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Review

Reticulon proteins: emerging players in neurodegenerative diseases

R. Yan*, Q. Shi, X. Hu and X. Zhou

Department of Neurosciences, Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195 (USA), Fax: +1 216 444 7927, e-mail: yanr@ccf.org

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Abstract. Reticulons (RTNs) are a group of integral membrane proteins that have a uniquely conserved C-terminal domain named RHD. In mammalian genomes, transcripts are produced from four genes, *rtn1* to *rtn4*, under the regulation of tissue or cell-type-specific expression. The presence of alternative promoters for gene expression and multiple cryptic splicing sites have resulted in large numbers of genes/proteins that are classified among the reticulon family. Although this family exists in almost all eukaryotes, only the *rtn4* gene product, Nogo (RTN4), has gained relatively more in-depth attention. Despite predominant localization in the endoplasmic reticulum, Nogo on the cell surface appears to play a critical role as an inhibitory molecule for axonal growth and regeneration in humans and rodents. Recently, studies have expanded the biological functions of RTNs to other facets including modulating the enzymatic activity of β-secretase in Alzheimer's disease. In this review, we summarize the accumulated findings concerning the structural and functional aspects of RTNs and speculate on their linkage to the pathogenesis of neurodegenerative diseases.

Keywords. Reticulon, Nogo, RHD, neurodegeneration, BACE1.

Introduction

Reticulons (RTNs) are membrane-bound proteins that have begun to attract more attention for their roles in various disorders, including neurodegenerative disease. The nomenclature of reticulons originally referred to a neuroendocrine-specific protein (NSP) anchored mainly on the membrane of smooth endoplasmic reticulum (ER) [1]. Subsequent molecular cloning and in silico search of expressed sequence tag (EST) databases have classified a long list of proteins belonging to the RTN family [2–7]. RTNs exist in plants, fungi and animals [7]. While the precise cellular functions of RTNs remain to be defined, RTNs have already been shown to regulate many different cellular processes, and to interact with diverse cellular proteins such as BACE1 [8], extracellular receptors [9–10], fusion and endocytic proteins [11]. Nevertheless, precisely how RTNs function at multiple cellular locales remains a major and interesting quest.

In mammalian genomes, four independent reticulon genes – *rtn1, rtn2, rtn3* and *rtn4* – have been identified as encoding a large number of gene products. Among them, RTN4 (more popularly known as Nogo) has been intensively investigated as a critical inhibitory molecule controlling axonal growth and regeneration (for reviews see [12–13]). Its longest isoform, RTN4-A, is a particularly potent inhibitor of neurite outgrowth after spinal cord injury [14]. Recent discoveries of RTNs in modulating β-secretase and vascular remodeling have further extended our understanding of the diverse functions of RTNs [15–16]. In this review, the connection of RTNs to the pathogenesis of human diseases, principally the neurodegenerative disorders, will be expounded. However, before focusing on this topic, we will first present an overview of the most recent findings

* Corresponding author.

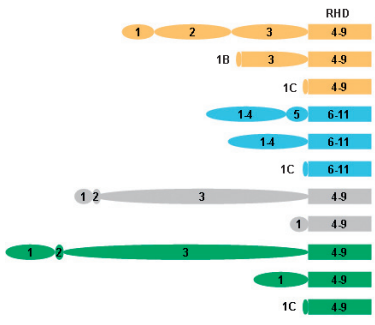
on the structural and functional aspects of mammalian RTNs; the growing knowledge of RTN in lower model organisms will be touched on in relevant sections.

Gene structure of mammalian RTNs

The genomic structure of RTN in mammalian systems has a common feature: it spans a region of considerable length and contains multiple large introns [7]. The large first intron in all mammalian RTNs typically contains promoters for alternative transcriptional initiation. Such gene structures are presumed to produce multiple transcripts divergent at the 5′ side. Indeed, all RTN genes give rise to multiple transcripts resulting from the use of different promoters (Fig. 1). Furthermore, the presence of multiple cryptic splicing sites within the first several introns/exons in each RTN gene appears to control the production of tissue or cell-type-specific spliced variants. Strikingly, an abnormal splicing event appears to occur in some diseases, for example in epilepsy [16–17], implicating the existence of a regulated splicing event. In contrast to these complicated splicing events, which occur within the first few introns/exons, the genomic compositions coding for the C-terminal reticulon homology domain (RHD) are quite stable. Accordingly, these events have created spliced variants divergent in the N-terminal region and a highly conserved C-terminal RHD (Fig. 1).
Original studies have already documented the presence of multiple spliced variants of *rtn1, rtn2* and *rtn4*, while only one *rtn3* gene product was reported initially [3]. However, recent genome searches and biochemical analysis have suggested the presence of several scarce RTN3 isoforms in humans and rodents [7, 18]. This situation has created a confusing nomenclature for RTN3 isoforms. In Figure 1, two RTN3 splicing variants are included and renamed, consistent with the structural rule that has previously been applied to the other subfamilies. Since the other RTN3 isoforms do not appear in concordant when human and mouse EST databases are searched for comparison [W. He, Q. Shi and R. Yan, unpublished observation], we have provisionally excluded these rare isoforms in Figure 1 until further evidence is available.
In humans and rodents, the RHD of RTN1, RTN3 and RTN4 is encoded by exons 4–9, whereas RHD of RTN2 is encoded by exons 6–11 (Fig. 1), suggesting that RTN1, RTN3 and RTN4 are more similar to each other than to the RTN2 gene. Phylogenic comparison based on sequence homology is consistent with this structural organization [7]. Although only human and mouse genomic structures are depicted in Figure 1, similar genomic organizations of four RTN paralogues are extendable to other mammalian species. RTN in mammalian genomes appears to quadruplicate from an early ancestor; for example, only one RTN gene is found in nematodes [7]. Moreover, it is worth pointing out that recent studies in fish have revealed even more complicated pictures: fugu has six RTN genes, while zebrafish has seven RTN genes; and two fish RTN genes appear to cluster with one mammalian RTN subfamily according to phylogenic analysis [19]. The presence of an extra set of RTN genes in fish is consistent with the hypothesis of genome duplications in fish [20–21]. The presence of a larger RTN family in mammalian and vertebrate species may imply the need for many RTNs in order to accommodate more complicated and diversified cellular processes in mammals.

Structural organization of RTNs and the RHD

The highly constrained genomic structure coding for the C-terminal RHD throughout its evolution has preserved the traits of the RTN family. Although the size of RHDs range from as short as 186 amino acids in zebrafish to



RTN Family	Alternative symbols	Amino acids		Chromosome		Ref.
		Human	Mouse	Human	Mouse	
RTN1-A	Nsp-A	776	780	14q23.1	12C3	2
RTN1-B	Nsp-B	356	ND			
RTN1-C	Nsp-C	208	208			
RTN2-A	Nspl1-A	545	ND	19q13.32	7A2	45-46
RTN2-B	Nspl1-B	472	471			
RTN2-C	Nspl1-C	205	204			
RTN3-A	RTN3-A4b	1032	964	11q13	19A	7, 15
RTN3-B	RTN3, RTN3-A1	236	237			
RTN4-A	Nogo-A	1192	1162	2p16.3	11A3.3	4-7, 31
RTN4-B	Nogo-B, ASY	373	356			
RTN4-C	Nogo-C	199	199			

Figure 1. Schematic depictions of human and mouse RTNs. RTNs are transcribed from four *rtn* genes and each has multiple transcripts. The N-terminal domain is depicted as ovals and the conserved reticulon homologous domains (RHD) as boxes. The number corresponds to each exon. The major alternative nomenclatures are also listed in the table. The numbers of amino acids are calculated based on the protein sequence database of the National Center for Biotechnology Information (NCBI). The chromosomal locations of the reticulon genes are provided through the NCBI Map Viewer. The amino acid information is adopted from either the NCBI protein database or publications [3, 5, 18, 48, 50, 89].

276 amino acids in *Saccharomyces cerevasie*, its overall structural organization is the same. RHD possesses one hydrophilic loop of 66 amino acids flanked by two putative transmembrane segments plus a hydrophilic tail (Fig. 2a). The conservation of RHD has already been shown to confer common functions upon RTNs, such as localizing RTNs to the appropriate membranes and/or mediating protein interactions.

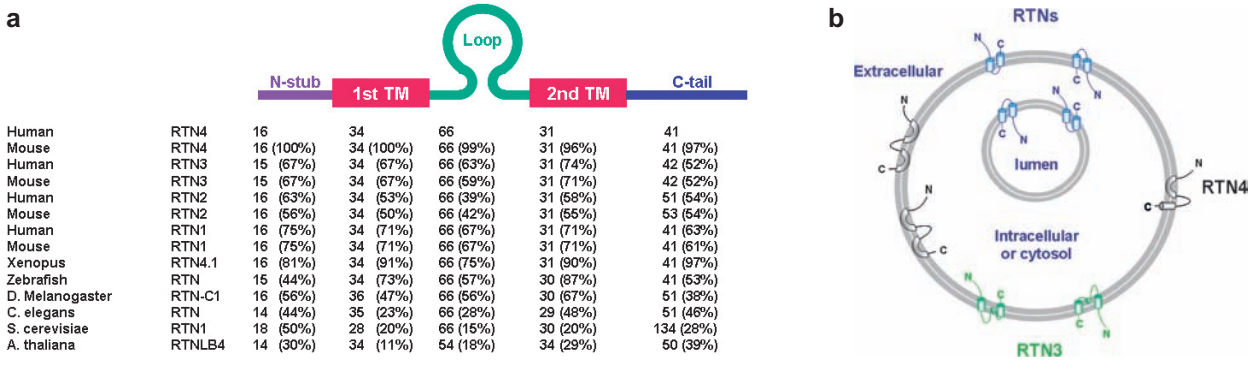
Two transmembrane domains

Based on the entire RHD sequence, a computer search predicts the presence of two presumable transmembrane domains. In order to span a phospholipid bilayer, a typical transmembrane domain must possess a helical hydrophobic segment comprising 21–25 amino acids [22]. Mammalian RTNs as well as their homologues in lower organisms contain two long stretches (28–36 amino acids) of totally hydrophobic residues (Fig. 2a); each is sufficient to embed RTNs in the membrane. The features present in these two long hydrophobic stretches most likely govern the cellular localization of RTNs and their topology on the membrane. Bioinformatic analysis indicates that most RTNs adopt a horse shoe-like orientation in the membrane; but several RTN members, including human RTN3, may also adopt an alternative pattern with a small flip-flop region within the membrane (Fig. 2b). Studies using antibodies that recognize the RTN4 N-terminal domain have indicated that orientation of the N-terminal domain on a plasma membrane is bilateral [23], suggesting that the transmembrane domain, mainly the first one, may adopt a flexible mechanism to either extend across both leaflets or to flip back from one leaflet (Fig. 2b). The presence of the N-terminus or the loop region on the cell surface is consistent with the potential functions of RTN4, particularly RTN4-A, as an inhibitor of neurite extension.

Unlike a type I transmembrane protein, the N-terminal region in RTNs has no signal peptide sequence. It is possible that the signal peptide sequence in RTN may be concealed within the two putative transmembrane domains. The low sequence identity of these two domains between vertebrate and invertebrate RTNs suggests that a structural feature, rather than the sequence motif, potentially aids in targeting RTNs to the membrane. Moreover, mutagenesis studies have revealed that the integrity of these two hydrophobic stretches is also important for the stability of RTNs [W. He, Q. Shi and R. Yan, unpublished observations]. Disruption of the second segment has already been shown to affect the localization of RTN4 in the ER compartments [23]. Therefore, the putative transmembrane domains are critical components for the proper function of RTNs.

The N-stub

The N-stub within RHD is short and relatively conserved among various species. However, the structural informa-



Human	RTN4	16	34	66	31	41
Mouse	RTN4	16 (100%)	34 (100%)	66 (99%)	31 (96%)	41 (97%)
Human	RTN3	15 (67%)	34 (67%)	66 (63%)	31 (74%)	42 (52%)
Mouse	RTN3	15 (67%)	34 (67%)	66 (59%)	31 (71%)	42 (52%)
Human	RTN2	16 (63%)	34 (53%)	66 (39%)	31 (58%)	51 (54%)
Mouse	RTN2	16 (56%)	34 (50%)	66 (42%)	31 (55%)	53 (54%)
Human	RTN1	16 (75%)	34 (71%)	66 (67%)	31 (71%)	41 (63%)
Mouse	RTN1	16 (75%)	34 (71%)	66 (67%)	31 (71%)	41 (61%)
Xenopus	RTN4.1	16 (81%)	34 (91%)	66 (75%)	31 (90%)	41 (97%)
Zebrafish	RTN	15 (44%)	34 (73%)	66 (57%)	30 (87%)	41 (53%)
D. Melanogaster	RTN-C1	16 (56%)	36 (47%)	66 (56%)	30 (67%)	51 (38%)
C. elegans	RTN	14 (44%)	35 (23%)	66 (28%)	29 (48%)	51 (46%)
S. cerevisiae	RTN1	18 (50%)	28 (20%)	66 (15%)	30 (20%)	134 (28%)
A. thaliana	RTNLB4	14 (30%)	34 (11%)	54 (18%)	34 (29%)	50 (39%)

Figure 2. Conserved RHD and its topology in the membranes. (*a*) As schematically illustrated, the RHD contains two putative transmembrane domains (TM1 and TM2), a loop region and a short C-terminus. The unusually long C-terminal tail of yeast RTN1P is due to the presence of duplicated sequence. The RHD is linked to the N-terminal region of the protein through an N-stub. The length of each protein segment is indicated with the numbers of aa residues. The sequence identity shown in parenthesis was determined based on pairwise comparison of amino acid sequence to the RHD from RTN4 (Blast 2 software from NCBI). Only human, mouse and selected RTNs from lower organisms are listed. The transmembrane domain is predicted using the online Transmembrane Prediction Server at http://www.sbc.su.se/~miklos/DAS/. The NCBI accession numbers for RTNs (genus and species) are listed as the following: human (*Homo sapiens*) RTN4, AAH07109; mouse (*Mus musculus*) RTN4, NP_077188; human RTN3, DAA01943; mouse RTN3, DAA01968; human RTN2, NP_996784; mouse RTN2, DAA01963; human RTN1, AAH00314; mouse RTN1, NP_001007597; xenopus (*Xenopus laevis*) RTN4, AAQ82641; zebrafish (*Danio rerio*) RTN1, NP_955946; fruit fly (*Drosophila melanogaster*) RTN1P-C1 mRNA, AY164770; nematode (*Caenorhabditis elegans*) RTN, NP_506658; yeast (*Saccharomyces cerevisiae*) RTN1P mRNA, AY164798; cress (*Arabidopsis thaliana*) RTNLB4, NP_198975. (*b*) Topology of RTN proteins. Although likely topological orientations of RTN4 have been suggested, the precise topology of RTNs in the lipid bilayer has not been resolved. Based on predictions by computer programs, human, mouse and *Xenopus* RTN3 as well as *Xenopus* and zebrafish RTN2 (in green) may adopt a different folding that may flip-flop in the lipid bilayer due to the presence of charged residues with TM2. Since both TM1 and TM2 in all RTNs are long enough to loop back within the lipid bilayer, we also illustrated other potential orientations of the RTN proteins in the membrane. Future biochemical studies will surely pinpoint which is the authentic topology of RTNs.

tion in this region has not yet been described because it is mainly regarded as a part of the conserved sequence in RHD. This region appears to serve as a structural linker between the N-terminal domain and the first transmembrane domain. A pull-down assay using a 41-amino acid RTN1-C N-terminal fragment that includes this stub has been shown to mediate interaction of RTN1-C with syntaxin 1 [24]. However, whether this N-stub is required for the above interaction has not been unambiguously determined.

The loop region

The loop region is predicted to be hydrophilic, and this domain may face the cytoplasm or lumen/extracellular spaces as mentioned earlier (Fig. 2b). This region in each RTN potentially exerts specific functions involving intercellular, and possibly also intracellular, communications. Indeed, the loop of RTN4 (Nogo), known as the Nogo-66 loop, specifically mediates the binding of RTN4 to its partners such as Nogo receptor (NgR) [9] and Caspr [10]. When compared with the loop region among human RTNs, RTN1 is closer to RTN4 with 67% identity at the amino acid level, while RTN2 is only 39% identical to RTN4 (Fig. 2). However, the loop in RTN1 does not interact with NgR despite the high homology [9]. Further mapping of this loop region indicates that the N-terminal 40 amino acids of Nogo-66 (NEP1-40) are sufficient to bind to NgR [25]. In an *in vitro* assay of axonal outgrowth, a synthetic peptide corresponding to NEP 1-40 functions as an antagonist [25]. The interaction between the Nogo-66 loop and NgR evidently transduces signals from oligodendrocytes to neurons for inhibiting axonal regeneration (for reviews see [26–29]). In addition to the signaling pathway that is initiated from Nogo-66, the interaction of the Nogo-66 loop in oligodendrocytes with Caspr in axons underscores an additional importance of RTN4 in the control of K^+ channel localization at axonal paranodes [10]. The binding partners for the loops of the other three RTNs are currently highly sought after. Future identification of receptors for the other loops will not only extend the scope of signaling pathways involving RTN members but also provide better clues to understand the general function of the RTN family.

The C-terminal tail

The sequence in the short C-terminal tail has over 50% identity among mammalian RTNs. Since it contains a

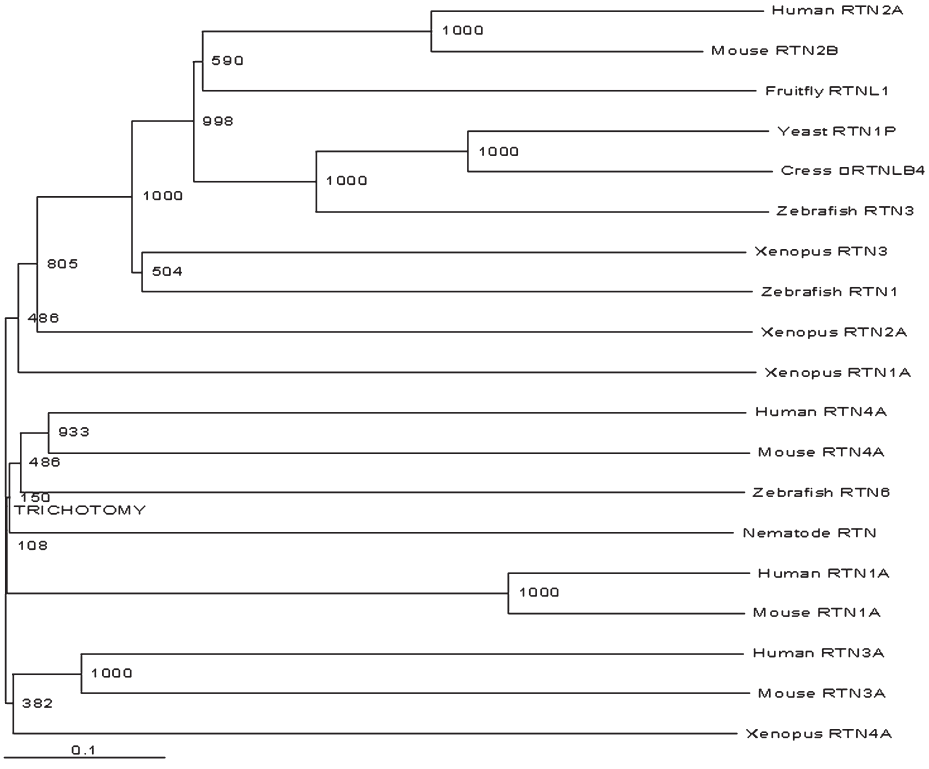


Figure 3. Phylograms of RTN homologues. The phylogenic analysis of RTN long isoforms based on the N-terminal regions of RTN using the online software of ClustalW (version 1.82) at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). Human and Mouse RTN paralogues are chosen to represent mammalian RTNs. *S. cerevisae* RTN1P is selected to represent yeast RTNs. The NCBI accession numbers for RTNs (genus and species) are the same as those listed in Figure 2. The branch lengths of the phylogenic tree are proportional to the amount of inferred evolutionary change. The number indicates the bootstrap values that reflect branching reliability. The higher number suggests higher reliability.

highly conserved KKXX motif, a typical ER retrieve signal that retains RTN proteins in the ER [30], this short C-tail would logically be expected to face the cytosol. In fact, however, this ER retention motif is less effective in restricting localization of RTNs solely in the ER. In accordance with dual possible orientations on a membrane, a portion of RTN that does not face the cytosol is unlikely to be retrieved. Confocal examinations using anti-RTN antibodies have already revealed the presence of RTNs in the Golgi, cell surface, dendrites and along the axons [1, 8, 21, 31]. The function of the C-terminal tail is beginning to be understood. Recent mapping studies showed that this C-terminal tail interacts with BACE1 (W. He and R. Yan, unpublished observations]. Deletion of this tail abolishes the RTN interaction with BACE1 and inhibitory activity of the RTN on BACE1. Future studies are expected to reveal additional functions of this C-tail.

Divergent N-terminal domains in RTNs

As demonstrated in their genomic organization, the N-terminal domains of four mammalian RTN paralogues are completely divergent. This divergence potentially allows each N-terminal domain to exert its specific function. It has been noted that, even though quite different among paralogues, the N-terminal sequences among RTN orthologues exhibit clear homology. For instance, the N-terminal domains of RTN4-A have about 60% amino acid identity between human and mouse orthologues and about 40% identity between human and chicken orthologues. However, the homology between human and frog orthologues is reduced to 30% identity, reflecting the distances in evolution. Using the N-terminal domain of each long isoform for phylogenic construction (Fig. 3), Zebrafish RTN6, which has a relatively longer N-terminal domain than the Zebrafish RTN4 and is believed to be a duplicate of the RTN4 subfamily [19], clearly clusters with the mammalian RTN4 subfamily. Nevertheless, this fish RTN4 orthologue, like Zebrafish RTN4, completely lacks the mammalian RTN4-A unique region [19]. These properties suggest that each RTN subfamily in mammalian species may have gained certain biological functions during evolution, particularly when compared with their orthologues in fish and lower organisms.

In the RTN family, the function of the N-terminal domain in RTN4-A has been extensively studied. Early experiments that examined neurite outgrowth and fibroblast outspread led to the discovery of a neutralizing antibody named IN-1 that was found to de-repress an inhibitory signal to axonal regeneration after injury [32]. Molecular cloning using IN-1 reveals that IN-1 recognizes an epitope within the N-terminal domain of RTN4-A. Further mapping study suggests that a region unique to RTN4-A (NiG Δ20; aa 544–725) appears to play a critic inhibitory function in neurite outgrowth [4, 23]. Since both fish RTN4 orthologues lack this critical region [19], that may explain the fact that fish, but not mammalian species, have the capacity of axonal regeneration after injury. Supporting this persuasive argument further, Diekman et al. found that the recombinant fragment corresponding to NiG Δ20 inhibited axonal growth when fish axons were explanted on coverslips coated with NiG Δ20 [19]. Even though the mechanism has not yet been fully revealed, activation of a small GTPase RhoA, which in turn inhibits Rac activity, has been implicated in driving the inhibition of neurite outgrowth [33].

While the role of the N-terminal domain is still being refined, the most recent binding study suggests that a 24-amino acid peptide named Nogo-A-24 (aa 995–1018 in the RTN4-A unique region), which is immediately adjacent to the N-stub in RHD, binds to NgR with high affinity [34]. Unlike binding of Nogo-66 to NgR, binding of Nogo-A-24 to NgR fails to alter cell spreading or axonal growth. This observation suggests that multiple sites in the N-terminal domain mediate protein-protein interactions [34]. In addition to the above observations, several biochemical studies also indicate that the N-terminal domain interacts with cellular trafficking proteins (see further discussion below).

Rich expression of RTNs in brain

Data from EST databases and Western analysis indicate that mammalian RTNs are ubiquitously expressed in all tissues [11], but each RTN member exhibits a unique expression pattern that prefers certain tissues or even cell types. Here, we limited our summary to the expression of RTNs in human and rodent brains and found that all four mammalian RTNs are expressed in both. The rich expression of RTNs in mammalian brains are consistent with the sources that led to original discoveries or molecular cloning of RTNs [11]. However, one surprising observation is the failure to detect RTN messengers in the brain of *Caenorhabditis elegans* by in situ hybridization [35]. Accordingly, it is speculated that the function of RTNs in the brain has likely evolved for more complicated networking in higher organisms. The fact that fluctuations of RTN levels, particularly RTN4 variants, have been found to disrupt normal brain function in schizophrenia and several neurodegenerative disorders agrees with this supposition [8, 16, 36–37]. On the other hand, mice lacking one particular RTN member, for example RTN4, are viable [38–40]. Therefore, each RTN in the brain may be needed only for precise functions rather than for vital functions. At the same time, the expression of more than one RTN member in the same cells may signify the necessity for both complementary effects and more diverse cellular processes.

Table 1. Expression of RTNs in human and rodent brain regions.

		Cerebral cortex	Hippo-campus	Olfactory bulb	Dience-phalon	Brain stem	Cere-bellum	Spinal cord	Reference
RTN1-A	mRNA	m, r	m, r	r	r	r	m, r	r	44, 45, 91, 95, 96
	protein	h	r	m			h, m, r		1, 89–91, 94
RTN1-B*	mRNA								42, 100
	protein								
N1-C	mRNA	h, m, r	m, r	r	r	r	h, m, r	r	43–45, 91, 95–97
	protein	h	r				h		24, 90
RTN2-A/B	mRNA	h	h		h	h	h	h	49
	protein								
RTN2-C	mRNA	–	–		–	–	–	–	48, 49
	protein								
RTN3-A	mRNA	m					m	m	18
	protein	m	m		m		m, h	m	8, 18, a
RTN3-B	mRNA		h		h		h		3
	protein	m, r	m	m, r	m	m	m	m	8, 93, a
RTN4-A	mRNA	r	r	r	r		r	h, r	4, 5, 85, 92, 98, 99
	protein	m, r	m, r		m, r		m, r	m, r	31, 85, 92, 99
RTN4-B	mRNA	r	r				r	r	4, 5, 85
	protein	r	r				r	r	85
RTN4-C	mRNA	r					r	r	4, 85
	protein	r	r				r	r	4, 85

(–) Not detectable.
(*) Only reported in human NCI-H82 cell line [42, 100].
a Shi and Yan, unpublished observation.

The expression of RTNs in a specific CNS cell type has not been fully characterized. However, RTN1-C appears to express in neurons [42, 43], but not glial cells [43]. Nogo-A has been found in oligodendrocytes and neurons, but not in astrocytes and microglia [98]. RTN3-B is notable in neurons and minor in glial cells [8]. The cellular locations of RTN2 isoforms are not yet available, and their expression in brains is mainly revealed by Northern analysis [45].

Table 1 summarizes the expression of RTN variants in different brain regions. RTN1, initially considered to be a neuroendocrine-specific protein [41–42], is actually expressed in neurons of various brain regions [1, 43–46]. RTN1-C has also been considered to be a marker for neuronal differentiation [47]. The RTN2-B isoform is detectable in both human and mouse brain regions, but RTN2-C may exist predominantly in muscles and other tissues [48–49]. Searches of EST databases and Western analysis have revealed at least six potential RTN3 spliced variants [7, 18], but not all forms have been indisputably confirmed by Western analysis in comparison to samples from various mammalian species [Q. Shi, W. He and R. Yan, unpublished observation]. RTN3-B, the first reported and the most abundant RTN3 isoform [3], has the highest expression in the brain, primarily in neurons of hippocampal, cortical and cerebellar regions [8, 18].

Similarly, up to 10 different RTN4 spliced variants may exist, but only 3 protein isoforms have been confirmed by Western analysis [50]. RTN4-A is predominantly expressed in brain, predominately in oligodendrocytes and present in the inner and outer loops of the myelin sheath in the adult CNS [31]. The messenger RNA (mRNA) coding for all RTN4 isoforms in normal neurons seems detectable [17, 51–52], but the expression of RTN4-A/B isoforms is significantly lower than that in oligodendrocytes when examined in parallel in immunohistochemical staining [8]. On the other hand, the neuronal expression of RTN4 variants appears to be increased in certain dis-

ease states such as epilepsy [16] and several other neurodegenerative diseases [53–54]. The consequence of this induction to the etiology of disease is still unclear, but the increased presence of an inhibitory molecule of neurite growth such as RTN4-A is unlikely to have beneficial effects in these diseases.

RTNs in cellular trafficking

The common biological function of the RTN family has always been an intriguing question. Thus far, this question has not yet been satisfactorily answered. From an evolutionary viewpoint, analysis of RTN origins suggests that the RTN family may have arisen during early eukaryotic evolution in conjunction with the establishment of the endomembrane system [7]. Although evidence assigning RTNs to the category of cellular trafficking proteins is lacking, the patterns of RTNs localizing in the ER, Golgi and plasma membranes imply the probable existence of trafficking functions of RTNs in the secretory compartments. Accordingly, interactions of cellular trafficking proteins with different RTN homologues have already been demonstrated using such approaches as yeast two-hybrid fishing and co-immunoprecipitation. For instance, human RTN1-A/B, but not RTN1-C, is found to bind to AP50, a component of the AP-2 adaptor complex that mediates endocytosis [55]. Stable expression of RTN1-A or RTN1-B in CV-1 cells moderately affects AP50-associated internalization of clathrin-coated vesicles. On the other hand, human RTN1-C mainly forms a complex with SNARE proteins such as syntaxin 1, syntaxin 7, syntaxin 13 and VAMP [24]. Even though only a minor fraction of a given protein is in this complex, ectopic expression of RTN1-C in neuronal PC12 cells enhances secretion of growth hormone, suggesting its potential role in exocytosis. The role of RTN1-C in exocytosis is consistent with its localization in a nocodazole-sensitive, but calreticulin-negative domain of the ER. Consistent with the observed involvement of mammalian RTNs in cellular trafficking, a study in *C. elegans* has also shown an interaction of nematode RTN-C with RME-1, a protein with a role in endocytic recycling [35]. All these observations suggest that RTN proteins are significantly involved in endocytosis and exocytosis along the secretory compartments. Although the relationship between the altered RTN expression and the potential effects on cellular trafficking is still being investigated, abnormality in exocytosis or endocytosis has already been suggested to be one of the mechanisms that could lead to neurodegenerative diseases [56–58].

RTNs in Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease. Although the etiology of AD has not yet been fully elucidated, the aberrant accumulation of β-amyloid peptide (Aβ) is known to cause the formation of amyloid plaques that may lead to a cascade of damage, including neuronal death in human brains [59]. Aβ is released from a large amyloid precursor protein (APP) through sequential cleavage by two endopeptidases: β- and γ-secretase. The β-secretase, also known as BACE1 for β-site APP cleaving enzyme, has been shown by co-immunoprecipitation to interact with all four human RTN proteins [8]. Increased expression of a single member of RTNs in HEK-293 cells that stably express human Swedish mutant APP significantly reduces the levels of Aβ, the culprit that causes Alzheimer's pathology in patients' brains. Conversely, reduction of RTN3 in APP-expressing cells through RNAi-mediated knockdown increases the levels of Aβ. Further mechanistic studies indicate that increased interaction between an RTN with BACE1 sequesters BACE1 from accessing the APP substrate, thereby reducing the processing of APP by BACE1 [8].

Despite the fact that RTNs appear to function as negative modulators of BACE1 via the shared C-terminal RHD, the physiological significance of the observed cellular interaction between RTNs with BACE1 is still an enigma. In relating the potential role of RTNs in cellular trafficking, we speculate that RTNs may mediate trafficking of BACE1, but not APP, between the ER and Golgi compartments. BACE1, a type I transmembrane aspartyl protease, has already been ascertained to localize in the late and trans Golgi network as well as in the ER and endosomal compartments in cells [60–63]. Now, although our speculation remains to be confirmed, it constitutes a logical step to further explore the function of RTNs in trafficking. Future *in vivo* studies including the usage of RTN mouse models will be essential for revealing the exact role of RTNs in AD pathogenesis.

RTNs in other neurodegenerative diseases

Recent discovery of RTNs as BACE1 interacting proteins has raised the interesting question of whether RTNs also play roles in other neurodegenerative diseases. A search of the published literature reveals numerous studies that link the expression of RTNs to many diseases. For instance, high expression of RTN1 is associated with small cell lung carcinoma with neuroendocrine differentiation [64], and increased expression of RTN3 has been reported in patients with astrocytoma [65] and macular degeneration [3]. Increased expression of RTN4 in neurons is associated with the pathogenesis of several neurodegenerative diseases, discussed below. While there is not enough evidence to establish a causal role for increased expression of RTN4 in several neurodegenerative diseases, the presence of higher levels of this inhibitory mol-

ecule nevertheless would likely worsen pathological conditions owing to inert axonal regeneration.

Altered expression of RTN4 in ALS

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease with clinical characteristics of selective degeneration of motor neurons and skeletal muscular fibers. In an attempt to identify highly demanding biomarkers for the disease, Dupuis et al. found that RTN4-A (Nogo-A) was significantly elevated, whereas the protein level of RTN4-C is inversely decreased in postmortem muscular samples of ALS patients [53]. Similar changes have also been observed in a transgenic mouse model for ALS [53]. These changes in protein levels correlate with their mRNA levels, stemming potential alterations in gene expression patterns from switching events in splicing. These observations suggest that induced expression of RTN4-A in neurons plays a role in ALS even though RTN4-A is largely expressed in oligodendrocytes. Potentially, increased expression of RTN4-A in muscle is a diagnostic biomarker for ALS. More interestingly, this change positively correlates to ALS severity in patients [37]. It has been known that slow-twitch type I fibers and fast-twitch type II fibers are differentially impaired during the progression of ALS. At earlier stages of ALS, slow-twitch type I fibers are more obviously affected than fast-twitch type II fibers. Increased expression of RTN4-A is exclusively detected in slow-twitch type I fibers, and more important, all RTN4-A-positive type I fibers show atrophy [53]. It remains unclear how upregulated expression of RTN4-A is associated with the atrophy of specific fibers in ALS. A possible connection to these observations is the finding of significantly increased expression of RTN4-A in the surgically denervated skeletal muscle of mice [66]. Speculatively, increased expression of RTN4-A in ALS muscle would likely lead to deterioration of the disease because the denervated muscle may be prevented from reinnervating in ALS.

Autoimmune response to RTN4 in MS. Multiple sclerosis (MS) is a chronic, progressive and degenerative disorder that is characterized by intermittent episodes of demyelination and axonal loss or damage in the central nervous system (CNS) [67]. Although the etiology is still uncertain, MS has been regarded as a disease associated with chronic inflammatory disorders resulting from an autoimmune response against myelin components. Autoantibodies recognizing CNS myelin proteins have been detected in MS patients [68]. Autoantibodies recognizing RTN4-A, a component in CNS myelin, have also been detected in serum and cerebrospinal fluid (CSF) from patients with MS, but not in controls [69]. These autoantibodies are detected more frequently in patients with relapsing-remitting compared with chronic progressive MS, suggesting that the elevated production of these antibodies may manifest protective autoimmune responses to the injury. Recently, the role of immune response to RTN4 in MS-like animal models has also been explored [70]. Mice immunized with myelin oligodendrocyte glycoprotein (MOG, residues 35–55) develop a chronic form of experimental autoimmune encephalomyelitis (EAE) that mimics some of the pathogenic responses in MS. Further vaccination of EAE mice with antigen derived from RTN4-A (residues 623–640) robustly suppresses inflammatory responses in these animals [70], suggesting that anti-RTN4-A has protective effects on the initiation and/or progression of EAE. Consistent with this observation, Karnezis et al. further noticed that mice deficient in RTN4 significantly delayed onset of EAE when RTN4 KO mice were immunized with MOG (35–55) [70]. Passive immunization with anti-Nogo immunoglobulin Gs (IgGs) also suppresses the inflammatory responses in these EAE animal models [70]. Therefore, the autoantibodies produced in patients and administration of anti-Nogo-A IgG in EAE mice suggest that RTN4 is an important modulator of the immune response to antoimmune-mediated demyelination. Although the mechanism is obscure, this study may cumulatively imply that the blockage of RTN4-A, a protein important for neurite growth in brains, may help to maintain and/or restore neuronal integrity after autoimmune insults in diseases such as MS [70].

In addition to the possible general functions of RTNs in cellular trafficking, a growing body of evidence has already linked certain specific functions of RTNs to potential pathological consequences in human diseases associated with the central or peripheral nerve systems. Neurodegenerative diseases are often concomitant with neuronal death, and the possible role of RTNs in apoptosis has been reported. Axonal malfunction is one of the pathological features of neurodegenerative diseases, and the role of RTNs in regulating neurite growth and axoglial interactions is thus relevant. We now discuss these functional aspects of RTNs from a cellular biology viewpoint for their possible connection to neurological disorders.

Regulation of neurite outgrowth

Ample evidence has supported RTN4, especially the RTN4-A isoform, as an important agent in inhibiting neurite outgrowth and axonal regeneration [4–6, 38–39, 69–70]. Increased expression of RTN4-A, an inhibitory molecule, is logically regarded as a detrimental event in several neurodegenerative diseases. However, the biochemical mechanism of this inhibition has not been fully elucidated. Three regions in RTN4, residues 59–172 (common to RTN4-A/B), 544–725 (unique to RTN4-A) and the loop 66 amino acids (Nogo-66), appear to be responsible

for the inhibitory effects [9, 23]. The current model suggests that these regions of RTN4 on the surface of oligodendrocytes can face the extracellular space and may *in trans* transduce inhibitory signals to axons via either interaction of the N-terminal region of Nogo-A with an unknown receptor or binding of Nogo-66 to NgR/p75 receptor complexes [11, 71]. Interestingly, only less than 5% of total RTN4 in oligodendrocytes is localized on the cell surface [15, 23], suggesting that the levels of RTN4 on the surface may be particularly sensitive to the inhibitory signaling pathway networking between cells. Therefore, the increased presence of RTN4 on the surface of CNS oligodendrocytes or peripheral nervous system (PNS) Schwann cells will likely enhance inhibitory effects, while reducing the interaction of RTN4 with its receptor complexes will antagonize inhibition. Accordingly, only RTN4 expressed in mature oligodendrocytes, but not in neurons, is seen to exert inhibitory effects on chick neuronal regeneration [72].

Despite all the elegant *in vitro* studies, the *in vivo* inhibitory role of RTN4 in axonal growth appears to be subtle. It has been reported that the RTN4/NgR signaling pathway in neuronal regeneration has minimally affected another line of RTN4 null mice generated by a different targeting approach [39, 73]. It is likely that many factors, including genetic background issues, may be sufficient to account for the experimental discrepancy [74]. To offer an alternative explanation to the above weak phenotypic changes, we speculate the presence of certain compensatory factors in mice with the loss of either RTN4 or NgR. For example, the potential trafficking role of RTN4 may need to be tested to mechanistically address its inhibitory effects on neurite outgrowth. One highly speculative but testable hypothesis is to assume the presence of a more critical inhibitory molecule for axonal growth. This molecule may likely be regulated through RTN4-mediated trafficking, while other RTNs insufficiently mediate this trafficking process. However, in RTN4 knockout mice, other RTNs may potentially compensate for the loss of RTN4 in this function. The proposal of a potential trafficking role of RTN4 is along the consideration of RTNs as cellular trafficking molecules. Future studies of RTN4 in trafficking may provide more definite clues regarding this molecule, and perhaps other RTN members as well, in neuronal regeneration and probably the relevant neurodegeneration.

Role of RTNs in apoptosis

Apoptotic events are directly related to the pathogenesis of diseases, including cancers and neurodegenerative disorders. Increased expression of RTNs has been suggested to trigger apoptosis. When Bcl-x_L was used as bait to search for interacting proteins, both RTN1-C and RTN4-B were identified as binding partners [75]. Bcl-2 and its family members are mainly localized in the mitochondria and nuclear envelope in order to exert their anti-apoptotic activity [76]. Increased interaction of Bcl-2 with RTN4-B reduces anti-apoptosis by preventing the translocation of Bcl-2 to the mitochondria from the ER. Disruption of Bcl-2 translocation by specific RTNs is also consistent with the biochemical function of RTNs in cellular trafficking. Similarly, RTN1-C has been shown to interact with glucosylceramide synthetase, the key enzyme in the biosynthesis of glycosphingolipids at the Golgi/ER interface, and to modulate its catalytic activity in situ [77]. This interaction enhances a p53-independent apoptotic pathway that renders human neuroepithelialoma CHP-100 cells transfected with RTN1-C, but not with RTN3, more sensitive to fenretinide-induced apoptosis [77].

Alternatively, increased expression of RTN proteins in certain cells causes changes in the cellular response to ER stress [78–80]. Transient expression of RTN4-B and RTN3 in cancer cell lines such as HeLa cells has been shown to induce apoptosis. Deletion of the second transmembrane domain reduces induction of apoptosis [80]. It has been speculated that the induced ER overload response pathway is an attributable apoptotic event [80]. In HeLa cells, ectopically expressed RTN3 causes elevation of cytosolic Ca^{2+} through increased releasing from the ER. This elevated cytosolic Ca^{2+} in turn elicits activation of caspase 12 and subsequent mitochondria dysfunction.

Despite encouraging observations of certain RTNs in pro-apoptosis events, such studies have not been made in other cell lines expressing RTN3 or RTN4-B [8, 81]. Increased expression of RTN1-C in rat PC12 and human hNT2 cells has been more directly correlated to their neuronal differentiation instead of apoptosis [47]. The major caveat of these studies is that apoptosis was only observed under the 'ectopically overexpressing' condition in specific cell lines, and the percentage of the dead cells only accounts for less than 20% of the total cells. Although the role of RTNs in apoptosis may not be the primary effect of RTNs in neurodegeneration, the fact that fluctuations in RTN protein levels may cause perturbed ER function remains a testable hypothesis. In particular, ER dysfunction may cause altered metabolism of membrane protein such as APP in AD [82–84].

Modulation of axon-glial junction architecture

Clustering of the axonal domain constituents and the exact localization of ion channels such as K^+ channels are dependent on communication between axons and oligodendroglia. A co-immunoprecipitation approach has revealed specific *in trans* interaction of RTN4-A with Caspr [10]. Mammalian RTN4-A (Nogo-A) is predominantly localized in CNS oligodendrocytes [4–6], specifically in the cell bodies, innermost and outermost loops of myelin membrane [85], while Caspr is a transmembrane

adhesion molecule that constitutes the major paranodal axonal component with F3/contactin and neurofascin 155 [86]. This axoglial crosstalk, mediated by the loop region of RTN4-A, modulates localization of K^+ channels at both paranodal and juxtaparanodal regions during developmental stages through on-and-off interactions between Caspr and Kv1.1 and Kv1.2, the components in the K^+ channels. Studies using dysmyelinating mouse models, such as those deficient in myelin-related and axonal proteins like ceramide galactosyl transferase and myelin basic protein (*Shiverer* mice), provide a more interesting observation: the change of RTN4-A distribution is correlated with improper K^+-channel localization at juxtaparanodes in the above pathological models that display paranodal junctional defects. It is therefore suggested that RTN4-A may complement or regulate the action of Caspr in the organization of mature axonal domains. This process aids in the coordinated localization of K^+ channels to juxtaparanodes during development [10].

In MS, Caspr localization is abnormal, and paranodal junctions on some myelinated axons in the borders of lesions of patients with chronic progressive MS are no longer intact [87]. It will be interesting to test whether RTN4-A antagonist would actually affect the RTN4-A-Caspr interaction and how alteration in this axoglial talk may impact MS pathogenesis. On the other hand, it will be equally interesting to evaluate whether this axoglial talk mediated by the loop of RTN4-A or even other RTN isoforms will affect Alzheimer's pathology. This is because numerous glial cells have been detected in areas surrounding Alzheimer's amyloid plaques that are mainly present in fiber-enriched regions [88], and these cells express RTNs. Future studies extending similar inter-cellular interactions will be particularly informative.

Future perspectives

There is no doubt that cellular homeostasis of RTNs is important for normal cellular function. Changes in the levels of RTNs, particularly increased levels, have been associated with the reported disease processes. Regardless of the delayed full understanding of RTNs in general cellular function, growing interest in RTNs in axonal regeneration, neurodegeneration and other diseases has nevertheless begun to uncover important relationships between their biochemical functions and relevant underlying mechanisms. For instances, the loop region of RTN4 has been connected to attractive or repulsive pathways transducing signals to neighboring neurons and probably even non-neuronal cells. Identification of loop regions of other RTNs in similar signaling pathways may attribute reticulon proteins, the so-called residents of the ER, to function as surface signaling molecules. On the other hand, the preserved structural organization of RTNs from lower organisms to humans implies the presence of a common essential biological function of RTNs during evolution. It is expected that the unambiguous revelation of RTNs in cellular function will be central to the future elucidation of the role of RTNs in the pathogenesis of neurodegenerative diseases. Since fluctuations in RTN levels or RTN dysfunction will affect cellular trafficking, another interesting question is whether this alteration disrupts other cellular functions that ultimately lead to the pathogenesis of human disease. For this endeavor, approaches employing the tools of biochemistry, cell biology and genetics will expand our understanding of this protein family. More important, the knowledge gained from future studies may enable the design of drugs targeting RTNs for therapeutic applications.

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