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Emerging themes of ER organization in the development and maintenance of axons

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Abstract

The endoplasmic reticulum (ER) is a continuous membrane system comprising the nuclear envelope, polyribosome-studded peripheral sheets, and a polygonal network of smooth tubules extending throughout the cell. Though protein biosynthesis, transport, and quality control in the ER have been extensively studied, mechanisms underlying the heterogeneous architecture of the ER have been clarified more recently. These insights have increased interest in ER morphology changes associated with the development of neuronal axons and dendrites as well as their integration with pre- and postsynaptic signaling pathways. A number of proteins involved in shaping and distributing the ER network are mutated in neurological disorders, particularly the hereditary spastic paraplegias, emphasizing the importance of proper ER morphology for the establishment and maintenance of highly-polarized neurons.

Keywords

endoplasmic reticulum; hereditary spastic paraplegia; morphology; reticulon; shaping

Introduction

Cellular organelles have diverse but characteristic morphologies that are evolutionarily conserved, indicating that the form of an organelle is fundamentally related to its function. The endoplasmic reticulum (ER) is among the most visually striking of all organelles for its large size and morphological heterogeneity. Though a continuous membrane system, it comprises the distinct shapes of the nuclear envelope, peripheral sheet-like structures studded with polyribosomes, and a network of interconnected smooth tubules distributed extensively throughout the cell. In step with this structural heterogeneity, the ER is a multifunctional organelle involved in the synthesis, modification, quality control, and trafficking of integral membrane and secreted proteins. In addition, it is critical for the sequestration and regulated release of Ca^{2+} , sterol synthesis, and lipid synthesis and distribution (1–4). In neurons in particular, the ER plays a crucial role in the massive membrane expansion that occurs during dendrite and axon formation and as a Ca^{2+} store tightly integrated with pre- and postsynaptic

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signaling mechanisms. Local variations in ER morphology also correlate with dendritic spine density and maturation, linking ER morphology to changes in synaptic organization and function (5–8).

The ER interacts with, and is highly dependent upon, the microtubule cytoskeleton in animal cells. This relationship is particularly important for highly-polarized neurons, which utilize microtubules extensively for the proper distribution of organelles and molecules. Thus, mechanisms must exist not only to maintain and regulate the shapes of various ER domains but also to position spatially the proper type of ER throughout the neuron. Identifying the molecular machineries that mediate ER shape and interactions with other cellular elements is critical, with important implications for neuronal development, formation of axons and dendrites, synaptic function, and neurological disease (9).

As in other eukaryotic cells, the ER appears continuous within neurons (10,11), extending promiscuously from the nuclear envelope in the soma into peripheral dendrites as well as into axon growth cones and terminals. Within axons and dendrites smooth ER (SER) tubules predominate, and rough ER (RER) sheets and the nuclear envelope are most prominent within the soma (12). The morphological heterogeneity of ER in neurons likely plays a crucial role in the spatial segregation of its different functions. In fact, it appears that sheets are mostly associated with protein synthesis, quality control, and trafficking, and tubules with other functions such as lipid and cholesterol synthesis and distribution as well as regulating Ca^{2+} dynamics. RER, Golgi outposts, and components of the early secretory pathway have been identified within dendrites, and the involvement of ER dynamics in dendritic function has been widely-studied and recently reviewed (13–15). Here, we present emerging themes of ER function in the development and maintenance of axons, emphasizing recent discoveries of protein families that specify the shapes of different ER domains, particularly tubules. Investigations of proteins mutated in the hereditary spastic paraplegias (HSPs), a class of neurological disorders affecting some of the longest axons in the body, have generated unexpected insights into the important roles of ER morphology in axon formation and maintenance.

Main text of review

Shaping rough sheets and smooth tubules

Perhaps unexpectedly given its highly heterogeneous appearance, for many years the means by which the ER is shaped remained unclear (3). However, in recent years several classes of proteins have been identified that appear necessary and sufficient for the generation of SER tubules (16–18), which represent the predominant form of ER in axons. These ER-shaping proteins comprise at least two protein families, the DP1/REEP/Yop1p and reticulon proteins. Though these families have little overall sequence homology to one another, they exhibit a common structural feature – elongated hydrophobic segments that appear to form paired hairpin domains that partially span the phospholipid bilayer (16). With the majority of the transmembrane portion predicted to reside in the outer leaflet of the bilayer, ER-shaping proteins may generate curvature through hydrophobic wedging (Figure 1). Since they form large, immobile oligomers in cells (18), scaffolding may play a role in curvature generation and stabilization as well (19).

The maintenance of different ER domains also requires proper segregation of proteins, and the hydrophobic hairpins appear to function as curvature-sensing motifs. In fact, the ER shaping proteins are excluded from peripheral sheets and the nuclear envelope even when highly overexpressed, and the hydrophobic hairpins are required and sufficient for this distribution (16,18). Though the hairpins can mediate homo- and heterotypic interactions necessary for the

formation of large oligomeric structures (18), specificity may also be provided by interactions among their cytoplasmic domains.

ER sheets have similar curvature along their edges as ER tubules, likely mediated by ER-shaping proteins localized at the edges of ER sheets (19). However, mechanisms to form and stabilize their sheet-like morphology are less well understood. The consistent luminal thickness of extensive ER sheets may be maintained via polyribosome complexes or intraluminal bridges formed via proteins such as cytoskeleton linking membrane protein of 63 kDa (CLIMP63), which forms multimeric complexes in ER sheets and harbors a coiled-coil domain that assembles into ~90 nm rod-like oligomers (19,20). Stacks of organized SER sheets may be stabilized through protein interactions at their cytoplasmic face (21), but these may employ mechanisms distinct from those of RER sheets. The nuclear envelope has the appearance of a sheet, except in areas contacting the nuclear pore complexes, which are highly curved. ER-shaping proteins are required for nuclear pore formation (22), likely due to their membrane-curving functions. Flatter, sheet-like areas of the nuclear envelope are stabilized by interactions of nuclear membrane proteins with the nuclear lamina and chromatin (23).

Distribution of the ER Network

Shaping the different ER domains is not sufficient to explain fully the formation of the interconnected network visualized in cells. Recent work has highlighted the important role of the atlastin family of dynamin-related GTPases in ER network formation (24–27). In mammals, there are three closely-related atlastin proteins -- atlastin-1, -2, and -3, and these oligomeric, integral membrane GTPases localize predominantly to the tubular ER. Atlastin-1 is highly localized to the central nervous system, while the others are enriched in peripheral tissues (24). The atlastins appear to be functional orthologs of Sey1p in the yeast *S. cerevisiae* and RHD3 in the flowering plant *Arabidopsis* (26) (Box 1). All harbor an N-terminal GTP-binding domain and two very closely-spaced hydrophobic segments near the C-terminus (Figure 1) (24,26). These large GTPases interact with ER-shaping proteins of both DP1/REEP/Yop1p and reticulon families and are required for the formation of three-way junctions in ER, likely by mediating homotypic fusion of ER tubules (24,26,27). Consistent with their role in ER network formation, atlastins/Sey1p localize to distinct puncta along ER tubules, including at three-way junctions (Figure 2) (24,26,28).

BOX 1

Polarized cell expansion: lessons from *Arabidopsis*

In *Arabidopsis*, root hairs are long processes formed by tip growth of specific root epidermal cells. Reminiscent of neuronal axon growth, root hair tip growth is an extreme form of polarized cell expansion that depends on signaling molecules, Ca^{2+} flux, cytoskeletal dynamics, small GTPases of the Rab, Arf, and Rho/Rac families, and reactive oxygen species. The importance of ER morphology in the formation and maintenance of root hairs in *Arabidopsis* has been revealed by loss-of-function mutations in the atlastin/Sey1p ortholog root hair defective 3 (RHD3) (55). These *rh3* plants have short, wavy root hairs and abnormal appearing, tubular ER bundles, with an unusually large number of vesicles in the subapical region of the root hairs -- such vesicles are typically in apical regions. This results in defective polarized cell expansion, possibly due to reduced or uneven deposition of secretory vesicles during root hair elongation. Though ER in plants is typically oriented along actin fibers, root hair tip growth also depends on microtubules (56), and the morphology of the ER changes noticeably during the elongation phase of root hair growth (57). Thus, long cellular protrusions such as plant root hairs and neuronal axons are highly

dependent on the dynamic morphology of the tubular ER, and studies of root hair elongation may provide important insights into mechanisms of axon growth.

An important theme in ER distribution within cells is its intimate interaction with the cytoskeleton. Proteins such as CLIMP63 may be important for shaping ER sheets as well as mediating their interaction with the microtubule cytoskeleton (3). In ER tubules, atlastins interact directly with the AAA ATPase spastin that binds and severs microtubules (29–31). Spastin exists as two isoforms generated through the use of different initiator Met residues, M1 or M87, and the interaction with atlastins occurs through a predicted hydrophobic hairpin present in only the larger M1 isoform (28). In addition to interactions with reticulons, atlastins and M1 spastin also interact with DP1/REEPs (26,28). These latter proteins comprise 6 different members in humans -- REEP1–6 (REEP5 is also known as DP1). Until recently, these were considered to be functionally redundant. However, phylogenetically and structurally there are clear differences between REEP1–4 and REEP5–6 (Figure 1) (28). In particular, REEP1–4 proteins harbor hydrophobic hairpins but also interact with microtubules through an extended C-terminal cytoplasmic domain (28). Interactions among REEPs, atlastins, and spastin via hydrophobic hairpins provide a compelling mechanism for coupling ER membrane remodeling to microtubule cytoskeletal dynamics. In nearly all cases, alterations in these proteins affect axon branching or elongation. Other proteins are also likely involved in mediating ER and microtubule interactions in neurons. For example, the large p600 protein associates with both ER and microtubules, and its depletion in primary neurons impairs neurite extension and ER distribution (32).

The interaction of the ER-associated proteins M1 spastin, CLIMP63, REEP1–4, and p600 (among other proteins) with microtubules prompts an interesting question. Why is it important to couple the ER to the microtubule cytoskeleton? One well-established reason is to traffic pleiomorphic transport carriers in the early secretory pathway from the ER to the Golgi apparatus (33). Another is the formation of the ER itself, since in animal cells the ER is formed along microtubules by a number of distinct mechanisms (34,35). These include formation of ER tubules by membranes sliding along microtubules or attached to polymerizing microtubules. In this context, tip attachment complexes in association with polymerizing microtubules pull the membrane in the plus-end direction, while motor proteins drive transport along established microtubules. Though the microtubule cytoskeleton is not required for ER network formation *in vitro* (16), cytoskeletal interactions are important for the characteristic appearance of ER in cells. In fact, disruption of the microtubule cytoskeleton using nocodazole results in collapse of the ER by retraction from the cell periphery and conversion of peripheral ER tubules to extended sheet-like structures (36). Spastin and the REEP1–4 class of proteins likely mediate the interaction of ER tubules with the microtubule cytoskeleton (28). In a complementary manner, CLIMP63 may mediate attachment of the sheets to the microtubules (20). Interestingly, CLIMP63 interacts with MAP2, a microtubule-associated protein enriched in the neuronal soma and dendrites (37), prefiguring distinct modes of ER-microtubule interactions in axons and dendrites. Additional stabilization of the ER network may occur through interactions with other cellular organelles. Such interactions are likely important for the interorganellar exchange of lipids, and a number of proteins have been suggested to participate in such interactions. The mitochondrial-ER attachment sites have been among the most intensively studied, since these organelles are among the most abundant in cells and the interorganellar transfer of Ca^{2+} and phospholipids between them has been an area of intense interest (38). Similarly, interactions between the ER with the plasma membrane are of interest for their role in responding to the depletion of ER Ca^{2+} stores. In particular, the ER protein STIM1 acts as a Ca^{2+} sensor that oligomerizes in response to Ca^{2+} depletion, translocates to the plasma membrane via a polybasic C-terminal domain, and finally stimulates store-activated Ca^{2+} influx to replenish stores (39). It will be important to establish how connections with the

ER are mediated and regulated for other organelles as well since such interactions are a powerful means for the ER to communicate with the rest of the cell (14).

Dynamics changes in ER shape

The ER is highly dynamic, undergoing fusion reactions to form three-way junctions as well as regulated changes in morphology. The Ca^{2+} sensor STIM1 in fact appears to play a role in shaping the ER, since STIM1 overexpression strongly stimulates ER extension occurring through the microtubule tip attachment complex (40). Another example of dynamic regulation of ER morphology was presented by Banno and Kohno (41) a number of years ago; they showed that intraventricular application of a metabotropic glutamate receptor agonist induced conformational changes of the SER to form lamellar bodies in Purkinje cell dendrites. More recently, differential distribution of ER has been shown to control metabotropic signaling and plasticity at hippocampal synapses (42). In non-neuronal cells, prominent changes in ER morphology occur during cell division, though recent studies have proposed different shape transitions. While Puhka *et al.* (43) reported that the ER of Chinese hamster ovary cells loses its sheet-like structure and becomes almost completely tubular during mitosis, a more recent study by Lu *et al.* (36) demonstrated that from prometaphase to telophase in a variety of mammalian cell types, most of the ER is organized as extended cisternae. This tubule-to-sheet transition is reminiscent of the changes in ER morphology observed upon nocodazole-induced microtubule depolymerization in interphase cells, providing additional support for a fundamental role of the microtubule cytoskeleton in regulating ER morphology and distribution (36). Though these shape transitions occur without disrupting the continuity of the ER, there are limited instances where the ER can be fragmented. These include a Ca^{2+} -dependent process in starfish eggs after fertilization (44) and the application of Ca^{2+} ionophores in non-neuronal cells and glutamate in cultured neurons (45,46). It will be important to confirm any physiological fragmentation of the ER in neurons, since the connectivity of the ER is one of its defining properties.

One of the hallmarks of neuronal development is the polarized cell enlargement that results in the most striking morphological feature of neurons, their elaborate dendritic arborizations and long axons. A differential role for the proteins of the early secretory pathway has been suggested in the development of axons and dendrites. For instance, *Drosophila* orthologs of the secretory components Sec23, Sar1, and Rab1 were identified in a screen for dendritic growth defects, and whereas limiting ER-to-Golgi transport caused decreased membrane supply from soma to dendrites, membrane supply to axons remained sustained (47). More recent studies have indicated that axons also have ER exit sites and components of the secretory pathway during stages of development characterized by selective axon growth (48,49), emphasizing the role of proper ER type and distribution in neuronal development.

ER network defects and neurological disease

A remarkable example of the importance of ER morphology in highly-polarized cells is provided by a class of disorders known as the HSPs. Though these disorders comprise over 40 distinct genetic loci (*SPG1-46*), the most common forms, representing ~60% of affected individuals, are due to autosomal dominant mutations in one of three homomeric proteins: spastin (SPG4), atlastin-1 (SPG3A), and REEP1 (SPG31) (50). These proteins interact with one another and localize to the tubular ER in corticospinal neurons, where they mediate ER shaping and interactions of the tubular ER network with the microtubule cytoskeleton (28, 51).

Evidence supports a role for ER shaping mechanisms in other related neurologic disorders. In the superoxide dismutase 1 (SOD1) G93A transgenic mouse model for amyotrophic lateral sclerosis (ALS), which involves both corticospinal and lower motor neurons, the ER-shaping

protein reticulon-4A selectively redistributed the ER chaperone protein disulfide isomerase intracellularly (52), reminiscent of earlier studies in non-neuronal cells (17), and reticulon-4A overexpression protected against neurodegeneration (52). Conversely, knock out of reticulon-4A, B on this SOD1 G93A background worsened disease (52). Interestingly, oligodendrocyte reticulon-4A (also known as Nogo-A) has long been implicated in negative regulation of regeneration and plasticity in the adult central nervous system, via signaling mediated by the Nogo receptor (NgR) on the plasma membrane (53). Since the vast majority of reticulon proteins in cells are found intracellularly within the tubular ER, advances in our understanding of the role of reticulons in controlling ER structure may help to identify functional correlations between these seemingly disparate processes.

Further supporting a role for ER morphogenesis in neurologic disorders, the vesicle-associated membrane-protein associated protein B (VAP-B) mutant P56S that underlies a autosomal dominant form of familial ALS is associated with the production of a novel form of organized SER (54). The specificity and significance of this morphological change are not yet clear, but the fact that ER shaping defects are increasingly emerging as a pathogenic mechanism for related disorders such as the HSPs will certainly spur studies investigating the effects of this novel organized SER on motor neuron function and survival.

Taken together, these studies support a converging neurologic disease mechanism comprising alterations of ER shape and distribution within the cell. We speculate that highly-polarized neurons are most affected because they rely on a carefully coordinated and selective distribution of different types of ER over long distances.

Conclusions

With several protein families recently identified that play fundamental roles in ER network formation and organization, the stage is set for not only the identification of additional proteins that collaborate in shaping ER morphology but also for investigations into the functional regulation of ER network distribution within neurons. It seems likely that signaling pathways linked to modifications of these proteins will have key roles in modulating the appearance and function of ER within cells. In fact, investigation of the dynamic morphology of ER in neurons, particularly within axons, will be an important area for future work. Such studies take on more urgency and importance since ER morphology defects appear to be the predominant pathogenic mechanism underlying the length-dependent corticospinal axonopathies known as HSPs, and possibly other inherited axonopathies. The identification of additional genes for such disorders may provide us with new tools to deconstruct the neuronal ER network and better understand the roles of ER shape and distribution for normal neuronal function as well as neuropathologic states.

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Figure 1.

Schematic diagram of ER tubule shaping proteins. Predicted membrane topologies of proteins involved in shaping the tubular ER network. All of these proteins are oligomeric *in vivo* (not shown). Abbreviations: AAA, ATPases associated with a variety of activities domain; GTP, GTP-binding domain; MIT, present in microtubule-binding and trafficking proteins; MTB, microtubule-binding domain.

Figure 2.

Interactions of the tubular ER network with the microtubule cytoskeleton. Myc-atlastin-1 overexpressed in COS7 cells shows punctate enrichment along ER tubules (green; top) in the cell periphery, including at three-way junctions. Microtubules are identified by co-immunostaining for β -tubulin (red; bottom). Adapted from Park *et al.* (28).