Neuronal endoplasmic reticulum architecture and roles in axonal physiology

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ABSTRACT

The endoplasmic reticulum (ER) is the largest membrane compartment within eukaryotic cells and is emerging as a key coordinator of many cellular processes. The ER can modulate local calcium fluxes and communicate with other organelles like the plasma membrane. The importance of ER in neuronal processes such as neurite growth, axon repair and neurotransmission has recently gained much attention. In this review, we highlight the importance of the ER tubular network in axonal homeostasis and discuss how the generation and maintenance of the thin tubular ER network in axons and synapses, requires a cooperative effort of ER-shaping proteins, cytoskeleton and autophagy processes.

1. Introduction

The endoplasmic reticulum (ER) is a large, singular, organelle composed of tubules and sheet-like areas of membrane. We are now starting to appreciate the complexity of the ER network, its dynamics, the existence of various ER subdomains, and to understand cell-specific functions. The ER in neurons is especially striking, given that the ER populates even the most distant neuronal compartments (Fig. 1). It forms specialized subdomains, such as the ribosome-rich rough ER (RER) in the somatodendritic area, smooth ER (SER) tubules in axons and synapses, peripheral cisternae, and many contact sites with other organelles. The sheet-like RER in neuronal cell bodies is mainly involved in the production, folding and modification of approximately one-third of all proteins. For a long time, axonal building blocks were believed to be entirely cell body-derived. A more recent view, however, supports the notion that proteins (and lipids) can be locally produced, notably in metabolically very active sites such as branch points and axonal growth cones (Grochowska et al., 2022). Most tubular SER is located in axons and dendrites and plays an important role in the storage of calcium ions (Ca2+). The regulated release of Ca2+ from the ER lumen is a universal and versatile mechanism involved in a wide range of processes, including gene expression, cellular secretion, cytoskeletal dynamics, but also specific neuronal processes like plasticity (Baker et al., 2013; Karagas and Venkatachalam, 2019). The SER also acts as the main site of synthesis for several important lipids, including cholesterol, phospholipids, and neutral lipids. At membrane contact sites (MCSs), ER-produced lipids can be exchanged with the plasma membrane (PM) or other organelles, for instance mitochondria, that lack lipid-producing capabilities. MCSs between the ER and other membranes also play crucial roles in regulating organelle biogenesis, function and membrane repair (Wu et al., 2018). All these ER compartments are not static in time but continuously undergo rearrangements, with growing and retracting tubules, sheets-to-tubules and tubules-to-sheets transformations, that are believed to impact local ER function (Arruda and Parlagkül, 2023; Verweij et al., 2022). The shape of the ER is determined by ER-resident transmembrane proteins. For example, the ER-shaping protein CLIMP63 determines the lumen width in ER sheets (Shibata et al., 2010), whereas proteins such as Reticulon (Rtns) and REEPs promote high curvature of ER membranes to form tubules (Voeltz et al., 2006). These differences in ER curvature not only parallel the many functions of ER, but might also dictate the partitioning of proteins and lipids to create distinct ER subdomains (Puhka et al., 2012; Westrate et al., 2015; Zamponi et al., 2022).

In early developing cultured rat neurons, ER tubules are present in the growing neurites and especially enriched in axonal growth cones (Zhu et al., 2006; Farias et al., 2019). In Drosophila neurons, ER accumulates in the tips of regenerating axons (but not of regenerating...
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The architecture of the neuronal endoplasmic reticulum (ER). ER (blue) is the largest organelle and continuous from the soma into neurites, pre- and postsynapses. (1) The somatodendritic compartment contains the ribosome-containing RER. The ER network in axons and synapses consists of very thin, smooth, tubules with a sparse presence of small cisternae at presynaptic terminals and branch points. ER-bound ribosomes are mostly absent from the axonal ER network but they have been found on branchpoint cisternae. (2) ER network dynamics are involved in a variety of cellular processes, including synaptic transmission. At the postsynapse, ER has been observed to selectively and dynamically enter active spines. (3) The ER also interacts through membrane contact sites with a diverse set of other organelles, i.e. mitochondria (a), endo-lysosomes (b) and the plasma membrane (c).
3. Axonal ER-shaping and positioning

Shaping of axonal ER allows subcompartimentalization and dynamic adaptations to environmental cues. Two factors known to contribute to ER organization are the ER-shaping proteins and the continuous interaction of the ER with the cytoskeleton. Considering that axonal ER is mostly composed of tubular ER, the high curvature generating proteins ER organization are the ER-shaping proteins and the continuous interactions with microtubules and the motor proteins Kinesin (Kif5A) and Dynein, respectively. ER-localized protein P180 can contact microtubules, inducing stabilization of ER-microtubule interactions (Reggio et al., 2021; Shibata et al., 2008) (Fig. 2). Whereas in yeast, the absence of both proteins converts tubules into filaments (Voeltz et al., 2006), mammalian REEPs (REEP1–6) and Rtn (RTN1–4) proteins could be redundantly required for peripheral ER tubule formation. Recent work on axonal ER in cultured hippocampal neurons, indicated that knockdown of RTN4 (the most abundant Rtn isoform in neurons) together with REEP5 results in a significant reduction of axonal ER tubules, based on GFP-Sec61b localization in developing rat neurons (DIV1–7) (Farias et al., 2019). REEPs and Rtns work together with Atlastin (ATLs), which are dynamin-like GTPases involved in generating ER tubular networks by facilitating homotypic fusion of the tubules, highlighted by its enrichment at axonal branch points (Zhu et al., 2006). Disease-causing mutations in these ER-shaping proteins emphasize the importance of axonal ER shape and function in the axon. Indeed, mutations in ATL-1, Rtn, and REEP proteins all result in neurological problems and hereditary spastic paraplegia (HSP), a disease characterized by progressive spasticity and weakness of the lower limbs originating from axon degeneration (Blackstone, 2012). Studies in Drosophila have shown that HSP-related mutations in ATL1, Rtn and REEP result in a shortened fly lifespan associated with ER network disruption, perturbations in Ca\textsuperscript{2+} handling in the distal axon and impaired evoked neurotransmitter release (Napoli et al., 2019; Oliva et al., 2020; Summerville et al., 2016). Similarly, fragmentation of axonal ER in mammalian neurons also affects the Ca\textsuperscript{2+}-driven cycling of synaptic vesicles (Lindhout et al., 2019), supporting the idea that ER-shaping processes are important modulators of axonal homeostasis. Mouse models of HSP typically have mild phenotypes. Nonetheless, a recent HSP mouse model harboring homozygous mutations of both ATL1 and REEP1 shows progressive defects in motor function (Zhu et al., 2022). Electron microscopy (EM) revealed ER morphological changes in the corticospinal axons of these mice correlating with behavioral changes (Zhu et al., 2022). Intriguingly, the ER in those mature mutant corticospinal axons expands transversely and periodically to create a ladder-like appearance similar to what is observed in developing axons of cultured hippocampal neurons (Zamponi et al., 2022). The mechanism underlying the periodicity is unclear but indicates that ER-shaping processes are distributed non-homogenously along the axonal ER tubular network.

Whereas the actin cytoskeleton seems imperative for dynamics of the spine apparatus, several lines of evidence suggest that microtubules (MTs) and associated motor proteins provide the structural backbone for axonal ER tubule localization and movements. Cryo-electron tomography experiments show that axonal ER tubules are in close proximity to MTs, and that nocodazole-induced MT depolymerization disrupts axonal ER network architecture (Farias et al., 2019; Zamponi et al., 2022). Transport of the ER along MTs is driven by motor proteins. Kif5A, a MT plus-end motor and Dynein, a minus-end motor, are required for the antero- and retrograde transport of ER tubules within the axon. ER-MT interactions are further stabilized by P180, which is enriched in axonal ER tubules and promotes axon specification and growth (Farias et al., 2019). In addition, P180-mediated ER tubule–MT interactions can locally promote kinesin-1-powered lysosome fission and the subsequent axonal translocation of these organelles (Ozkan et al., 2021). Next to P180, the Ca\textsuperscript{2+} sensor STIM1 is another ER-protein that interacts with MTs. STIM1 tracks along the MTs by interacting with EB1/EB3 proteins at the MT-plus ends. This interaction was demonstrated to remodel both MTs and ER in navigating growth cones (Pavez et al., 2019), again indicating that dynamics and function of both the ER and axonal MT network rely on their tight connection.

4. The role of ER tubules and their MCSs in regulating presynaptic function

Local availability of ER tubules not only instructs axon formation but can also regulate neurotransmission, synaptic vesicle release, and synaptic plasticity (Kuijpers et al., 2021; Singh et al., 2021). Synaptic vesicle fusion and neurotransmitter release relies on the rapid Ca\textsuperscript{2+} influx through membrane-resident voltage-gated Ca\textsuperscript{2+} channels (VGCCs) (Fig. 3). In the presynapse, mitochondria and ER can act as Ca\textsuperscript{2+} buffers and sources and are shown to modulate neurotransmission in a variety of model synapses (Chanaday and Kavalali, 2022; Datta and Jaiswal, 2021). ER Ca\textsuperscript{2+} efflux is mediated by release channels located in...
the ER membrane, such as inositol 1,4,5-trisphosphate receptors (IP3Rs) and Ryanodine Receptors (RyRs), whereas sarco/endoplasmic reticulum calcium buffering proteins (e.g. Calreticulin). (1) ER- Ca2+ release channels (RyRs and IP3R) and uptake transporters (SERCA) can influence cytosolic [Ca2+] and thereby modulate local processes such as synaptic vesicle exocytosis. (2) Refilling of the ER, upon a drop in [Ca2+], is mediated by store-operated Ca2+ entry (SOCE) through the clustering and interaction of the ER Ca2+ sensor stromal interaction molecules 1 and 2 (STIM1/2) and the PM Ca2+ channel Orai1. In neurons, STIM2-mediated SOCE enhances spontaneous excitatory neurotransmission. (3) STIM1 proteins are also involved in the modulation of synaptic vesicle exocytosis, presumably by modulating voltage-gated Ca2+ channels (VGCC) at the PM. (4) MCSs between ER and mitochondria are proposed to deliver Ca2+ to the mitochondria for optimal ATP production.

Fig. 3. The role of presynaptic ER in calcium homeostasis and neurotransmission. Ca2+ in the ER is present both as free ion as well as bound to calcium buffering proteins (e.g. Calreticulin). (1) ER- Ca2+ release channels (RyR and IP3R) and uptake transporters (SERCA) can influence cytosolic [Ca2+] and thereby modulate local processes such as synaptic vesicle exocytosis. (2) Refilling of the ER, upon a drop in [Ca2+], is mediated by store-operated Ca2+ entry (SOCE) through the clustering and interaction of the ER Ca2+ sensor stromal interaction molecules 1 and 2 (STIM1/2) and the PM Ca2+ channel Orai1. In neurons, STIM2-mediated SOCE enhances spontaneous excitatory neurotransmission. (3) STIM1 proteins are also involved in the modulation of synaptic vesicle exocytosis, presumably by modulating voltage-gated Ca2+ channels (VGCC) at the PM. (4) MCSs between ER and mitochondria are proposed to deliver Ca2+ to the mitochondria for optimal ATP production.

5. ER degradation

The post-mitotic nature, unique polarization and high-energy demand of neurons requires specialized mechanisms to manage local and timely changes in protein and organelle distribution. This is especially true for distant synapses, where local environments can modulate changes in protein, lipid and organelle composition to alter neurotransmitter release, and synapse remodeling. To fine-tune ER activities, the abundance of specific ER-proteins needs to be controlled and, additionally, the ER must turnover actively to cope with stress conditions such as protein aggregation, aging, or Ca2+ disturbances. Considering the relatively short half-lives of (smooth) ER membrane lipids and proteins (3–5 days) (Fornasiero et al., 2018; Omura et al., 1967), tubular ER and associated proteins have to be degraded in a very efficient manner. Recent findings have revealed that degradation by autophagy (or ‘ER-phagy’) is a key ER remodeling process (Gubas and Dikic, 2022).

The autophagy pathway utilizes double membrane vesicles, called autophagosomes, which sequester components such as damaged proteins, organelles, and delivers these to the lysosome for degradation and recycling. In neurons, this final step in the pathway mostly happens in somata, where the majority of degradative-competent lysosomes are located. The so-called autophagy-related (ATG) genes and proteins are central to the autophagy process. Briefly, upon autophagy initiation, the ULK1 complex (FIP200–ATG13–ATG101–ULK1) translocates to an autophagy initiation site where it forms assemblies that recruit downstream autophagy proteins. In later maturation stages, ubiquitin-like conjugation systems (such as the ATG16-ATG12-ATG5 complex) control the lipidation and membrane targeting of ATG8/LC3 which further drives autophagosome assembly and substrate recruitment (Stavoe and Holzbaur, 2019). Aside from the ATG proteins, a growing set of adaptors, receptors and co-receptors (hereafter all called receptors) have been identified in non-neuronal cells that mediate distinct types of ER-phagy (Gubas and Dikic, 2022). These ER-localized receptors can bind (via their so-called LIR domains) to lipidated ATG8/LC3 on the autophagic membrane and direct specific ER subdomains, for instance tubules and sheets, to the autophagic pathway. Importantly, mutations in ER-phagy receptors FAM134B and ATL3 that impair the ATG8/LC3
interaction, lead to autosomal recessive hereditary sensory and autonomic neuropathy (HSAN), a disease characterized by axonal abnormalities and degeneration (Hubner and Dikic, 2020; Kurth et al., 2009), indicating the importance of ER degradation in neuronal axons. FAM134 family proteins (FAM134A, FAM134B, and FAM134C) contain a reticulon-homology domain and are presumed to induce high-membrane curvature, thereby generating small ER-membrane vesicles which are engulfed by the growing pre-autophagosomal membrane (Reggio et al., 2021) (Fig. 4). Disruption of FAM134B in mice indeed inhibits ER turnover, leading to ER expansion and eventually degeneration of sensory neurons (Khamnits et al., 2015). In Drosophila, HSAN-causing mutated ATL3 is excluded from entering the axon, causing a reduction of axonal autophagosomes which may contribute to neurodegeneration (Behrendt et al., 2021). Whereas in non-neuronal cells ER-phagy can counteract protein aggregation (Cai et al., 2019), clear disease causing collagen (Forrester et al., 2019) or to help ER recovery after stress (Fumagalli et al., 2016), the implications of neuronal ER-phagy disturbances on axonal maintenance are not fully understood. Recent studies in autophagy-deficient mice lacking neuronal ATG5 revealed defective selective degradation of axonal tubular ER, indicating that the tubular ER is a major substrate for neuronal autophagy. Defective ER degradation resulted in increased excitatory neurotransmission through excessive Ca\(^{2+}\) release from accumulated ER stores and associated RyRs (Kuijpers et al., 2021). The identity of the ER-phagy receptor involved needs to be determined but, considering the variety of ER-subsdomains and the functional redundancy of ER-phagy receptors (Chino et al., 2019), more than one receptor might mediate axonal ER-phagy in neurons. Another open question is what triggers, local or global, degradation of axonal ER. In mammalian cells, ER-phagy constitutively occurs at a basal level but is enhanced upon nutrient withdrawal (Liang et al., 2018) or ER-stress (Smith et al., 2018). Interestingly, recent work by Zheng and colleagues shows that autophagy initiation by nutrient starvation elicits local ER-mediated Ca\(^{2+}\) transients that trigger the condensation of FIP200 and the formation of ULK1 assemblies (Zheng et al., 2022). It would be interesting to see if other stimuli, such as oxidative stress and perhaps neuronal activity, can trigger similar responses.

6. Outlook

During decades of research, we have acquired a wealth of detailed knowledge on synapses and neurotransmitter release, even to the point that we know copy numbers of individual proteins in single synapses and synaptic vesicles. In great contrast, we know surprisingly little about the axonal and synaptic contribution of the largest organelle in neurons: the ER. For a long time, a lack of molecular markers and imaging techniques made it very difficult to study such a dynamic and narrow organelle. Recent developments in super-resolution light microscopy and in particular cryo-fixation and EM imaging, have opened up possibilities to study the fine ultrastructural details and dynamics of ER subdomains in neurons and other cell types.

In neurons, besides the nuclear envelope and wide ER sheets in the somatodendritic area, axons contain a particular thin form of tubular ER. Here, the ER lacks sheets (except in branch points) and can adopt a ladder-like structure where ER-proteins such as STIM1 are segregated into specific subdomain. Defects in neuronal ER-shaping and ER-phagy mainly affect axons, suggesting that there are different mechanisms in place for maintaining axons versus somatodendritic regions. It is however unclear what the exact molecular mechanisms are that define these differences. A plethora of studies have linked mutations in ER-shaping proteins to ER morphology changes in HSP, a group of clinically and genetically diverse disorders characterized by degeneration of long upper motor neuron axons (axonopathy) in the brain and spinal cord. Mutations in SPG4, encoding the ER-shaping protein Spastin (Fig. 2), are the most common cause of HSP, counting for 40 % of the autosomal dominant cases. Other ER-resident hairpin-containing proteins, such as Atlastin-1 (HSP subtype SPG3A), RREEP1/2 (HSP subtype SPG31 and SPG72), Reticulon-2 (HSP subtype SPG12) and Prorudin (HSP subtype SPG33) are also commonly found mutated in patients, although their exact roles in HSP-onset are still investigated (Martignoni et al., 2008; Sonda et al., 2021). Other neurological disorders have also been linked to mutations in genes encoding for ER-proteins. For instance, one of the genes whose mutations are responsible for early-onset Parkinsonism is VPS13C, a lipid transfer protein that localizes at contact sites between the ER and late endosomes/lysosomes (Kumar et al., 2018). Mutations in the ER Ca\(^{2+}\)-release channel IP3R type 1, the dominant IP3R subtype in the central nervous system, are linked to spinocerebellar ataxia, pontine cerebellar hypoplasia and Gillespie syndrome (Hishatsume and Mikoshiba, 2017). Mutations in ER transmembrane protein TMCO1, proposed to facilitate Ca\(^{2+}\) leak upon ER overload, cause cerebrofacialotoric dysplasia, a developmental disorder characterized by distinctive craniofacial dysmorphism, cognitive impairments and sometimes epilepsy (Ratnayake et al., 2022). In addition, a variety of neurological diseases, including AD and ALS, are associated with, or arise from, abnormally shaped ER (Sharoo et al., 2016; Teuling et al., 2007), disrupted ER-organelle contact sites (Kim et al., 2022) or ER Ca\(^{2+}\) dyshomeostasis (Kovace et al., 2021; Sharoo et al., 2016; Teuling et al., 2007), suggesting that ER dysfunction could be a common pathway. ER-stress and associated signaling events, such as the unfolded protein response and ER-associated protein degradation, also play important roles in the pathology of neurological diseases such as AD, ALS and prion disease (Roussel et al., 2013). To date, it is still unclear where many of these ER-associated processes take place and if they are segregated in certain ER subdomains i.e. somata, dendritic and/or axonal compartments. Ribosomes, mRNA, translation and folding machineries are present in the axon and can locally synthesize proteins (Hafner et al., 2019). What role the axonal ER plays in the modification, processing and degradation of these locally produced proteins is a topic for future research.

Due to the involvement of ER proteins in many hereditary neurological diseases, a further understanding of specific disease mutations on

**Fig. 4.** The process of Endoplasmic Reticulum (ER)-phagy. Turnover of ER membrane and proteins is important for axon homeostasis. Selective removal of certain ER domains via autophagy (ER-phagy) has emerged as a major ER remodeling process. Formation of the autophagosome, a double-membrane structure that encloses cargoes targeted for degradation, is driven by the ATG proteins. Cargo selection is mediated by ER-phagy receptors that bind ATG8/LC3 proteins on the pre-autophagosomal membrane and physically link the cargo to the forming autophagosome. FAM134 and ATL3 are examples of ER-phagy receptors that are implicated in a group of axon degeneration disorders. FAM134 proteins contain a reticulon-homology domain and induce high-membrane curvature upon local clustering, resulting in small ER-derived vesicles. ATL3 acts downstream of FAM134 and its GTPase activity allows it to fuse tubules and separate pieces from the ER. During ER-phagy, a subsection of the ER will eventually be pinched off and incorporated into the autophagosome which will be transported to the neuronal soma, to fuse with degradative lysosomes.
ER structure, function and turnover could open new exciting pathways for drug development. However, to understand how these ER protein disruptions eventually result in neurodegeneration it is imperative to understand in what way ER composition and dynamics contribute to neuronal function. Many questions concerning the role of ER in neurons remain currently unanswered. How does ER-shapings and dynamics affect presynaptic strength? Are ER proteins, such as ER-Ca$^{2+}$ channels, segregated into spatial domains or perhaps synaptic “hot-spots”? What triggers ER turnover at specific compartments? Future studies on how local and global changes in axonal ER status affect axon homeostasis and neurotransmission will provide us with basic insights into how axons and synapses are formed, maintained and function.

**Declaration of competing interest**

The authors declare that they have no conflict of interest.

**Data availability**

No data was used for the research described in the article.

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**Even though the text was extracted correctly, the references section seems to be missing. It is important to provide all the references for completeness and accuracy.**