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-
Effect of RTN3 Expression on Axonal Transport of BACE1

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)H2O: 274 mM NaCl (catalog no. S7653, Sigma Aldrich), 10 mM KCl (catalog no. P9333, Sigma Aldrich), 1.4 mM Na2HPO4.7H2O (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-N3 was made by subcloning BACE1 cDNA amplified from the previous construct in between the BamH1/Age1 sites of pcGFP-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/EcoRI 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 pMDL 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), Synaptin1 (catalog no. 3288S, Cell Signaling Technology), EE1 (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 sizes 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. Co-localization of BACE1 and synaptophysin was defined as overlapped red or blue peaks. Quantification analysis was performed by counting co-localized 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.

**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. 1A) 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. 1C).

**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 calcinein (Fig. 2A, 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. 2E). Hence, RTN3 in cultured neuronal soma is also localized in the tubular ER.

Noticeably, in the neuritic region, RTN3-eGFP was rarely colocalized with calcinein (Fig. 2A, 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. 2C).

**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. 3A). This partial colocalization is likely related to the colo-
localization 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. 3A). Interestingly, although both RTN3 and BACE1 are localized in the axon, RTN3 was only moderately colocalized with BACE1 (Fig. 3B). 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. 4A, 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. 4B). 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. 4C, 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. 5A). In BACE1-mRFP- infected neurons, RTN3 colocalizes with BACE1 in the soma. Cultured mouse neurons were cotransfected 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.
colocalization of BACE1-mRFP with synaptophysin was evident because some BACE1-mRFP particles visibly overlapped with synaptophysin-containing vesicles (Fig. 5A), 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. 5B). Unlike RTN3, BACE1-mRFP was clearly present in the dendritic spines (Fig. 5C), 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. 6C, 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. 7C; 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. 7D; 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. 8A). 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. 8B; 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,
which were increased from 59.22 ± 2.84% in BACE1-transfected neurons to 74.50 ± 3.44% in BACE1- and RTN3-coexpressing neurons (Fig. 8D, *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. 8E, ***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. 8F).

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. 9A). From the quantification, we observed no significant
changes in axonal transport of APP-eGFP vesicles (Fig. 9B; 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. 9C) 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.
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

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 ± 3.96% versus 65.47 ± 3.28% stationary; **, p > 0.05, Student’s t test.

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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 calysteginin 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 activated, and such an increase generates higher levels of BACE1-cleaved APP C-terminal fragments (APP-C99) (58–60). We

In summary, decreasing axonal transport of BACE1 by modulating 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.

REFERENCES

1. Selkoe, D. J., Yamazaki, T., Citron, M., Podlisny, M. B., Koo, E. H., Teplow, D. B., and Haass, C. (1996) The role of APP processing and trafficking pathways in the formation of amyloid β-protein. Ann. N.Y. Acad. Sci. 777, 57–64

2. Sisodia, S. S., and St George-Hyslop, P. H. (2002) γ-Secretase, Notch, Aβ and Alzheimer’s disease. Where do the presenilins fit in? Nat. Rev. Neurosci. 3, 281–290

3. Haass, C. (2004) Take five. BACE and the γ-secretase quartet conduct the transmembrane aspartic protease with Alzheimer’s disease. J. Biol. Chem. 281–290

4. Hussain, I., Powell, D., Howlett, D. R., Tew, D. G., Meek, T. D., Chapman, C., Gloger, I. S., Murphy, K. E., Southan, C. D., Ryan, D. M., Smith, T. S., Simmons, D. L., Walsh, F. S., Dingwall, C., and Christie, G. (1999) Identification of a novel aspartic protease (Axp 2) as β-secretase. Mol. Cell Neurosci. 14, 419–427

5. Vassar, R., Bennett, B. D., Babu-Khan, S., Kahn, S., Mendiz, E. A., Denis, P., Teplow, D. B., Ross, S., Amarante, P., Loefel, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M. A., Biere, A. L., Curran, E., Burgess, T., Louis, J. C., Collins, F., Treanor, J., Rogers, G., and Citron, M. (1999) β-Secretase cleavage of Alzheimer’s amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286, 735–741

6. Yan, R., Bienkowski, M. J., Shuck, M. E., Miao, H., Tory, M. C., Pauley, A. M., Brasier, J. R., Stratman, N. C., Mathews, W. R., Buhl, A. E., Carter, D. B., Tomasselli, A. G., Parodi, L. A., Heinrichs, R. L., and Gurney, M. E. (1999) Membrane-anchored aspartyl protease with Alzheimer’s disease β-secretase activity. Nature 402, 533–537

7. Sinha, S., Anderson, J. P., Barbout, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Doyee, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Soumenaari, S. M., Wang, S., Walker, D., Zhao, J., McConlogue, L., and John, V. (1999) Purification and cloning of amyloid precursor protein β-secretase from human brain. Nature 402, 537–540

8. Liu, X., Koelsch, G., Wu, S., Downs, D., Dashki, A., and Tang, J. (2000) Human amyloid protease memapsin 2 cleaves the β-secretase site of β-amyloid precursor protein. Proc. Natl. Acad. Sci. U.S.A. 97, 1456–1460

9. Cumming, J. N., Iserohu, O., and Kennedy, M. E. (2004) Design and development of BACE-1 inhibitors. Curr. Opin. Drug Discov. Devel. 7, 536–556

10. DiSich, B., and Lichtenhaller, S. F. (2012) The membrane-bound aspartyl protease BACE1. Molecular and functional properties in Alzheimer’s disease and beyond. Front. Physiol. 3

11. Luo, X., and Yan, R. (2010) Inhibition of BACE1 for therapeutic use in Alzheimer’s disease. Int. J. Clin. Exp. Pathol. 3, 618–628

12. Ghosh, A. K., Brindisi, M., and Tang, J. (2012) Developing β-secretase inhibitors for treatment of Alzheimer’s disease. J. Neurochem. 120, 71–83

13. Vassar, R., Kovacs, D. M., Yan, R., and Wong, P. C. (2009) The β-secretase enzyme BACE in health and Alzheimer’s disease. Regulation, cell biology, function, and therapeutic potential. J. Neurosci. 29, 12787–12794

14. Tan, J., and Evin, G. (2012) B-site APP-cleaving enzyme 1 trafficking and Alzheimer’s disease pathogenesis. J. Neurochem. 120, 869–880

15. Siegenthaler, B. M., and Rajendran, L. (2012) Retromers in Alzheimer’s disease. Neurodegener. Dis. 10, 116–121

16. Finan, G. M., Okada, H., and Kim, T. W. (2011) BACE1 retrograde trafficking is uniquely regulated by the cytoplasmic domain of sortilin. J. Biol. Chem. 286, 12602–12616

17. Wen, L., Tang, F. L., Hong, Y., Luo, S. W., Wang, C. L., He, W., Shen, C., Jung, J. U., Xiong, F., Lee, D. H., Zhang, Q. G., Brann, D., Kim, T. W., Yan, R., Mei, L., and Xiong, W. C. (2011) VPS35 haploinsufficiency reduces Alzheimer’s disease neuropathology. J. Cell Biol. 195, 765–779

18. Tesco, G., Koh, Y. H., Kang, E. L., Cameron, A. N., Das, S., Sena-Esteves, M., Hiltunen, M., Yang, S. H., Zhong, Z., Shen, Y., Simpkins, J. W., and Tanzi, R. E. (2007) Depletion of GGA3 stabilizes BACE and enhances β-secretase activity. Neuron 54, 721–737

19. Kang, E. L., Cameron, A. N., Piazza, F., Walker, K. R., and Tesco, G. (2010) Ubiquitin regulates GGA3-mediated degradation of BACE1. J. Biol. Chem. 285, 24108–24119

20. He, X., Li, F., Chang, W. P., and Tang, J. (2005) GGA proteins mediate the recycling pathway of memapsin 2 (BACE). J. Biol. Chem. 280, 11696–11703

21. Wahle, T., Prager, K., Raffler, N., Haass, C., Fanulok, M., and Walter, J. (2005) GGA proteins regulate retrograde transport of BACE1 from endosomes to the trans-Golgi network. Mol. Cell Neurosci. 29, 453–461

22. Santosa, C., Rasche, S., Barakat, A., Bellingham, S. A., Ho, M., Tan, J., Hill, A. F., Masters, C. L., McLean, A., and Evin, G. (2011) Decreased expression of GGA3 protein in Alzheimer’s disease frontal cortex and increased co-distribution of BACE with the amyloid precursor protein. Neurobiol. Dis. 43, 176–183

23. He, W., Wu, Y., Qahwash, I., Xu, Y., Chang, A., and Yan, R. (2004) Reticulin family members modulate BACE1 activity and amyloid-β peptide generation. Nat. Med. 10, 959–965

24. Murayama, K. S., Kamefushi, F., Saito, S., Kume, H., Akiyama, H., and Araki, W. (2006) Reticulins RTN3 and RTN4-B/C interact with BACE1 and inhibit its ability to produce amyloid β-protein. Eur. J. Neurosci. 24, 1237–1244

25. Shi, Q., Prior, M., He, W., Tang, X., Hu, X., and Yan, R. (2009) Reduced amyloid deposition in mice overexpressing RTN3 is adversely affected by preformed dystrophic neurites. J. Neurosci. 29, 9163–9173

26. Yan, R., Shi, Q., Hu, X., and Zhou, X. (2006) Reticulin proteins. Emerging players in neurodegenerative diseases. Cell Mol. Life Sci. 63, 877–889

27. Yang, Y. S., and Strittmatter, S. M. (2007) The reticulins. A family of proteins with diverse functions. Genome Biol. 8, 234
Effect of RTN3 Expression on Axonal Transport of BACE1

28. Oertle, T., Klinger, M., Steurmer, C. A., and Schwab, M. E. (2003) A reticular rhabdomy. Phylogenetic evolution and nomenclature of the RTN/Nogo gene family. *J. Mol. Biol.* 17, 1238–1247

29. Teng, F. Y., and Tang, B. L. (2005) Nogo signaling and non-physiological injury-induced neuronal system pathology. *J. Neurosci. Res.* 79, 273–278

30. Iwashashi, J., Hamada, N., and Watanabe, H. (2007) Two hydrophobic segments of the RTN1 family determine the ER localization and retention. *Biochem. Biophys. Res. Commun.* 355, 508–512

31. Voeltz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M., and Rapoport, T. A. (2006) A class of membrane proteins shaping the tubular endoplasmic reticulum. *Cell* 124, 573–586

32. He, W., Shi, Q., Hu, X., and Yan, R. (2007) The membrane topology of RTN5 and its effect on binding of RTN3 to BACE1. *J. Biol. Chem.* 282, 29144–29151

33. Prior, M., Shi, Q., Hu, X., He, W., Levey, A., and Yan, R. (2006) Mapping of interaction domains mediating binding between BACE1 and RTN/Nogo proteins. *J. Mol. Biol.* 363, 625–634

34. Kume, H., Murayama, K. S., and Araki, W. (2009) The two-hydrophobic domain tertiary structure of reticulon proteins is critical for modulation of β-secretase BACE1. *J. Biol. Chem.* 87, 2963–2972

35. Shi, Q., Hu, X., Prior, M., and Yan, R. (2009) The occurrence of aging-dependent reticulon 3 immunoreactive dystrophic neurites decreases cognitive function. *J. Neurosci. Res.* 29, 5108–5115

36. Araki, W., Oda, A., Motoki, K., Hattori, K., Itoh, M., Yusa, S., Konishi, Y., Shin, R. W., Tamaoka, A., and Ogino, K. (2012) Reduction of β-amyloid accumulation by reticulon 3 in transgenic mice. *Curr. Alzheimer Res.* 10, 135–142

37. Wojcik, S., Engel, W. K., Yan, R., McFerrin, J., and Askanas, V. (2007) NOGO is increased and binds to BACE1 in sporadic inclusion-body myositis and in Aβ PP-overexpressing cultured human muscle fibers. *Acta Neuropathol.* 114, 517–526

38. Zhao, B., Pan, B. S., Shen, S. W., Sun, X., Hou, Z. Z., Yan, R., and Sun, F. Y. (2013) Diabetes-induced central nervous system pathology. *J. Biol. Chem.* 288, 15590–15599

39. Prior, M., Shi, Q., Hu, X., He, W., Levey, A., and Yan, R. (2010) RTN/Nogo in forming Alzheimer’s neuritic plaques. *Neurobiol. Aging* 31, 1201–1206

40. Hu, X., Shi, Q., Zhou, X., He, W., Yi, H., Yin, X., Gearing, M., Levey, A., and Yan, R. (2007) Transgenic mice overexpressing reticulin 3 develop neuritic abnormalities. *EMBO J.* 26, 2755–2767

41. Pidoux, G., Witzczak, O., Jarnaes, E., Myrvold, L., Urlaub, H., Stokke, A. J., Küntziger, T., and Taskén, K. (2011) Optic atrophy 1 is an A-kinase anchoring protein on lipid droplets that mediates adrenergic control of lipolysis. *EMBO J.* 30, 4371–4386

42. Shibata, Y., Voss, C., Rist, J. M., Hu, J., Rapoport, T. A., Prinz, W. A., and Voeltz, G. K. (2008) The reticulin and DPM1/Yoplp1 proteins form immobile oligomers in the tubular endoplasmic reticulum. *J. Biol. Chem.* 283, 18892–18904

43. Koo, E. H., Sisodia, S. S., Archer, D. R., Martin, L. J., Weidemann, A., Beyreuther, K., Fischer, P., Masters, C. L., and Price, D. L. (1990) Precursor protein and regulates Aβ-deposition in vivo. *J. Cell Biol.* 168, 291–302

44. Lazarov, O., Morfini, G. A., Pigino, G., Gadadhar, A., Chen, X., Robinson, J., Ho, H., Brady, S. T., and Sisodia, S. S. (2007) Impairments in fast axonal transport and motor neuron deficits in transgenic mice expressing familial Alzheimer’s disease-linked mutant presenilin 1. *J. Neurosci.* 27, 7011–7020

45. Szodorai, A., Kume, H., Yu, H., Shihata, S., Kato, K., Schätzle, P., Ludwig, A., Tagaya, M., Kunz, B., and Sonderegger, P. (2012) Calyculin-1-bands APP from proteolytic processing during anterograde axonal transport. *Biol. Open* 1, 761–774

46. Troyanovsky, V., Varvel, N. H., Lamb, B. T., and Muresan, Z. (2009) The cleavage products of amyloid-β precursor protein are sorted to distinct carrier vesicles that are independently transported within neurites. *J. Neurosci. Res.* 29, 3565–3578

47. Zhang, B., Pan, B. S., Shen, S. W., Sun, X., Hou, Z. Z., Yan, R., and Sun, F. Y. (2013) Diabetes-induced central nervous system pathology. *J. Clin. Invest.* 128, 2501–2509

48. Rodrigues, E. M., Weissmiller, A. M., and Goldstein, L. S. (2012) Enhanced β-secretase processing alters APP axonal transport and leads to axonal defects. *Hum. Mol. Genet.* 21, 4587–4601