-mRFP- infected neurons,

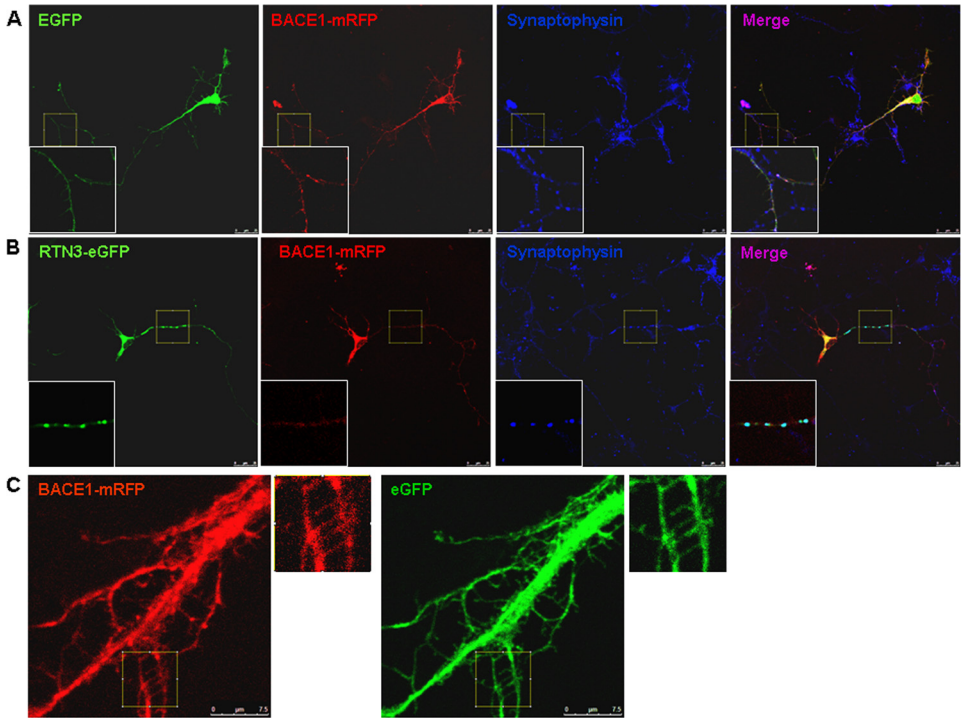

FIGURE 5. **RTN3 reduces colocalization of BACE1 with synaptophysin.** Cultured neurons were infected with the BACE1-mRFP lentivirus-expressing construct together with either lentiviral vector expressing eGFP (*A*) or RTN3-eGFP (*B*) for 48 h. After transfection, neurons were fixed and examined for BACE1 colocalization with synaptophysin. *C*, an enlarged view was captured to determine BACE1-mRFP in spines. The *boxed area* is enlarged and shown as an *inset*. *Scale bars* = 10 μm and 7.5 μm in *C*.

colocalization of BACE1-mRFP with synaptophysin was evident because some BACE-1mRFP particles visibly overlapped with synaptophysin-containing vesicles (Fig. 5*A*), indicating the presence of BACE1 in presynaptic vesicles. However, BACE1 in presynaptic vesicles appeared to be markedly reduced when RTN3 was overexpressed because less BACE1-mRFP beads were visible along axons (Fig. 5*B*). Unlike RTN3, BACE1-mRFP was clearly present in the dendritic spines (Fig. 5*C*), indicating that BACE1 is present in both pre- and postsynaptic compartments.

To confirm this reduction of BACE1 in synaptic compartments by overexpressed RTN3, we further quantified the colocalization of BACE1 with synaptophysin in the presence or absence of overexpressed RTN3. BACE1-mRFP particles were randomly picked, and line profiles were plotted on merged images for the selected BACE1 particles (see the example in Fig. 6, *A* and *B*). In the cultured neurons transfected with only BACE1-mRFP, about 31.61 ± 3.44% in axons was colocalized with synaptophysin, and this colocalized BACE1-mRFP with synaptophysin was reduced to 2.28 ± 1.34% if RTN3 was coexpressed (Fig. 6*C*, $n = 118$ BACE1 particles, $p < 0.001$, Student's *t* test).

In fact, when RTN3 was co-overexpressed with BACE1, BACE1-mRFP particles in neurites exhibited a visible reduction (Fig. 7, *A* and *B*). Quantification showed that BACE1-mRFP particles in these two different transfected neurons were reduced by about 1-fold (Fig. 7*C*; 1.28 ± 0.15 particles/unit length in BACE1 and eGFP-transfected neurons *versus* 0.62 ± 0.07 in BACE1 and RTN3 coexpressed neurons; ***, $p < 0.001$; Student's *t* test with Welch's correction). We also analyzed the size of BACE1-mRFP puncta in neuronal somata by measuring the average size of the five biggest aggregates in one neuron. The size of BACE1-mRFP puncta was increased (Fig. 7*D*; 133.7 ± 15.02 pixels in BACE1-transfected neurons *versus* 272.6 ± 36.95 pixels in BACE1- and RTN3-coexpressed neurons; $n = 25$ neurons from three independent experiments; **, $p = 0.0015$; Student's *t* test). Together, these results could explain the reduced colocalization of BACE1 with synaptophysin in neurites.

Axonal Transport of BACE1 Is Altered by Overexpressed RTN3—In addition, we investigated why increased RTN3 reduces the colocalization of BACE1 with synaptophysin by asking whether RTN3 affects axonal transport of BACE1. A prior study suggested that BACE1 undergoes axonal transport via kinesin1 light chain-mediated vesicles (43). Cultured neurons were, therefore, transfected with BACE1-eGFP together with either empty vector or RTN3-myc in separate vials, and axonal trafficking of BACE1-eGFP particles in live neurons was imaged and analyzed with ImageJ software. We found that axonal BACE1-eGFP particles were readily mobile, as shown in representative kymographs (Fig. 8*A*). It was clear that anterograde trafficking of BACE1-eGFP-containing vesicles was visibly decreased when RTN3 was cotransfected because less BACE1-eGFP-containing vesicles were detected in the anterograde direction.

Additional quantification revealed that about 22.51% BACE1-eGFP underwent anterograde transport and that this was reduced to 8.45% when RTN3 was overexpressed (Fig. 8*B*; 457 vesicles from BACE1 + RTN3-cotransfected cells and 583 vesicles from BACE1 + empty pcDNA3.1 vector-cotransfected cells; $n = 22$ axons; **, $p = 0.0014$; Student's *t* test). The reduction of BACE1 axonal transport by overexpression of RTN3 was mostly due to an increase in stationary BACE1-mRFP beads,

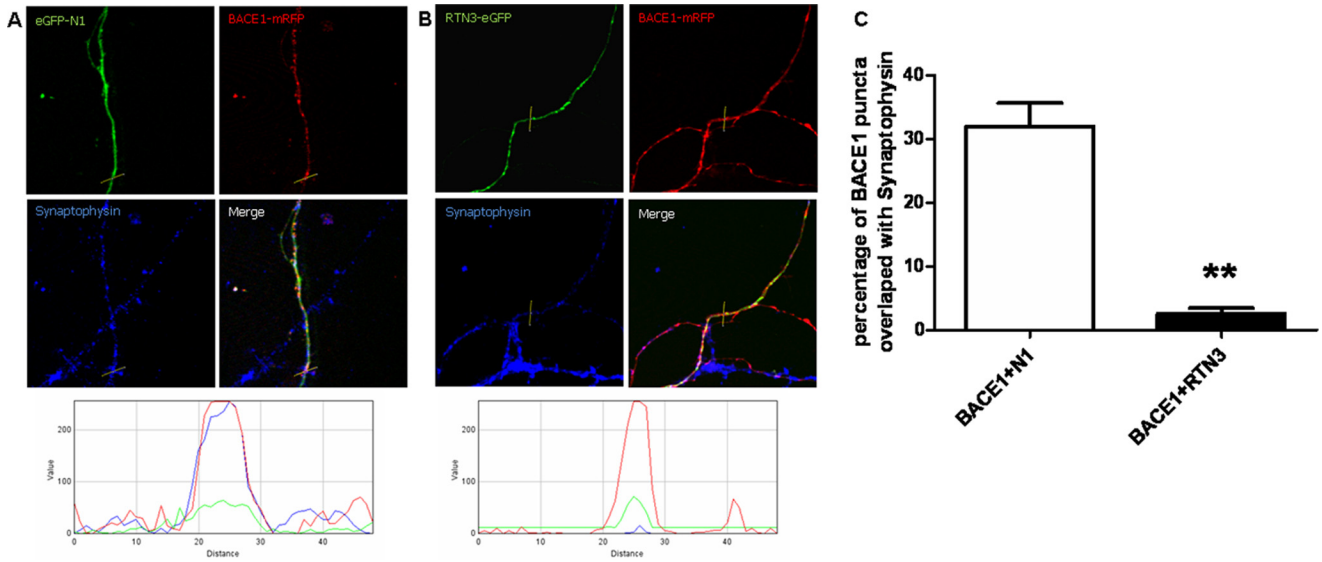

FIGURE 6. **Quantitative analysis of the colocalization of BACE1 with synaptophysin.** Neurons were infected with BACE1-mRFP together with a vector expressing either control eGFP (*A*) or RTN3-eGFP (*B*). BACE1-mRFP fluorescent particles were selected randomly. Line profiles were plotted on merged images for the same BACE1 particles (*lower panels*). The *x* axis represents the distance along the line, and the *y* axis represents the pixel intensity. Colocalization of BACE1 and synaptophysin at each point was defined as overlapping red and blue peaks. *C*, a total of 118 BACE1-mRFP fluorescent particles from 20 axons were analyzed for colocalization with synaptophysin. Results are summarized from three independent experiments. **, $p = 0.0014$; Student's *t* test.

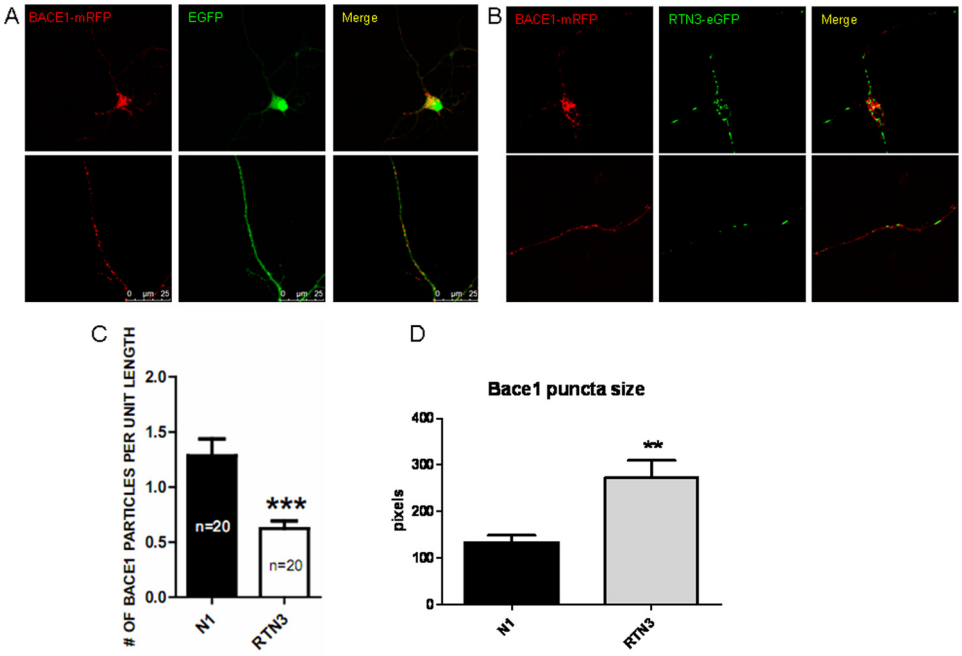

FIGURE 7. **Overexpression of RTN3 reduces BACE1 particles scattered along the neurites.** Neurons were infected with the indicated lentiviral expression constructs, and neurons with coexpression of BACE1-mRFP and eGFP (*A*) or BACE1-mRFP and RTN3-eGFP (*B*) were analyzed. The images of neurites from the corresponding neurons were enlarged and are presented in the *lower panels*. *Scale bar* = 25 μm. *C*, a statistical analysis of a total of 20 neurites from three independent experiments was conducted. The number of BACE1 particles in BACE1-mRFP/eGFP (*N1*) coinfected neurons was decreased from 1.28 particles/unit length in the neurites to 0.62 particles in the RTN3 overexpression condition. ***, $p = 0.0005$; Student's *t* test with Welch's correction. *D*, analysis of BACE1-eGFP puncta size in the neuronal somata was performed by measuring the average size of the five biggest aggregates in one neuron, and a total of 25 neurons from three independent experiments were chosen for quantification. **, $p = 0.0015$; Student's *t* test with Welch's correction.

which were increased from 59.22 ± 2.84% in BACE1-transfected neurons to 74.50 ± 3.44% in BACE1- and RTN3-coexpressing neurons (Fig. 8*D*, $p = 0.0014$). Further quantification indicated that the rate of BACE1 anterograde trafficking was decreased from 15.85 μm/min to 6.68 μm/min in the presence of overexpressed RTN3 (Fig. 8*E*; ***, $p < 0.0001$; $n = 20$ cells; Student's *t* test with Welch's correction). The difference in retrograde trafficking rate was not statistically significant (Fig. 8*F*).

To examine the potential specific effect of RTN3 on BACE1 axonal transport, we conducted a parallel experiment by coexpressing RTN3 with APP-eGFP, which has been demonstrated to be axonally transported (43). We found that small numbers of APP-eGFP-containing vesicles underwent anterograde axonal transport, whereas a majority of APP-eGFP-containing vesicles were in a stationary position in our cultured condition (Fig. 9*A*). From the quantification, we observed no significant

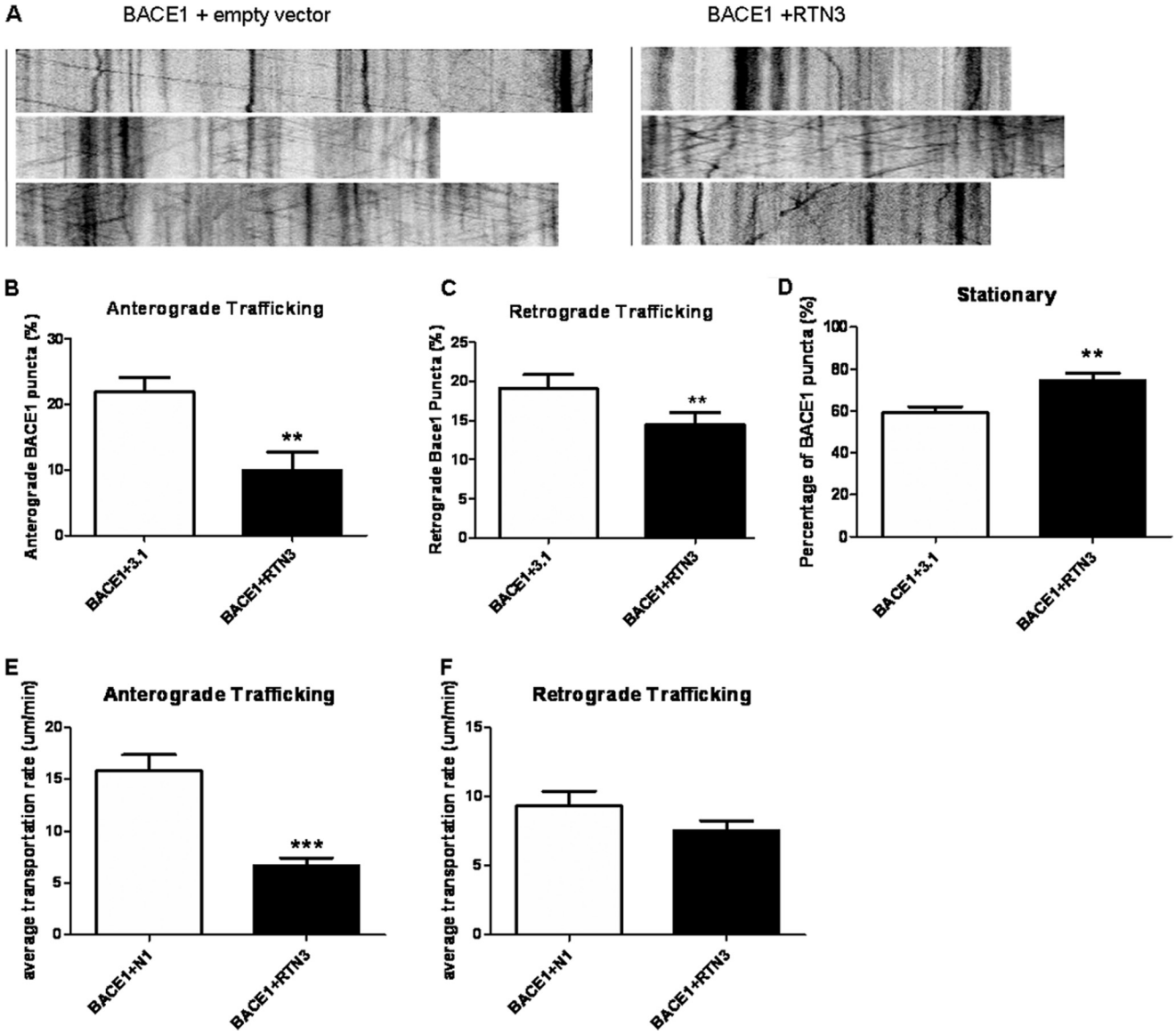

FIGURE 8. **Reducing anterograde transport of BACE1 in axons by overexpressed RTN3.** *A*, representative kymographs of BACE1-eGFP-containing vesicles in the axon. Cultured neurons at DIV7 were transfected with BACE1-eGFP together with either pcDNA3.1 empty vector (*3.1*) or RTN3-myc for 48 h. Live cells were imaged at 1 s/interval for 1 min using confocal microscopy with ×630 magnification. Kymographs of BACE1-positive vesicles trafficking along axons were analyzed by ImageJ. Right or left descending particles represent anterograde or retrograde transport in axons. *B–D*, axonal transport of BACE1-containing vesicles was quantitatively compared with or without RTN3 overexpression. Flux rates of BACE1-eGFP-containing vesicles were measured. Of three independent experiments, a total of 457 BACE1-eGFP vesicles from 22 RTN3 cotransfected neurons and 583 vesicles from 22 neurons transfected with BACE1-eGFP and empty vector were quantified. **, $p = 0.0014$; Student's *t* test. *E* and *F*, the trafficking rate of BACE1-eGFP vesicles in either the anterograde or retrograde direction was calculated. Anterograde trafficking of BACE 1 was decreased from 15.85 μm/min to 6.68 μm/min in the presence of overexpressed RTN3. ***, $p < 0.0001$; $n = 20$ cells; Student's *t* test with Welch's correction (*E*). On the other hand, trafficking of BACE1-eGFP vesicles was not significantly affected in the anterograde direction (*F*).

changes in axonal transport of APP-eGFP vesicles (Fig. 9*B*; 17.13 ± 2.70% in neurons cotransfected with empty vector *versus* 18.93 ± 1.77% in neurons coexpressing RTN3; $p = 0.58$; Student's *t* test). Similarly, no significant changes in retrograde transport (Fig. 9*C*) or stationary (*D*) APP-eGFP vesicles in both conditions were detected, indicating that overexpressing RTN3 has no effect on axonal transport of APP.

Thus, RTN3 overexpression appears to reduce the anterograde transport of BACE1 along axons, and this reduction could imply the decreased presence of BACE1 in synapses. In our separate recording experiments, we noticed that most of the RTN3-eGFP bead-like structures, which were much larger in size than the BACE1-eGFP bead-like structures, released small vesicles along the axon (data not shown). We also noticed that the "beads" themselves appeared to be moving slowly during our recording and were not affected by overexpression of BACE1. Hence, our results indicate that overexpression of RTN3 specifically regulates the axonal transport and presynaptic localization of BACE1.

DISCUSSION

Aβ is generated from the sequential cleavages of APP by BACE1 and γ-secretase, and BACE1 has been experimentally confirmed as the sole enzyme for initiating the cleavage of APP

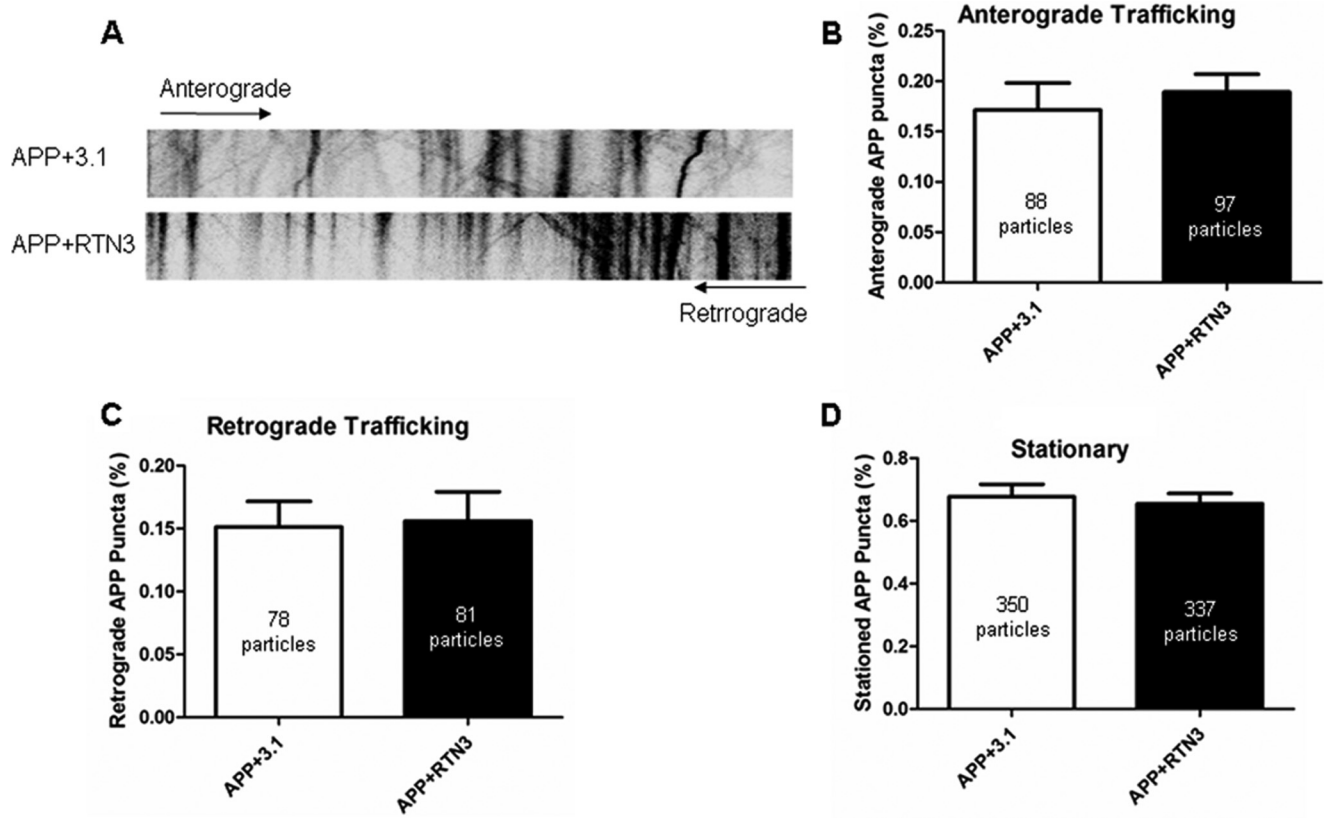

FIGURE 9. **RTN3 has no effects on axonal transport of APP.** *A*, representative kymographs of APP-eGFP-containing vesicles in the axon. The Swedish mutant version of APP was fused to eGFP to generate APP-eGFP, which was transfected together with either RTN3-myc or its empty vector into neurons at DIV7 for about 48 h. Cells were then imaged at 1 s/interval for 1 min. *B–D*, RTN overexpression does not significantly alter the percentage of stationary or mobile APP-eGFP vesicles. A total of 515 APP-eGFP vesicles from 20 neurons cotransfected with APP and RTN3 and 516 vesicles from 20 neurons transfected with APP-eGFP and empty vector were quantified to determine the percentage of vesicles either being transported or stationary (17.13 ± 2.67% *versus* 18.93 ± 1.77% in anterograde transport; 15.13 ± 2.02% *versus* 15.61 ± 2.31% in retrograde transport; 67.74 ± 3.98% *versus* 65.47 ± 3.28% stationary; **, $p > 0.05$, Student's *t* test.

that releases Aβ peptides. Fluctuation of BACE1 activity in neurons is, therefore, a predetermined critical factor for Aβ generation and oligomerization in the human brain. Because excessive Aβ accumulation is linked to the onset of amyloid deposition in the brains of Alzheimer patients, inhibition of BACE1 activity is a practical approach for preventing or reducing pathological damage. We show here that BACE1 resides not only in the neuronal somata, including the TGN and endosomal compartments, but also in synapses. A recent study also shows BACE1 in presynaptic terminals by immune-EM (44), and our result is consistent with this *in vivo* result. The localization of BACE1 in the synaptic region supports a prior study that suggested differential generation of Aβ from secretory compartments and synaptic terminals (45). Although BACE1 is mostly colocalized with its negative regulator RTN3 in the soma, increased expression of RTN3 can also reduce BACE1 in synapses, and this reduction is expected to decrease BACE1 activity. Hence, this study is the first to demonstrate the regulation of BACE1 in synapses by RTN3.

Aβ released from synaptic terminals appears to be more correlated with amyloid deposition (46). To release Aβ from the neuronal presynaptic terminals, APP, BACE1, and γ-secretase need to be axonally transported to allow sequential cleavages to occur locally. APP has long been shown to undergo axonal transport (43), although mechanistic explanations of its axonal transport remain to be fully elucidated (45, 47–50). Presenilin 1, a proteolytic component in the γ-secretase, has also been shown to be axonally transported (51). BACE1 has been found recently to be in the same vesicle with APP during anterograde axonal transport (52). By taking advantage of a fluorescent tag fused with BACE1, we investigated the transport of BACE1 in axons. Indeed, we confirmed the axonal transport of BACE1 in this study. Although RTN3 is recognized as an ER tubular structure-shaping protein, our data clearly showed that RTN3 was also present in the axon and dendrite. Our results further demonstrate that the transport of BACE1 is inversely affected by overexpression of RTN3 because the anterograde transport of BACE1 was decreased significantly upon RTN3 overexpression. We found that RTN3-eGFP and BACE1-mRFP were not fully colocalized along axons, and we do not yet know the routes along which these two proteins were transported. BACE1 has been suggested to be in the same calsyntenin 1-containing vesicles with APP via kinesin 1-mediated axonal transport (52, 53). Although future studies will be aimed at investigating whether RTN3 transport is mediated by kinesin 1, our previous study has shown that increased expression of RTN3 enhances retention of BACE1 in the ER (25) and that this enhanced ER retention could cause a decrease in available BACE1 for exiting from the late Golgi and TGN compartments for axonal transport. We have also shown that overexpressing RTN3 had a weak

effect on axonal transport of APP. Nevertheless, RTN3 is not physically associated with APP (23).

Previous studies have also demonstrated that the level of Aβ released from synapses has a direct impact on amyloid deposition and synaptic functions (54, 55). Modulating the localization of BACE1 in synaptic terminals is an alternative approach for decreasing amyloid deposition. Recent studies showed that either increased axonal transport of BACE1 by calsyntenin 1 (53) or retrograde transport by vps35 significantly altered amyloid deposition (56). Transgenic mice overexpressing RTN3 have reduced amyloid deposition (23, 35, 36, 57), and this reduction is partially attributable to reduced BACE1 in presynaptic terminals in response to overexpression of RTN3 in mouse neurons.

Reduced levels of BACE1 in axonal transport will also result in decreased levels of BACE1-cleaved APP C-terminal fragments (APP-C99). In Alzheimer brains, BACE1 levels are elevated, and such an increase generates higher levels of BACE1-cleaved APP C-terminal fragments (APP-C99) (58–60). We found that overexpressing RTN3 in cultured neurons significantly reduced the level of APP-C99 (data not shown), and this observation was consistent with previous reports (23–25, 25, 36). Recent studies, using a knock-in mouse model developed to mimic human familial Danish dementia (61, 62), showed synaptic and memory deficits that are dependent on altered levels of APP-C99 (63). On the other hand, a high level of APP-C99, because of enhanced BACE1 cleavage of APP, can affect the anterograde axonal transport of APP and lead to axonal defects (64). Hence, reducing the level of APP-C99 by RTN3 overexpression may affect not only axonal function associated with kinesin 1-mediated abnormal axonal transport but also synaptic function.

In summary, decreasing axonal transport of BACE1 by moderately increased expression of RTN3 will reduce Aβ generation and APP-C99 levels. This study also offers a strategy to explore drugs that can reduce axonal transport of BACE1 to reduce amyloid deposition.

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