Published in final edited form as:

Neuropathol Appl Neurobiol. 2009 April ; 35(2): 178–188. doi:10.1111/j.1365-2990.2008.00974.x.

Expression of reticulon 3 in Alzheimer's disease brain

Hideaki Kume^{1,*}, Yoshihiro Konishi^{2,*}, Kiyoko S. Murayama¹, Fuyuki Kametani³, and Wataru Araki¹

¹Department of Demyelinating Disease and Aging, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodara, Tokyo, Japan

²Department of Clinical Research, National Tottori Medical Center, Mitsu, Tottori, Japan

³Tokyo Institute of Psychiatry, Tokyo Metropolitan Organization for Medical Research, Setagaya, Tokyo, Japan

Abstract

Aims—Reticulon 3 (RTN3), a member of the reticulon family of proteins, interacts with the β -secretase, BACE1, and inhibits its activity to produce β -amyloid protein. The aim of the present study was to clarify the biological role of RTN3 in the brain and its potential involvement in the neuropathology of Alzheimer's disease (AD).

Methods—We performed immunohistochemical and biochemical analyses using a specific antibody against RTN3 to investigate the expression and subcellular localization of RTN3 in control and AD brain tissue samples.

Results—Western blot analysis revealed no significant differences in the RTN3 levels between control and AD brains. Immunohistochemical staining showed that RTN3 immunoreactivity was predominantly localized in pyramidal neurons of the cerebral cortex. The patterns of RTN3 immunostaining were similar in control and AD cerebral cortices, and senile plaques were generally negative for RTN3. Biochemical subcellular fractionation disclosed that RTN3 co-localized with BACE1 in various fractions, including the endoplasmic reticulum and the Golgi apparatus. Double immunofluorescence staining additionally indicated that RTN3 was localized in both endoplasmic reticulum and Golgi compartments in neurons.

Conclusions—These results show that RTN3 is primarily expressed in pyramidal neurons of the human cerebral cortex and that no clear difference of RTN3 immunoreactivity is observable between control and AD brains. Our data also suggest that there is considerable co-localization of RTN3 with BACE1 at a subcellular level.

Keywords

Alzheimer's disease; β -amyloid; BACE1; reticulon 3; subcellular localization

INTRODUCTION

β -Amyloid protein (A β) is the main component of senile plaques, the major pathological feature in brains of Alzheimer's disease (AD) patients. A β is derived from amyloid precursor protein (APP) through sequential proteolytic cleavage by β - and γ -secretases. The accumulation and deposition of A β is thought to play a crucial role in the pathogenesis of AD [1]. β -secretase

Correspondence to: Wataru Araki, Department of Demyelinating Disease and Aging, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodara, Tokyo 187-8502, Japan. E-mail: araki@ncnp.go.jp.

*These authors contributed equally to this work.

has been identified as a novel membrane-bound aspartyl protease BACE1 (β -Site APP cleaving enzyme 1) [2–6]. Studies of *in vivo* functions of BACE1 have been conducted by using BACE1-deficient and BACE1-transgenic mice. In BACE1-deficient mice, A β generation is eliminated [7–11]. Conversely, transgenic mice overexpressing BACE1 display elevated A β production and deposition [12–14]. These findings clearly indicate that BACE1 is essential for A β production. Several recent reports also confirm that BACE1 expression is increased in AD brains [15–18], implying that BACE1 participates in the pathology of AD. Since mice lacking BACE1 do not display overt abnormalities [7–11,19], inhibition of BACE1 is considered as an effective therapeutic strategy. In fact, attempts are being made to develop small-molecule inhibitors of BACE1 [20–22].

The reticulon (RTN) family proteins are integral membrane proteins with characteristic topological features. Their conserved C-terminal domain is called the reticulon homology domain (RHD), which include two hydrophobic segments and a hydrophilic loop [23]. There exist four paralogues of mammalian RTNs, designated RTN1, RTN2, RTN3 and RTN4 (or Nogo). The physiological functions of RTN family proteins have been poorly understood, although RTN4-A (Nogo-A) is known to have a neurite-inhibitory activity [23]. However, recent studies have begun to disclose important novel functions of these proteins [24,25].

Elucidation of the *in vivo* mechanisms that regulate BACE1 is important for developing novel methods to suppress its activity. Recently, two laboratories including ours conducted proteomic analyses to search for proteins interacting with BACE1, which identified reticulon 3 (RTN3) and reticulon 4-B/C (RTN4-B/C or Nogo-B/C) as BACE1-associated membrane proteins [26,27]. RTN3 and RTN4-B/C interact with both BACE1 and its homologue BACE2 [3,28, 29] in cultured cells, and inhibit BACE1 activity, consequently suppressing A β production [26,27]. The RHD in these RTN proteins most likely mediates the interaction with BACE1 [26,27]. These findings indicate that RTN3 and RTN4-B/C are important regulators of BACE1 activity. However, it is currently unclear whether RTN3 and RTN4-B/C are involved in the pathological mechanisms of AD. In order to clarify the role of RTN3 in the brain and its potential involvement in AD pathology, we analyzed the expression and subcellular distribution of RTN3 proteins in control and AD brain tissues. We show here that RTN3 is primarily expressed in pyramidal neurons in the human cerebral cortex and that senile plaques are generally negative for RTN3. Our analyses also suggest that RTN3 localizes in both ER and Golgi compartments in neurons.

MATERIALS AND METHODS

Antibodies

Rabbit polyclonal RTN3 antibody (RN3-C) was raised against residues 217–232 of human RTN3 and affinity-purified, as described previously [27]. Rabbit polyclonal BACE1 antibody (NBA) has been described in a previous report [30]. Mouse monoclonal BACE1 (MAB9311) and rabbit polyclonal BACE1 antibody (AB5832) were obtained from R&D systems (Minneapolis, MN, USA) and Chemicon International (Temecula, CA, USA), respectively. Rabbit polyclonal APP antibody (R37) was generated by Kametani *et al.* [31]. Mouse monoclonal β -actin antibody was purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal antibodies to calnexin, β -COP, and early endosomal antigen 1 (EEA1) were obtained from BD Biosciences (San Jose, CA, USA). Mouse monoclonal microtubule-associated protein 2 (MAP2) and A β antibodies (4G8) were acquired from Chemicon International.

Human brain tissue

Human autopsy brains were received from the Sun Health Research Institute Brain Donation Program of Sun City, Arizona, U.S.A. Age, gender, postmortem interval (PMI), disease duration, and neuropathological diagnosis of 5 control and 5 AD cases examined are summarized in Table 1. All AD patients were diagnosed neuropathologically as having definite AD according to the CERAD neuropathological criteria [32]. Non-demented control cases were selected based on prior medical records with antemortem neuropsychological test scores and postmortem neuropathological records with the CERAD criteria.

Western blotting

Human postmortem brains were removed within 3.3 h of death, sectioned coronally at 1–2 cm, immediately frozen directly on slabs of dry ice, and stored at -80°C . Brain tissues from the temporal cortex were homogenized with 10 volumes of Tris-saline buffer (10 mM Tris, pH 7.4, 150 mM NaCl) containing protease inhibitors. The homogenate was clarified by centrifugation at 1,000 g for 10 min, and the supernatant spun at 100,000 g for 1 h. The pellet was extracted with RIPA buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 1% Na deoxycholate, 5 mM EDTA and 0.1% SDS) containing protease inhibitors. Brain homogenates were also prepared in buffer containing 0.5% NP-40. The protein content was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were mixed with 2x Laemmli sample buffer and incubated at 37°C for 10 min.

Western blotting was performed, as described previously [33,34]. Proteins were separated on 12% or 5–16% polyacrylamide gels and blotted onto polyvinylidene difluoride membranes. Blots were blocked in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T) and 5% non-fat dry milk, and incubated with primary antibodies in PBS-T containing 1% bovine serum albumin. The membranes were subsequently incubated with peroxidase-labeled anti-rabbit or anti-mouse IgG, and protein expression detected using a chemiluminescence reagent (PerkinElmer, Boston, MA, USA). The Can Get Signal Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan) was occasionally used for incubation with primary and secondary antibodies. Quantification of protein bands was achieved with an image analyzer, LAS-1000 Plus (Fuji Film, Tokyo, Japan).

Subcellular fractionation

Human brain tissues from the temporal cortex were homogenized by 10 passages through a 25-gauge needle in 20 volumes of homogenization buffer (10 mM Tris, pH 7.5, 0.25 M sucrose, 1 mM EDTA) containing protease inhibitors. Homogenates were centrifuged at 500 g for 10 min to remove nuclei and unbroken cells. The pellets were rehomogenized and centrifuged. The post-nuclear supernatants were centrifuged at 65,000 g for 1 h, and the pellets were resuspended in 0.2 ml of homogenization buffer. Discontinuous gradients of iodixanol (Optiprep; Axis-Shield PoC, Oslo, Norway) were prepared according to Xia *et al.* [35] with some modifications. Iodixanol was diluted to a final concentration of 50% iodixanol in a solution containing 0.25 M sucrose, 10 mM Tris, pH 7.5 and 1 mM EDTA, and the resulting solution was used as the gradient stock solution. Different densities of iodixanol were established by diluting the stock with homogenization buffer. Gradients were set up in Beckman SW60 centrifuge tubes by overlaying from the bottom to top as follows: 30% iodixanol, 72 μl ; 20%, 120 μl ; 17.5%, 120 μl ; 15%, 480 μl ; 12.5%, 120 μl ; 10%, 480 μl ; 7.5%, 480 μl ; 5%, 480 μl ; and 2.5%, 240 μl . The samples were loaded on top of the gradients and centrifuged in a SW60 rotor at 270,000 g for 2.5 h. Fractions (240 μl) were collected and diluted with 3 volumes of 10 mM Tris, pH 7.5 and 1 mM EDTA. After centrifugation at 100,000 g for 30 min, the pellets were treated with Laemmli sample buffer and analyzed by Western blotting.

Co-immunoprecipitation

Cerebral cortical tissues from 1-month-old C57BL/6 mice were homogenized with 10 volumes of Tris-saline buffer (10 mM Tris, pH 7.4, 150 mM NaCl) containing protease inhibitors. After centrifugation at 100,000 g for 1 h, the pellet was solubilized with lysis buffer (10 mM Tris, pH 7.4, 0.5% NP-40, 2 mM EDTA, 10% Glycerol) containing protease inhibitors on ice for 1 h. Extracts were incubated overnight with monoclonal BACE1 antibody and protein G-agarose (Roche Applied Science, Mannheim, Germany). Immunoprecipitated materials were washed, eluted with Laemmli sample buffer, and subjected to Western blot analysis.

Immunohistochemistry

For tissue staining, brains were removed within 3.3 h of death, sectioned coronally at 1 cm intervals, fixed for 48 h in ice-cooled 4% buffered paraformaldehyde (pH 7.4), and cut into 40 μ m sections on a freezing microtome. For single immunostaining with a rabbit anti-RTN3 polyclonal antibody, fixed tissue sections were treated with 3% hydrogen peroxide in water for 10 min, 4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, anti-RTN3 antibody diluted 1:1,000 with PBS containing 4% BSA, and 0.3% Triton X-100 for 24 h at 4°C, and goat anti-rabbit IgG conjugated to biotin (Jackson Immuno-Research Laboratories, Bar Harbor, ME, USA). Subsequently, sections were reacted with the avidin-biotin-horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA), and peroxidase activity visualized with 0.05% 3, 3'-diaminobenzidine. For double immunofluorescence analysis, fixed tissue sections were treated with 4% BSA in PBS for 30 min, and incubated with anti-RTN3 polyclonal and anti-MAP2 monoclonal antibodies, respectively, for 24 h at 4°C. Then, the sections were incubated with goat anti-rabbit IgG conjugated to biotin (Jackson) and goat anti-mouse IgG conjugated to Alexa Fluor 488 (Molecular Probes, Leyden, The Netherlands), and with Cy 3-conjugated streptavidin (Jackson Immuno-Research Laboratories). Thereafter, sections were treated with 0.5% Sudan Black B in 70% ethanol to reduce autofluorescence, and Alexa Fluor 488 and Cy 3 fluorescence detected using a confocal laser-scanning system (Radiance 2000, Bio-Rad, Hercules, CA, USA) mounted with a Nikon Eclipse E2000 microscope. In parallel, adjacent brain sections were treated with non-immune rabbit and mouse sera instead of the anti-RTN3 and anti-MAP2 antibodies, respectively, to confirm staining specificity. Double immunofluorescence using anti-RTN3 and anti-A β antibodies was performed as described above. Double immunofluorescence using anti-RTN3 and anti-calnexin antibodies, or anti-RTN3 and anti- β -COP antibodies was performed by the same method as above, except that goat FITC-conjugated anti-mouse IgG antibody (Jackson Immuno-Research Laboratories) followed by donkey FITC-conjugated anti-goat IgG antibody (Jackson Immuno-Research Laboratories) were used in place of goat anti-mouse IgG conjugated to Alexa Fluor 488.

RESULTS

RTN3 interacts with BACE1 in mouse brain

We previously showed that RTN3 interacts with BACE1 in cultured cells co-transfected with both genes [27]. To investigate the interactions between RTN3 and BACE1 in brain, we performed co-immunoprecipitation experiments with extracts of mouse brain cortex. Using an anti-RTN3 antibody (RN3-C), we detected a ~28 kDa RTN3 protein band in the membrane fraction of mouse brain extracts (Fig. 1A). As shown in Fig. 1B, RTN3 co-immunoprecipitated with endogenous BACE1, confirming interactions between these two membrane proteins in the mouse brain.

Total RTN3 protein expression in AD brains

Since RTN3 inhibits the β -secretase activity of BACE1 to produce A β , it is possible that alterations in the expression of this protein are involved in pathological A β accumulation in AD. Accordingly, we compared the RTN3 expression levels in 5 control and 5 AD brains by Western blotting. The details of these cases are summarized in Table 1. The RTN3 antibody detected a ~28 kDa RTN3 band in membrane extracts of the human cerebral cortex (Fig. 2A). Densitometric analysis of bands revealed no significant differences in the levels of RTN3 between control and AD brains (Fig. 2B). Thus, the total expression levels of RTN3 in AD brains are comparable to those in controls.

Immunohistochemical analysis of RTN3 expression in AD brains

We conducted RTN3 immunostaining of the frontal and temporal cortices in 5 AD and 5 control brains. RTN3 immunolabeling was observed in cells located in layers II, III, V and VI of the frontal and temporal cortices, with the strongest signal in layer III. Based on morphological features and distribution patterns, the majority of immunoreactive cells were identified as pyramidal neurons. Staining was evident in the cell bodies and processes of these cells (Fig. 3A–D). The distribution patterns and intensity of RTN3 immunoreactivity were similar in AD patients and controls. No RTN3 immunoreactivity was observed when the primary antibody against RTN3 was replaced with non-immune rabbit IgG (data not shown).

We performed double immunofluorescence analysis for RTN3 and MAP2 in the frontal and temporal cortices of 5 AD and 5 control brains. In confocal images, RTN3 immunoreactivity was largely evident in MAP2 positive cells (Fig. 3E–G). RTN3 displayed punctuate staining, while diffuse staining was observed for MAP2. No immunoreactivity was evident when the primary antibodies against RTN3 and MAP2 were replaced with non-immune rabbit and mouse sera, respectively (data not shown). In addition, double immunofluorescence staining for RTN3 and A β disclosed that senile plaques labeled with monoclonal 4G8 antibody were generally not immunopositive for RTN3. These double immunofluorescence results were similar to those obtained in our re-examination, as described below.

During the preparation of our manuscript, Hu *et al.* [36] reported abnormal RTN3 immunoreactivity surrounding senile plaques. Since the morphology was similar to the dystrophic neurites, and only small portion of the immunoreactivity of RTN3 was overlapped with those of phosphorylated tau and neurofilaments, they suggested that the abnormal structures may represent a distinct population of dystrophic neurites. They additionally reported that high molecular weight RTN3 protein bands were increasingly detectable in the AD brain samples compared with the control brain samples on Western blots [36]. Therefore, we carefully re-examined brain sections of the 5AD cases and additional 6 AD cases by single or double immunostaining for RTN3 or RTN3 and A β , respectively. In this re-examination, we performed antigen retrieval with microwave irradiation and applied sensitive immunostaining methods using tyramide signal amplification (TSA). We reconfirmed the major RTN3-immunoreactivity present in neurons (Fig. 3H), and observed that senile plaques were predominantly negative for RTN3 (Fig. 3I–K). In accordance with these immunohistochemical results, we failed to detect high molecular weight RTN3 bands on Western blots of homogenates of the 5 AD and 5 control cerebral cortices, unless samples were heat-denatured (data not shown).

Subcellular localization of RTN3 in human brains

Next, we analyzed the subcellular localization of RTN3 in two control and two AD brain samples by biochemical fractionation. Membrane proteins from cerebral cortical tissues were fractionated using an iodixanol gradient, followed by Western blot analysis. Representative data of a control brain sample are shown in Fig. 4A. β -COP, a Golgi marker protein, was mainly

present in fractions 2–7, whereas calnexin, an ER marker protein, was identified in fractions 9–12. EEA1, a marker for endosomes, was detected in fractions 1 and 2. RTN3 was broadly distributed from ER to Golgi fractions. Mature BACE1 was also distributed in various fractions, including ER and Golgi. APP exhibited a similar, but relatively broader distribution. Similar data were obtained with AD brain samples.

We then performed double immunofluorescence labeling of human cerebral cortical sections of three control and three AD cases with anti-RTN3 and anti-calnexin or anti-RTN3 and anti- β -COP. As shown in Fig. 4B–D, the immunoreactivities of RTN3 and calnexin partially overlapped in pyramidal neurons. Similarly, the immunoreactivity of RTN3 was partially colocalized with that of β -COP (Fig. 4E–G). These observations confirm the localization of RTN3 in multiple compartments including ER and Golgi in pyramidal neurons.

DISCUSSION

RTN family proteins have received considerable attention since the recent discovery that RTN3 and RTN4-B/C are novel regulators of BACE1 [24,26,27]. However, the relevance of these RTNs to AD pathology remains to be established. In the present study, we focus on RTN3, since it is selectively expressed in the brain and has a relatively potent inhibitory effect on BACE1. Western blotting and immunohistochemical analysis were performed to determine the expression levels and patterns of RTN3 in human brains of control and AD cases. We further analyzed the subcellular distribution of RTN3 in brain.

Initially, co-immunoprecipitation experiments were performed to confirm the interactions between RTN3 and BACE1 in mouse brain. RTN3 co-immunoprecipitated with BACE1 in brain extracts, suggestive of physiological interactions between the endogenous proteins in brain. Next, we performed Western blot analysis to compare RTN3 protein expression levels in control and AD brains. There were no significant differences in the total levels of RTN3 protein between control and AD brains. A recent report using cDNA subtraction methodology disclosed downregulation of RTN3 expression in AD [37]. However, these results appear preliminary, since the group used only a small number of brain samples. Further investigation using a large number of brain samples is needed for precise comparison of RTN3 expression levels in control and AD brains.

Immunohistochemical analysis of post-mortem human brains reveals that RTN3 is expressed predominantly in the pyramidal neurons of the human cerebral cortex. This finding is consistent with previous reports showing neuronal expression of RTN3 [26,38,39]. Similarly, BACE1 is expressed primarily in pyramidal neurons of the cerebral cortex [2,40–42]. Thus, it is possible that RTN3 co-localizes with BACE1 in pyramidal neurons and regulates BACE1 function. In addition, no remarkable differences were evident in the distribution patterns and staining intensity of RTN3 immunoreactivity between AD and control brains. During the preparation of our manuscript, as noted in the results section, Hu *et al.* [36] have reported that they detected abnormal RTN3-immunopositive structures around senile plaques that resemble dystrophic neurites. Although we re-examined 11 AD cases by addition of microwave irradiation and TSA, we observed that only a small number of senile plaques were weakly positive for RTN3, as demonstrated in Fig 3I–K. In only 2 AD cases with severe neuronal loss, such positive staining in senile plaques was relatively observable in the limited cortical areas. These results suggest that RTN3 does not play a significant role in the formation of senile plaques. One apparent difference between our data and those of Hu *et al.* [36] is that our immunostaining results showed clear RTN3 immunoreactivity in neurons, whereas theirs showed only mild immunoreactivity. Additionally, our Western blots of AD brain samples did not show the high molecular weight RTN3 bands observed by Hu *et al.* [36]. It remains unclear whether these discrepancies were the result of methodological differences.

Subcellular fractionation of human brain tissues shows that RTN3 co-localizes with BACE1 in both ER and Golgi fractions. Similar results were obtained with mouse brain tissues and rat primary neurons (data not shown). These findings are in agreement with a recent study showing co-localization of BACE1 and RTN3 in HEK293 cells overexpressing BACE1 [26]. Moreover, our double immunofluorescence analysis confirmed that RTN3 localizes in ER and Golgi in pyramidal neurons in the cerebral cortex. Thus, it is likely that in pyramidal neurons the A β -generating activity of BACE1 is regulated by RTN3 in particular compartments including Golgi/*trans*-Golgi network, which is the major site of A β production [43].

In conclusion, our results demonstrate that RTN3 is abundantly expressed in pyramidal neurons in the human brain. Our data also suggest that there is considerable co-localization of RTN3 with BACE1 at a subcellular level. Further clarification of the biological roles of RTN3 in the brain may contribute to the development of novel therapeutic strategies for AD.

Acknowledgments

We are grateful to the Sun Health Research Institute Brain Donation Program of Sun City, Arizona, U.S.A. for the provision of post-mortem human brain tissues. The Brain Donation Program is partially supported by a National Institute on Aging grant (P30 AG19610 Arizona Alzheimer's disease Core Center). This work was supported, in part, by a grant from the Ministry of Health, Labour and Welfare of Japan, a Grant-in-Aid for Scientific Research (C) 19591025 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a research grant from the Mitsubishi Pharma Research Foundation.

REFERENCES

1. Selkoe DJ. Deciphering the genesis and fate of amyloid beta-protein yields novel therapies for Alzheimer disease. *J Clin Invest* 2002;110:1375–1381. [PubMed: 12438432]
2. Vassar R, Bennett BD, Babu-Khan S, Kahn S, Mendiaz EA, Denis P, Teplow DB, Ross S, Amarante P, Loeloff R, Luo Y, Fisher S, Fuller J, Edenson S, Lile J, Jarosinski MA, Biere AL, Curran E, Burgess T, Louis JC, Collins F, Treanor J, Rogers G, Citron M. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science* 1999;286:735–741. [PubMed: 10531052]
3. Yan R, Bienkowski MJ, Shuck ME, Miao H, Tory MC, Pauley AM, Brashier JR, Stratman NC, Mathews WR, Buhl AE, Carter DB, Tomasselli AG, Parodi LA, Heinrikson RL, Gurney ME. Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. *Nature* 1999;402:533–537. [PubMed: 10591213]
4. Sinha S, Anderson JP, Barbour R, Basi GS, Caccavello R, Davis D, Doan M, Dovey HF, 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, Suomensari SM, Wang S, Walker D, Zhao J, McConlogue L, John V. Purification and cloning of amyloid precursor protein beta-secretase from human brain. *Nature* 1999;402:537–540. [PubMed: 10591214]
5. Hussain I, Powell D, Howlett DR, Tew DG, Meek TD, Chapman C, Gloger IS, Murphy KE, Southan CD, Ryan DM, Smith TS, Simmons DL, Walsh FS, Dingwall C, Christie G. Identification of a novel aspartic protease (Asp 2) as beta-secretase. *Mol Cell Neurosci* 1999;14:419–427. [PubMed: 10656250]
6. Lin X, Koelsch G, Wu S, Downs D, Dashti A, Tang J. Human aspartic protease memapsin 2 cleaves the beta-secretase site of beta-amyloid precursor protein. *Proc Natl Acad Sci USA* 2000;97:1456–1460. [PubMed: 10677483]
7. Luo Y, Bolon B, Kahn S, Bennett BD, Babu-Khan S, Denis P, Fan W, Kha H, Zhang J, Gong Y, Martin L, Louis JC, Yan Q, Richards WG, Citron M, Vassar R. Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nat Neurosci* 2001;4:231–232. [PubMed: 11224535]
8. Roberds SL, Anderson J, Basi G, Bienkowski MJ, Branstetter DG, Chen KS, Freedman SB, Frigon NL, Games D, Hu K, Johnson-Wood K, Kappenman KE, Kawabe TT, Kola I, Kuehn R, Lee M, Liu W, Motter R, Nichols NF, Power M, Robertson DW, Schenk D, Schoor M, Shopp GM, Shuck ME, Sinha S, Svensson KA, Tatsuno G, Tintrup H, Wijsman J, Wright S, McConlogue L. BACE knockout

- mice are healthy despite lacking the primary beta-secretase activity in brain: implications for Alzheimer's disease therapeutics. *Hum Mol Genet* 2001;10:1317–1324. [PubMed: 11406613]
9. Cai H, Wang Y, McCarthy D, Wen H, Borchelt DR, Price DL, Wong PC. BACE1 is the major beta-secretase for generation of A β peptides by neurons. *Nat Neurosci* 2001;4:233–234. [PubMed: 11224536]
 10. Ohno M, Sametsky EA, Younkin LH, Oakley H, Younkin SG, Citron M, Vassar R, Disterhoft JF. BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron* 2004;41:27–33. [PubMed: 14715132]
 11. Dominguez D, Tournoy J, Hartmann D, Huth T, Cryns K, Deforce S, Serneels L, Camacho IE, Marjaux E, Craessaerts K, Roebroek AJ, Schwake M, D'Hooge R, Bach P, Kalinke U, Moechars D, Alzheimer C, Reiss K, Saftig P, De Strooper B. Phenotypic and biochemical analyses of BACE1- and BACE2-deficient mice. *J Biol Chem* 2005;280:30797–30806. [PubMed: 15987683]
 12. Chiocco MJ, Kulnane LS, Younkin L, Younkin S, Evin G, Lamb BT. Altered amyloid-beta metabolism and deposition in genomic-based beta-secretase transgenic mice. *J Biol Chem* 2004;279:52535–52542. [PubMed: 15452128]
 13. Mohajeri MH, Saini KD, Nitsch RM. Transgenic BACE expression in mouse neurons accelerates amyloid plaque pathology. *J Neural Transm* 2004;111:413–425. [PubMed: 14991462]
 14. Willem M, Dewachter I, Smyth N, Van Dooren T, Borghgraef P, Haass C, Van Leuven F. beta-site amyloid precursor protein cleaving enzyme 1 increases amyloid deposition in brain parenchyma but reduces cerebrovascular amyloid angiopathy in aging BACE x APP[V717I] double-transgenic mice. *Am J Pathol* 2004;165:1621–1631. [PubMed: 15509532]
 15. Holsinger RM, McLean CA, Beyreuther K, Masters CL, Evin G. Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann Neurol* 2002;51:783–786. [PubMed: 12112088]
 16. Fukumoto H, Cheung BS, Hyman BT, Irizarry MC. Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. *Arch Neurol* 2002;59:1381–1389. [PubMed: 12223024]
 17. Li R, Lindholm K, Yang LB, Yue X, Citron M, Yan R, Beach T, Sue L, Sabbagh M, Cai H, Wong P, Price D, Shen Y. Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. *Proc Natl Acad Sci USA* 2004;101:3632–3637. [PubMed: 14978286]
 18. Zhao J, Fu Y, Yasvoina M, Shao P, Hitt B, O'Connor T, Logan S, Maus E, Citron M, Berry R, Binder L, Vassar R. Beta-site amyloid precursor protein cleaving enzyme 1 levels become elevated in neurons around amyloid plaques: implications for Alzheimer's disease pathogenesis. *J Neurosci* 2007;27:3639–3649. [PubMed: 17409228]
 19. Laird FM, Cai H, Savonenko AV, Farah MH, He K, Melnikova T, Wen H, Chiang HC, Xu G, Koliatsos VE, Borchelt DR, Price DL, Lee HK, Wong PC. BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *J Neurosci* 2005;25:11693–11709. [PubMed: 16354928]
 20. Chang WP, Koelsch G, Wong S, Downs D, Da H, Weerasena V, Gordon B, Devasamudram T, Bilcer G, Ghosh AK, Tang J. In vivo inhibition of A β production by memapsin 2 (beta-secretase) inhibitors. *J Neurochem* 2004;89:1409–1416. [PubMed: 15189343]
 21. Asai M, Hattori C, Iwata N, Saido TC, Sasagawa N, Szabó B, Hashimoto Y, Maruyama K, Tanuma S, Kiso Y, Ishiura S. The novel beta-secretase inhibitor KMI-429 reduces amyloid beta peptide production in amyloid precursor protein transgenic and wild-type mice. *J Neurochem* 2006;96:533–540. [PubMed: 16336629]
 22. Hussain I, Hawkins J, Harrison D, Hille C, Wayne G, Cutler L, Buck T, Walter D, Demont E, Howes C, Naylor A, Jeffrey P, Gonzalez MI, Dingwall C, Michel A, Redshaw S, Davis JB. Oral administration of a potent and selective non-peptidic BACE-1 inhibitor decreases beta-cleavage of amyloid precursor protein and amyloid-beta production in vivo. *J Neurochem* 2007;100:802–809. [PubMed: 17156133]
 23. Oertle T, Schwab ME. Nogo and its paRTNers. *Trends Cell Biol* 2003;13:187–194. [PubMed: 12667756]
 24. Yan R, Shi Q, Hu X, Zhou X. Reticulon proteins: emerging players in neurodegenerative diseases. *Cell Mol Life Sci* 2006;63:877–889. [PubMed: 16505974]

25. Yang YS, Strittmatter SM. The reticulons: a family of proteins with diverse functions. *Genome Biol* 2007;8:234. [PubMed: 18177508]
26. He W, Lu Y, Qahwash I, Hu XY, Chang A, Yan R. Reticulon family members modulate BACE1 activity and amyloid-beta peptide generation. *Nat Med* 2004;10:959–965. [PubMed: 15286784]
27. Murayama KS, Kametani F, Saito S, Kume H, Akiyama H, Araki W. Reticulons RTN3 and RTN4-B/C interact with BACE1 and inhibit its ability to produce amyloid beta-protein. *Eur J Neurosci* 2006;24:1237–1244. [PubMed: 16965550]
28. Acquati F, Accarino M, Nucci C, Fumagalli P, Jovine L, Ottolenghi S, Taramelli R. The gene encoding DRAP (BACE2), a glycosylated transmembrane protein of the aspartic protease family, maps to the down critical region. *FEBS Lett* 2000;468:59–64. [PubMed: 10683441]
29. Bennett BD, Babu-Khan S, Loeloff R, Louis JC, Curran E, Citron M, Vassar R. Expression analysis of BACE2 in brain and peripheral tissues. *J Biol Chem* 2000;275:20647–20651. [PubMed: 10749877]
30. Murayama KS, Kametani F, Araki W. Extracellular release of BACE1 holoproteins from human neuronal cells. *Biochem Biophys Res Commun* 2005;338:800–807. [PubMed: 16243299]
31. Kametani F, Tanaka K, Ishii T, Ikeda S, Kennedy HE, Allsop D. Secretory form of Alzheimer amyloid precursor protein 695 in human brain lacks beta/A4 amyloid immunoreactivity. *Biochem Biophys Res Comm* 1993;191:392–398. [PubMed: 8460999]
32. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, Vogel FS, Hughes JP, van Belle G, Berg L. CERAD Neuropathologists. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 1991;41:479–486. [PubMed: 2011243]
33. Araki W, Yuasa K, Takeda S, Takeda K, Shirotani K, Takahashi K, Tabira T. Pro-apoptotic effect of presenilin 2 (PS2) overexpression is associated with down-regulation of Bcl-2 in cultured neurons. *J Neurochem* 2001;79:1161–1168. [PubMed: 11752057]
34. Takeda K, Araki W, Tabira T. Enhanced generation of intracellular Abeta42 amyloid peptide by mutation of presenilins PS1 and PS2. *Eur J Neurosci* 2004;19:258–264. [PubMed: 14725619]
35. Xia W, Zhang J, Ostaszewski BL, Kimberly WT, Seubert P, Koo EH, Shen J, Selkoe DJ. Presenilin 1 regulates the processing of beta-amyloid precursor protein C-terminal fragments and the generation of amyloid beta-protein in endoplasmic reticulum and Golgi. *Biochemistry* 1998;37:16465–16471. [PubMed: 9843412]
36. Hu X, Shi Q, Zhou X, He W, Yi H, Yin X, Gearing M, Levey A, Yan R. Transgenic mice overexpressing reticulon 3 develop neuritic abnormalities. *EMBO J* 2007;26:2755–2767. [PubMed: 17476306]
37. Yokota T, Mishra M, Akatsu H, Tani Y, Miyauchi T, Yamamoto T, Kosaka K, Nagai Y, Sawada T, Heese K. Brain site-specific gene expression analysis in Alzheimer's disease patients. *Eur J Clin Invest* 2006;36:820–830. [PubMed: 17032350]
38. Kumamaru E, Kuo CH, Fujimoto T, Kohama K, Zeng LH, Taira E, Tanaka H, Toyoda T, Miki N. Reticulon3 expression in rat optic and olfactory systems. *Neurosci Lett* 2004;356:17–20. [PubMed: 14746891]
39. Di Scala F, Dupuis L, Gaiddon C, De Tapia M, Jokic N, Gonzalez de Aguilar JL, Raul JS, Ludes B, Loeffler JP. Tissue specificity and regulation of the N-terminal diversity of reticulon 3. *Biochem J* 2005;385:125–134. [PubMed: 15350194]
40. Sun A, Koelsch G, Tang J, Bing G. Localization of beta-secretase memapsin 2 in the brain of Alzheimer's patients and normal aged controls. *Exp Neurol* 2002;175:10–22. [PubMed: 12009756]
41. Leuba G, Wernli G, Vernay A, Kraftsik R, Mohajeri MH, Saini KD. Neuronal and nonneuronal quantitative BACE immunocytochemical expression in the entorhinohippocampal and frontal regions in Alzheimer's disease. *Dement Geriatr Cogn Disord* 2005;19:171–183. [PubMed: 15677864]
42. Singer O, Marr RA, Rockenstein E, Crews L, Coufal NG, Gage FH, Verma IM, Masliah E. Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. *Nat Neurosci* 2005;8:1343–1349. [PubMed: 16136043]
43. LaFerla FM, Green KN, Oddo S. Intracellular amyloid-beta in Alzheimer's disease. *Nat Rev Neurosci* 2007;8:499–509. [PubMed: 17551515]

Fig. 1. Interactions between RTN3 and BACE1 in the brain. (A) Western blotting with the anti-RTN3 antibody (RN3-C) led to the specific detection of RTN3 proteins (~28 kDa) in mouse brain extracts. (B) Membrane extracts of mouse cerebral cortical tissues were immunoprecipitated with monoclonal BACE1 antibody (MAB9311). Precipitated proteins were immunoblotted with anti-RTN3 antibody (RN3-C). The membrane was reprobed with polyclonal BACE1 antibody (NBA).

Fig 2.

RTN3 protein expression in control and AD brains. (A) Membrane proteins from control and AD brains were subjected to immunoblotting with RTN3 antibody. The membrane was reprobed with β -actin antibody. (B) Band intensities in (A) were quantified by densitometric analysis, and the relative expression levels of RTN3 were calculated. β -Actin was used for normalization. Data are presented as means $\pm$ SEM (n=5).

Fig. 3.

Immunohistochemical analysis of RTN3 expression in the cerebral cortices of AD and control brains. (A–D) Distribution of immunoreactivity for RTN3 in the temporal cortices of AD (A, B) and control (C, D) brains. RTN3 immunoreactivity was observed mainly in cells located in layers II–III of the temporal cortex. No significant differences in cellular distribution of RTN3 immunoreactivity were observed between AD (A, B) and control (C, D) patients. Panels B and D represent higher magnification views of panels A and C, respectively. Scale bars indicate 100 μ m (A, C) and 25 μ m (B, D). (E–G) Confocal images of double immunofluorescence for RTN3 and MAP2. Neuronal localization of RTN3 immunoreactivity was observed in the temporal cortex of a control case. RTN3 immunoreactivity (detected with Cy 3 as a red fluorescence; F) was predominant in cells containing MAP2 immunoreactivity (detected with Alexa Fluor 488 as a green fluorescence; E). Panel G is an overlaid image of panels E and F. Scale bars indicate 25 μ m. Similar results were observed from double immunofluorescence analysis of the temporal cortex of other control and AD cases. (H–K) By addition of microwave irradiation and TSA, single immunoperoxidase staining for RTN3 (H) and double immunofluorescence for RTN3 and 4G8 (I–K) were re-performed on 11 AD cases. Panels H–K are representative images in the temporal cortex of an AD case. The single immunostaining (H) revealed that RTN3-positive cellular distribution was similar to that shown in panels A–D. The double immunofluorescence for RTN3 and A β (I–K) revealed that RTN3 immunoreactivity (detected with Alexa Fluor 488 as a green fluorescence; I) was present largely in pyramidal neurons, while senile plaques were clearly labeled with 4G8 antibody (detected with Cy 3 as a red fluorescence; J). As depicted in the overlaid image (K), senile plaques were generally negative for RTN3, although a small number of senile plaques were sometimes weakly immunopositive. Scale bars indicate 25 μ m.

A

Fig. 4.

Analyses of the subcellular distribution of RTN3 in human cerebral cortical tissues (A) Cerebral cortical tissues from a control case were subjected to biochemical subcellular fractionation with iodixanol, followed by Western blotting with an anti-RTN3 antibody. The membrane was reprobed with the different antibodies specified in the panel. Calnexin, β -COP, and EEA1 are markers for ER, Golgi, and endosomes, respectively. (B–D) Confocal images of double immunofluorescence for calnexin and RTN3. Calnexin and RTN3 immunoreactivities were detected with FITC (green; B) and Cy 3 (red; C), respectively. The merged image (D) indicates that RTN3 and calnexin immunoreactivities partially overlapped in neurons in the temporal cortex from a control case. (E–G) Confocal images of double immunofluorescence for β -COP and RTN3. β -COP and RTN3 immunoreactivities were detected with FITC (green; E) and Cy 3 (red; F), respectively. The merged image (G) indicates the partial colocalization of RTN3 and β -COP immunoreactivities in neurons. Scale bars indicate 25 μ m. Similar results were observed from double immunofluorescence analysis of the temporal cortex of other control and AD cases.

Table 1

Summary of cases examined.

Group	Age (years)	Gender	PMI (h)	Disease duration (years)	Neuropathological diagnosis
Control					
1	85	M	3.2		Control
2	86	F	2.5		Control
3	82	F	2.3		Control
4	84	M	3.0		Control
5	91	M	1.5		Control
AD					
1	78	M	1.8	8	Definite AD
2	87	F	3.3	7	Definite AD
3	85	F	1.5	10	Definite AD
4	70	M	2.3	11	Definite AD
5	75	M	3.0	7	Definite AD

PMI, postmortem interval.