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Pharmacological regulation of endoplasmic reticulum structure and calcium dynamics: importance for neurodegenerative diseases

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Endoplasmic Reticulum Pharmacology in Neurodegeneration

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ATF	activating transcription factor
BHQ	2,5-di-t-butyl-1,4-benzohydroquinone
CICR	Ca^{2+} -induced Ca^{2+} release
DAG	diacylglycerol
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERGIC	ER-Golgi intermediate compartment
GPCR	G-protein coupled receptor
hK2	protease human glandular kallikrein 2
MAM	mitochondrial-associated membrane

MANF	mesencephalic astrocyte-derived neurotrophic factor
MERC	mitochondria-ER contact
MERS	mild ER stress
NAADP	nicotinic acid adenine dinucleotide phosphate
IP3R	inositoltriphosphate receptor
IRE1	inositol-requiring kinase 1
ISR	integrative stress response
ORAI1	Ca^{2+} release-activated calcium channel protein 1
PDI	protein disulfide isomerase
PERK	double-stranded RNA-activated protein kinase-like ER kinase
PLC	phospholipase C
RyR	Ryanodine receptor
SERCA	type P sarco/endoplasmic reticulum Ca^{2+} ATPase
SIGMAR1	sigma non-opioid intracellular receptor 1
SOCE	store-operated Ca^{2+} entry
SPCA	secretory pathway Ca^{2+} ATPase
STIM	stromal interaction molecule
TRP	transient receptor potential
UPR	unfolded protein response
VAPB	vesicle-associated membrane protein-associated protein B

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Abstract

The endoplasmic reticulum (ER) is the largest organelle of the cell, composed of a continuous network of sheets and tubules, and is involved in protein, calcium (Ca^{2+}) and lipid homeostasis. In neurons, the ER extends throughout the cell, both somal and axodendritic compartments, and is highly important for neuronal functions. A third of the proteome of a cell, secreted and membrane-bound proteins, are processed within the ER lumen and most of these proteins are vital for neuronal activity. The brain itself is high in lipid content and many structural lipids are produced, in part, by the ER. Cholesterol and steroid synthesis are strictly regulated in the ER of the blood-brain barrier protected brain cells. The high Ca^{2+} level in the ER lumen and low cytosolic concentration is needed for Ca^{2+}-based intracellular signaling, also for synaptic signaling and Ca^{2+} waves, as well as preparing proteins for correct folding in the presence of high Ca^{2+} concentrations to cope with the high concentrations of extracellular milieu. Particularly, ER Ca^{2+} is controlled in axodendritic areas for proper neurito- and synaptogenesis and synaptic plasticity and remodeling. In this review, we cover the physiological functions of the neuronal ER and discuss it in context of common neurodegenerative diseases focusing on pharmacological regulation of ER Ca^{2+}. Furthermore, we postulate that heterogeneity of the ER, its protein folding capacity and ensuring Ca^{2+} regulation is a crucial factor for the aging and selective vulnerability of neurons in various neurodegenerative diseases.

Significance statement

ER Ca^{2+} regulators are promising therapeutic targets for degenerative diseases for which efficacious drug therapies do not exist. The use of pharmacological probes targeting maintenance and restoration of ER Ca^{2+} can provide restoration of protein homeostasis, e.g. folding of complex plasma membrane signalling receptors and slow down the degeneration process of neurons.

Visual abstract.

A. In a healthy neuron, ER comprises of continuous network of ribosome-covered sheets and smooth tubules in the soma, and interconnected tubules in dendrites and axon all the way to the axonal presynaptic terminals. B. Based on the studies mostly done on non-neuronal cells and immortalized cell lines, putative/predicted/possible morphological changes upon ER stress may include: a.) formation of ER swirls or whorls, b.) fragmentation and discontinuation of tubular ER in the axon, c) disorganization of the ER in presynaptic terminals leading to reduced contacts between ER and plasma membrane, d) normal ER sheets (in middle) become dilated (left-side) or super-thin (on right), and e) formation on branched tubular ER clusters in soma or in the axon.

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ER morphology and overview of Ca^{2+}-related ER functions

Recently, we have reviewed the morphological features of the ER in neurons (Sree et al., 2021). In brief, the ER comprises a continuous network of sheets and tubules. Sheets are covered with ribosomes and contain fenestrations, and dynamic three-way junctions are connecting the sheets and tubules to each other. ER expands throughout the cell, including polarized cells such as neurons. Based on the cell type and its physiological state, the ER structure differs in its sheet-tubule ratio and in ribosome density. For example, the active secretory pancreatic beta-cells present more ribosome-bound sheets. In contrast, enormous sterol producing cells or xenobiotics detoxifying hepatocytes exhibit more tubules. For neurons, the ER is particularly important with regards to calcium (Ca^{2+}) signaling and, thus, they have tubules extending in their neurites for efficient synaptic signaling from the axodendritic compartments. The ER in axons exhibits distinct and highly specific morphological features including narrow tubules and formation of an ER ladder composed of rails and rungs. The ER ladder affects trafficking of associated vesicular axonal cargoes and facilitates ER-plasma membrane contacts upon ER-luminal Ca^{2+} depletion. The ER specifically in axons have unique features not seen in other cells or other parts of neurons. For example, the tubules have been reported to be much narrower and in developing axons the ER may form a ladder - composed of rails and rungs – structure which affects trafficking of associated vesicular axonal cargoes and helps regulate ER contacts to the plasma membrane upon luminal Ca^{2+} depletion (Terasaki, 2018; Zamponi et al., 2022). In this review, we focus on the pathophysiology of ER-Ca^{2+} signaling pathways in context of neurodegenerative diseases. In addition, we summarize state of art methods and probes for studying Ca^{2+} signaling.

Cell organelles are unique and perform their specific functions in coordination with each other to maintain cell growth and survival. The ER is responsible for many functions, which are evolutionarily conserved in eukaryotes, and certain functions happen in all eukaryote cells, regardless of the originating species or cell type. It is important to realize that no organelle works in isolation and is always connected or regulated by another organelles (Spang, 2018). The ER is the largest organelle in the cell and essentially has some crosstalk with all other organelles. However, the extent of this crosstalk and the subsequent underlying mechanisms, especially in highly compartmentalized cells like neurons, remain elusive. The advent of new methods and technologies has enabled us to investigate these complex mechanisms more efficiently. The environment in the ER lumen differs from the cytosol in several ways. It is very oxidative to enable efficient formation of disulfide bonds and contains high concentration of Ca^{2+} that functions as a buffer for the folding chaperones and enzymes in addition to carrying out cellular signaling (Stutzmann and Mattson, 2011). As such, it resembles the conditions of the extracellular milieu effectively making sure that proteins transferred to the extracellular matrix can endure its harsh conditions and high Ca^{2+} concentration. The high gradient of Ca^{2+} and distribution of it between other organelles and the cytoplasm is especially important for the ER to be able to carry out its functions (Burdakov et al., 2005).

Ca^{2+} is one of the most ubiquitous ion in all cell types. A myriad of proteins throughout the cell, and especially in the ER lumen, require Ca^{2+}-binding for their efficient folding, transport, and function. Ca^{2+} is involved in cellular processes as is demonstrated by the wealth of proteins encoded by the human genome that contain Ca^{2+} binding moieties or by the amount of Ca^{2+} binding proteins alone in the ER (Coe and Michalak, 2009). Protein folding is one of the most error prone processes in gene expression due to the environmental requirements and complexity. In fact, a significant number of proteins designated for the ER do not get folded into their native conformations and are degraded, arrested in the ER lumen, or stored in lysosomes (Schubert et al., 2000). Large proteins take more time to be synthesized and folded. For example, monomeric globular proteins can fold in a millisecond to second timescale, while larger multihelical fibrous proteins such as collagen can take from minutes to hours to fold (Baum and Brodsky, 1997). Mutations in the protein may increase the folding time (Hartmann and Zacharias, 2021).

Humans have about 20000 protein coding genes from which a cell produces approximately 100000 different protein isoforms, based on estimations of mRNA splice variants and post-translational modifications (Brett et al., 2002). Of these, about a third are transmembrane proteins or proteins secreted to the extracellular milieu (Medus et al., 2017; van Anken and Braakman, 2005), which are synthesized and processed in the ER. Proteins are synthesized and undergo cotranslational translocation through the translocon channel complex at the ER membrane. The folded proteins are further modified before entering the secretory pathway (Lang et al., 2017). The ribosome-translocon complex facilitates a passive leakage of free Ca^{2+} from the ER lumen (Van Coppenolle et al., 2004). The prolonged ER-Ca2+ leakage can lead to a chronic disease. To counter this problem, cells compensate the free ER-Ca^{2+} leakage by Ca^{2+} pumps and channels to keep the ER replenished.

The ER is involved in the synthesis of structural phospho- and sphingolipids, storage lipids, such as triacylglycerols and sterols, most importantly cholesterol, and is the main regulator of cellular lipid homeostasis (Higgins, 1974; Ikonen, 2008). It provides other organelles with lipids and lipid metabolism is done in tandem with mitochondria and Golgi depending on the lipid species. Thus, mitochondria-ER contact sites (MERCs) are highly enriched with cholesterol (Hayashi and Fujimoto, 2010). As the ER is responsible for generating membrane lipids for the cell, its own membrane lipid composition, in terms of synthesis, degradation and export needs to be tightly regulated (McMahon and Gallop, 2005). MERCs are also involved in Ca^{2+} transfer between the ER and mitochondria (Wang et al., 2021; Ziegler et al., 2021).

The ER is in contact with all other main organelles of the cell, i.e. the nucleus, Golgi apparatus, mitochondria, lysosomes, and the cytoskeleton (Spang, 2018). The nuclear and Golgi contact areas are large, due to their cooperative function in the regulation of protein synthesis. With other organelles the contact sites are smaller and much more dynamic, i.e. the amount of contact sites change constantly (Guo et al., 2018; Luarte et al., 2018). The ER regulates Ca^{2+} homeostasis in conjunction with the Golgi and mitochondria. The cytoskeleton is involved in ER motility and lysosomes are trafficked with the coordination of these two organelles. In addition, the ER also seems to regulate membraneless organelles, biological condensates, specifically Processing bodies (P-bodies) (Lee et al., 2020; Lee and Bahmanyar, 2020).

The dynamic relationship especially between the ER and mitochondria has received a lot of attention. This is mainly due to the findings that MERCs, or mitochondrial-associated membranes (MAMs) are involved especially in aging-related neurodegenerative diseases, such as Amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) (Bernard-Marissal et al., 2018; Gomez-Suaga et al., 2018; Moltedo et al., 2019). Mutations in proteins tethering mitochondria and ER can cause

neurodegeneration, such proteins e.g., vesicle-associated membrane protein-associated protein B (VAPB) and sigma non-opioid intracellular receptor 1 (SIGMAR1), which are implicated in heritable ALS (Nishimura et al., 2004). Mitochondrial proteins regulate the ER and *vice versa*, such as the opposite strand protein 1 of phosphatidylinositol glycan anchor biosynthesis class B which is a small microprotein located in the mitochondrial outer membrane and regulates the ER-related unfolded protein response (ER-UPR) from MERCs (Chu et al., 2019). MERCs seem to be involved to some extent in Alzheimer's disease (AD) pathogenesis as well, as changes in apposition of ER and mitochondria have been reported in various AD models (Dentoni et al., 2022).

The ER regulates apoptosis independently or in concert with mitochondria. Ca^{2+} is transferred between these organelles through MERCs and mediated by many factors such as inositol-requiring kinase 1 (IRE1) or the previously mentioned SIGMAR1 (Carreras-Sureda et al., 2019; Nishimura et al., 2004). Ca^{2+} transfer affects apoptotic factors such as Bcl-2, and *vice versa*. Thus, as a balance in ionic homeostasis is of utmost importance, finding regulators of this homeostasis – and the factors contributing to enhance it – will be critical when searching for new therapeutics targeting ER Ca^{2+}.

Specified functions of ER calcium in neurons and regulatory targets

A reservoir for Ca^{2+} ions

The ER is the largest intracellular Ca^{2+} store. Ca^{2+} concentrations within the ER can be 5000 times greater than in the cytoplasm. ER Ca^{2+} fluxes are regulated by Ca^{2+}-induced Ca^{2+} release (CICR) and store operated Ca^{2+} entry (SOCE), which regulates the Ca^{2+} efflux and influx channels (Majewski and Kuznicki, 2015; Verkhratsky and Shmigol, 1996). The most important influx channel for upkeeping the concentration gradient is the type P sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, which transports two Ca^{2+} ions in exchange of one ATP molecule (Guerrero-Hernandez 2010). There are three genes coding for SERCA proteins, SERCA 1, 2 and 3, and 10 isoforms of these proteins (Periasamy and Kalyanasundaram, 2007). SERCA1 is expressed primarily in skeletal muscle, SERCA1a in adult tissue and SERCA1b in neonates. While SERCA2a is highly expressed in cardiac myocytes, SERCA2b is expressed ubiquitously. SERCA3 is also expressed in multiple tissues and in neurons, SERCA2a and SERCA3 are found co-expressed in cerebellar Purkinje neurons (Periasamy and Kalyanasundaram, 2007). The maintenance of ER Ca^{2+} levels require a high amount of energy as muscle cells may use up to 25-50 % of ATP on the SERCA pumps solely (Smith et al., 2013). SERCA is coupled to the Ca^{2+} sensors, stromal interaction molecule (STIM) and calcium release-activated calcium channel protein 1 (ORAI1), which are a part of SOCE in upkeeping the Ca^{2+} homeostasis (Jousset et al., 2007; Tian et al., 2016).

Aged neurons have a decline in SERCA function (Pottorf et al., 2000) and adding to the hypothesis that age-dependent neurodegeneration is caused by Ca^{2+} disbalance, aged neurons also display impaired function of the plasma membrane Ca^{2+} ATPase (Michaelis et al., 1996). Furthermore, regucalcin, a calcium-binding protein, is expressed in neurons and increases expression of SERCA, while a deficiency in regucalcin, which decreases SERCA levels, has been linked to age-dependent disbalance in Ca^{2+} homeostasis (Lai et al., 2011).

The secretory pathway Ca^{2+} ATPases (SPCAs) together with SERCAs modulate the uptake of Ca^{2+} to Golgi complex and the secretory compartment. In addition to calcium, SPCAs transports Magnesium (Mn^{2+}) to the Golgi lumen to regulate both the physiology and pathophysiology of the cells (Brini and Carafoli, 2009; Shull, 2000; Vangheluwe et al., 2009; Vanoevelen et al., 2007). As with the ER, the specific roles of SPCAs in neurons have not been studied extensively, but a study suggests that they

may have a neuroprotective role, at least in a model of heat stroke-induced neurodegeneration (Kourtis et al., 2012).

Efflux is regulated by Ca^{2+} channels, mainly ryanodine receptors (RyR) and inositoltriphosphate receptors (IP3R), but also by nicotinic acid adenine dinucleotide phosphate (NAADP) receptors, and polycystin-2 channels to some extent (Koulen et al., 2002; Patel et al., 2010; Santulli et al., 2017). The IP3R and RyR form large tetrameric channels, which are some of the largest protein complexes in a cell, with their atomic mass upwards to the megadalton range comparable to muscle proteins such as nebulin and titin which are known as some of the largest proteins produced by animal cells (Meyer and Wright, 2013; Woll and Van Petegem, 2022).

There are three different isoforms of RyR, and although all of them have been found to be expressed in neurons, they are differentially expressed within brain regions and during development (Zalk et al., 2007). For example, RyR3 has been found in the hippocampus, striatum, and diencephalon (Abu-Omar et al., 2018). With regards to their sub-cellular localization, RyRs have been detected in all parts of a neuron. There are three IP3R isoforms with different sensitivities to Ca^{2+}. IP3R1 is the main isoform found in neurons of the brain, and IP3R3 is primarily expressed in the spinal cord and glial cells (Berridge, 1998).

Due to the unique morphology and signaling of neurons, i.e., long extensions and synaptic signaling, ions need to be mobilized efficiently throughout the cell. Therefore, Ca^{2+} tunnelling may be critical for neuronal function. As the ER is a continuous connected network, Ca^{2+} tunnelling is used to distribute Ca^{2+} to different sites in the cell throughout the ER lumen to confer local needs (Choi et al., 2006; Petersen et al., 2017). For example, depletion of local dendritic Ca^{2+} stores with caffeine in midbrain dopaminergic neurons does not cause severe store depletion. Moreover, photobleaching experiments show that a dye targeted to the ER lumen may diffuse quickly up to 90 μm from the soma to the dendrites (Choi et al., 2006). This suggests that the large somatic ER compartments act as a reservoir for Ca^{2+}, which can be efficiently moved according to local needs throughout the ER network. However, complicating matters is a recent finding that challenges the concept of the ER being a continuous network and operating as a unified Ca^{2+} store, as hippocampal pyramidal neuron ER seems to have two distinct Ca^{2+} regulating compartments, one containing IP3Rs and the other RyRs (Chen-Engerer et al., 2019). Furthermore, there are other indications on the existence of various compartments as different neurons have different responses to ER Ca^{2+} regulating compounds, e.g. dorsal root ganglion neurons which may have thapsigargin-insensitive ER Ca^{2+} stores (Liu et al., 2003). Nevertheless, the extent of this phenomena within different neuronal subtypes and its relation to other cellular processes has not been extensively studied and warrants further investigation.

Ca^{2+} signalling

Ca^{2+}, being a versatile signaling molecule, regulates a multitude of cellular processes including gene transcription, respiration, metabolism, growth, and cell division. Ca^{2+} channels, pumps and transporter proteins facilitate a strict regulation of intracellular Ca^{2+} $[Ca^{2+}]i$, to maintain the homeostasis. However, a dysregulation of $[Ca^{2+}]i$ is associated with several pathological states including neurodegenerative diseases. Ca^{2+} concentrations are highly regulated in cells. Ca^{2+} concentration is 20000-fold lower in the cytoplasm than the extracellular compartment. In comparison, Na^{+} and K^{+} have only a 10 to 30-fold difference (Surmeier et al., 2017). Hence, changes in Ca^{2+} concentration can be used very broadly and efficiently for signaling.

In neuronal tissue, Ca^{2+} signaling is highly important as it regulates signaling at the synaptic level, i.e. neurotransmission, which does not occur in other tissues (Berridge 1998). In neurons, the $[Ca^{2+}]i$ levels fluctuate between the low hundreds of nanomolars up to tens of micromolars range in different

compartments of the cells, or microdomains. The ER is largest intracellular Ca^{2+} store with a concentration range of 100-800 μM (Samtleben et al., 2013). This helps in regulating not only inter neuronal signaling by the release of synaptic vesicles, but in intraneuronal signaling on growth, development, and synaptic plasticity (Augustine et al., 2003; Parekh, 2008). Other ER- Ca^{2+} - dependent processes are ubiquitous across cell types, however, neurons may have differences in these processes within their microdomains.

Neurons rely on action potentials for signaling through axons, and synaptic vesicle-mediated transmission of signals between neurons. Three main classes of plasma membrane proteins are involved in synaptic activity, which trigger transient elevations in cytosolic Ca^{2+}; voltage-dependent Ca^{2+} permeable ion channels (L-type, N-type, P/Q-type, R-type, and T-type), ionotropic receptors gated by neurotransmitters (e.g., nicotinic acetylcholine receptors), and G_q-linked G-protein coupled receptors (GPCRs), which are activated by neurotransmitters (e.g., metabotropic glutamate receptors) (Figure 1). GPCRs do not flux Ca^{2+} themselves but via ligands for receptors that release Ca^{2+} from intracellular stores (Berridge, 1998; Brini et al., 2014; Zampese and Surmeier, 2020). These are differentially regulated in neuronal subtypes for specific type of activity, such as the autonomous pacemaking of substantia nigra dopamine neurons, which express R-type channels contributing to somatic Ca^{2+} oscillations during spiking. Synaptic vesicle release is triggered by a Ca^{2+} signal generated by the action potential, and this is where ER Ca^{2+} is of high importance. Store-operated ORAI1 channels and transient receptor potential (TRP) channels participate in neuronal Ca^{2+} signaling. Action potentials can cause propagating Ca^{2+} waves by CICR, which are a major cause of Ca^{2+} fluctuations from and into the ER (Karagas and Venkatachalam, 2019; Llano et al., 1994; Shmigol et al., 1995). In the SN dopamine neurons, due to their pacemaking, and low intrinsic Ca^{2+} buffering, the high Ca^{2+} transients trigger CICR through RyRs, which are coupled to L-type channels specifically expressed by these neurons. Ca^{2+} signaling in these neurons triggered by the pacemaking regulates dopamine synthesis and mitochondrial oxidative phosphorylation, which are beneficial for their high anabolic and metabolic demands (Zampese and Surmeier, 2020). CICR is tightly regulated by IP3R and RyrRs by their biphasic nature as they have both inhibitory and activation sites for Ca^{2+}. To keep Ca^{2+} homeostasis intact, when cytosolic Ca^{2+} levels go below a threshold, the high affinity sites are dominant and Ca^{2+} is released from the channels (Stutzmann and Mattson, 2011). Conversely, when cytosolic Ca^{2+} levels are high, the inhibitory sites are dominant, and the channels are blocked. CICR is also regulated by TRP channels and VGCCs, and alterations in mitochondrial Ca^{2+} uptake. Ca^{2+} waves have differing effects depending on the neuron and the spatiotemporality, i.e. where they happen and how frequently. For example, synaptically induced Ca^{2+} waves are linked to synaptic plasticity and LTP and are initiated at dendritic branching points through IP3Rs (Ross, 2012). Furthermore, Ca^{2+} buffering (proteins capable of binding Ca^{2+}) proteins control the amplitude, kinetics, and distribution of these Ca^{2+} transients mediated by the aforementioned proteins. Some Ca^{2+} binding proteins act as sensors and effectors for precise regulation of the transients' spatiotemporality (Schwaller, 2010). In dopamine neurons, differential regulation of Ca^{2+} buffering proteins may contribute to their susceptibility towards neurodegeneration, as the ventral tegmental area dopamine neurons, which have a higher intrinsic Ca^{2+} buffering capability than the neighboring SN dopamine neurons, do not degenerate as extensively in PD. As Ca^{2+} signaling is involved in almost all neuronal cell functions, Ca^{2+} levels must be stringently regulated to avoid uncontrolled apoptosis or other pathologies leading to cell death run amok.

Ca^{2+} signalling Mechanisms

An external stimulus, such as neurotransmitter, hormone, sphingolipid, or growth factor, binds to GPCRs or protein tyrosine kinase-linked receptors that couple to phospholipase C (PLC) isoforms PLCβ or PLCγ, respectively. Active PLC catalyses the hydrolysis of phosphatidylinositol 4,5-

bisphosphate (PIP2) to form two distinct second messengers i-e, diacyglycerol diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG potentially activates Ca^{2+} channels, such as transient receptor potential canonical (TRPC) channels, present on the cell membrane and induces a direct influx of Ca^{2+} into the cells known as receptor-operated Ca^{2+} entry. IP3 diffuses in the cytoplasm and binds to the IP3-receptors (IP3R) present on the ER membrane to evokes a robust release of Ca^{2+} from ER Ca^{2+} stores into the cytoplasm, known as emptying of ER Ca^{2+}. The low ER Ca^{2+} level is sensed by STIM1 protein localized in the ER membrane, which in response gets activated, forms oligomers and makes membrane contact sites with plasma membrane by recruiting ORAI1 Ca^{2+} channels to induce an influx of Ca^{2+} into the cells (Collins et al., 2022; Putney, 2007)**.** Furthermore, in excitable neuronal cells, the voltage-operated Ca^{2+} channels are activated by the depolarization of the plasma membrane and facilitate Ca^{2+} entry into these cells. Taken together, the elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]$i binds to several Ca^{2+} binding proteins and regulates a multitude of cellular functions. The resting level of free Ca^{2+} in the cytoplasm is low (~100 nM) and maintained by a precise regulation of Ca^{2+} channels, pumps, and Ca^{2+}-binding proteins. In neuronal cells, the disruption of Ca^{2+} homeostasis leads to severe neurodegenerative disorders such as Alzheimer's, Huntingtin's, and Parkinson's diseases (Callens et al., 2022; Collins et al., 2022). Lithium has been proposed as a potential drug for neuropathies as it hampers the free inositol relocation to the membrane and thus inhibiting the agonist evoked emptying of the ER via IP3/ Ca^{2+} signaling pathway (Berridge, 2016; Berridge et al., 1989).

One of the emerging therapeutic targets for neurodegenerative diseases are the TRP cation channels which are predominantly expressed in human brain cells and participate in both neuronal health and disease. TRP canonical (TRPC, 1-7 except -2), TRP vanilloid (TRPV, 1 and 4), TRP melastatin (TRPM, 2,3,4 and 7), and TRP ankyrin (TRPA,1) have been shown to participate in neurodegenerative diseases. In neurons, the cation TRPC channels evoke membrane depolarization and induce an influx of Ca^{2+} into the cells to give rise an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]$i) that modulates normal functions of these cells. Conversely, a downregulation or inhibition of these channels has been associated with neuropathies (Grueter et al., 2010; Koivisto et al., 2022; Mezey et al., 2000; Moran et al., 2011; Shibasaki et al., 2010; Shibasaki et al., 2015; Sukumaran et al., 2017).

Neuron specific ER requirements

As the ER has the largest surface area of the organelles in the cell, and can be up to ten times larger than the plasma membrane, and considering that neurons have a surface area easily over 10 times compare to smaller non-polarized cells, upkeeping the ER functional in a neuron is not an easy task (Horton and Ehlers, 2003a; b).

Of all the organs in humans, the brain has the highest amount of cholesterol (Zhang and Liu, 2015). Since lipoproteins do not cross the blood-brain barrier, most of cholesterol is synthesized by the brain cells *de novo*. Most of the cholesterol is in oligodendrocytes which produce the myelin sheath that surrounds and insulates axons. In neurons, cholesterol is enriched in synapses and growth cones, particularly at MERCs and it is one of the rate-limiting factors affecting neurite outgrowth (Funfschilling et al., 2012).

As the brain requires large amount of lipids and especially cholesterol, it suggests that disbalances of ER lipidostasis may have a significant role in neurodegeneration (Jung et al., 2017). Hence, the cholesterol hypothesis has been studied extensively in aging-related neurodegeneration (Petrov et al., 2016). Alzheimer's disease (AD) and altered lipid metabolism, in particular, have received attention, due to associations between AD risk genes and cholesterol levels (Banerjee and Mukherjee, 2018).

As mentioned, the ER contains different Ca^{2+} channels, buffers, and sensors to regulate Ca^{2+} homeostasis. Neuronal function relies especially in ER Ca^{2+} sequestration, as the stores are regulated by neuronal stimuli for transducing signals (Brini et al., 2014). It is important to note that although the ER contains a plethora of Ca^{2+} sequestering proteins, their affinity for Ca^{2+} may be much lower than contemporary cytosolic partners. In neurons, some of the most abundant ER Ca^{2+} buffering proteins are calreticulin, calnexin and calsequestrin. Other ER resident Ca^{2+} buffering proteins include BiP/grp78, endoplasmin and proteins of the CREC family.

For neurons, the trafficking of proteins through the cytoskeleton is especially crucial to work efficiently due to the long distances between the soma and the tips of the neurites. As such, the relation of protein trafficking and ER dynamics in neurites have received much warranted attention. Studying ER dynamics in the axons of dorsal root ganglion neurons has shown that SERCA pumps and IP3 receptors move bidirectionally at 0.1 μm/s in a predominantly non-vesicular and microtubule-dependent manner (Petersen and Verkhratsky, 2007; Ramirez and Couve, 2011). In dendrites, as well, ER motility is dependent on microtubules and kinesins and is involved in the transport of ER-resident proteins.

The importance of axonal ER in regulating action potentials was discussed previously, but more recent studies have started to uncover the extent of its regulation in Ca^{2+} mediated release of synaptic vesicles. (de Juan-Sanz et al., 2017) found that axonal ER acts as an actuator of plasma membrane function to control neurotransmitter release mediated by STIM1-sensing. As such, the ER is paramount for proper synaptic function.

ER is also involved in regulating growth cones in tandem with the cytoskeleton (Gasperini et al., 2017; Luarte et al., 2018). During axon guidance the cytoskeleton controls growth cone motility through Ca^{2+} signaling, specifically Ca^{2+}-induced phosphorylation, which is also linked to ER Ca^{2+} regulation. The interactions of the cytoskeleton and ER have been shown to be mediated by, at least, myosin-VA and RyRs (Wada et al., 2016). Activation of CICR causes myosin-VA to dissociate from RyRs with preferential spatial localization. This regulates release of vesicles from the part of the growth cone where CICR is induced promoting growth cones to grow towards respective cues.

Although it has been long known that regulating Ca^{2+} stores affect neuritogenesis and the amount and size of dendritic spines, e.g. Ca^{2+} released from the ryanodine-sensitive stores by caffeine increases the size of spines of cultured hippocampal neurons (Korkotian and Segal, 1999; Lankford et al., 1995), the exact effects on ER morphology has been less studied than overall effects on spines or the cytoskeleton within. Dendritic ER is especially interesting as it has a high turnover rate (Toresson and Grant, 2005). Moreover, ribosomes and components of the translocon complex, such as Sec61, have been found in the spines. This was under speculation for quite a while as Nissl staining stains primarily the ribosomal ER and stains dendrites of different neuronal subtypes (Palay and Palade, 1955). The dendritic ER is highly susceptible to synaptic activity. For example, in primary hippocampal cultures, activating N-methyl-D-Aspartate (NMDA) glutamate receptors changes the morphology of dendritic ER by fragmenting and redistributing it (Kucharz et al., 2009; Valenzuela et al., 2014). Also, activation of type I metabotropic glutamate receptors results in a rearrangement of tubular ER into lamellar bodies in Purkinje cell dendrites. As synaptic activity modulates the morphology of the ER in dendrites, and dendritic ER morphology affects local Ca^{2+} signaling, it has intriguing implications on how local supply of proteins and lipids are regulated from the ER. Furthermore, recent studies have shown that these changes happen rapidly and often (Perez-Alvarez et al., 2020). The ER can fragment and reinstate continuity reversibly and repeatedly within a neuron due to synaptic activity. The dynamics of ER fission/fragmentation is regulated by atlastins, discussed

previously (Sree et al., 2021). However, these events have also been linked to P-bodies, but the exact mechanisms are unclear and how they influence neuronal function and their relevance to disease is yet to be explored (Lee et al., 2020).

ER morphology is related to function (Cui-Wang et al., 2012; Fowler et al., 2019; Westrate et al., 2015). Changes in ER morphology (sheet to tubule ratio, amount of sheet fenestrations, distribution and packing of sheets) are seen in context of differences in function of different cells or even metabolic state of the same cell. Differences are also seen in the ER morphology in different regions of the same cell. A multitude of factors interact to render flexibility to the ER architecture. The difference in morphology and ratio of different forms is tightly regulated as they respond to differences in growth cues and conditions of the cell (Fowler et al., 2019; Luarte et al., 2018; Schwarz and Blower, 2016). The sheets are predominant in secretory cells like in plasma cells (Shibata et al., 2010), the tubules are abundant in muscle cells (Schwarz and Blower, 2016). Within a cell itself, variations can be seen in distribution such as in neurons, ribosome studded ER sheets are prominent in the somatodendritic region while a major share of ER in the axons is composed of narrow tubules (Wu et al., 2017). Also, the same type of structure might have different types of functions in different cellular contexts. For example, the ER tubules participate in detoxification in liver cells but is mainly concerned with Ca^{2+} homeostasis in muscle cells (Voeltz et al., 2006). ER structural changes in response to external stimuli is for example seen as the conspicuous changes that occur in ER due to glutamate receptor activation in hippocampal neurons. In this study glutamate stimulation triggered changes in dendritic ER from a continuous network to a collection of fragmented compartments as observed using confocal microscopy (Ramirez and Couve, 2011). Phenotypic analysis of ER morphology to find new drugs would be an important future endeavour.

ER stress in neurons

In general, ER stress is defined as a disbalance in the homeostasis of the ER, where a disruption in any of the ER's functions diminish the proper folding and secretory capacity of the ER causing one or multiple mechanisms aimed at restoring the homeostasis to be activated (Costa-Mattioli and Walter, 2020; Hetz and Saxena, 2017; Mercado et al., 2013; Mou et al., 2020; Needham et al., 2019). These mechanisms include ER Ca^{2+} homeostasis restoring mechanisms, the integrative stress response (ISR), ER-associated degradation (ERAD) and the unfolded protein response (UPR). The functions of these mechanisms are to restore folding conditions and/or retain unfolded proteins exiting from the ER, try to refold them, and if not possible then degrade them. This is done by selectively slowing down protein synthesis and upregulating chaperones, which assist in protein folding, and proteins, which assist in degrading terminally misfolded proteins. These integrated quality control systems are interrelated as they all may be activated due to ER stress, whether it is intrinsically or extrinsically caused, but not necessarily interdependent as the extent of their crosstalk or dependence is yet to be elucidated (Hwang and Qi, 2018). For example, while the UPR controls the expression of ERAD genes, ERAD is also involved in controlling the turnover of the most conserved arm of the UPR, IRE1. ERAD, in general, is responsible for recognizing and moving terminally unfolded proteins from the ER for the cytosolic ubiquitin-proteasome system for degradation. ERAD is composed of various chaperones and lectins, e.g. BiP and EDEM, which recognize and move the unfolded proteins within the ER lumen to ERAD adaptors, e.g. Erlins and Insigs, located in the ER membrane. Proteins then undergo retrotranslocation through channels such as Hmg-CoA reductase degradation ligase to the cytosol to be polyubiquitinylated and degraded by 26S proteasomes. The ISR comprises all signaling leading to the activation of the translation initiation factor 2 (eIF2α)-ATF4-CHOP pathway in response to various stress, notably oxidative stress, amino acid and glucose deprivation, viral infection, or misfolded proteins (Costa-Mattioli and Walter, 2020).

The UPR has three different sensors mediating effects aimed at restoring ER homeostasis through their respective signaling pathways. The three sensors are the double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and IRE1 (Cox et al., 1993; Harding et al., 1999; Haze et al., 1999; Liu et al., 2002). In healthy conditions they all bind BiP (GRP78), which is an abundant luminal ER chaperone, but when a misfolded protein occurs BiP binds to it and dissociates from the sensors causing them to transduce their signals to the transcription factors, which control the expression of genes that aid in restoring the functionality of the ER. ER stress signaling, as many other cellular signaling, may also be induced in a non-canonical manner, such as through the AGGF1–- miR-183-5p axis, which directly regulates CHOP, a downstream target of PERK-mediated UPR signaling (Yao et al., 2017). It is also now widely accepted that UPR transducers are not only involved in protein misfolding, but they also function as Ca^{2+} regulators having direct effects on interorganelle communication and bioenergetics of the cell (Carreras-Sureda et al., 2019; Carreras-Sureda et al., 2018). In neurons, the UPR is also important for the developing cortex as it is involved in regulating neuronal migration and neurite extension (Tseng et al., 2018; Tseng et al., 2017).

The UPR is not only activated in the somal ER, as ER stress sensors have been found in the dendrites of primary mouse neurons. When ER stress is induced, GRP78/BiP and phosphorylated eIF2α are induced also in the dendrites besides the soma (Murakami et al., 2007). Additionally, XBP1 mRNA and phosphorylation of IRE1 have been found in the proximal dendrites.

ER stress can have different consequences on cell fate depending on the disease context and which responses are activated and to which extent. This is especially apparent in the UPR pathways, as modulation of its three different sensors may have opposing effects (Hetz and Saxena, 2017).

Changes in ER structure during ER stress

As ER sheets are the main site of protein synthesis, the major structural change during ER stress triggered by accumulation of unfolded or misfolded proteins is ER expansion through increased generation of ER sheets (Bernales et al., 2006; Schuck et al., 2009). ER stress evidently changes the protein and RNA content of ER, but the exact changes in ER structure and the stage at which these occur need further investigation (Hollien and Weissman, 2006; Schwarz and Blower, 2016; Yanagitani et al., 2009).

Detrimental effects of ER stress in neurons

If the UPR cannot endure the stress caused by the unfolded protein load, it can eventually lead the cell to apoptosis (Galehdar et al., 2010). Neurons have a relatively small secretory and protein folding capacity compared to specialised secretory cells and the proteins they secrete are large and complex (multisubunit) extracellular proteins and receptors. Since neurons are post-mitotic, neurons undergone apoptosis can only be replaced to certain extent, involving the neurogenesis observed in the dentate gyrus. However, most neurodegeneration happens in areas of the brain and in cell types which cannot be replaced, and ER stress induced apoptosis may exacerbate progression of the diseases (Mercado et al., 2013; Mou et al., 2020). Although cell death mediated by ER stress has been unequivocally linked to activation of the intrinsic mitochondrial apoptotic pathway, reports have also shown that ER stress can also induce necroptosis (Saveljeva et al., 2015). Particularly, in the context of neurodegeneration, intriguing is that the necroptosis machinery has been found to mediate axonal degeneration in a model of Parkinson's Disease (PD) (Onate et al., 2020).

ER stress has been linked to aged neurons and longevity, especially to age-associated neurodegenerative diseases (Hetz and Saxena, 2017; Wang and Kaufman, 2016). One non-pharmacological remedy to this calamity which has been studied quite extensively is exercise. For

example, in aged presenilin 2 mutant mice, regular exercise on the treadmill reduces amyloid beta induced ER stress by modulating the UPR (Kang et al., 2013). The role of exercise and UPR is compactly reviewed in (Estebanez et al., 2018).

Neurotropic viruses that target the ER deserve a mention due to the SARS-CoV-2 virus that spread globally at the end of 2019. Like the Zika virus, coronaviruses are neurotropic viruses, meaning they infect neurons, although not exclusively, and they use the ER for their replication (Phillips and Weiss, 2011; Tardieu et al., 1986). Indeed, microcephaly, ER stress, and Zika virus has been correlated (Alfano et al., 2019; Beaufrere et al., 2019; Gladwyn-Ng et al., 2018), and also ER lumen protein homeostasis disturbance is associated with slower cortex development in mouse (Tseng et al., 2017). Many enveloped viruses use the ER-Golgi intermediate compartment (ERGIC) for assembly before budding (Fehr and Perlman, 2015). This triggers ER stress, and SARS-CoV, which was responsible for the 2002-2003 epidemic, has been shown to take advantage of the UPR to enhance and prioritize production of viral proteins instead of the cells own proteins (Chan et al., 2006; Minakshi et al., 2009). Thus, the UPR is an important drug target for many viral infections, including SARS-CoV-2 and efforts to target the host-derived ERGIC proteins incorporated into the virus membrane are under way. These proteins are much more preserved, and less variable compared to the highly mutable proteins originating from the viral genome.

Beneficial effects of ER stress in neurons

Mild ER stress (MERS), also known as ER hormesis, may offer protection in neurodegenerative diseases as has been observed in multiple studies (Matus et al., 2012; Matus et al., 2011). ER hormesis is a cellular response which preconditions the cells' stress responses by making the cell more resistant to further stress when encountered first with lower levels of stress. For example, ablation of an ER Ca^{2+} regulating protein, Herp, can upregulate autophagy (Quiroga et al., 2013). Especially fine-tuning of the UPR may be the key in therapeutic strategies for neurodegenerative diseases (Medinas et al., 2017; Mou et al., 2020). Nevertheless, as prolonged activation of the different arms of the UPR in regulating apoptosis is still quite poorly understood, developing drugs with regards to their half-lives, dosing regimens, therapeutic windows and timing related to disease progression will be challenging.

For example, ATF6 activation has been studied as therapeutic approach as (Blackwood et al., 2019). ATF6 signaling can be inhibited by α-synuclein and ATF6 protects against toxin induced degeneration of dopamine neurons and is thus of interest particularly in the treatment of PD (Credle et al., 2015; Egawa et al., 2011). Small molecule activators specifically targeting ATF6 have been developed with inactive enantiomers, which enables the controlled studying of ATF6 activation to treat disease (Plate et al., 2016). As ATF6 contributes to enhancing ER folding capacity by expanding the ER membrane, compounds directly affecting these should therefore affect ER morphology (Bommiasamy et al., 2009; Maiuolo et al., 2011). AA147 is an ATF6 inducer which works by blocking covalent bond formation of protein disulfide isomerases (PDIs) (Paxman et al., 2018; Plate et al., 2016). It has an inactive enantiomer called RP22. The changes in ER morphology can be analyzed with thin section transmission electron microscopy (TEM) of ventral midbrain neuronal cultures and this enables label free techniques. An example of midbrain neurons treated with 10 μM AA147 or 10 μM RP22 can be seen in Figure 2 and Supplemental video 1 (AA147) video 2 (RP22)). In Figure 2 and Video 1-2 one can focus particularly on TH positive neurons and there are not any significant morphological changes to the ER after 24 h of exposure to these compounds.

ER and Ca^{2+} dynamics in neurodegeneration

ER stress has been studied extensively in various diseases, mainly metabolic diseases such as diabetes, liver diseases and heart diseases. Recently links between ER stress and neurodegeneration have been found, and the importance of the ER in these diseases is more appreciated (Gerakis and Hetz, 2018; Hetz and Saxena, 2017; Jung et al., 2017; Medinas et al., 2019; Mou et al., 2020). Especially the most common neurodegenerative diseases all have a large amount of supporting evidence from preclinical models all the way to human post-mortem samples on the role of ER stress in these diseases (Gerakis and Hetz, 2018; Jaronen et al., 2014; Martinez et al., 2019; Medinas et al., 2019; Salminen et al., 2020; Santos and Ferreira, 2018). Neurodegenerative diseases are often referred to as protein misfolding disorders due to the common pathology of accumulation of misfolded proteins. The accumulation of the misfolded proteins leads to generation of ER stress and activation of UPR, which has been linked to the pathophysiology of protein misfolding disorders (Hetz and Saxena, 2017). Changes in the Ca^{2+} homeostasis has also been widely linked to the pathology of many of the neurodegenerative diseases (LaFerla, 2002; Rivero-Rios et al., 2014). Many of the genetic mutations leading to familial forms of the diseases are linked to changes in the Ca^{2+} dynamics, giving support to the idea that these changes have a crucial role in the neurodegeneration and pathology of the diseases.

Parkinson's disease

Parkinson's disease (PD) is a heterogeneous disorder with widespread pathology. One of the most common characteristics of the disease is the degeneration of dopaminergic neurons in the substantia nigra paras compact and the presence of Lewy bodies, which consist of misfolded proteins, such as α-synuclein. Changes in the ER and Ca^{2+} homeostasis have also been linked to the pathogenesis (Vila et al., 2008). Several studies indicate a role of Ca^{2+} signaling in PD pathogenesis, and some also link it to the selective neuron degeneration seen in the disease (Korecka et al., 2019; Rivero-Rios et al., 2014; Surmeier et al., 2017). Substantia nigra pars compacta dopamine neurons have a unique pace-making activity driven partly by voltage dependent L-dependent Ca^{2+} channels (Grace and Bunney, 1983; Grace and Onn, 1989; Overton and Clark, 1997). Compared to ventral tegmental area dopamine neurons they also have lower levels of Ca^{2+} buffering protein calbindin and higher levels of L-type Ca^{2+} channels (German et al., 1992; Khaliq and Bean, 2010; Rivero-Rios et al., 2014). Altered ER Ca^{2+} control has been observed in stem cell-derived neurons from genetic PD patients harboring a mutation in leucin repeat rich kinase 2 (Korecka et al., 2019).

α-synuclein has been shown to disrupt the connection between the mitochondria and ER by binding to VAPB, which is a protein that together with PTPIP51 acts as scaffolds that link the organelles (Paillusson et al., 2017). An overexpression of familial and wild-type PD mutant α-synuclein disrupts the VAPB-PTPIP51 connections between the organelles, which loosens the ER-mitochondria connections. This leads to a decreased level of Ca^{2+} exchange and ATP production in the neurons, which can be fatal for the sensitive substantia nigra dopamine neurons (Paillusson et al., 2017).

The UPR has been linked to PD by studies showing increased levels of UPR activation in PD *post mortem* brain tissues. A link between UPR activation markers and diffuse α-synuclein staining shown on in vitro PD models indicate that UPR activation is an early event in the disease (Hoozemans et al., 2007; Hoozemans et al., 2012). Mutations in SNCA, a gene that produces α-synuclein, was the first mutation linked to PD and has also been linked to the ER's effect on the disease (Venda et al., 2010). Studies have shown that overexpression of α-synuclein inhibits COPII vesicles trafficking of proteins between ER and Golgi apparatus (Cooper et al., 2006; Credle et al., 2015). This has also been shown to modulate UPR, by inhibiting ATF6 production, as it uses COPII for the ER-Golgi transport (Credle et al., 2015).

Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease and the leading cause of dementia. The incidence is increasing exponentially, with the number of patients doubles in every 20 years (Mayeux and Stern, 2012; Prince et al., 2013). The disease is heterogeneous and often classified by the beta-amyloid plaques and neurofibrillary tangles of hyperphosphorylated tau proteins, but ER stress and Ca^{2+} homeostasis misbalance has also strongly been linked to the pathophysiology (Hoozemans et al., 2012; LaFerla, 2002). It has been shown that there is an early activation of UPR in AD brains and an indication that the prolonged UPR activation is involved in the tau phosphorylation and the neurodegeneration in AD pathology (Hoozemans et al., 2009; Hoozemans et al., 2005).

There are many hypotheses to the pathology of AD and one of them is the so-called Ca^{2+} hypothesis, which claims that dysregulation of the intracellular Ca^{2+} has a central role in the neurodegeneration (Popugaeva et al., 2018). One of the strongest supports to this hypothesis is the finding that a mutation in the presenilin (PS1 and PS2), which is linked to familial AD, affect the ER Ca^{2+} movement (LaFerla, 2002). Presenilin has also shown to be enriched at the MAMs and its function to produce Ca^{2+} -permeable ion channels on the ER that leak Ca^{2+} into the cytosol is impair in AD, leading to an increased level of Ca^{2+} in the ER (Area-Gomez et al., 2009; Tu et al., 2006).

Connections have also been made between the protein dysfunction and the Ca^{2+} dysregulation. For example, a high production of β-Amyloid (Aβ) has been detected at MAMs, and this has shown to increase ER-mitochondria tethering, effecting the transport of Ca^{2+} and lipids (Hedskog et al., 2013; Schreiner et al., 2015). *In vivo* experiments also indicate that Aβ plaques impair the Ca^{2+} homeostasis in the neurons (Kuchibhotla et al., 2008). The Ca^{2+} levels in the cytosol are also believed to be increased due to Aβ oligomers producing channels that lead to an extracellular influx of Ca^{2+} (Demuro and Parker, 2013). In addition, many of the proteins that are involved in the ER Ca^{2+} signaling have been linked to the AD pathogenesis (Ozturk et al., 2020). In neurons, the amyloidogenic pathway is activated by hydrolysis of amyloid precursor protein which results in Aβ accumulation. This stimulates production of IP3 and elevates cytosolic Ca^{2+} concentration via IP3/Ca^{2+} signaling pathway. At the initial stage, it induces a decline in memory, however, a prolonged raise in cytosolic Ca^{2+} causes death of the neurons (Berridge, 2013; Green and LaFerla, 2008; Stutzmann, 2005). As mentioned previously, changes in the proximity of ER to mitochondria has been reported in AD models and the AD pathogenesis-associated proteins amyloid-beta precursor protein and tau have been found to be enriched in MERCS (Dentoni et al., 2022).

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease that causes degeneration in the lower and upper motor neurons controlling voluntary muscles. This leads to the progressive weakness and loss of muscles and eventually paralysis (Hardiman et al., 2017). The main neuropathological hallmark of the diseases is the accumulation of protein aggregates that form ubiquitinated proteinaceous inclusions in motor neurons. Although the pathology of the disease still is quite unknown, mutations that lead to familial ALS link ER and ER-mitochondria dysfunctions to the disease.

A mutation in VAPB is linked to ALS and its expression is reduced in ALS patients (Nishimura et al., 2004; Teuling et al., 2007). As mentioned earlier, VAPB is present on the ER membrane taking part in the tethering between ER and other organelles and is important in the formation of MAMs. The P56S mutant VAPB that is linked to ALS results in the formation of cytosolic aggregates in cultured

cells. Also, neurons expressing the mutated VAPB had a higher rate of cell death (Teuling et al., 2007). Another gene mutation linked to familial ALS is mutations of TDP-43 and accumulation of TDP-43 is a hallmark pathology of ALS. Mutations of TDP-43 reduces the ER-mitochondria interactions by disturbing the binding of VAPB and PTPIP51 (Stoica et al., 2014). This is linked with an increased cytosolic Ca^{2+} level and a decreased level of Ca^{2+} in mitochondria due to IP3R-mediated Ca^{2+} release from ER, which also can be linked to the disruption of the ER-mitochondria association (De Vos et al., 2012; Stoica et al., 2014).

The Ca^{2+} homeostasis and ER-mitochondria crosstalk is also disrupted by mutations of ER chaperone SIGMAR1 (Bernard-Marissal et al., 2015; Prause et al., 2013; Vollrath et al., 2014). A mutation in SIGMAR1 has been linked to juvenile ALS. SIGMAR1 is localized at MAMs and together with IP3R regulates Ca^{2+} signalling and cell survival (Hayashi and Su, 2007). Ataxin-2 is another protein that takes part in the tubular ER morphogenesis and dynamics and a mutation in the gene coding this protein has been linked to ALS (Del Castillo et al., 2019; Elden et al., 2010).

Huntington's disease

Huntington's disease (HD) is an autosomal dominant disease caused by an expanded CAG trinucleotide repeat in the HTT1 gene leading to cognitive decline and defects in motor coordination (Bates et al., 2015). The disease-causing glutamine expansion leaves the proteins prone to misfolding and the forming of aggregates, which is the main pathological finding in HD. UPR activation has been shown in HD post-mortem brains and many studies suggest that ER stress can contribute to the disease (reviewed in (Carnemolla et al., 2009; Matus et al., 2011)).

Mutant Huntingtin (mHTT) protein has in many ways been shown to interfere with ER. For example, mHTT can bind to IP3R, leading to an increased Ca^{2+} release from the ER to the cytosol (Tang et al., 2003). The mHTT protein strongly binds to C-terminal region of IP3R1, making this receptor more sensitive to IP3, thus generating high Ca^{2+} signals which disrupt neuronal function and persistent increase of Ca^{2+} leads to cell death (Bezprozvanny, 2007; 2011). In the HD mouse model, TRPC5 has been shown to mediate the glutathionylation evoked increase in Ca^{2+} that resulted in the death of striatal neurons. However, this effect was abolished on decreased expression of TRPC5 on the plasma membrane through depalmitoylation (Hong et al., 2020; Hong et al., 2015).

Pharmacological tools to modulate ER function

Canonical ER stressors and their effects in neurons

There are a variety of pharmacological tools affecting the ER, which can have substantial changes in the morphology and its functions. Bona fide ER stressors include tunicamycin, brefeldin A and thapsigargin, which are commonly used to model ER stress and used as stressors in survival experiments (Chmielarz et al., 2017; Hetz et al., 2019). They have different primary mechanisms of actions on how they induce ER stress. The wide variety of compounds affecting the ER allow mechanistic studies in probing the function of the ER and ER stress pathways. Other compounds and investigated drugs targeting ER stress, specifically UPR modulators are well reviewed in (Gonzalez-Teuber et al., 2019; Hetz et al., 2019).

Tunicamycin, a metabolite with antibiotic properties produced by *Streptomyces lysosuperificus*, causes ER stress by blocking N-linked glycosylation.Some proteins that are to be folded by disulfide bond stabilization by PDI enzymes can be glycosylated as the chaperones linking them to PDIs use

the sugar moieties as ligands. If for some reason, a protein that should be glycosylated is not, PDI enzymes cannot act on them, and this will trigger the UPR.. Tunicamycin causes apoptosis in neurons through rapid induction of ATF4 (Galehdar et al., 2010), and can trigger apoptosis pathways also directly (Reimertz et al., 2003) so, with very high concentrations there can be several cell death pathways. Besides being used in modelling ER stress, tunicamycin may have also therapeutic value as, for example, it has been used to produce MERS in a mouse and fly model of PD displaying neuroprotective properties (Fouillet et al., 2012).

Brefeldin A, an antiviral fungal metabolite produced by *Penicillium brefeldianum*, is a common probe used to study ER-Golgi interactions as it blocks trafficking of proteins from the ER to the Golgi (Ulmer and Palade, 1991). Brefeldin A is used in neurons to model features of ALS pathophysiology as it induces oxidative stress-related neurotoxicity in cultured spinal neurons of rat spinal cord with motor neurons being most vulnerable to its effects (Kikuchi et al., 2003). Phenyl-2-decanoyl-amino-3-morpholino-1-propanol-hydrocholride (PDMP), a sphingolipid synthesis inhibitor, causes temporal ER fragmentation which can be partially reversed by a Ca^{2+} chelating agent (Sprocati et al. 2006). PDMP interferes with brefeldin A-induced retrograde transport from the Golgi to the ER (Kok et al. 1998). Brefeldin A potently disassembles golgi stacks and vesiculo-tubular remnants observed as puncta in normal rat kidney cells (Seemann et al., 2000). As brefeldin A disrupts the golgi stacks, golgi enzymes are left over in small vesicular-tubular golgi clusters and uptaken by the ER, which may contribute to the ER expansion observed in brefeldin A induced ER stress.

Thapsigargin, a sesquiterpene lactone derived from the plant *Thapsia garganica*, is a irreversible SERCA inhibitor, which blocks all three SERCA isoforms (Rossier et al., 1993). Thapsigargin and its analogs administered in low (0.1 μM) concentrations for 24h cause cellular death via ER Ca^{2+} depletion and the UPR independent of the SOCE (Sehgal et al., 2017). Although thapsigargin indirectly activates the UPR as the depletion of ER Ca^{2+} causes the activity of Ca^{2+}-dependent chaperones to diminish leading to increased amounts of unfolded proteins, it has also been shown to activate ATF6 directly, so it may induce ER stress via multiple mechanisms (Li et al., 2000). However, cell death caused by thapsigargin has also been linked to nitric oxide production (Canova et al., 2007). Besides convergence of apoptotic pathways, it is not clear which of its actions is dominant in causing cellular death. This may also be dependent on the cell type or cell line as in neurons, thapsigargin treatment has also been shown to induce ATF4-CHOP pathway mediated apoptosis similar to tunicamycin (Galehdar et al., 2010). Thapsigargin is widely used as a research tool, but only its peptide-linked derivative, mipsagargin (G-202) has been tested in clinical settings for cancer. This prodrug is activated by the protease human glandular kallikrein 2 (hK2), which is expressed by prostate cancer cells and thus can be targeted specifically to these cells (Janssen et al., 2006). However, mipsagargin has also been studied in patients with glioblastoma multiforme, suggesting that it could be targeted to neurons (Pagliaro et al., 2021). Certain hK2 splice variants are expressed in brain cancer tissue which may explain the positive responses observed in the clinical trial (Adamopoulos et al., 2019). Using this strategy to find neuronal populations which express proteases that could activate thapsigargin specifically in the brain could be exploited in treatment of neurodegenerative diseases. Furthermore, as submaximal inhibition, i.e therapeutic doses, of SERCA by thapsigargin and BHQ have been demonstrated to increase the lifespan of C. elegans, SERCA inhibition may indeed have therapeutic benefit beyond cancers (Garcia-Casas et al., 2018).

ER Ca^{2+} depletion and modulators of ER Ca^{2+} in neurons

Depletion of ER Ca^{2+} has been linked to multiple diseases (Karagas and Venkatachalam, 2019; Mekahli et al., 2011; Nikoletopoulou and Tavernarakis, 2012). For example, it has been linked to inflammatory diseases due to increased phagocytic uptake of cells causing the secretion of abundant ER chaperones including calreticulin, BiP, gp96 and PDI (Peters and Raghavan, 2011). In addition, other diseases such as heart failure, and many neurodegenerative diseases including AD and PD have been linked to ER Ca^{2+} mediated secretion of ER luminal proteins. Recently this phenomenon was further characterized by the discovery that depleting ER Ca^{2+} causes ER resident proteins that have a ER retention signal (ERS) to secrete out of the cell (Henderson et al., 2021; Trychta et al., 2018). This event has been termed as "ER exodosis". It was found that there are over 70 human proteins in the ER lumen that exit the cells upon ER lumen Ca^{2+} depletion (Trychta et al., 2018). Previous studies have shown with studying the secretion of ER lumen protein MANF -an UPR responsive gene- that the thapsigargin-induced secretion occurs via classical secretion pathway (Henderson et al., 2014). Also, KDEL receptors act differently to thapsigargin-induced UPR. KDELR2 and KDELR3, but not KDELR1, are UPR response genes and they are upregulated as a cell protective response (Trychta et al., 2018). Thus, when exodosis occurs and there is exit of KDEL retrieval sequence containing ER luminal proteins there is isoform-specific control of the KDEL receptors. ER Ca^{2+} leakage, regardless of the secretion of ER resident proteins, is highly implicated in aging-related neurodegenerative disease in general (Nikoletopoulou and Tavernarakis, 2012). Also, more recently it was identified several FDA-approved drugs that can prevent thapsigargin-induced exodosis and these include bromocriptine -dopamine receptor agonist used for PD -, dextromethorphan – a cough suppressant-, dantrolene – a muscle relaxant -, verapamil – heart specific Ca^{2+} channel blocker-, and diltiazem – Ca^{2+} channel blocker and hypertension drug (Henderson et al., 2021). Moreover, bromocriptine and its derivatives have protective effects in various cell-based models of exodosis and *in vivo* animal models of stroke and diabetes (Henderson et al., 2021).

ER Ca^{2+} depletion also causes changes in the morphology of organelles; however, reports of the morphological changes have varied greatly. Bravo (Bravo et al., 2011) showed that thapsigargin causes redistribution of the ER towards the nucleus and an increase in contact sites between the ER and mitochondria in HeLa cells. Furthermore, also in HeLa cells, a study by Numata (Numata et al., 2013) indicated that thapsigargin and tunicamycin treatments do not seem to affect ER morphology as based on PDI, calreticulin, and GRP78 staining. However, Varadarajan and others found in a series of papers a novel ER stress response involving the reorganization and aggregation of ER tubules, induced by many compounds including thapsigargin, in various immortalized cell lines (Varadarajan et al., 2012; Varadarajan et al., 2013; Yedida et al., 2019). We, on the other hand, studying the effects of thapsigargin in postnatal dopaminergic neuronal cultures, using TEM that enables label-free image analysis and accurate ultrastructure analysishave observed that thapsigargin at a concentration of 250 nM does not seem to have an ER reorganization effect, or any other gross morphological changes, shown by thin slice TEM (Figure 3). Although the treatment times were similar, with either 4 h, 8 h or 24 h of exposure, it is important to note that the concentrations used in the studies greatly varied. Whereas Varadarajan used very high concentration of 10 μM, and others have used mainly concentrations ranging from low nanomolar to 1 μM which would be more physiologically relevant (Numata et al., 2013; Trychta et al., 2018). As previously noted, thapsigargin can cause cell death already at low doses (100 nM) at time exposures beyond 24 h (Sehgal et al., 2017), whereas exodosis can be seen already with 10 nM. Nevertheless, regarding the possibility of thapsigargin-insensitive ER Ca^{2+} stores, it would be of great importance to study time and concentration dependent effects of thapsigargin in a multitude of models, especially in neuronal cell (Liu et al., 2003).

Most studies that have looked at the relationship between ER morphology and ER Ca^{2+} depletion have used thapsigargin as means to deplete the ER Ca^{2+} stores. Nevertheless, there are multiple other exogenous and endogenous regulators of ER Ca^{2+} stores (Figure 1). In fact, studying ER Ca^{2+} regulation is convenient with available pharmacological agents, as there are plenty of both Ca^{2+} influx and efflux channel inhibitors and activators available. For example, other SERCA blockers include, cyclopiazonic acid and 2,5-di-t-butyl-1,4-benzohydroquinone (BHQ) which are reversible inhibitors. Caffeine, one of the most used and studied psychoactive plant metabolites, is a RyR agonist, in addition to ryanodine itself, while dantrolene is an antagonist. IP3R can be inhibited by Xestospongin C and heparin or activated by Adenophostin A (DeLisle et al., 1997; Solovyova et al., 2002). There are also plenty of Ca^{2+} ionophores, such as ionomycin, to inflict similar changes in cytosolic Ca^{2+} as with certain ER Ca^{2+} depletors. Endogenous factors include, e.g., phospholamban and sarcolipin, which are small proteins regulating SERCA. Many hormones and neurotransmitters also regulate ER Ca^{2+} levels, and directly or indirectly SERCA function, such as acetylcholine and cholecystokinin, which evoke Ca^{2+} release through IP3 and NAADP (Cancela et al., 2000). Additionally, various small-molecule compounds such as dynarrestin, pridopidine, metformin, sulforaphane, trolox, luteolin and urolithin A may affect function of MERCs, either by restoring their function and connectivity or tethering, or increasing or decreasing their apposition. (Dentoni et al., 2022). However, most of their molecular targets within the MERCs are not known and how they affect ER calcium homeostasis. Herp is an endogenous membrane-associated ER Ca^{2+} stabilizer which is also an integral ERAD component (Eura et al., 2012). Herp has been shown to prevent the ER-stress mediated death of dopaminergic neurons caused by α-synuclein, one of the main proteins that aggregate and form the hallmark Lewy bodies in PD. α-synuclein itself has also been shown to increase the activity of SERCA (Betzer et al., 2018). Mesencephalic astrocyte-derived neurotrophic factor (MANF), which is a luminal ER chaperone, has also been linked to ER Ca^{2+} regulation by blocking leakage of the RyR2 Ca^{2+} channel in podocytes (Matlik et al., 2015; Park et al., 2019). MANF is involved in protein homeostasis as it is upregulated upon ER stress, interacts with BiP and is secreted upon ER Ca^{2+} depletion (Glembotski et al., 2012; Petrova et al., 2003). As MANF has pleiotropic actions in regulating ER homeostasis factors, including the UPR and Ca^{2+} channels, it has been studied also extensively as a therapeutic approach for many ER stress related diseases, such as Wolfram disease, diabetes, stroke, hearing loss and Parkinson's disease (Airavaara et al., 2010; Airavaara et al., 2009; Albert and Airavaara, 2019; Glembotski et al., 2012; Herranen et al., 2020; Lindahl et al., 2014; Mahadevan et al., 2020; Voutilainen et al., 2015; Voutilainen et al., 2009).

With regards to PD, depletion of ER Ca^{2+} stores have been observed in both familial and sporadic PD cases. It has been linked to several different mechanisms regulating ER Ca^{2+}. For example, RyR agonism by caffeine, its metabolite paraxanthine and other RyR-acting compounds exhibit neuroprotective effects in dopaminergic neurons, enhancing their survival and secretory capacity (Guerreiro et al., 2008; Le Douaron et al., 2016; Xu et al., 2010). Moreover, BST1, an adenosine diphosphate ribose (ADP) cyclase that can regulate Ca^{2+} mobilization from the ER through the RyR is a gene associated with sporadic PD (Bruzzone et al., 2003; Saad et al., 2011). Furthermore, considering the latest findings on MANFs beneficial effects in the treatment of PD and its function in the regulation of RyR channels, there is now even more precedent for studying the role of RyR modulation in the treatment of PD. However, considering RyRs in AD, things get complicated as

increased expression and activity of RyRs has been reported in preclinical AD models and proposed treatment of AD with RyR antagonist such as dantrolene (Wu et al., 2013). The association of RyR and AD are linked to presenilins, which have a dual action in regulating ER Ca^{2+}. Presenilins regulate SERCA and increase Ca^{2+} in the ER, which has been shown to make cells more susceptible to apoptosis (Green et al., 2008). Conversely, they can also cause ER Ca^{2+} leakage, which also may cause apoptosis through ER stress. Thus, it is important to keep in mind the multitude of contributing factors and the net effect of targeting a specific Ca^{2+} regulator for determining the overall outcome on ER Ca^{2+} regulation and furthermore the therapeutic effect of the approaches. As another example, downregulation of SOCE has been associated with PD and enhancing it seems to be neuroprotective, while this may be opposite in other neurodegenerative states, such as ischemia (Berna-Erro et al., 2009; Selvaraj et al., 2012). What is the right target and for which neurodegenerative disease remains to be elucidated, but various components of the SOCE, the Ca^{2+} release activated channels, STIM1/2 and their isoforms or ORAI1 and other TRPs besides the main influx (SERCA) and efflux (RyR) channels provide optional targets for drug development projects aiming to correct ER Ca^{2+} homeostasis (Majewski and Kuznicki, 2015; Tian et al., 2016).

Other tools and methods to study ER calcium

Measurement of $[Ca^{2+}]i$, by classical methods such as use of Ca^{2+}-selective microelectrode, Ca^{2+}-activated photoproteins or bis-azo metallochromic Ca^{2+} dyes was intricate. However, the discovery of Fluorescent Ca^{2+} indicators has facilitated the accurate and precise measurement of $[Ca^{2+}]i$. There are various probes developed to specifically study ER Ca^{2+} modulation. These include dyes, fluorescent probes (small-molecule or antibody conjugates), and genetically modified tags (exogenous or endogenous). Some of the tools and methods with examples of newer ones specifically aimed to be used for neurons or neuronal tissue are listed in Table 1. In combination with electron microscopy, the changes in Ca^{2+} can be correlated to changes in the ER ultrastructure, combining morphology with function.

Concluding remarks

ER with its different structural functionally significant domains is integral to the survival of a cell. Many membrane proteins are important for shaping these domains (Hübner and Kurth, 2014; Voeltz et al., 2006; Zhang and Hu, 2016). There is now ample evidence that changes in neuronal ER morphology affect many different functions of the ER which have been linked to neurodegeneration, most notably, functions of the ER related to Ca^{2+} regulation independently and in contact with other organelles. For example, as mentioned previously, reticulon 4 mediated shaping of the ER is critical for proper functioning of the SOCE and ER reorganization due to ionic imbalances may affect trafficking of protein to the plasma membrane (Jozsef et al., 2014; Varadarajan et al., 2013). Additional supporting evidence for the importance of morphology in Ca^{2+} regulation is the recent finding that the size of mitochondria directly promotes their capacity to uptake Ca^{2+} affecting overall cellular Ca^{2+} homeostasis (Kowaltowski et al., 2019). The authors also found that mitochondrial fission promotes basal Ca^{2+} levels and lower ER Ca^{2+} stores. These studies raise important questions; how much, and specifically what kind, changes in ER morphology and other organelles which

regulate Ca^{2+} in conjunction with the ER, affect cellular Ca^{2+} levels? Can changes in ER Ca^{2+} affect ER morphology even in the distal parts of the neurons significantly enough to cause ER stress up to the point of neurodegeneration? Do different neuronal subtypes have varying degrees of ER morphologies and/or ER Ca^{2+} regulation? As links between Ca^{2+} regulators, e.g. UPR transducers, and ER morphology are elucidated in different neurodegenerative states, comparative studies become of utmost importance in finding out which factors contribute to the different underlying vulnerabilities of certain neurons succumbing to neurodegeneration. Targeting ER homeostasis is already ripe common ground for developing new therapeutics to treat multiple neurodegenerative diseases and even more so if it turns out that inherent differences in neuronal responses to perturbations of ER homeostasis is the main culprit converging behind all sporadic neurodegenerations.

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Figure 1. Mechanisms of cellular Ca^{2+} signaling: Membrane depolarization (ΔmV), activates Voltage-gated Ca^{2+} channels (VGCCs) that induce a rapid influx of Ca^{2+} into the cells. An agonist binds to the G protein-coupled receptor (GPCR) and through G-protein subunits Gi, Gq or both, activates phospholipase C (PLC), stimulating production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG evokes receptor-operated Ca^{2+} entry by stimulating TRPC Ca^{2+} channels on the plasma membrane and subsequent Ca^{2+} influx into the cells. IP3 evokes store-operated Ca^{2+} entry by binding to the IP3 receptors present on the endoplasmic reticulum (ER) membranes that leads to a robust release of Ca^{2+} from ER- Ca^{2+} stores. Altogether, these mechanisms rapidly increase cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]i$). In response to the ER- Ca^{2+} stores depletion, STIM1 molecules present on the ER membrane rapidly tetramerize and bind to ORAI1 Ca^{2+} channels by forming ER-membrane contact site, which results in the opening of ORAI1 channels and a huge influx of Ca^{2+} into the cells, referred to as store-operated Ca^{2+} entry. In addition**,** the expression of ER-stress proteins including BiP, Canexin, Calreticulin, and Herp modulates the ER stress pathway. To avoid persistent overload of ($[Ca^{2+}]i$) in the cytoplasm, the pumps present on the ER (SERCA) or plasma membrane (NCX and PMCA) are activated and Ca^{2+} is shuttled back into the ER to refill the stores, or exported out from the cells, respectively. In addition, direct ER Ca^{2+} regulators modulating the function of the ER influx (SERCA) and efflux (RyR and IP3R) channels are shown. Additionally, plasma membrane Ca^{2+} regulators (Orai, CALHM), leaky channels (Pannexin, Presenilin, Translocon, LRRC8B), Ca^{2+} buffering (BiP, calnexin, calreticulin) proteins, and Ca^{2+} stabilizers (Herp) contribute to ER Ca^{2+} levels.

Figure 2. Effect of ATF6 inducer on ventral midbrain dopaminergic neurons. Cultured dopaminergic neurons from postnatal (P1-3) mice treated for 24 hours with an ATF6 inducer (AA147) and its inactive control (RP22) with no gross effects on the endoplasmic reticulum (ER) ultrastructure. Yellow arrowheads point to the ER. Scale is 500 nm.

Figure 3. Effect of thapsigargin on ventral midbrain dopaminergic neurons. Cultured dopaminergic neurons from postnatal (P1-3) mice treated for 1, 3, or 24 hours of 250 nM thapsigargin or vehicle (DMSO) with no significant effects observed on the ultrastructure of the endoplasmic reticulum. Scale bar is 1 μm.

Table 1. Calcium probes and methods

Method	Compound(s)	Imaging Technique	References
Non- Fluorescent Molecules and Chelators	NAADP-AM, BAPTA AM, EGTA-AM	Plate Reader CCD Camera One photon microscopy	(Pafumi et al., 2017; Urabe et al., 2022; Zhang et al., 2022a)
Fluorescent Single wavelength Ca^{2+} Indicators	Fluo-8, Cal-520, Calbryte 520, Mag-Fluo-4 AM	Plate Reader CCD Camera Fluorescent microscopy	(Bo et al., 2020; Kielbik et al., 2020; Rossi and Taylor, 2020; Zhang et al., 2022b)
Fluorescent Ratiometric $[Ca^{2+}]i$ Indicators	Fura-2, Fura-8, Indo-1, CalRed R525/650, Fluo-3, Fluo-4, Calcium Green-1	Plate Reader CCD Camera Fluorescent microscopy, Confocal microscopy	(Barreto-Chang and Dolmetsch, 2009; Diszhazi et al., 2021; Wang et al., 2000)
Fluorescent-tagged pharmacological agents	Fluorescent thapsigargin SERCA pumps inhibitor	Plate Reader CCD Camera Fluorescent microscopy	(Hua et al., 1995)
Luminescent Ca^{2+} Indicators	Aequorin, Coelenterazine EGFP-Aequorin	Luminometry	(Aulestia et al., 2018; Pulli et al., 2015)
Luminescent Ca^{2+} Chelator	TPEN	CCD camera Luminometry	(Cui et al., 2021)
Secreted Gaussia luciferase (GLuc)–based ER calcium-monitoring proteins (SERCaMPs)	SERcAMP	Plate Reader Luciferase assay	(Henderson et al., 2014)
Fluorescent GECIs for ER lumen ER membrane Golgi apparatus Mitochondria	YC3er, YC4er, YC3.3er, DIER, TIER, GCEPIA1-$SNAP_{ER}$ GCaMP6f-T/J GT-YC3.3 YC2.mt, YC2.1mt, YC3mt, YC4mt	Förster resonance energy transfer (FRET) Confocal Microscopy One-Photon and Two-Photon Microscopy	(Hossain et al., 2018; Luo et al., 2019; Suzuki et al., 2016; Zhang et al., 2021)

Figure 1

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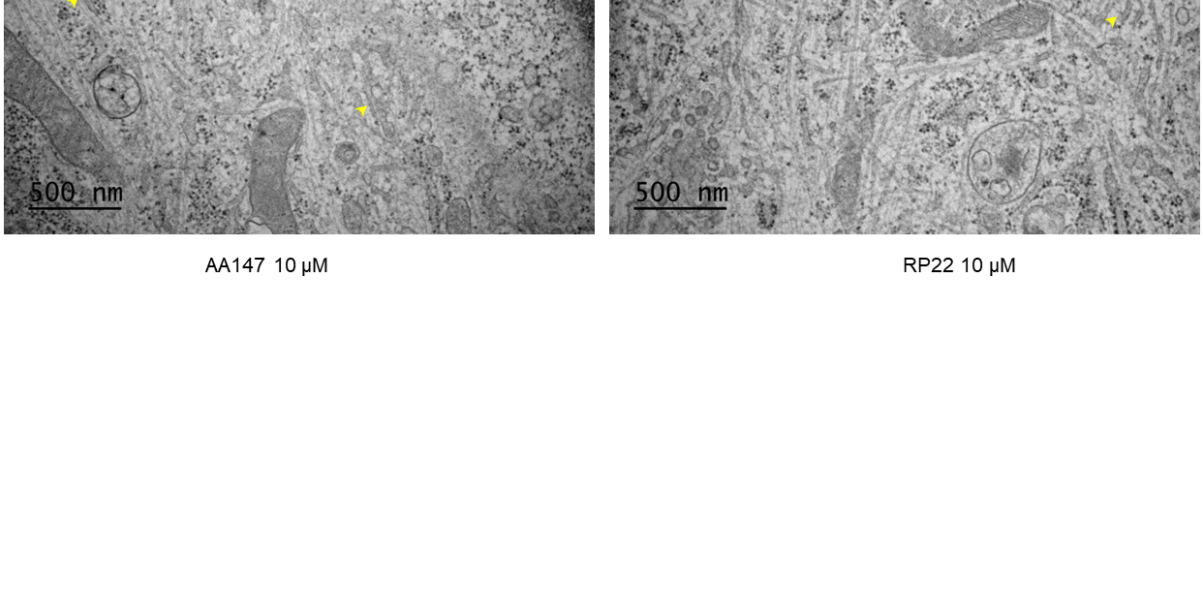

AA147 10 µM

RP22 10 µM

Figure 2

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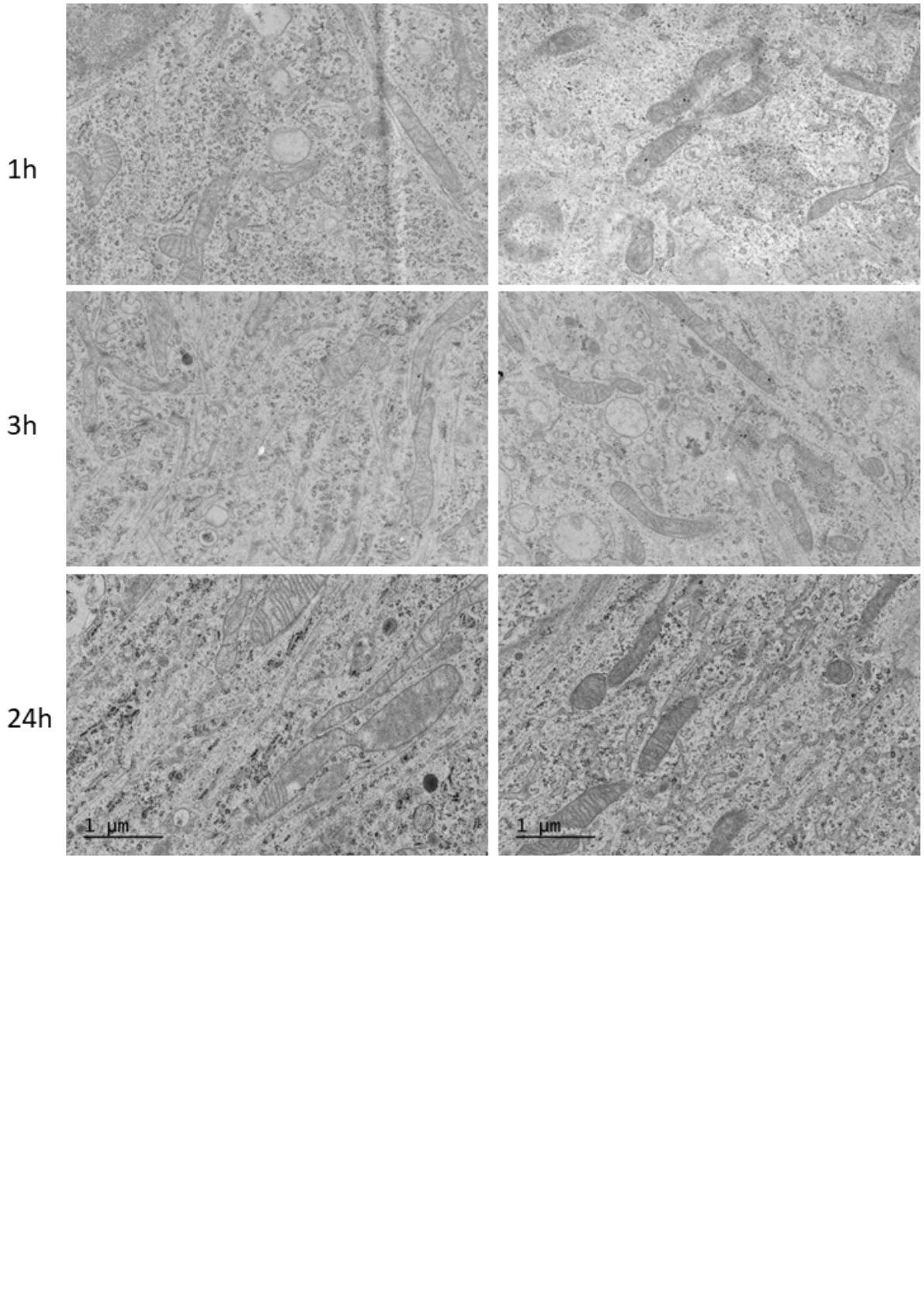

Figure 3

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