The Axonal Endoplasmic Reticulum: One Organelle—Many Functions in Development, Maintenance, and Plasticity

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ABSTRACT: The endoplasmic reticulum (ER) is highly conserved in eukaryotes and neurons. Indeed, the localization of the organelle in axons has been known for nearly half a century. However, the relevance of the axonal ER is only beginning to emerge. In this review, we discuss the structure of the ER in axons, examining the role of ER-shaping proteins and highlighting reticulons. We analyze the multiple functions of the ER and their potential contribution to axonal physiology. First, we examine the emerging roles of the axonal ER in lipid synthesis, protein translation, processing, quality control, and secretory trafficking of transmembrane proteins. We also review the impact of the ER on calcium dynamics, focusing on intracellular mechanisms and functions. We describe the interactions between the ER and endosomes, mitochondria, and synaptic vesicles. Finally, we analyze available proteomic data of axonal preparations to reveal the dynamic functionality of the ER in axons during development. We suggest that the dynamic proteome and a validated axonal interactome, together with state-of-the-art methodologies, may provide interesting research avenues in axon physiology that may extend to pathology and regeneration. © 2017 Wiley Periodicals, Inc. Develop Neurobiol 78: 181–208, 2018

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INTRODUCTION

Neurons are excitable, polarized, and large cells that control the generation, propagation, and integration of electrical signals in the nervous system. These properties underlie relevant brain outputs such as autonomic functions, sensory/motor coordination, and higher cognitive processes like learning and memory (Varela et al., 2001).

Two domains comprise the morphofunctional organization of mature neurons, the somatodendritic, and axonal compartments (Dotti et al., 1988). Dendrites are branched extensions that project radially from the soma, receiving synaptic inputs, whereas axons are longer and thinner projections that generate action potentials and propagate them to the presynaptic terminal for neurotransmitter release.

Multiple microdomains are essential for axon development, physiology, and repair. For example, growth cones in developing axons determine guidance and the wiring of the nervous system and play key roles after damage (Hur et al., 2012; Tamariz and Varela-Echavarría, 2015). In like manner, in mature

neurons axon initial segments (AIS) determine the threshold of axon potential firing and maintain neuron polarity (Buffington and Rasband, 2011). Axons are also classified as myelinated or nonmyelinated depending on whether they contain or not a myelin sheath that increases conduction velocity. In myelinated axons, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) provide the specialized myelin sheath that surrounds the axon and generate interspaced nodes of Ranvier, naked segments of the axonal plasma membrane or axolemma enriched in ion channels (Cai and Sheng, 2009; Tuck and Cavalli, 2010; Von Bartheld and Altick, 2011; Arancibia-Carcamo and Attwell, 2014).

Intracellular organelles sustain axon and microdomain function. Importantly, evidence from eukaryotic cells suggests that the ER plays major roles in controlling the intracellular levels of calcium, lipid synthesis, protein translation, quality-control and trafficking, in addition to a variety of interactions with other membrane-bound organelles such as mitochondria, vesicles and endosomes (Friedman and Voeltz, 2011; Westrate et al., 2015; Phillips and Voeltz, 2016; Valm et al., 2017). However, the role and relevance of the multiple functions of the ER in axons are only beginning to emerge.

In this review, we discuss the structure of the ER in axons of CNS and PNS neurons. We examine the multiple functions of the ER and their potential local contribution to axonal physiology. We emphasize the emerging role of the ER in axonal biosynthesis, quality control, and trafficking of transmembrane proteins, calcium dynamics, and interactions with other organelles. We also analyze available proteomic data of pure axonal preparations to reveal the dynamic functionality of the axonal ER during development.

THE STRUCTURE OF THE ER IN AXONS

General Features of the ER Structure in Neurons

The structure of the ER is highly conserved in eukaryotes and neurons are no exception. The ER harbors the largest surface area of all eukaryotic organelles, and may represent up to 10 times the surface area of the plasma membrane. Thus, considering that the surface area of neurons is on average 4 orders of magnitude larger than other small and less polarized cells, the extension of the neuronal ER constitutes a remarkable evolutionary achievement (Bolender, 1974; Horton and Ehlers, 2003b).

The neuronal ER is a lipid and protein network, enclosing a continuous lumen that expands throughout the cell (Deitch and Banker, 1993). Its continuity has been demonstrated by filling cerebellar Purkinje neurons with a fluorescent dye (Terasaki et al., 1994). As in other cell types, the neuronal ER is composed of the nuclear envelope, the ribosome-rich rough ER (RER) and the smooth ER (SER) mostly devoid of ribosomes.

In neurons, the nuclear envelope is constituted mainly by ER devoid of ribosomes and formed by a double lipid bilayer. The inner membrane is smooth and nearly 130 Å thick whereas the outer membrane comprises subtle undulations and tubular protrusions into the cytoplasm (Palay and Palade, 1955). The nuclear envelope regularly fuses its membranes to form pores of approximately 200 Å. The envelope is continuous with the RER network composed of flat sheets organized in cisternae that are decorated with ribosomes and interconnected by tubules (Palay and Palade, 1955). The RER is the major component of the Nissl staining, abundant throughout the soma and in dendrites of different neuronal subtypes (Palay and Palade, 1955). Cisterna in the soma are in close apposition with the plasma membrane (≤ 30 nm) (Wu et al., 2017). Nearly 40% of cisternae are tight with a completely occluded lumen, whereas the remaining 60% accommodate a 20 to 100 nm lumen (Wu et al., 2017). In other eukaryotic cells proteins of the translocon complex, such as Dad1, TRAPα, and Sec61 are abundant in ER sheets. The preferential distribution of TRAPα in sheets is dependent on the direct or indirect association with translating polysomes (Shibata et al., 2010). Interestingly, ribosomes and components of the translocon complex, such as Sec61, have been identified in dendrites and even inside dendritic spines (Pierce et al., 2000). Additionally, cytoskeleton-linking membrane protein 63 (CLIMP-63) holds membrane sheets together in cisternae by forming coiled-coil domain-dependent clusters in the ER lumen serving as spacers of approximately 50 nm between sheets that are relevant for ER morphology in hippocampal neurons (Shibata et al., 2010; Cui-Wang et al., 2012).

The neuronal SER, partially devoid of ribosomes, predominates in distal dendrites and axons. It is composed of approximately 80 nm diameter tubules and occasionally organize as edges of irregular polygons resembling three-way junctions (Wu et al., 2017).

The ER in dendrites establishes close contacts with the plasma membrane, forming sub-surface cisternae (Rosenbluth, 1962; Herndon, 1963; Henkart et al., 1976; Buschmann, 1979). The distances of these cisternae to the plasma membrane range from 20 to 80 nm, and their morphology suggests that both membranes are

frequently bound (Rosenbluth, 1962). Intriguingly, the ER also reaches deep into dendritic branches and penetrates into spines establishing continuity with the spine apparatus (Spacek and Harris, 1997). The ER content of dendritic spines may be related to calcium signaling in hippocampal neurons and is highly dynamic, as only a fraction contains the organelle at any given moment (Toresson and Grant, 2005; Holbro et al., 2009; Segal and Korkotian, 2014). The dendritic ER can lose continuity as a result of synaptic activity. For example, activation of NMDA receptors induces a redistribution and fragmentation of the dendritic ER in cultured hippocampal neurons (Kucharz et al., 2009; Valenzuela et al., 2014). However, the roles of these morphological changes are still unclear.

The surface/volume ratio of the axonal ER is the largest among all known cellular compartments, but it is not entirely clear how the enormous amount of ER membrane is organized in axons to sustain function (Horton and Ehlers, 2003b; Pfenninger, 2009). Next we examine the structure of the ER in axons.

Morphology of the ER in Axons

The ER in PNS and CNS axons is mostly constituted by a continuous, branched, and anastomosed tubular SER (Droz et al., 1975; Broadwell and Cataldo, 1984; Deitch and Banker, 1993). However, axon initial segments of some CNS neurons, such as cortical principal cells, spiny stellate cells, or dentate granule cells, also contain ER cisternae-like stacked sheets (Peters et al., 1968; Kosaka, 1980; Takei et al., 1992; Benedeczky et al., 1994; Berridge, 1998). Interestingly, a dynamic tubular network of ER is present in growth cones, and thin tubular projections move to the periphery in parallel to microtubule bundles, preceding the movement of vesicular elements during extension (Dailey and Bridgman, 1989). In PNS axons the tubular SER ramifies and extends throughout the axolemma, forming periodically spaced networks of tubules or sub-axolemmal plates (Droz et al., 1975). In addition, densely packed ER tubules have been described in sensory axons, which are anastomosed into beaded-like structures, especially near nodes of Ranvier (González et al., 2016). A similar continuous and anastomosed structure is found in CNS axons of the nucleus accumbens, where a single ER tubule frequently runs parallel to the axolemma in narrow axonal portions (Wu et al., 2017). These ER tubules may be profusely branched in synaptic varicosities in the distal axon, resembling the ER within axon growth cones of developing hippocampal neurons *in vitro* (Deitch and Banker, 1993; Wu et al., 2017).

In neurons of the nucleus accumbens membrane appositions of less than 30 nm between the ER and the plasma membrane are far more frequent in the soma than in dendrites or axons, covering 12% of the surface in the soma and less than 2% in the latter compartments (Wu et al., 2017). Small portions of the axonal ER may be discontinuous, although the possibility that these discrete structures correspond to endosomes has not been ruled out (Wu et al., 2017).

Besides contacts with the plasma membrane, the axonal ER associates closely with several membrane-bound organelles, including mitochondria, vesicles, and endosomes. For example, the ER forms a continuous network that surrounds mitochondria, synaptic vesicles, as well as other organelles including multivesicular bodies (MVBs) (Wu et al., 2017). Interestingly, the proportion of contacts is different in the axonal compartment than in the soma, with ER-mitochondria contacts covering more than 4% of the axonal ER surface (Wu et al., 2017). In agreement with these observations, mitochondria intermingle with a dense network of ER tubules in the proximity of nodes of Ranvier of sensory axons (Tsukita and Ishikawa, 1980; González et al., 2016). The tight association of the axonal ER with mitochondria has also been described in mouse spinal cord neurons, in synaptosomal preparations from rat brain, and in rat pyriform cortex presynaptic terminals (Bird, 1978; McGraw et al., 1980; Westrum and Gray, 1986). These observations suggest a great interdependence of energetic demands or calcium buffering in axons.

The large and complex architecture of the ER in axons requires interactions with the cytoskeleton and distinct membrane shaping mechanisms, which are carried out by ER shaping proteins. Although interactions with the cytoskeleton are crucial for the dynamic morphology of the ER, these will not be covered here as they have been thoroughly reviewed elsewhere (Gurel et al., 2014). ER-shaping proteins are described in the next section.

The Role of ER-Shaping Proteins in Axons

The structure of the ER is mainly determined by interactions with integral ER membrane proteins referred to as ER-shaping proteins. ER-shaping proteins belong to a group that includes atlastins, receptor expression-enhancing proteins (REEPs), spastin, ADP-ribosylation factor-like 6 interacting protein 1 (ARL6IP1), family with sequence similarity 134 (FAM134), and reticulons (Hübner and Kurth, 2014). All of them contain a conserved C-terminal structural domain consisting of two transmembrane segments

that partially span the ER membrane, forming a hairpin-like structure asymmetrically associated to one face of the lipid bi-layer. These membrane segments are responsible for the induction of membrane bending, favoring tubular shapes or curved edges, such as borders of cisternae (Westrate et al., 2015). In addition to the conserved C-terminal domain with shaping properties, their N-termini interact with a variety of partners, including other organelles such as mitochondria, trafficking vesicles and the cytoskeleton (Steiner et al., 2004). Accordingly, mutations in different domains of ER-shaping proteins result in perturbations of trafficking and in the aberrant distribution of membrane-bound organelles that may contribute to pathogenicity in Hereditary Spastic Paraplegias and Amyotrophic Lateral Sclerosis (Chiurchiù et al., 2014).

Hereditary spastic paraplegias constitute a genetically diverse group of neurological disorders in which the primary symptoms are weakness and spasticity of the lower limbs caused by a length-dependent axonopathy of neurons of the corticospinal tract. Interestingly, nearly 50% of Hereditary spastic paraplegias are associated to dominant autosomal mutations on genes coding ER-shaping proteins (Mccorquodale et al., 2011). Mutations in spastin (spg4) account for 40% of all the cases, followed by atlastin-1 (spg3A) that accounts for nearly 10%. A remaining fraction is associated to mutations in other genes for ER-shaping proteins such as REEP1 (spg31) (Mccorquodale et al., 2011).

Spastin is a microtubule-severing ATPase with sequence similarity to katanin associated with diverse cellular activities (Lumb et al., 2012). Spastin severs microtubules *in vitro*, induces a decrease in the content of the microtubule cytoskeleton in *Drosophila* muscle cells and helps concentrate the ER at tips of regenerating axons (Sherwood et al., 2004; Roll-Mecak and Vale, 2005; Rao et al., 2016). Atlastin-1 is a dynamin-like protein that contains an ER-shaping domain and a GTPase domain necessary for the homotypic fusion of ER membranes (Orso et al., 2009). Atlastin-1 is frequently located at the three-way junctions of the ER network (Hu et al., 2009). REEP1 promotes the formation of the ER network *in vitro*, and induces the alignment of the ER with the microtubule cytoskeleton (Park et al., 2010). Spastin and atlastin-1 interact in COS7 cells (Evans et al., 2006). Intriguingly, a clinically relevant mutation does not disrupt the GTPase function of atlastin-1, but prevents the interaction with spastin, suggesting a relevant role for their coupling in axonal physiology (Evans et al., 2006). Accordingly, spastin, atlastin-1 and REEP1 interact in cortical neurons, although the

functional relevance of this interaction for axonal physiology is only beginning to emerge (Park et al., 2010).

Despite the importance of ER-shaping proteins for neuronal physiology, it is still not clear how their different mechanisms contribute to axonal function. However, emerging evidence on the role of reticulons, the best characterized members of ER-shaping proteins, is beginning to illustrate their relevance.

Four reticulon genes are present in mammals (*RTN1*-3 and *RTN4/Nogo*). They code several isoforms generated through alternative splicing, namely reticulons 1A–C, 2A–C, 3A and B, and 4/NogoA–C (Westrate et al., 2015). All reticulons contain a highly conserved reticulon homology domain (RHD) located at their C-terminal region that is composed of two relatively long hairpin transmembrane motifs separated by a small hydrophilic loop facing the cytosol. RHDs induce membrane curvature depending of the length of their transmembrane domains (Zurek et al., 2011). All reticulon isoforms are highly enriched in ER tubules in an RHD-dependent manner, which contributes to stabilize the tubular structure and decrease the proportion of peripheral cisternae (Zurek et al., 2011). How tubule stabilization affects the function of the axonal ER is still an open question. However, absence of reticulon 1 and both versions of REEP proteins (A and B) result in ER fragmentation on distal portions of motor axons in *Drosophila* (Yalçın et al., 2017). Additionally, downregulation or overexpression of Atlastin in motor neurons result in locomotor deficits in larvae and adult *Drosophila*, and correlate with defects in axonal secretory organelle and presynaptic protein distribution. Furthermore, synaptic vesicle release is also impaired after atlastin downregulation (De Gregorio et al., 2017).

Assuming invariable axon thickness, the tubular shape of the ER avoids interference with the function of other organelles, a requirement for axonal extension (English and Voeltz, 2013a,b; Sackmann, 2014). Thus, ER-shaping proteins that promote tubule-like structures, such as reticulons, favor axonal growth. Indeed, ER-shaping proteins such as atlastin-1 and reticulon-1 are enriched in axonal growth cones of cultured cortical neurons, and their depletion reduces axonal length during early development (Zhu et al., 2006; Nozumi et al., 2009). Similarly, the ER concentrates in growth cones during axonal regeneration in *Drosophila*, but mutant animals defective for atlastin and spastin show no enrichment of the ER at the axonal growth cones as well as impaired regeneration (Rao et al., 2016). Interestingly, ER enrichment is selectively present at regenerating axons, but not in dendritic growth cones. The selective enrichment of

atlastin-1, reticulon-1, and the ER structure is also observed in developing axons of rat cortical neurons (Deitch and Banker, 1993; Zhu et al., 2006; Nozumi et al., 2009) Abundance of the ER in axonal growth cones is a potential source of membranes (Deitch and Banker, 1993; Craig et al., 1995). Indeed, nanometric appositions of the ER with the plasma membrane are necessary for proper axonal growth in cultured rat cortical neurons (Petkovic et al., 2014). Thus, these observations strongly suggest that the tubular ER plays relevant roles under conditions of rapid demand of biomolecules in growth cones.

In addition to the ER-shaping properties of reticulons, intriguing connections with the activity of the secretory pathway have been reported (Chiurchiù et al., 2014). Overexpression of reticulon 2B increases the delivery of the neuronal EAAC1 glutamate transporter from the ER to the plasma membrane (Liu et al., 2008), while overexpression of reticulon 3 interferes with ER to Golgi and Golgi to ER transport (Wakana et al., 2005). Additionally, reticulon 1C interacts with soluble NSF attachment protein (SNAP) receptor proteins (SNAREs), which have major roles in secretion. In fact, a fragment of this protein increases the rate of growth hormone release in a neuronal cell line (Steiner et al., 2004).

Reticulon 1A also directly associates with ryanodine receptor (RyR) 2, regulating intracellular calcium buffering by reducing oscillations mediated by RyR2 on rat Purkinje neurons (Kaya et al., 2013). Reticulon 1A may additionally raise the surface/volume ratio of the ER favoring calcium release. Although these roles have not been described in axons they provide interesting insights for further research.

Recent super-resolution imaging in tissue culture cells demonstrates that the tubular shape of the ER may be ubiquitous because some structures originally described as cisternae by diffraction-limited microscopy are in fact a high-density tubular network (Nixon-Abell et al., 2016). The consequences of this discovery might be crucial to unravel the role of ER tubules on axonal function and also emphasize the need of high-end super-resolution techniques to explore the structural relationships of the ER structure and axonal physiology.

THE MULTIPLE FUNCTIONS OF THE AXONAL ER

Despite detailed morphological descriptions of the axonal ER, we are only beginning to understand the multiple functions of the organelle in axons. Next we explore some conserved mechanisms and discuss unique functional/structural relationships of the axonal ER during development, maintenance and special forms of plasticity such as regeneration (Chen and Zheng, 2014; Jamann et al., 2017). These emerging functions are summarized in Fig. 1.

Biosynthesis

The somato-dendritic ER is a major biosynthetic site in neurons. Therefore, neuronal components such as lipids and transmembrane or secreted proteins may originate in the soma and travel to axons via fast vesicular transport (Twelvetrees et al., 2012; Roy, 2014). However, the timely delivery of components may be challenging in distant neuronal compartments, especially under rapid metabolic demands during axonal development, plasticity or regeneration. Thus, the astonishing extension and complexity of axons may be supported by the local synthesis of lipids and secreted and transmembrane proteins (Alvarez et al., 2000; Brittis et al., 2002; Merianda et al., 2012; Cornejo et al., 2017).

Lipid Synthesis. The ER is the major site of lipid synthesis and the starting point for lipid targeting. The predominant phospholipid in the plasma membrane and intracellular organelles of mammalian cells is phosphatidylcholine. Accordingly, phosphatidylcholine constitutes more than half of the lipid content of neurons (Paoletti et al., 2011). Phosphatidylcholine is synthesized via the Kennedy pathway and the final reaction is catalyzed by 1,2-diacylglycerol cholinephosphotransferase (CPT), an enzyme mainly located in the ER (Paoletti et al., 2011). Cholesterol is another membrane lipid relevant for neuronal physiology, contributing to several structural and functional features of cell membranes such as cell signaling and ion permeability (Martín et al., 2014). Neuronal cholesterol is enriched in synapses and growth cones, and its availability determines the extent of neurite growth (Fünfschilling et al., 2012). Cholesterol synthesis occurs in the soma and the rate-limiting step of its biosynthetic pathway is also catalyzed by an ER-membrane enzyme, namely 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (Goldstein and Brown, 1990). Thus, key reactions of lipid metabolism in neurons depend on ER resident enzymes.

The lipid content of the soma differs from axons. For example, in cultured neurons from dorsal root ganglia the total lipid content of the soma is around 37% of dry weight and the most abundant lipids are phospholipids (57.1%), cholesterol (5.4%), and
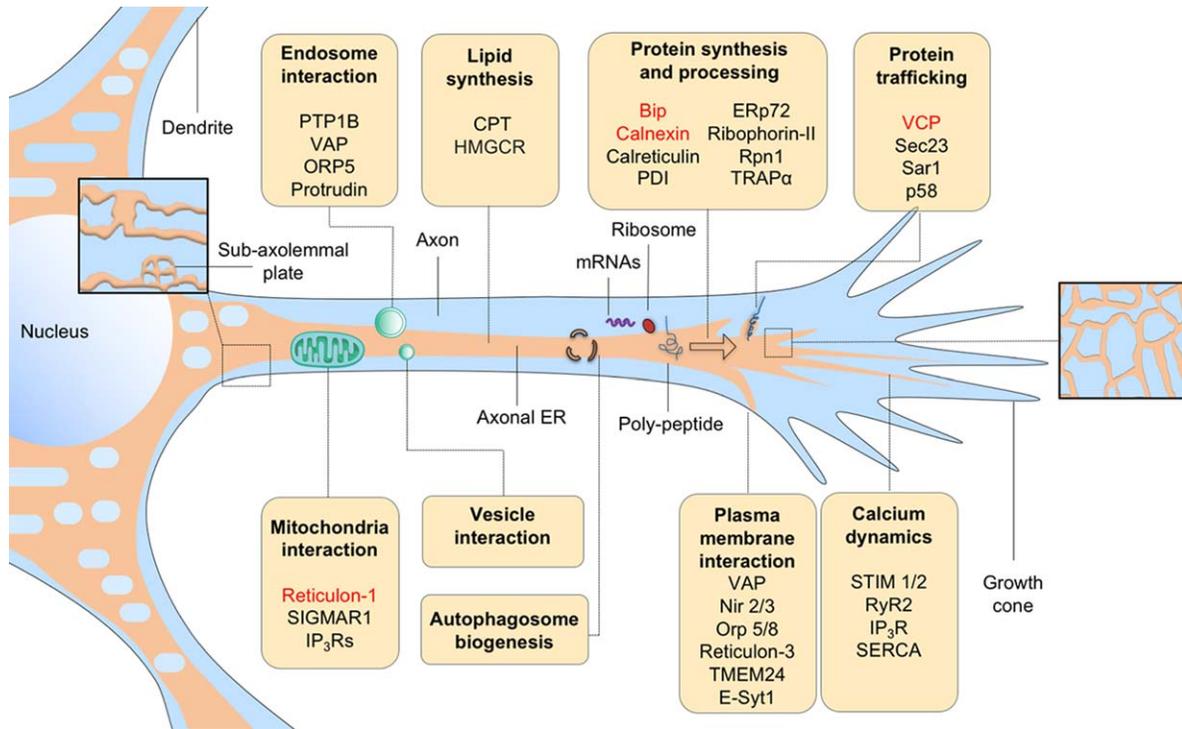
Figure 1 Summary of emerging functions of the axonal ER. The figure illustrates a section of a typical neuron containing the axon and part of the somatodendritic compartment (not to scale). Rounded rectangles highlight the multiple functions of the ER, organelle interactions and some ER-proteins involved in the corresponding category. Proteins in red are enriched in growth cones of developing axons. Two structural boxes indicate (i) the tubular structure of the ER in the axonal shaft, including sub-axolemmal plates, and (ii) the profuse and dense network of the ER in growth cones. Some additional features are indicated. [Color figure can be viewed at wileyonlinelibrary.com]

galactolipid (4.8%). The total lipid content of axons is substantially lower, corresponding to 15% of dry weight, and the composition is different, with similar levels of phospholipids (56.4%), but a higher content of cholesterol (22.1%) and galactolipids (7.7%) (Calderon et al., 2002). It is currently not known to what extent this differential composition results in specialized axonal functions.

The precise mechanisms for lipid transport to axons have not been fully addressed. It is well established that the bulk of transport depends on lipid-containing vesicles from the soma (Pfenninger, 2009). However, evidence also supports local lipid remodeling and local synthesis in axons. For example, axons locally synthesize major phospholipids, including phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, as well as fatty acids in cultured rat sympathetic neurons (Vance et al., 1991, 1994). In fact, nearly 50% of axonal phosphatidylcholine is locally synthesized in distal axons and local synthesis is required for axonal growth (Posse de Chaves et al., 1995). Intriguingly, despite the fact

that cholesterol is prominent in axons, this compartment is unable to synthesize it (Vance et al., 1994). Thus, axons depend on the exogenous uptake of cholesterol for regeneration (Posse de Chaves et al., 1997). Together with lipid uptake, the lipid biosynthetic capacity of the axonal ER may constitute a rate-limiting step to sustain the expansion of the axolemma.

Protein Translation and Processing. The ER is the site of synthesis of most secreted and transmembrane proteins, as well as the source of proteins that reside throughout the secretory and endocytic pathways, including the ER itself. These proteins account for approximately 30% of the eukaryotic proteome (Anken and van Braakman, 2005). Although components of the secretory route have been described in axons, their contribution to a functional pathway is still under investigation. Next, we briefly describe core biosynthetic and secretory elements of the ER as well as protein folding and glycosylation components in axons. We then discuss evidence supporting local

translation and trafficking of transmembrane proteins and their functional implications.

Proteins that enter the secretory pathway contain a signal peptide that binds the cytoplasmic signal recognition particle (SRP). The SRP targets nascent polypeptides to the SRP receptor at the ER membrane. After release from the SRP receptor polypeptides are transferred to the Sec61$\alpha\beta\gamma$ heterotrimer, the central element of the translocon complex (Osborne et al., 2005). This molecular machinery promotes co-translational translocation of proteins into the lumen or the ER membrane. A subset of proteins, on the contrary, are translocated post-translationally (Johnson et al., 2013).

Besides this crucial function in translation, the ER plays fundamental roles in co- and post-translational modifications and quality control. Post-translational modifications include signal-peptide removal, disulfide bond formation, and glycophosphatidylinositol (GPI)-anchor addition (Ellgaard and Helenius, 2003). However, the most ubiquitous co-translational modifications of secreted and transmembrane proteins are *N*-glycosylations, which occur through three major steps, namely synthesis of precursor oligosaccharide linked to dolichol, transfer of primary oligossaccharide to specific protein residues, and processing of the oligosaccharide in the ER and the Golgi apparatus. Addition of the mannose-rich primary oligosaccharide at the ER lumen is mediated by oligosaccharyl transferase. Glucose units are removed by glucosidase-I and glucosidase-II, resulting in a sequence recognized by two quality-control molecular chaperones, the integral membrane protein calnexin and the soluble protein calreticulin. Nascent polypeptides enter a calnexin/calreticulin folding cycle in the ER lumen with their associated co-chaperone ERp57, a thiol-disulfide oxidoreductase glycoprotein. Folding-incompetent proteins are targeted for ER-associated degradation (ERAD), translocated out of the ER and subsequently degraded by the ubiquitin-proteasome machinery (26S proteasome) (Preston and Brodsky, 2017). ERAD is part of a general response mechanism produced by the overload of unfolded proteins in the ER, which is termed the unfolded protein response (UPR) (Preston and Brodsky, 2017). The UPR controls RNA stability and increases protein translation of components involved in virtually every step of the secretory pathway, ERAD and protein folding (Hetz and Saxena, 2017). Abnormal function of these mechanisms may lead to the loss of temporal and spatial regulation of protein levels or proteostasis, potentially participating in the aethiology of neurodegenerative diseases such as Parkinson disease or Amyothrophic Lateral Sclerosis (Ciechanover and Kwon, 2017).

Abundance of axonal transcripts for transmembrane proteins supports ER-dependent local translation. Indeed, in addition to mRNAs coding for mitochondrial proteins, ribosomal components, cytoskeleton, and transcription factors, *in silico* analyses of mRNAs from pure axonal samples of different developmental stages and species have revealed a diversity of transcripts coding for transmembrane proteins (Willis et al., 2007; Taylor et al., 2009; Zivraj et al., 2010; Gumy et al., 2011; Shigeoka et al., 2016; Cornejo et al., 2017). Interestingly, these are more represented than mRNAs coding for transcription factors, such as ATF4, whose axonal translation and impact in degeneration have been well documented *in vivo* (Baleriola et al., 2014). Among the transmembrane protein transcripts, those for guidance receptors, cell adhesion molecules and ion channels are highly represented. For example, the cell adhesion molecules neogenin 1 (NEO1), neuronal cell adhesion molecule (NCAM1), and neurexin-3 (NRXN3) have been identified in a transcriptomic study in preinjured cortical axons from embryonic rats (Taylor et al., 2009). In addition, roundabout homolog 1 (ROBO1), a guidance receptor relevant for development, as well as potassium/sodium hyperpolarization-activated cyclic nucleotide-gated ion channel (HCN2) and potassium voltage-gated channel subfamily A member 2 (KCNA2) have also been found in a pure axonal screen (Taylor et al., 2009). Enrichment of mRNAs coding for protein synthesis, mitochondria, extracellular matrix, growth signals, peroxidase family, and the use of alternative long of 3′UTRs were also recently observed in preinjured axons of rat dorsal root ganglia (Hirai et al., 2017). Importantly, 3′UTR lengthening was reported for voltage-gated sodium channel NaV1.8, including alternative cleavage and polyadenylation, which may provide a novel mechanism to control the abundance of ion channels in neuropathic pain (Hirai et al., 2017). Thus, reproducible identification of transcript families for transmembrane proteins supports the contribution of the axonal ER biosynthetic machinery during development or damage.

Direct evidence for the synthesis of transmembrane or secreted proteins in axons is still fragmentary. However, ribosomes and components of the translational machinery have been reported in CNS and PNS axons (Tennyson, 1970; Zelena, 1972; Bunge, 1973; Steward and Ribak, 1986; Deitch and Banker, 1993; Bassell et al., 1998; Kalinski et al., 2015). Pioneering electron microscopy (EM) studies of the axonal ER did not reveal ribosomes or RER (Lasek et al., 1973), but sparse ribosomes have now been documented in a number of developing and

mature model systems and may participate in local translation (Zelená, 1970, 1972; Pannese and Ledda, 1991; Kun et al., 2007; Twiss and Fainzilber, 2009). For example, polysomes were identified in developing axons from cultured neurons of rat superior cervical ganglia (Bunge, 1973). In addition, axonal growth cones from embryonic chick spinal cord show polysomes, although less abundant when compared to dendritic growth cones (Skoff and Hamburger, 1974). Ribosome-like bodies were also reported in adult dorsal root ganglion neurons (Zelená, 1970, 1972). Furthermore, conspicuous ribosomal domains have been located in axons from spinal nerve roots of adult rabbits and rats (Koenig et al., 2000). Additionally, the ribosomal 5.8S non-coding rRNA and the 60S subunits were recently identified in axons of mature hippocampal neurons by super-resolution microscopy (Younts et al., 2016). Furthermore, regenerating sensory axons from adult rats contain several ribosomal proteins such as P0, L4, L29, L17, and RPP, in addition to translation initiation factors eIF2α, eIF4e, and eIF5, the signal recognition particle 54 (SRP54) and translocon-associated protein α (TRAPα) (Merianda et al., 2009; Nozumi et al., 2009; Gumy et al., 2010; Merianda and Twiss, 2013). Ribosomes in the axon shaft of the optic nerve head, optic nerve and presynaptic terminals in the superior colliculus were recently reported in mice (Shigeoka et al., 2016). Finally, ribosomes are transported into developing axons in a microtubule-dependent fashion in *Caenorhabditis elegans* (Noma et al., 2017). Whether some of these ribosomes associate with the ER is still an open question, but cerebellar axons include cisterna-like structures with partial presence of ribosomes in close apposition to the axolemma (Henkart et al., 1976). Perhaps the application of a recently described technique to visualize mRNA translation in living cells could help directly probe the local functionality of ribosomes in axons (Morisaki et al., 2016).

Accumulating evidence also sustains the local capacity of the axonal ER for post-translational modifications, such as glycosylation. Synaptic cell adhesion molecule 1 (SynCAM 1) is localized in growth cones of developing neurons and helps establishing adhesive clusters between axo-dendritic compartments (Stagi et al., 2010). The lack of glycans at the asparagine sites Asn[70]/Asn[104] reduces the homophilic interactions of SynCAM 1 by approximately 50%, highlighting the functional relevance of *N*-glycans in the establishment of pre-synaptic terminals (Fogel et al., 2010). In addition, *N*-glycosylation modulates the biophysical properties of ion channels. For example, glycans at the pore of ion channels control gating activity (Scott and

Panin, 2014). Indeed, *N*-glycosylation of TRPM8, a transient receptor potential (TRP) channel expressed on a subset of cold-responding sensory neurons in the trigeminal ganglion (Thut et al., 2003), affects temperature threshold without affecting membrane location or availability, suggesting that this effect is mainly produced by the regulation of its biophysical properties (Pertusa et al., 2012). Importantly, dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (or ribophorin II), a subunit of the *N*-oligosaccharyl transferase complex that mediates the transfer of a high mannose oligosaccharide to an asparagine residue within the Asn-X-Ser/Thr consensus motif in nascent polypeptides has been reported in rat peripheral sensory axons (Merianda et al., 2009). In addition, dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 1 (Rpn1), another subunit of the *N*-oligosaccharyl transferase complex, has been described in growth cones of developing rat hippocampal cultured neurons (Estrada-Bernal et al., 2012). Although it is still early, the function of ion channels and adhesion molecules enriched in sensory terminals or growth cones may depend on local *N*-glycosylation, impacting neuron physiology.

In principle, local translation and processing of transmembrane proteins also require a protein quality-control system in the axonal ER. Activation of the UPR is mainly coordinated by three ER stress sensors, namely PERK, ATF6, and IRE1α (Scheper and Hoozemans, 2015; Hetz and Saxena, 2017). These proteins transduce signals that control expression of UPR genes aimed at restoring proteostasis. The activity of the ER stress sensors is controlled by the binding of the ER chaperone immunoglobulin-binding protein (BiP), also known as 78 kDa glucose-regulated protein (GRP-78) (Scheper and Hoozemans, 2015). BiP is a calcium-binding chaperone component of the translocation machinery that imports proteins into the ER, mediates ERAD and may induce autophagy to assist cell survival (Wang et al., 2017). Interestingly, BiP is enriched in growth cones of developing axons from cultured rat cortical neurons, suggesting that an axonal ER protein-quality control operates at least in the early stages of development (Nozumi et al., 2009). Additionally, the content of BiP and the activity of IRE1 are significantly increased 8 days after mouse sciatic nerve crush in distant nerve portions (Oñate et al., 2016). Although most of this increase is due to glial cells, a local increase of ER stress factors in axons cannot be ruled out. Other proteins involved in folding have been described in axons. These include protein disulfide-isomerase (PDI), ER-protein 72 (ERp72), and calnexin (Merianda et al., 2009; Nozumi et al., 2009; Merianda and Twiss, 2013).

The Axonal ER and Protein Trafficking. Properly folded proteins leave the ER in COPII vesicles that assemble in ER exit sites (ERES) at the ER membrane. They transit to the ER-to-Golgi intermediate compartment (ERGIC) and later to the golgi apparatus where mannose is trimmed and other sugars are added, generating complex *N*-glycans from the initial core-glycosylated protein. Proteins are then sorted to the plasma membrane, targeted for secretion, the endosomal system, or other membranous compartments, including the ER (Horton and Ehlers, 2003b).

Although early electron microscopy studies failed to show a classical secretory route in axons (Zelená, 1972; Spencer et al., 2000; Zheng et al., 2001), a growing number of secretory components have been described recently (Merianda and Twiss, 2013; González et al., 2016). For example, KDEL-receptor and Sec23 have been reported in growing as well as in mature peripheral sensory axons (Merianda et al., 2009; Merianda and Twiss, 2013; González et al., 2016). Interestingly, some of these components increase significantly after injury, suggesting an increased secretory capacity to support development and repair (Merianda et al., 2009). Regarding CNS neurons, the ER protein, transitional ER ATPase (TERATPase), also known as valosin-containing protein (VCP), which participates in the generation of transitional vesicles between the ER and the Golgi, is enriched in axonal growth cones of developing rat cortical neurons (Nozumi et al., 2009).

The abundance of axonal ER may correlate with neurite outgrowth. For example, ER proteins such as calnexin are enriched in axons of cultured CNS neurons at early developmental stages. However, as neurons mature, calnexin becomes evenly distributed in dendrites and axons (Krijnse-Locker et al., 1995; Nozumi et al., 2009). In agreement with these observations, Sar1, an ERES component, is selectively enriched in axons of 2 days *in vitro* cultured neurons but axonal enrichment decreases as polarization proceeds (Aridor and Fish, 2009). These results suggest that the local function of the axonal ER may be differentially relevant during development.

Accumulating evidence also suggest that the axonal ER participates in delivery of transmembrane proteins. The axolemma is enriched in ion channels and receptors that mediate the propagation of electrical and chemical signals and/or axon guidance during development. Isolated invertebrate axons deliver a G protein-coupled receptor to the plasma membrane after axonal injection of mRNA, and chick axons deliver *de novo* synthesized EphA2 receptors with a potential role in axon guidance during development (Spencer et al., 2000; Brittis et al., 2002). Similar results were obtained *in vitro* with the neuronal membrane protein 35 (NMP35) in a process that contributes to axonal regeneration in dorsal root ganglion neurons (Merianda et al., 2012). Furthermore, local ER to plasma membrane trafficking of voltage-gated sodium channels has been described in peripheral nerves from rat sensory cultured neurons (González et al., 2016).

Cell adhesion molecules interact with the extracellular matrix to promote growth, fasciculation and navigation of axons (Pollerberg et al., 2013). Axonal growth cones are also enriched in cell adhesion molecules, which mediate intracellular signaling during development and regeneration. Importantly, activated leukocyte cell adhesion molecule (ALCAM) is locally synthesized and delivered to the plasma membrane in axonal growth cones of retinal ganglion cells, participating in axonal growth *in vitro* (Thelen et al., 2012). Likewise, stimulating the local synthesis of the adhesion molecule NF-protocadherin (NFPC) controls guidance of retino-tectal projecting axonal growth cones (Leung et al., 2013). In addition, local synthesis of Down syndrome cell adhesion molecule (DSCAM) takes place in growth cones of cultured hippocampal neurons, with potential relevance during axonal growth (Zhu et al., 2011). Combined, these results highlight the functional relevance of the biosynthetic and export capacity of the axonal ER during development and regeneration. However, the mechanisms governing the distribution of locally translated proteins along the axonal ER, or their export mechanisms have not been elucidated.

A noncanonical mechanism for confinement of secretory cargo, namely ER complexity, has been described for the ER in dendrites. The structural complexity of the ER network, defined as the relative abundance of tubules, three-way junctions and cisterna, may influence cargo diffusion and the site of delivery of dendritic proteins that enter the secretory pathway. ER structural complexity has been positively correlated with diminished lateral mobility of ER-integral membrane proteins such as Sec61β (Cui-Wang et al., 2012). Interestingly, GluA1 subunits, a subtype of ionotropic glutamate receptors, increase their dwell time in the ER prior to local delivery to dendritic spines in response to activity (Cui-Wang et al., 2012). The tubular SER in PNS axons ramifies and extends throughout the axolemma with domains of higher complexity such as sub-axolemmal plates (Droz et al., 1975). Thus, it is tempting to speculate that the site for local delivery of components to axonal microdomains may also be controlled by the structural complexity of the ER in a similar fashion to dendrites. Interestingly, the ER mobility of axonal

GABA$_B$ receptors revealed a kinesin-1 dependent pre-Golgi mechanism of transport in hippocampal neurons (Valdés et al., 2012). Some GABA$_B$ receptors move in synchrony with the ERGIC, suggesting a non-cannonical local mechanism for delivering receptors to the axolemma. Remarkably, ERGIC concentrates at discrete puncta in the Nodes of Ranvier of peripheral nerves, constituting a potential site for local delivery of transmembrane proteins (González et al., 2016).

Post-ER Trafficking in Axons. Post-ER trafficking components such as ERGIC marker p58, GM130, Giantin, and TGN38/TGN46 have been described in PNS axons (Merianda et al., 2009; Merianda and Twiss, 2013; González et al., 2016). Similar results have been described for CNS axons from cultured rat hippocampal neurons, which express Giantin, GM130, clathrin (TGN), and AP1 (TGN) at early developmental stages (Aridor and Fish, 2009). Conventional Golgi structures have not been identified in axons, so these components may participate in unique mechanistic solutions.

In dendrites, local processing of core glycosylated transmembrane proteins impact the structural and functional maturity of ER client proteins (Mikhaylova et al., 2016). Indeed, atypical glycosylation of surface dendritic proteins may be widespread and may be attributed to complete or partial bypass of the Golgi apparatus (Hanus et al., 2016). Interestingly, noncanonical dendritic Golgi structures distribute far from the cell body may contribute to intracellular trafficking of transmembrane proteins. These include Golgi outposts and Golgi satellites. Golgi-like structures in dendrites were first described in rat CA1 hippocampal neurons (Pierce et al., 2001). Golgi outposts, involved in the local trafficking of transmembrane proteins and brain derived neurotrophic factor (BDNF), were identified in primary branches of a subset of cultured hippocampal neurons (Horton and Ehlers, 2003a; Horton et al., 2005). More recently, ubiquitous Golgi satellites were identified using pGOLT, a recombinant tracer which contains a transmembrane domain of the calcium sensor protein calneuron-2 (Mikhaylova et al., 2016). In contrast to Golgi outposts, Golgi satellites are present in virtually all dendrites of cultured hippocampal neurons. They contain elements of the glycosylation machinery, but without canonical Golgi cisternae, and distribute in close proximity to the ERGIC and the retromer complex, possibly mediating the trafficking of cargo from endosomes to the trans-Golgi network (TGN) (Bhalla et al., 2012). Importantly, synaptic proteins such as GluN2B may traffic anterogradely to Golgi satellites as well as retrogradely from the plasma membrane back to Golgi satellites (Mikhaylova et al., 2016). Thus, a recycling microsystem may control the availability and function of synaptic proteins locally in dendrites. It is not known whether a Golgi satellite-ERGIC-retromer recycling complex is also present in axons, and whether it operates similarly to dendrites. However, the presence of calneuron-1 has been reported in axons *in vivo* (Hradsky et al., 2015). In addition, the core retromer complex has been identified in CNS axons and TGN components have been described in peripheral axons (Bhalla et al., 2012; González et al., 2016). Interestingly, delivery of cystic fibrosis transmembrane conductance regulator (CFTR) may use only trans-Golgi compartments to reach the plasma membrane in baby hamster kidney cells (BHK), supporting the partial by-pass of Golgi structures (Yoo et al., 2002). It is tempting to speculate that the trafficking of transmembrane proteins in axons may rely on similar mechanisms. Indeed, recent evidence indicates that Kv2.1 channels may be targeted to the axon initial segment by a nonconventional trafficking pathway independent of the Golgi apparatus (Jensen et al., 2017).

Sorting and targeting newly synthesized proteins may also rely on an endocytic carrier system (Grieve and Rabouille, 2011). This mechanism may potentially operate with cell adhesion molecules of the L1 family. Accordingly, L1/NgCAM is firstly targeted to the plasma membrane of the somatodendritic compartment and then transferred by the endocytic pathway to the axolemma (Wisco et al., 2003; Winckler and Choo Yap, 2011). Tubular-shaped endosomes carry L1/NgCAM to axons (Yap et al., 2008a,b). Interestingly, L1/NgCAM bearing a specific deletion comprising the first 45 aminoacids of its cytoplasmic tail is enriched in the axolemma by-passing somatodendritic targeting (Yap et al., 2008a). However, this construct contains a tyrosine-based endocytic motif (YRSL). Thus, although L1/NgCAM may directly exit the axonal ER, it is still unclear if and how endocytic adaptors participate in trafficking to the axolemma. It is tempting to speculate that the axonal ER may deliver axonal membrane proteins directly or through endocytic components, but the relevance of these mechanisms in physiology or during injury and repair remain for the most part unexplored.

Calcium Dynamics

Calcium is a ubiquitous second messenger with multiples roles in neuronal physiology such as axon guidance and extension, neurotransmitter release, activity-driven

transcription, activity-dependent modification of synaptic efficacy, and excitotoxicity (Berridge et al., 2003). The ER constitutes a major player in axonal calcium dynamics.

Regulation of Calcium Sources. Different mechanisms converge to account for the homeostasis of calcium levels in neurons. Cytosolic levels at rest are in the nanomolar concentration range, but during neuronal activity this value may raise three orders of magnitude to approximately 100 μM in spatially restricted microdomains. The intracellular concentration of calcium is governed by the influx of extracellular ions across the plasma membrane and by the flux of calcium from intracellular stores, in a complex balance controlled by channels, pumps, exchangers, and binding proteins. These dynamic changes in the cytosolic concentration of calcium underlie a wide spectrum of signaling processes relevant for axonal physiology.

In axons, calcium influx is determined by permeable channels present at the axolemma. These include voltage-operated calcium channels (VOCCs), which allow vesicle fusion at presynaptic terminals to trigger neurotransmitter release, store-operated calcium channels (SOCCs), which mediate calcium flux through the axolemma in response to ER calcium depletion, receptor activated calcium channels (RACCs), which respond to neurotransmitters such as glutamate, and transient receptor potential canonical channels (TRPCs) (Antkiewicz-Michaluk, 1999; Ouardouz et al., 2009a,b; Nilius and Owsianik, 2011; Majewski and Kuznicki, 2014).

Calcium can also be released from the ER through several mechanisms, including RyRs, inositol (1,4,5)-trisphosphate receptors (IP$_3$Rs), and passive leakage (Stutzmann and Mattson, 2011). IP$_3$Rs act as coincidence detectors during synaptic plasticity (Simpson et al., 1995). In addition, small sub-threshold increases on InsP$_3$ enhance calcium sensitivity, contributing to the formation of regenerative calcium waves that may travel along the ER (Lorenzon et al., 1995). Importantly, regenerative calcium waves mediate the regenerative response in axons from *C. elegans* after laser axotomy (Knight and Bass, 2001).

Calcium entry into the ER is carried out by pumps such as sarco-endoplasmic reticulum calcium-adenosine ATPases (SERCAs) that uptake calcium from the cytosol (Vandecaetsbeek et al., 2011). SERCAs are present in the axon initial segment of cortical principal neurons, in close proximity to synaptic junctions of GABAergic axo-axonic terminals (Benedeczky et al., 1994). In addition, IP$_3$Rs and SERCAs have been described in axons of chicken Purkinje cells (Takei et al., 1992).

Mitochondria and the ER behave as reservoirs that uptake calcium and decrease cytosolic levels (McGraw et al., 1980). Additionally, cytosolic calcium levels may also be determined by calcium binding proteins that sequester ions lowering free cytosolic levels. Indeed, calcium binding proteins such as S100 β, calmodulin, calsequestrin and neurocalcin 3 have been found in axonal growth cones of cultured chick forebrain neurons (Takei et al., 1992; Alexanian et al., 2001).

SOCE and Implications for Axons. Extracellular calcium may also refill the ER through capacitive calcium entry (CCE) or store-operated calcium entry (SOCE) (Majewski and Kuznicki, 2014). CCE occurs when ER calcium levels decline, generating the activation of plasma membrane channels that increase calcium entry. A similar mechanism, SOCE, occurs by an increase in the calcium influx across the plasma membrane after the ER calcium reservoir is emptied by the activation of RyRs and/or IP$_3$Rs (Majewski and Kuznicki, 2014). Stromal interaction molecules (STIMs) and Orai proteins are the main components of SOCE. STIM1 is a transmembrane protein present at the ER or plasma membranes, depending on *N*-glycosylation of aminoacids Asn131 and Asn171 (Williams et al., 2002). The intraluminal region of STIM1 inside the ER contains a canonical EF-hand domain (cEF), which functions as a calcium sensor. Orai1 belongs to a group of calcium release-activated channels (CRACs) and is located at the plasma membrane with both N- and C- termini facing the cytoplasm. When a low ER calcium threshold is reached, calcium dissociates from the cEF domain of Stim1, generating the multimerization and concomitant binding of Orai1 to produce calcium influx (Potier and Trebak, 2008; Shim et al., 2015). Interestingly, SOCE may also occur by the influx of calcium through TRPC channels triggered by STIM1 after sensing low ER calcium levels (Yuan et al., 2007). Next, we discuss the emerging evidence and relevance of SOCE in axonal development and physiology.

SOCE operates in excitable cells such as cultured rat hippocampal neurons (Bouron, 2000). Here, in the absence of external calcium, the cholinergic agonist carbachol, which induces intracellular calcium increase due to phosphatidylinositol hydrolysis, and the SERCA inhibitor thapsigargin elevate calcium levels transiently. Then, extracellular calcium produces a second cytosolic calcium increase as a result of ER depletion (Bouron, 2000). Additionally, SOCE is abolished by transfection of a presenilin 1 version containing a familial Alzheimer's disease-associated mutation in

mouse cortical neurons (Yoo et al., 2000). These observations suggest that calcium entry is involved in the aethiology of neurodegenerative diseases.

STIM1 and Orai1 are present in cultured rat cortical neurons (Klejman et al., 2009). Over expression of a constitutively active version of STIM1 [YFP-STIM1 (D76A)] induces the recruitment of Orai1 (Klejman et al., 2009). Interestingly, both proteins co-distribute in puncta-like structures, suggesting that SOCE is functional in neurons (Klejman et al., 2009). Different functions have been reported for both STIM1 and STIM2 in cultured rat cortical neurons (Gruszczynska-Biegala et al., 2011). For example, SOCE operates only in neurons transfected with YFP-STIM1/ORAI1, but not with YFP-STIM2/ORAI1. Nevertheless, neurons transfected with YFP-STIM2/ORAI1 show an increase in constitutive calcium entry compared to neurons transfected with YFP-STIM1/ORAI1. Thus, STIM1 is the main activator of SOCE, whereas STIM2 may regulate basal calcium levels (Gruszczynska-Biegala et al., 2011). This result is coherent with others from non-neuronal cells, showing that STIM2 is mainly a sensor for cytosolic calcium, whereas STIM1 senses predominantly ER calcium levels (Liou et al., 2005; Brandman et al., 2007). Recent evidence shows that endogenous STIM2 and Orai1 interact in rat cortical neurons and mediate a specific SOCE that is insensitive to massive ER depletion through thapsigargin (Gruszczynska-Biegala and Kuznicki, 2013).

Functional Roles of the ER and Calcium in Axons. Calcium dynamics in growth cones has been implicated in axon pathfinding. For example, TRPC calcium channels mediate axonal guidance induced by brain-derived neurotrophic factor (BDNF) on cerebellar granule neurons (Li et al., 2005). Similar results were found for the chemotrophic response induced by netrin-1 on growth cones of cultured *Xenopus* neurons (Wang and Poo, 2005). Netrin-1 mediated guidance requires release of calcium from internal stores in *Xenopus* spinal neurons because a gradient of ryanodine replaces the netrin-1 gradient, generating attraction or repulsion, depending on the ryanodine concentration used (Hong et al., 2000). Additionally, the tight dependency of growth cone turning is mediated by a CaMKII (kinase)/calcineurin (phosphatase) molecular switch that controls attractive or repulsive responses in relation to the local calcium levels. Accordingly, larger local calcium levels activate CaMKII to induce attraction, whereas lower calcium levels activate calcineurin to induce repulsion. This mechanism has been further confirmed in growth cones from dorsal root ganglia, where depletion of the scaffolding and calcium regulating protein

Homer 1 inverted the turning response to BDNF and netrin-1 by changing the function of the CaMKII/calcineurin molecular switch (Gasperini et al., 2009). ER-dependent cytoplasmic calcium levels also regulate growth-cone extension *in vitro* as well as in *vivo* (Bixby and Spitzer, 1984; Bandtlow et al., 1993; Takei, 1998; Gomez and Spitzer, 1999).

Growing evidence involves axonal STIM1 in the control of calcium dynamics during neuronal development and plasticity. For example, STIM1 and Orai redistribute to discrete puncta after ER depletion and during growth cone turning in embryonic cultured sensory neurons from dorsal root ganglia (Mitchell et al., 2012). Interestingly, STIM1 localization is enriched towards the high calcium turning site of the growth cone and its knock-down alters guidance in response to gradients of BDNF and semaphorin-3a (Sema-3a) (Mitchell et al., 2012). Similarly, axonal growth cone turning towards netrin-1 depends on SOCE mediated by calcium entry through TRPC1 channels in *Xenopus* spinal growth cones *in vitro* (Shim et al., 2013). Importantly, disruption of STIM1 interfered with midline axon guidance of commissural interneurons of developing spinal cord *in vivo* (Shim et al., 2013). Atlastin-1 overexpression, depletion, or the expression of dominant-negative mutants, hinder SOCE, reducing neurite outgrowth induced by nerve growth factor (NGF) in PC-12 cells. These results suggest that atlastin-1 is critical to SOCE (Li et al., 2017). Accordingly, alterations in ER morphology mediated by absence of reticulon-4 also perturb SOCE in cultured mouse embryonic fibroblasts (Jozsef et al., 2014).

Action potentials travel along depolarizing axons to the presynaptic terminal, inducing a transient influx of calcium mediated by VOCCs enriched at synaptic boutons (Emptage et al., 2001). Calcium entry allows the fusion of synaptic vesicles with the synaptic membrane for neurotransmitter release. Presynaptic calcium levels may also fluctuate in the absence of stimulus to induce spontaneous neurotransmitter release (Emptage et al., 2001). Both evoked and spontaneous neurotransmitter release are modulated by intracellular calcium stores. The axonal ER contributes during evoked and spontaneous release either through calcium-induced calcium release (CICR) mediated by RyRs, or by SOCE (Emptage et al., 2001). Accordingly, CICR mediates spontaneous calcium transients as well as paired-pulse facilitation of excitatory postsynaptic potentials (EPSPs) in rat hippocampal neurons, whereas SOCE participates in spontaneous neurotransmitter release (Zucker, 1999; Emptage et al., 2001). Additionally, brief trains of 20 stimuli at 100 Hz induce early and

delayed calcium transients that depend on calcium release from the axonal ER in the rat hippocampus (Liang et al., 2002). Elegant fluorescence-based calcium measurements, performed selectively on the axonal ER, show a net calcium uptake by the ER during neuronal activity that shapes calcium transients only during prolonged firing (de Juan-Sanz et al., 2017). However, STIM1 mediated SOCE modulates release probability (de Juan-Sanz et al., 2017). Relevant issues remain to be solved, for example, what is the contribution of axonal calcium store operated mechanisms during learning and memory, or in neurodegenerative disorders. These and other fundamental functions may depend on the interactions of the axonal ER with other membrane bound organelles.

Contacts with Other Membrane Bound Organelles in Axons

The analysis of local contacts of the ER with other organelles in non-neuronal cells has revealed relevant functional relationships. For example, ER tubules circumscribe mitochondrial and endosome membranes, favoring organelle fission (Friedman et al., 2011; Rowland et al., 2014). Interestingly, many contacts are partially determined by protein interactions with ER resident proteins that function as tethers (Csordás et al., 2006). A summary of identified interactions between the ER and membrane bound organelles is presented in Table 1. Thus, the ER may be considered a center of a dynamic interacting network of organelles, including the Golgi apparatus, lysosome, peroxisome, mitochondria, autophagosomes, and lipid droplets (Valm et al., 2017). The ER makes extensive contacts with microtubules, which contribute to define structure and dynamics. Since these have been thoroughly reviewed elsewhere (Goyal and Blackstone, 2013; Joensuu and Jokitalo, 2015) here we will only focus on membranous organelles.

ER-Plasma Membrane Contacts. The abundant distribution of the axonal ER in growth cones of developing axons, together with the requirement of a narrow apposition between the ER and the plasma membrane, suggest a major role of these interactions during development (Deitch and Banker, 1993; Craig et al., 1995; Petkovic et al., 2014). Indeed, the interaction of the ER with plasma membrane is not only relevant for the control of calcium dynamics described earlier, but also for cell signaling and for determining the lipid composition of both compartments. In fact, the transport of lipids inside the cell may proceed with the help of tethering proteins that directly carry lipids between bilayers without the

need of membrane fusion (Holthuis and Menon, 2014). Considering the astonishing extensions of the axolemma and the axonal ER, it seems highly plausible that the local transfer of lipids between the ER and the plasma membrane also contribute to signaling and membrane addition during axonal growth. However, little is known about these contacts in axons, and most evidence comes from other cell types.

Contacts between the plasma membrane and the ER control their lipid composition. For example, the ER-integral membrane oxysterol-binding protein (OSBP)-related protein 5 and 8 (ORP 5/8) tether the ER to the plasma membrane through their pleckstrin domains by binding to phosphatidylinositol 4-phosphate (PI4P). These interactions allow their OSBP-related domains to exchange either PI4P or phosphatidylserine (PS) among both compartments, contributing to degradation of PI4P in the ER and for the enrichment of PS on the plasma membrane (Chung et al., 2015).

Different ER-plasma membrane contacts control cell signaling. For example, clathrin-independent epidermal growth factor (EGF)-induced endocytosis of EGF receptor (EGFR) requires ER-plasma membrane contacts mediated by reticulon 3 on HeLa cells (Caldieri et al., 2017). Additionally, ER-plasma membrane contacts may act as a sink to remove the excess of inositol 1,4,5-trisphosphate (IP_3) and diacyl-glycerol (DAG), accumulated in the plasma membrane after phospholipase C (PLC) hydrolysis of phosphatidylinositol-4,5-bisphosphate $PI(4,5)P_2$ (Saheki et al., 2016). This process requires the presence of extended synaptotagmin 1 (E-Syt1) in the ER, which contains a SMP lipid binding domain (synaptotagmin-like mitochondrial-lipid binding protein domain) that mediates DAG transfer from the plasma membrane to the ER, preventing its accumulation and providing a local signal (Saheki et al., 2016). A complementary mechanism for the control of IP_3 and DAG signaling is mediated by the Pyk2 N-terminal domain-interacting receptor 2 protein (Nir 2). Nir 2 is a lipid transfer protein that peripherally associates with the ER during PLC signaling (Kim et al., 2015). The association of Nir 2 with the ER is mediated by VAP-B (Amarilio et al., 2005; Chang and Liou, 2015). In order to sustain PLC signaling the association of the Nir 2/VAP-B complex with the plasma membrane provides a source of phosphatidic acid (PA) for the resynthesis of phosphatidylinositol by ER-associated enzymes, as well as a mechanism for delivering this phosphatidylinositol to the plasma membrane in order to replenish the levels of $PI(4,5)P_2$ consumed by PLC (Kim et al., 2015). Finally, TMEM24, which is reversible located at the ER or the plasma membrane depending on cytosolic calcium-dependent phosphorylation/dephosphorylation, mediates calcium oscillations that account for triggered

Table 1   ER Tethering Proteins Identified in Eukaryotic Cells

Interaction	ER Protein	Organelle Component	Cell Type	Reference
ER-LE	VAP-A	ORP1L	Mel JuSo	Rocha et al., 2009
ER-LE	VAP-A	STARD3/MLN64	HeLa	Alpy et al., 2013
ER-LE	VAP-A	STARD3NL/MENTHO	HeLa	Alpy et al., 2013
ER-LE	Protrudin	Rab7-PtdIns(3)P	HeLa, PC12	Raiborg et al., 2015
ER-LE/MVB	PTP1B	EGFR	HeLa	Eden et al., 2010
ER-EE/LE	Unknown	FAM-21[a]	COS-7	Rowland et al., 2014
ER-RETR	VAP-A/B	SNX2	HeLa	Dong et al., 2016
ER-PM	Reticulon-3	EGFR	HeLa	Caldieri et al., 2017
ER-PM	Extended synaptotagmin	PtdIns(4,5)P_2	HeLa	Saheki et al., 2016
ER-PM	VAP-B/Nir 2/3	PA, DAG	HeLa	Chang and Liou, 2015
ER-PM	ORP5/8	PtdIns4P	HeLa	Chung et al., 2015
ER-PM	TMEM24	acidic PM	COS-7, HeLa, INS-1	Lees et al., 2017
ER-MIT	Reticulon-1C	Mitochondrial ATP synthase	SH-SY5Y	Reali et al., 2015
ER-MIT	Reticulon-1A	Unknown	HeLa	Cho et al., 2017
ER-MIT	SIGMAR1; IP$_3$Rs	VDAC1	Primary motor neuron cultures	Bernard-Marissal et al., 2015
ER-MIT	SIGMAR1; IP$_3$Rs[a]	VDAC1[a]	CHO	Hayashi and Su, 2007
ER-MIT	VAP-B	PTPIP51	NSC-34	Stoica et al., 2014

[a]Potential interactor.
Organelle abbreviations: EE, early endosome; ER, endoplasmic reticulum; LE, late endosome; MIT, mitochondria; MVB, multivesicular body; PM, plasma membrane; RETR, retromer. Lipid abbreviations: DAG, diacylglycerol; PA, phosphatidic acid; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate. Cell line abbreviations: CHO cells, Chinese hamster ovary cell line; COS-7, Monkey kidney fibroblast cell line; HeLa, human cervical cancer cell line; INS-1, rat insulinoma cell line; Mel JuSo, human melanoma cell line; NSC-34, mouse motor neuron-like hybrid cell line; PC12, rat adrenal medulla cell line; SH-SY5Y, human neuroblast cell line.

insulin release (Lees et al., 2017). It is tempting to speculate that similar ER-mediated mechanisms operate locally in axons to control calcium oscillations and even neurotransmitter release.

ER-Endosome Contacts. Functional contacts between the ER and endosomes are relevant in neurons as in other eukaryotic cell types. Endosomes comprise different kinds of tubo-vesicular structures that belong to the endocytic pathway. Endocytosed material, engulfed via clathrin-dependent or clathrin-independent mechanisms, is initially found in Rab5-positive early endosomes, which constitute the first station for protein sorting. These are sent back to the cell surface in Rab4-positive vesicles or ubiquitinated and degraded in lysosomes. Alternatively, early endosomes mature to Rab7-positive late endosome, also known as multivesicular bodies (MVB), a vesicular structure containing small 10 to 50 nm diameter intraluminal vesicles (Sotelo and Porter, 1959). MVB can fuse with lysosomes continuing the degradative pathway, or with the plasma membrane, releasing their intraluminal vesicles, or exosomes, to the extracellular medium (Colombo et al., 2014). Additionally, endosome carriers mediate the transport

between early and late endosomes. Finally, endocytosed material may return to the plasma membrane in Rab11-positive late recycling endosomes (Scott et al., 2014). The ER and endosomes establish functional contacts, defined as 10 to 30 nm close interactions between two membranes, which increase in abundance as endosomes mature. Early endosomes interact with tubular ER membranes moving along microtubules (Friedman et al., 2010). Interestingly, a higher proportion of late endosomes remain attached to the ER. In fact, over 99% of late endosomes form contact sites with the ER, while only 50% of early endosomes maintain this association (Friedman et al., 2013).

Functions for ER-endosomes contact sites in mammalian cells include dephosphorylation of endosome membrane receptors by phosphatases present in the ER membrane (Eden et al., 2010), late endosome positioning in the cell, depending on cholesterol levels (Rocha et al., 2009), and late endosomes translocation to the plus-end of microtubules, positioning endosomes in the cell periphery and establishing fission sites (Rowland et al., 2014; Raiborg et al., 2015). These functions also depend on tethering proteins that mediate the structural coupling between

both organelles. Contacts between the ER and endosomes in axons are mostly unexplored, but they may have relevant functional consequences for cell physiology.

Endocytosed receptors in the presynaptic terminal may signal during their axonal retrograde transport to the soma. Retrograde signaling in axons is governed by endosomes that resemble late endosomes such as those positive for Rab7 (Zhou et al., 2012). Interestingly, signaling of late endosomes can be stopped by the sequestration of enzymes from their cytosolic surface. For example, enzymes from the Wnt pathway such as glycogen synthase kinase 3 (GSK3B) are located inside acidic intraluminal vesicles of late endosomes preventing encounters with their substrates (Taelman et al., 2010). Interestingly, the ER has the potential to control this process through resident enzymes with phosphatase activity. For example, the ER membrane phosphatase PTP1B interacts with the EGFR, inducing the incorporation of the receptor into intraluminal vesicles for lysosome degradation (Eden et al., 2010). It is interesting to speculate that similar mechanisms of control operate with signaling endosomes and the axonal ER.

Lipid transfer mediated by ER-endosome contacts may participate in relevant cellular processes. For example, VAP proteins in the ER control PI4P levels on endosomes and their WASH-dependent budding dynamics, modulating endosome-Golgi complex trafficking through their interaction with the SNX2 subunit of the retromer (Dong et al., 2016).

In HeLa cells low cholesterol levels induce a conformational change in the endosomal oxysterol-binding protein (ORP1L) that allows the interaction with Rab7 and VAP-A ([vesicle-associated membrane protein]-associated ER protein A) through an acidic tract motif (FFAT) to remove p150 (Glued) and its associated motors, favoring the movement of late endosomes to microtubule plus ends (Rocha et al., 2009). It is not known whether these interactions take place locally in axons. However, the *Drosophila* homolog of VAP-33, DVAP-33A, has been found in axons of motor neurons (Pennetta et al., 2002). Thus, although speculative, interaction of the ER with late endosomes may control their positioning along the axon depending on cholesterol levels (Rocha et al., 2009).

The transfer of cholesterol from endosomes may be relevant for their correct positioning along the nerve, but also a source of cholesterol for distal axons that do not synthesize it. These may be affected in lipid storage disorders such as Niemann-Pick type C disease, a fatal neurological disorder mostly explained by the loss of function of the NPC1

protein. NPC1 is located in the outer membrane of late endosomes and inserts cholesterol in endosomal membranes for export to other compartments such as the plasma membrane and the ER (Ikonen, 2008). Cholesterol transfer from low-density lipoprotein (LDL) reservoirs in late endosomes is facilitated by the cooperation of NPC1 with ORP5 in the ER (Du et al., 2011). Accordingly, cholesterol is accumulated in the soma and reduced in distal axons in NPC1-deficient neurons (Karten et al., 2002). Thus, abnormal cholesterol transfer from endosomes to the axonal ER, as in Niemann-Pick type C disease may partially mediate the negative outcome in axonal development and function (Karten et al., 2006; Neefjes and van der Kant, 2014).

The axonal ER may also determine the proportion of endosomes with tubular or vacuolar shape as well as their site of fission. Accordingly, over expression of STARD3NL, a cholesterol-binding protein present in the outer membrane of late endosomes, increases ER-endosome contacts and causes enlargement of lysosomes and endosomes positive for LAMP1 as well as a decrease in the overall number of tubular endosome carriers in HeLa cells (Alpy et al., 2013). The interaction of endosomes with the ER is also mediated by the tethering protein VAP-A, which interacts with STARD3 and STARD3NL present in late endosome membranes, which heterodimerize through their MENTAL domain by means of the FFAT motif (Alpy et al., 2013). Additionally, contact sites between ER tubules and endosomes, targeted by the retromer associated protein FAM-21, are necessary for early and late endosome fission (Rowland et al., 2014). Indeed, ER tubules are required to produce functional constriction of endosomal membranes and the overexpression of reticulon 4, that results in disorganization of the ER tubular network, inhibits this process (Rowland et al., 2014). However, it remains to be explored if similar interactions also hold true for the axonal ER.

Interaction of endosomes with the axonal ER may favor axonal elongation. For example, protrudin, an ER integral protein containing an FFAT motif, interacts directly with late endosomes by means of Rab7 and phosphatidylinositol 3-phosphate [PtdIns(3)P], inducing the formation of cellular protrusions (Shirane and Nakayama, 2006). Similar results have been found in neuroendocrine PC12 cells where the interaction of late endosomes with the ER favors their loading into kinesin-1 which moves to the plus-end of microtubules, mediating their anterograde transport to the neurite tip and their potential addition to axonal membranes (Raiborg et al., 2015). This process may also be of pivotal relevance in the assembly

of a new growth cone during regeneration (Bradke et al., 2012).

ER-Mitochondria. Contacts between mitochondria and the ER are relevant for axonal physiology. On virtue of these interactions, the ER modulates the output of virtually every cellular function accomplished by mitochondria. These include energetic handling, sterol synthesis, homeostasis of calcium, reactive oxygen species (ROS), and apoptosis (Saxton and Hollenbeck, 2012).

In eukaryotic cells nearly 20% of the mitochondrial surface is tightly opposed to ER membranes (<30 nm), forming mitochondria associated membranes (MAMs). Importantly, MAMs are particularly abundant in axons, suggesting a major interdependence of axonal function and mitochondrial dynamics (Bird, 1978; McGraw et al., 1980; Westrum and Gray, 1986; Wu et al., 2017). ER tethers include reticulon 1C, that interacts with components of MAMs regulating lipid exchange (Reali et al., 2015), and reticulon 1A, that promotes the formation of MAMs (Cho et al., 2017).

Axonal function relies heavily on energetic supply. In fact, action potential propagation and repolarization through Na^+/K^+ ATPase represent the largest energetic tasks accomplished by mitochondria in neurons (Lennie, 2003). Accordingly, mitochondrial density increases in axonal domains of higher energetic demands such as growth cones, nodes of Ranvier, and presynaptic terminals (Saxton and Hollenbeck, 2012). Furthermore, mutations affecting axonal energy supply may underlie neurodegenerative diseases (Sheng and Cai, 2012). For example, decrease in the axonal transport of mitochondria is associated to neuropathy with distal predominance in a *Drosophila* model of Friedreich's ataxia, and to axonal degeneration of retinal ganglion cells in animal models of glaucoma (Shidara and Hollenbeck, 2010; Takihara et al., 2015).

Deleterious effects on axons are likely mediated by impaired energy supply as a result of abnormal mitochondrial transport and biosynthetic function. Interestingly, tethering proteins mediating MAMs control mitochondrial motility as well as ATP production (Paillusson et al., 2016). Both functions are tightly dependent on mitochondrial calcium content, which in turn is regulated by the efflux from closely apposed ER calcium nanodomains (Brewster, 2017). For example, a mutation in Sigma 1 receptor (SIGMAR1), an ER-mitochondrial tethering protein that controls calcium transfer from the ER to mitochondria, has been identified in ALS patients and associated to locomotor deficits (Bernard-Marissal et al.,

2015). This calcium control is mediated by stabilizing the proximity between mitochondria and the IP_3Rs on the ER membrane (Hayashi and Su, 2007). Impaired SIGMAR1 function induces motor neuron loss in culture and alters mitochondrial dynamics, decreasing their size, distal localization, fission as well as retrograde and anterograde velocity in axons (Bernard-Marissal et al., 2015). Interestingly, when cytosolic calcium levels are diminished by chelation, the overall effects on mitochondrial dynamics are restored (Bernard-Marissal et al., 2015). IP_3R interacts with mitochondrial VDAC1, potentially mediating calcium transfer from the ER to mitochondria. Thus a tethering complex formed by SIGMAR1 and VDAC1/IP_3Rs mediates calcium transfer and impacts mitochondrial dynamics, which likely mediates motor neuron loss. Analogously, disruption of dynamin-1-like protein (DRP1), which mediates mitochondrial fission by wrapping around the scission site, leads to depletion of mitochondria on midbrain dopamine neurons, potentially participating in selective neuronal nigrostriatal degeneration associated to Parkinson's disease (Berthet et al., 2014).

Importantly, neuronal regeneration is also highly dependent on the transport of mitochondria in axons. In fact, mitochondrial transport in axons declines with neuronal maturation, potentially explaining the diminished regenerative capacity of mature neurons (Filbin, 2006). Accordingly, enhancing mitochondrial transport facilitates axonal regeneration, possibly due to increased ATP availability (Zhou et al., 2016).

Additionally, a mutant α-synuclein associated to Parkinson's disease as well as its wild-type version disrupt the interaction between the vesicle-associated membrane protein-associated protein B (VAP-B) and protein tyrosine phosphatase-interacting protein 51 (PTPIP51) loosening contacts between the ER and mitochondria (Stoica et al., 2014). These changes reduce calcium transfer from the ER with a concomitant decrease in mitochondrial ATP synthesis (Stoica et al., 2014). Accordingly, several enzymes of the tricarboxylic acid cycle increase their activity after calcium binding (Tarasov et al., 2012). However, prolonged calcium levels increase may lead to the opening of the mitochondrial transition pore (mPTP), inducing apoptosis (Pizzo and Pozzan, 2007). For example, calcium release from the axonal ER, through RyR and IP_3Rs, opens mPTP and induces axonal degeneration after mechanical and toxic insults (Villegas et al., 2014).

As far as we know, there is no assessment of the direct role of MAMs in axon development, plasticity or repair. However, the overall neuritic complexity of developing rat cortical neurons is diminished in cells

expressing a defective isoform of REEP1, which is associated to ALS and may be due to impaired MAMs, but this assumption needs to be confirmed (Lim et al., 2015).

ER-Autophagosomes. Neurons are long-lived postmitotic cells that critically depend on quality control mechanisms, such as autophagy (Maday, 2016). Autophagy is a conserved phenomenon where cytoplasmic proteins, protein aggregates, and organelles are engulfed in the cytosol by double membrane structures and degraded upon fusion with lysosomes (Kaur and Debnath, 2015). Autophagy is critical for axonal physiology as inhibition of autophagosome biogenesis in CNS neurons induces axonal degeneration and neuron death (Hara et al., 2006). Importantly, axonal autophagosomes may be continuous with the SER, suggesting ER related biogenesis (Bunge, 1973).

The autophagic process may be traced with a tagged version of microtubule-associated protein 1A/1B-light chain 3 (LC3), a well-established marker of autophagosomes (Kabeya, 2000). Enrichment of autophagic structures in axonal growth cones has been confirmed in rat hippocampal neurons using LC3 (Maday and Holzbaur, 2014). In developing neurons most autophagosomes originate in the distal tip of the axon and move retrogradely towards the soma and dendrites with a concomitant acidification of their lumen, which is compatible with lysosome association (Maday et al., 2012; Maday and Holzbaur, 2014). Importantly, the axonal ER is the main source of membranes for distal autophagic structures in CNS and PNS neurons (Maday and Holzbaur, 2014). These observations confirm previous EM evidence (Bunge, 1973). Therefore, the axonal ER is key for the emergence of autophagosomes that are critical for distal disposal. Most likely, this unique solution is also relevant during the rapid demands of plasticity or repair.

ER-Synaptic Vesicles. The involvement of the axonal ER in the biogenesis of synaptic vesicle precursors is still controversial. Likewise, it is still unknown whether synaptic vesicle recycling is influenced by the axonal ER. In this section we describe classic and recent evidence that supports both processes.

Functional synaptic vesicles require recycling from the plasma membrane or endosomal structures, as revealed by *in vitro* studies in vertebrate and invertebrate models, and by the localization and composition of nerve terminal derived vesicles (Heuser and Reese, 1973; Maycox et al., 1992; Faúndez et al., 1998; Di Paolo et al., 2002; Logiudice et al., 2009;

Watanabe et al., 2014). The relevance of recycling in synaptic vesicle maintenance was also demonstrated in *Drosophila* with the identification of a thermosensitive mutant in the dynamin gene, *shibire*. Exposing synapse to 29°C resulted in complete depletion of synaptic vesicles after transmitter release, while lowering the temperature to 19°C restored endocytosis and synaptic vesicle reformation. It is interesting to note that fast synaptic vesicle recovery occurred even after nerve severing, showing that membrane retrieval and synaptic vesicle reformation in the axon terminal is a local mechanism, independent of the cell body (Koenig and Ikeda, 1989).

Synaptic vesicle precursors may be recruited to a specific domain in the ER or the Golgi apparatus, and then co-transported in membrane carriers to the axonal terminal (Shim et al., 2004; Rizzoli, 2014). Indeed, mutations in proteins involved in the anterograde axonal transport correlate with focal axonal swellings, accumulation of vesicle components in the cell body, and a decrease in the number of synaptic boutons and synaptic vesicles in presynaptic terminals (Hall and Hedgecock, 1991; Kurd and Saxton, 1996; Pack-Chung et al., 2007). Although the subcellular components that give rise to synaptic vesicle precursors are still under investigation, tubovesicular carriers emerging from the axonal SER may associate to the formation of synaptic vesicles precursors (Korneliussen, 1972; Droz et al., 1975; Reinecke and Walther, 1978; Tsukita and Ishikawa, 1980; Mercurio and Holtzman, 1982; Matthew et al., 1999). During development, structures indistinguishable from synaptic vesicles establish contact with the SER and Golgi apparatus in different axonal segments and decrease their presence in nonterminal portions of axons as neurons mature, further suggesting that precursors may be produced by these organelles in immature neurons (Stelzner, 1971). In addition, in nucleus accumbens neurons, the axonal ER forms a web surrounding multiple organelles, including synaptic vesicles (Wu et al., 2017). Thus, the axonal ER may provide membrane and other macromolecules to synaptic vesicle precursors (Broadwell and Cataldo, 1984). In agreement with these observations, overexpression of the single *Drosophila* extended synaptotagmin ortholog, which is enriched in the axonal ER, promotes synaptic growth, increases neurotransmission, and sustains a functional synaptic vesicular pool during intense periods of activity. These observations suggest that synaptotagmin may supply synaptic membranes to the axon terminal and facilitate vesicle biogenesis (Kikuma et al., 2017).

## EXPLORING THE AXONAL PROTEOME OFFERS NEW RESEARCH PERSPECTIVES

To our knowledge two proteomic studies of growth cone fractions unravel their functional complexity. For example, analysis of growth cones from fetal brains revealed 2000 proteins (Estrada-Bernal et al., 2012). Relevant structural/functional classifications included ER to Golgi vesicles (including Arf1, Arl3, Copa, Sar1a, Sec31a, VCP), vesicle fusion (including snap25, Stx1b, VAP-A), protein translation (including Eif3a, Eef2, Rps3), folding (including calnexin, Hspa4, Hsp90ab1, Ppia, Pdia3), and components of the proteasomal complex and ubiquitin ligase activity (including Rad23b, Ubqln2, Ecm29, Cul3, Anapc1, Ube2o, Ubr4). Several candidates were further validated by immunofluorescence, demonstrating their localization in growth cones of hippocampal neurons *in vitro*. This was the case for proteins involved in translation (40s ribosomal protein s3, Rps 3), ER proteins such as protein disulfide isomerase A6 (Pdia6), dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 1 (Rpn1), and Golgi components such as alpha-mannosidase 2 (man2a1) (Estrada-Bernal et al., 2012). These results suggest that growth cones are equipped with a complex biosynthetic machinery.

A similar approach was performed to identify new growth cone markers by means of a quantitative proteomic analysis. Nine hundred forty-five proteins were found in growth cone fractions from P1-P2 rat forebrains (Nozumi et al., 2009). One hundred thirty candidates were selected because of their abundance and potential relevance, and validated by immunofluorescence in hippocampal neurons after 3 days *in vitro*. Interestingly, nearly 100 proteins were significantly enriched in axonal growth cones compared to the established marker neuromodulin (GAP-43). In addition, eight proteins corresponded to the ER compartment, namely calnexin, atlastin-1, VCP, Atp1a1, BiP, Mtdh, Rtn1 and Rtn4 and six of them were highly enriched in axonal growth cones, i.e. calnexin, VCP, Atp1a1, BiP, Rtn1, Rtn4. Seven proteins correspond to transmembrane proteins that face the ER lumen as well as the cytosol, which makes them suitable to act as potential tethers of the ER with functional complexes and organelles inside the axon.

To further explore these analyses we aimed at constructing an axonal ER interactome to account for relevant molecular interactions of the ER in growth cones. We selected ER components from the validated list of 130 growth cone proteins, namely calnexin, atlastin-1, VCP, Atp1a1, Mtdh, Rtn1, and Rtn4 (Nozumi et al., 2009). Taking advantage of the STRING platform, we performed an *in silico* analysis of the potential interactions of these ER proteins. Next, the protein interactors were classified by cellular compartment with gene ontology (GO) classification. From the analysis we found five relevant interaction categories: ER to golgi transport vesicle, mitochondria, proteasome complex, proteasome/ubiquitin degradation pathway (including ERAD) and endosomes, including retromer elements. These categories are expected be relevant in axons based on evidence from EM microscopy (Wu et al., 2017), immunofluorescence (Merianda et al., 2009; Estrada-Bernal et al., 2012; Merianda and Twiss, 2013) and the proteomic analysis of growth cones (Nozumi et al., 2009; Estrada-Bernal et al., 2012). However, since not all potential ER-interacting proteins identified by STRING localize to the axonal compartment, we marked those previously validated by the fetal brain proteomic analysis of growth cones (Fig. 2, blue) (Estrada-Bernal et al., 2012). Our analysis shows that in growth cones of developing axons the ER potentially interacts with a vast array of proteins that belong to organelles and pathways relevant for axonal physiology and plasticity. Most of these were discussed in this review, including ER-to-Golgi vesicle, proteasome, ERAD, retromer, calcium dynamics, and mitochondria.

We also analyzed how the representation of axonal ER proteins changed during development. We found that during P1, P3, and P10 the representation of ER proteins in axons remained relatively stable at approximately 9%. However, they decreased notoriously to approximately 3% at P15 (Fig. 3, insert). This observation is in agreement with the relative progressive decline of the ER in axons as neuronal polarization and development proceeds (Krijnse-Locker et al., 1995; Aridor and Fish, 2009). Next, we further classified the ER proteome at each developmental stage based on GO. Interestingly, categories including protein folding and organelle biogenesis were significantly reduced once callosal projections fully reached the contralateral cortex at P15, suggesting that they play preferential roles during axonal growth. ER stress and calcium homeostasis related proteins were more stable over time, possibly related to the absence of noxious stimuli or with their ubiquitous function in cell survival and function. Others such as redox, vesicle transport and ERAD increased at P15 suggesting their preferential role in the maintenance of mature axons. Surprisingly, sterol transport, autophagy, and apoptosis showed marked peaks at intermediate stages.
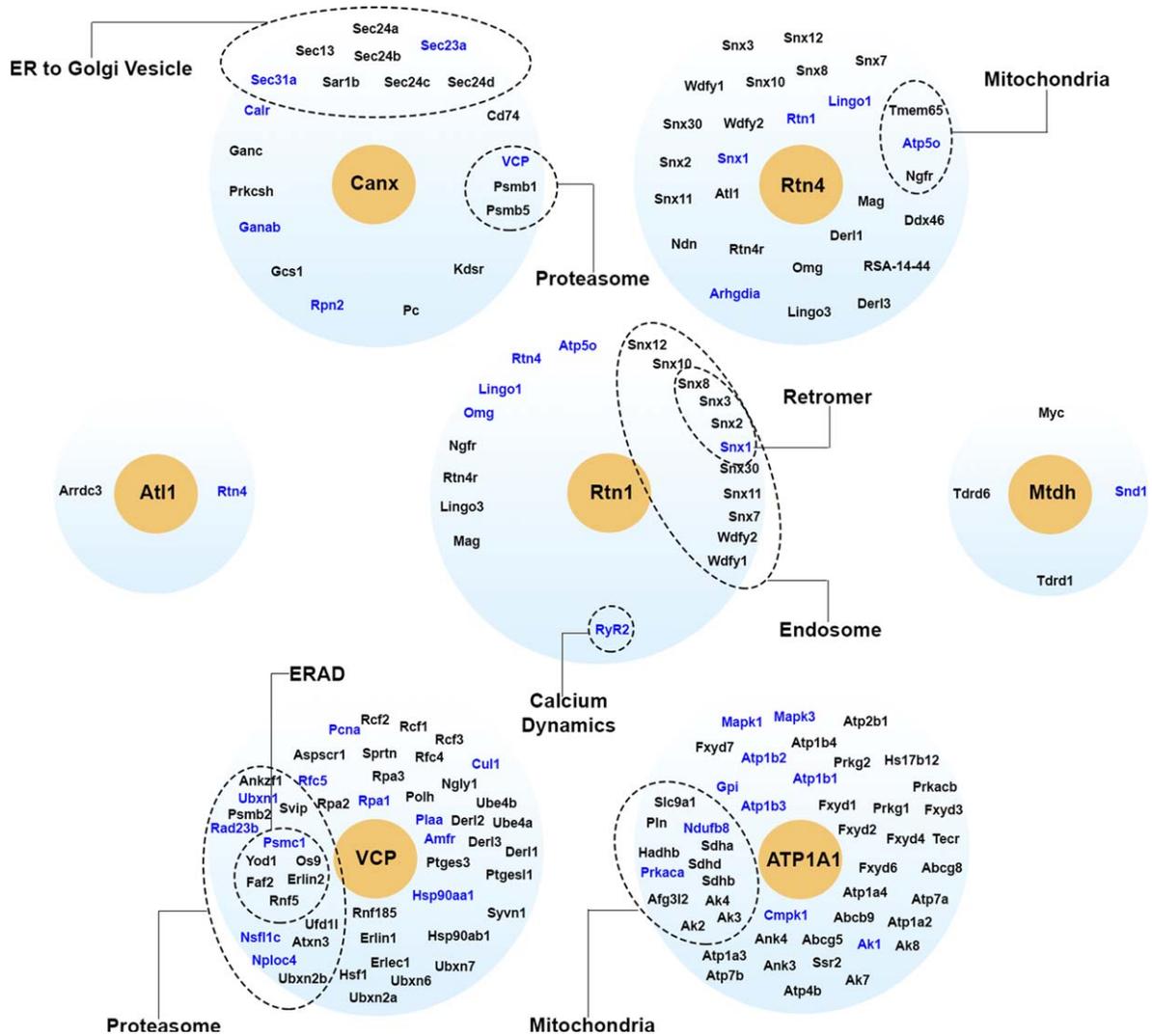
Figure 2 The potential interactome of the axonal ER during development. *In silico* analysis of potential interactions of ER proteins in growth cones of cultured cortical neurons. Proteins in orange circles are enriched in growth cones: reticulon-1 (Rtn1), reticulon-4 (Rtn4), Calnexin (Canx), ATP1A1, VCP. Atlastin-1 (Atl1), and Mtdh localize to growth cones but are not enriched in them (Nozumi et al., 2009). Proteins in blue indicate interactors validated in a proteomic study of axonal growth cones (Estrada-Bernal et al., 2012). Other interacting proteins are shown in black. Interacting proteins were classified by cellular compartment with gene ontology (GO) classification using AmiGO2 platform (Gene Ontology Consortium, 2015). Interaction categories are shown inside dotted ellipses. [Color figure can be viewed at wileyonlinelibrary.com]

Few other studies have addressed the changes in the axonal proteome during development and plasticity (Michaelevski et al., 2010; Yamatani et al., 2010). However, they do reveal specific proteomic signatures during different developmental stages and after injury. For example, the anterograde as well as retrograde transport categories change in response to injury in rat sciatic nerves, increasing the presence of 40S and 60S ribosomal components in the anterograde flux and potentially supporting local protein synthesis (Michaelevski et al., 2010). Additionally, maturation of lateral olfactory tract axons in mice is accompanied by the upregulation of calcium-dependent membrane-binding proteins, such as VILIP1, copine 6, neurocalcin delta, and annexin 6 (Yamatani et al., 2010). More recently, tagged ribosomes (ribo-tag) were selectively immunoprecipitated in axons from developing retinotectal projections in the mouse visual circuit, showing dynamic changes in the translational profile throughout development (Shigeoka et al., 2016).
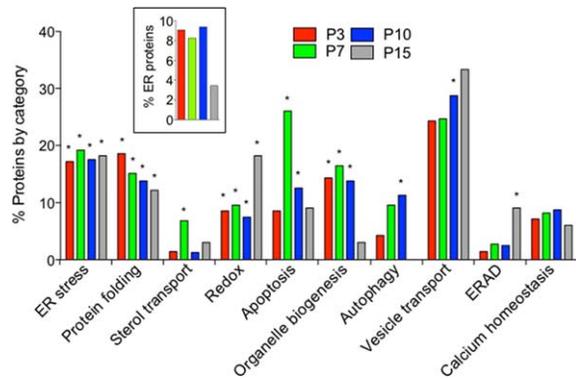
Figure 3 Dynamics of the ER proteome in developing axons. A mass spectrometry-based proteomic profiling was revisited to further characterize the dynamic functions of the axonal ER (Son et al., 2017). ER components were selected by criteria of gene ontology (GO). Insert: percentage of ER proteins compared to the universe of total proteins by day of development. Main panel: functional classification of ER proteins based in GO analysis: ER stress; protein folding; sterol transport; redox; apoptosis; organelle biogenesis; autophagy; vesicle transport; ERAD and calcium homeostasis. Significance of protein representation was compared to mice genome by Binomial statistic ($*p < 0.05$) (Cho, 2000). [Color figure can be viewed at wileyonlinelibrary.com]

The dynamic proteome of the axonal ER accounts for its multiple roles in axonal function. Furthermore, these functions rely on how proteins are organized into interacting networks. Thus, the dynamic proteome and the validated axonal interactome may provide interesting research avenues in axon physiology that may extend to pathology and regeneration. For instance, together with new super-resolution methodologies, these may help understand the role of ER-shaping proteins in defining the architecture of the ER and contacts with a variety of intracellular organelles. Additionally, combined with state-of-the-art methodologies to examine translation, processing, and trafficking they may contribute to complete our emerging understanding of the local synthesis of lipids and the local delivery of transmembrane or secreted proteins. Finally, combined with new fluorescence-based calcium reporters they may contribute to uncover fundamental mechanisms in axonal physiology and disease.

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