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FORUM REVIEW ARTICLE

Neuronal Calcium Homeostasis and Dysregulation

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Abstract

The calcium ion (Ca^{2+}) is the main second messenger that helps to transmit depolarization status and synaptic activity to the biochemical machinery of a neuron. These features make Ca^{2+} regulation a critical process in neurons, which have developed extensive and intricate Ca^{2+} signaling pathways. High intensity Ca^{2+} signaling necessitates high ATP consumption to restore basal (low) intracellular Ca^{2+} levels after Ca^{2+} influx through plasma membrane receptor and voltage-dependent ion channels. Ca^{2+} influx may also lead to increased generation of mitochondrial reactive oxygen species (ROS). Impaired abilities of neurons to maintain cellular energy levels and to suppress ROS may impact Ca^{2+} signaling during aging and in neurodegenerative disease processes. This review focuses on mitochondrial and endoplasmic reticulum Ca^{2+} homeostasis and how they relate to synaptic Ca^{2+} signaling processes, neuronal energy metabolism, and ROS generation. Also, the contribution of altered Ca^{2+} signaling to neurodegeneration during aging will be considered. Advances in understanding the molecular regulation of Ca^{2+} homeostasis and how it is perturbed in neurological disorders may lead to therapeutic strategies that modulate neuronal Ca^{2+} signaling to enhance function and counteract disease processes. *Antioxid. Redox Signal.* 14, 1261–1273.

Introduction

The extracellular free calcium (Ca^{2+}) concentration typically is 1.2 m*M*, whereas the typical resting cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) is approximately 100 n*M*. This 10,000-fold concentration gradient makes Ca^{2+} special when compared with the more abundant cations Na^+ and K^+, as it leads to a significant increase in $[Ca^{2+}]_{cyt}$ after depolarization, whereas influx of Na^+ and efflux of K^+ significantly affect the membrane potential, but cause only relatively minor changes in cytosolic ion concentration. During evolution, a plethora of Ca^{2+}-binding proteins and Ca^{2+}-dependent signaling pathways have arisen, making Ca^{2+} a key element that can relay information about the plasma membrane potential to the biochemical and metabolic machinery of a cell. While Ca^{2+} regulation is essential to any cell, it is obvious that in excitable cells the connection between electrochemical ion gradients and biochemical regulatory pathways is of special importance. One prime example of this connection is in myocytes, where upon depolarization and increased $[Ca^{2+}]_{cyt}$ the protein troponin C binds Ca^{2+} to initiate the protein conformation and protein–protein interaction changes that result in myocyte and muscle contraction (79). Coordinated contraction is the main function of muscles and the structure of myocytes (syncytial cells with triads of transverse tubules, sarcoplasmic reticulum, and contractile proteins) is clearly organized for excitation-contraction coupling. The main function of neurons is information processing (or integration) and transmission to effector cells that mediate behavioral responses (learning and memory, body movements, emotional responses, etc.). A typical neuron consists of complex dendritic arbors that receive electrochemical (synaptic) inputs from other neurons, a cell body (where genes are housed), and an axon that transfers signals to postsynaptic cells (neurons or muscle cells). In this (simplified) view of neurons, Ca^{2+} serves multiple complex and integrated functions, including the control of dendritic responses (morphological and functional) to neurotransmitters, signaling to the nucleus to regulate gene expression, and initiation of neurotransmitter release from presynaptic axon terminals.

Ca^{2+} influx into dendrites and the soma (cell body) is largely dependent on presynaptic neurotransmitter release and the membrane potential. The latter is mainly controlled by Na^+ and K^+ channels (37). Ca^{2+} is therefore a (second) messenger that transfers signals within the cell in response to membrane depolarization, thereby relaying information on neuronal activity status both locally (in a dendritic spine, for example) and globally (to initiate widespread changes in energy metabolism, for example) within the neuron. A major function of Ca^{2+} is therefore to regulate activity-dependent signaling. It is reasonable to assume that Ca^{2+} signaling helps a neuron adapt to its activity-dependent requirements and, with feed-back and feed-forward mechanisms, strengthens relevant synaptic connections, eliminates irrelevant

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connections, and avoids overexcitation. In these ways Ca^{2+} plays pivotal roles in controlling neuronal excitability. Ca^{2+} functions as a key regulator of electrochemical signaling, not only within individual neurons, but also among large populations of neurons that comprise neuronal networks.

The cost for extensive neuronal Ca^{2+} signaling is increased energy demand, as all Ca^{2+} that enters a neuron must be removed from the cytoplasm by ATP-dependent membrane Ca^{2+} "pumps" in order to maintain Ca^{2+} homeostasis. Therefore, Ca^{2+} signaling and excitability mutually regulate each other such that an optimal neuronal activity level is achieved for a given metabolic capacity. During aging and in pathological conditions, Ca^{2+} signaling and homeostasis will be affected and may thus change a neuron's excitability, which will in turn affect network activity and metabolism (47). As will be described below, neuronal Ca^{2+} homeostasis is altered during aging and in neurodegenerative conditions, and uncontrolled Ca^{2+} signaling may also actively contribute to neurodegenerative processes.

Because of the enormous database on the topic of Ca^{2+} in neuronal plasticity and disease, it is beyond the scope of any review to give an exhaustive overview on all Ca^{2+}-dependent signaling cascades. Instead, in this article we focus on mitochondria and endoplasmic reticulum (ER) to illustrate how neuronal activity, energy metabolism, and Ca^{2+} homeostasis are kept in balance and how this balance may be affected during aging and in neurodegenerative processes.

Ca^{2+} and Mitochondria

The main function of mitochondria is to produce high energy intermediates (NADH and ATP) through the tricarboxylic acid (TCA) cycle (Krebs cycle) and oxidative phosphorylation. Mitochondria also function as a Ca^{2+} buffer and are the main source of reactive oxygen species (ROS) (77). Mitochondria perform many more functions, but here we focus on the interrelationships between mitochondrial Ca^{2+} homeostasis, energy metabolism, and oxidative stress in the contexts of synaptic plasticity and neurodegenerative disorders.

Ca^{2+} Signaling and Energy Homeostasis

The TCA cycle feeds the electron transport chain, which in turn converts oxygen (O_2) to water and pumps protons from the mitochondrial matrix to the mitochondrial intermembrane space to generate the proton gradient. The proton gradient then is used by the F_0F_1-ATPase to generate ATP from ADP, and the adenosine nucleotide translocase (or transporter) (ANT) transports ATP from the matrix to the intermembrane space in exchange for ADP. Because proton pumping creates an inside-negative membrane potential, Ca^{2+} as a cation will tend to accumulate in the mitochondrial matrix (*i.e.,* the Ca^{2+} electrochemical gradient is the main driving force of Ca^{2+} influx in to the mitochondrial matrix) (77). The conductance of the uniporter is increased by high $[Ca^{2+}]_{cyt}$ and low ATP/ADP ratios (*i.e.,* in situations of high energy demand) but is low in situations of low $[Ca^{2+}]_{cyt}$ and high ATP/ADP ratios and may also depend on the mitochondrial membrane potential (1, 42, 46, 51, 56, 77). This mechanism ensures the unidirectional mode of transport. Increased mitochondrial matrix Ca^{2+} concentration ($[Ca^{2+}]_{mit}$) will increase activity of the three enzymes of the Krebs cycle: pyruvate dehydrogenase (indirectly via phosphatase activation), isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase (by binding directly to the enzyme), as well as the F_1F_0-ATPase, thus causing increased production of ATP and NADH (20, 21, 68). This is an elegant mechanism in which Ca^{2+} functions as an indicator of increased energy demand that signals to the mitochondria.

The following consideration outlines why mitochondrial buffering can be regarded as a mechanism that limits the maximal rate of O_2 consumption required to maintain Ca^{2+} homeostasis. Pumping out one Ca^{2+} ion from the cytoplasm across the plasma membrane or the to the endoplasmic reticulum (ER) membrane costs one ATP molecule, either directly via activity of the plasma membrane Ca^{2+} ATPase (PMCA) or the sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA), or indirectly by activity of the Na^+–Ca^{2+} exchanger (NCX) which exchanges three Na^+ for one Ca^{2+} and then the three Na^+ are pumped out by the Na^+/K^+ ATPase at the expense of one ATP. To restore the ATP level, three protons must be pumped by the ETC. If Ca^{2+} enters the mitochondrial matrix, this comes at the expense of only two protons (*i.e.,* 0.67 ATP molecules). Thus, mitochondrial Ca^{2+} buffering requires fewer protons to be pumped and accordingly less O_2 needs to be consumed. This is likely an important contribution to maintain energy homeostasis in neurons in situations of repeated axon potential generation, when cation influx and consequently ATP demand and O_2 consumption reach high levels. Importantly, maintenance of high ATP levels and a high ATP\ADP ratio is critical for neurons. If compared with a scenario where all Ca^{2+} is extruded through Ca^{2+} ATPases, mitochondrial Ca^{2+} buffering blunts the peak rate of O_2 that needs to be consumed in a situation of high ATP demand. This is important as neurons have high baseline O_2 consumption rates and only a limited potential to increase their O_2 consumption rate. While peak O_2 consumption is reduced, total O_2 consumption is in fact increased, as the extrusion of Ca^{2+} from the mitochondrial matrix to the cytosol, for example, by the mitochondrial sodium calcium exchanger (NCX) requires an extra proton to be pumped before Ca^{2+} can be extruded from the cytosol through Ca^{2+} ATPases. However, Ca^{2+} extrusion from mitochondria will occur at a timepoint when $[Ca^{2+}]_{cyt}$ is back to baseline levels and ATP demand and O_2 consumption are low.

Ca^{2+} is extruded from the matrix through the mitochondrial NCX (3 Na^+ for 1 Ca^{2+}) (48, 77) and is also believed to leave the mitochondrial matrix through a mitochondrial Ca^{2+} proton exchanger (2 or more H^+ for 1 Ca^{2+}) (95), and also through transient opening of the mitochondrial permeability transition pore (mPTP) (41) (Fig. 1). Each mechanism will directly or indirectly reduce the proton gradient and thus come at the expense of ATP. One interesting aspect is that the long sought after molecular identities and compositions of all three efflux pathways and the Ca^{2+} uniporter remain unclear despite intense search. This is remarkable in the era of genomics and proteomics. It may therefore be possible that, for example, sodium–calcium exchange and proton–calcium exchange are not performed by single molecules specifically dedicated to this task, but rather by proteins or protein complexes that have other primary tasks but have additional, as yet unrecognized capabilities in Ca^{2+} transport. Of note is that the maximal transport kinetics of mitochondrial sodium–calcium and proton–calcium exchange are slow compared

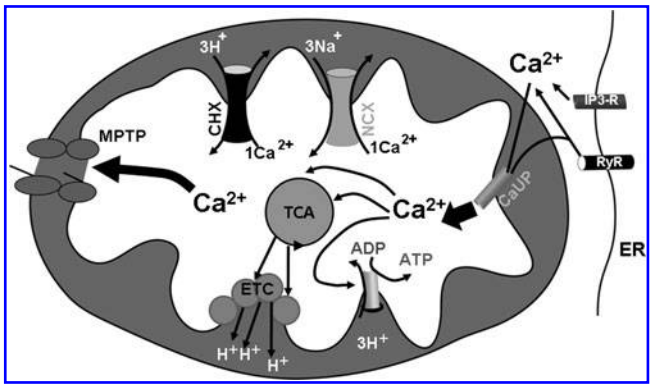

FIG. 1. Mechansims of mitochondrial Ca^{2+} influx and efflux. Ca^{2+} enters the mitochondria through the Ca^{2+} uniporter (CaUP). Close apposition of ER Ca^{2+} release channels and Ca^{2+} uniporter are likely to result in enhanced mitochondrial Ca^{2+} uptake. Inside the mitochondrial matrix, Ca^{2+} can increase the activity of dehydrogenases of the tricarboxic acid cycle (TCA), leading to enhanced feeding of the electron transport chain (ETC) and increased transfer of protons to the intermembrane space. Mitochondrial Ca^{2+} also activates the F1F0 ATPase to produce more ATP. Ca^{2+} is extruded from the mitochondrial matrix through a sodium-dependent mechanism (sodium calcium exchanger, NCX) and a membrane potential-dependent mechanism (calcium proton exchanger, CHX), as well as the permeability transition pore (mPTP). Note that the molecular identities of CaUP, mitochondrial NCX and CHX are unclear.

with the Ca^{2+} uniporter (96, 97). This would make mitochondria prone to Ca^{2+} overload. The existence of an additional high conductance Ca^{2+} efflux mechanism thus seems to be a physiological necessity. The mPTP fulfills all requirements for such an efflux route. Notably, mPTP opening is enhanced by high $[Ca^{2+}]_{mit}$. However, most of our knowledge of the mPTP stems from experiments with isolated mitochondria in pathological conditions (*e.g.*, Ca^{2+} overload or apoptosis). Our knowledge of mPTP functions and modes under physiological conditions is sketchier. Since a permanent mPTP opening is incompatible with continued mitochondrial ATP production, under physiological conditions transient opening of the mPTP has been suggested. But most of the evidence for such mPTP flickering stems from experiments with pharmacological mPTP inhibitors such as cyclosporin A or bongkrekic acid and thus is indirect and prone to artifacts (6–8, 36, 80). Also, the classical models for the molecular composition of the mPTP have been called into question through recent studies. For example, genetic ablation of the proposed mPTP component voltage-dependent anion channel (VDAC) did not result in any observable deficit in mPTP formation or resistance to apoptotic stimuli (2). Genetic ablation of the mPTP regulator and molecular target of cyclosporine A, cyclophilin D, resulted in a reduced sensitivity of mitochondria to oxidative stress and Ca^{2+} overload but not to apoptotic stimuli (3, 4, 65, 82). Cyclophilin D, therefore, may regulate the mPTP also under physiological conditions in a Ca^{2+}- and ROS-sensitive manner (see section on Ca^{2+} and ROS for details).

It should be noted that mitochondrial Ca^{2+} influx in itself is not a detrimental event; rather it is an essential step to stimulate mitochondrial ATP production. Therefore (periodic) mitochondrial Ca^{2+} influx is desirable. If a time delay is assumed between mitochondrial Ca^{2+} influx and efflux and in the kinetics of Ca^{2+}-dependent TCA cycle activation and deactivation, a model can be generated where periodic cytosolic Ca^{2+} influx will cause a periodic (but somewhat more protracted) mitochondrial Ca^{2+} influx, which in turn will lead to a nearly sustained enhanced activity of TCA cycle and oxidative phosphorylation and thus ensure high ATP and NADH levels (8, 35, 47) (Fig. 2). Such a model would be compatible with the observed beneficial effects of exercise or cognitive stimulation for mitochondrial and antioxidant function. Under pathological conditions with impaired mitochondrial capacity or low ATP levels, mitochondrial Ca^{2+} may pose more of a challenge to a neuron. But because Ca^{2+} is critical for activation of the TCA cycle and oxidative phosphorylation, inhibiting Ca^{2+} influx into mitochondria to reduce mitochondrial ROS generation will likely compromise energy production. Reducing $[Ca^{2+}]_{cyt}$ or scavenging ROS seems a more sensible approach.

Ca^{2+} and ROS

While the link between Ca^{2+} and energy homeostasis has been revealed in considerable molecular detail, the connection between ROS generation and Ca^{2+} is less well understood at the molecular level. The main (but not only) sources of ROS in the mitochondria are complexes I and III of the electron transport chain. The default reaction of these complexes is to transport electrons to the next link in the chain (*e.g.*, ubiquinone for complex I). Occasionally, however, the electron will be transferred onto an O_2 molecule, thereby generating one superoxide anion free radical ($O_2^{\cdot-}$). $O_2^{\cdot-}$ is a reactive membrane-impermeable ROS; it can be converted to H_2O_2 spontaneously or by manganese superoxide dismutase (SOD) in the mitochondrial matrix and copper/zinc SOD in the cytosol. H_2O_2 can then be converted by catalase (to H_2O and O_2)

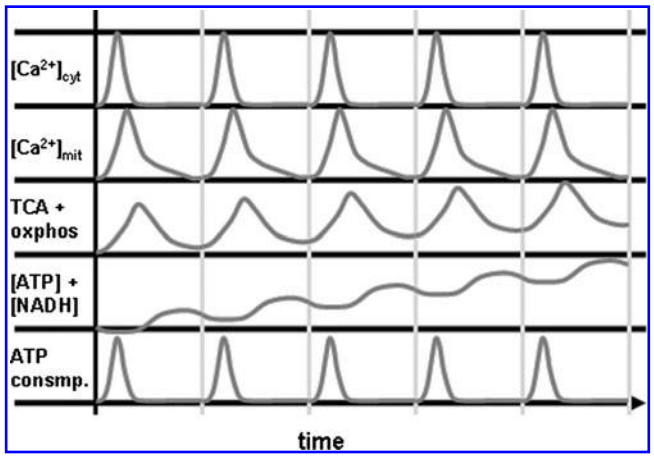

FIG. 2. Model for kinetics of mitochondrial Ca^{2+} flux, metabolism and energy levels. If a kinetic delay is assumed between $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$, then activity of the TCA cycle and oxidative phosphorylation will outlast the increase in $[Ca^{2+}]_i$ and cytosolic ATP demand, resulting in slightly higher levels of ATP and NADH at the end of a $[Ca^{2+}]_i$ transient. In this model, repeated $[Ca^{2+}]_i$ transients with the right frequency can therefore result in higher levels of ATP and NADH.

or peroxidases (to H_2O). Both SOD and catalase have a very high V_{max} that is essentially diffusion rate limited, and they do not need electron donors (12, 25, 58). Thus, the location of these enzymes near the source of ROS generation is as important as total enzyme availability. Peroxidases can detoxify H_2O_2 and other peroxides (ROOR), making peroxidases important cytoprotective enzymes. They require electron donors, such as glutathione in the case of glutathione peroxidases (9, 60, 70). Oxidized glutathione in turn is reduced by glutathione reductase at the expense of NADPH and so peroxidase-mediated detoxification is energy dependent. The rate of aberrant electron transfer in mitochondria has been estimated to be approximately 0.4% (53), but this estimate is based on measurements made in isolated mitochondria or in non-neuronal cells. It is generally accepted that $O_2^{\cdot-}$ generation increases if the proton gradient is high and electron transport occurs against a high energy gradient (therefore uncoupling proteins may reduce $O_2^{\cdot-}$ generation). Increased O_2 consumption by mitochondria naturally also will lead to more $O_2^{\cdot-}$ production (assuming a stable rate of 0.4% of aberrant electron transfer). At the same time increased TCA cycle activity will lead to increased NADH levels which in energized mitochondria can be used by nicotinamide nucleotide transhydrogenase to reduce $NADP^+$ to NADPH. NADPH can then reduce oxidized glutathione, which in turn can be used by peroxidases (40). Another mechanism that links increased TCA activity with increased NADPH production is via mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (40). An increase in $[Ca^{2+}]_{mit}$ will thus simultaneously increase $O_2^{\cdot-}$ generation and reducing equivalents. From a quantitative perspective the increase in reducing equivalents can compensate for the increased production of ROS. It can also be assumed that because of their high V_{max} SOD and catalase have reserve capacities to deal with an increase in ROS generation.

ROS Accumulation in Aging

Some ROS that escape the antioxidant system may activate cellular signaling pathways involved in a range of functions including cell survival and even synaptic plasticity (45, 93). Importantly, ROS, through oxidation and (subsequent) glutathionylation of thiol groups, also modulate kinetics of key proteins in ER and mitochondrial Ca^{2+} homeostasis, for example, mPTP, SERCA, inositol-3-phosphate receptors (IP_3-R), and ryanodine receptors (RyRs) (19). However, ROS may also react with lipids, proteins, or nucleic acids, leading in many instances to impaired biological function of the respective molecules. A large body of evidence has shown that such oxidative modifications of molecules (*e.g.*, hydroxynonenal, carbonylated proteins, 8-oxoguanine) accumulate during aging in mice as well as human and nonhuman primates in most tissues, including the brain (53). Studies in transgenic mice also indicate that the levels of antioxidant enzymes correlate negatively with the amount of ROS damage (64), and an increase in molecular oxidative modifications is found in most neurodegenerative diseases (23, 75, 85). For neuronal mitochondria, oxidative damage means less efficient ATP and NADH production, a reduced maximal respiratory capacity (reserve capacity), and likely an increased rate of $O_2^{\cdot-}$ generation (particularly if complex I is damaged by oxidative stress). Because Ca^{2+} entry into the mitochondria is dependent on the proton gradient, it is likely that the mitochondrial capacity to buffer Ca^{2+} decreases with increased oxidative damage. Such a gradual increase in ROS-mediated mitochondrial damage in neurons may first result in compensatory mechanisms. Such mechanisms could involve, for example, prolonged increases in $[Ca^{2+}]_{cyt}$ resulting in increased activation of calcineurin, which in turn dephosphorylates NMDA receptor proteins and thus causes a reduction in Ca^{2+} influx (55). The reduced ion influx would result in lower energy expenditure for ion homeostasis. The price for such a compensatory mechanism would be a reduction in information processing capacity, as maximal NMDA receptor activation would be impaired. In advanced stages of oxidative stress, ATP production may become so impaired that neurons become prone to excitotoxicity during periods of metabolic challenges (*e.g.*, during hypoglycemia) or even just through repeated axon potential firing.

A scenario of life-long accumulation of oxidative modifications resulting in impaired biological function of molecules and tissues is plausible, and constitutes the free radical theory of aging. It should be noted, however, that current evidence suggests that (at least in mice) the free radical theory of aging applies mostly to pathological conditions, and may be less important in determining maximal life span if aging occurs in optimal conditions (78). Also, evidence that antioxidant therapy can in fact improve aging-related declines in cognitive function in mice or humans is sparse (75, 76). Nevertheless, the existing data of increased ROS-mediated alterations in brain cells during aging and in neurodegenerative diseases suggest that ROS-induced impaired mitochondrial function and consequently impaired metabolic efficiency eventually will lead to impaired brain functions. An interesting and yet unresolved question is how neuronal excitability is affected on a network level by compensatory mechanisms that adapt a neuron to its impaired mitochondrial function. A detailed understanding of such mechanisms may lead to interventional strategies that aim at improving network efficiency (31).

ROS in Acute Stress

Less clear than ROS-mediated impairment of mitochondrial function during aging is the question of whether an acute increase in $[Ca^{2+}]_{cyt}$ or $[Ca^{2+}]_{mit}$ causes a lethal increase of ROS. While there is little doubt that in situations such as stroke or seizures, acute dramatic influx of Ca^{2+} will cause increased ROS production, it is not easy to reliably differentiate between ATP depletion and ROS generation as the primary cause for cell death because reduced ATP levels, increased ROS levels, and increased $[Ca^{2+}]_{cyt}$ occur concomitantly and amplify each other. One approach to address this problem is to measure neuronal O_2 consumption. The rationale here is that, in a scenario of ATP depletion, neurons would be expected to maximally increase their O_2 consumption, whereas in a scenario of mitochondrial impairment through ROS-mediated damage, mitochondrial ability to increase O_2 consumption maximally would be impaired. Studies that have used this approach have shown that in glutamate toxicity (the prototypical *in vitro* paradigm of acute neuronal Ca^{2+} overload) O_2 consumption increases maximally (2- to 2.5-fold over baseline), pointing to ATP depletion rather than ROS as a major cause of cell death (30, 69). Also, in the case of a purely ROS-mediated cell death, one would

expect a low level of mitochondrial uncoupling to improve cell survival, as mitochondrial uncoupling lowers the proton gradient and thereby facilitates the flow of electrons through the electron transport chain (ETC), while it will increase TCA cycle activity and thus NADH production. Indeed, several studies have provided evidence that increased mitochondrial uncoupling, induced either pharmacologically or by overexpression of uncoupling proteins, can protect neurons under excitotoxic conditions (14, 50, 57). However, at least in some cases, low level mitochondrial uncoupling in fact accelerates cell death during glutamate toxicity, again pointing to ATP depletion as the more relevant contributor to cell death (43). Moreover, in single neuron measurements it was found that maximal O_2 consumption is reached within 5 minutes after glutamate application (30). While it is not impossible, it would be surprising that in a healthy neuron a doubling of O_2 consumption (and thus ETC activity) for 5 minutes would result in a lethal dose of ROS (assuming a constant rate of O_2^- generation, *i.e.,* 0.4% of total O_2 consumption) that is capable of overwhelming the entire antioxidant system. *In vivo* the situation is different though, especially in conditions of reduced energy availability as occurs in stroke or traumatic brain injury.

During ischemia or hypoxia, ATP levels drop while NADH accumulates together with acyl esters of coenzyme A and carnitine (66). Acyl coenzyme A and acyl carnitine are believed to impair the functional and structural integrity of mitochondria (66). During reperfusion or reoxygenation, some neurons will display NADH hyperoxidation, which is frequently accompanied by peri-infarct depolarizations *in vivo* or hypoxic spreading depression *in vitro* (*i.e.*, prolonged depolarization of neurons starting in the brain regions close to the insult and spreading across the hemisphere) (26, 49). It is in this situation of structural mitochondrial damage combined with low NADH and high ATP demand in the reperfusion phase that the rate of $O_2^{\cdot-}$ generation will be higher than in physiological conditions. In addition, the antioxidant repair capacity is reduced due to a lack of reducing equivalents, as the transfer of hydrogen from NADH to NADP by nicotinamide nucleotide transferase is energetically not favored (40). In such a situation, it is more readily conceivable that acute ROS-mediated cell damage can contribute to neuronal cell death. Distinguishing between ATP depletion and ROS-induced cell damage is more difficult *in vivo* compared to cell culture glutamate toxicity models, because complex cellular interactions and vascular alterations occur in the intact brain. The role of reduced ATP levels, though, should not be underestimated or regarded as a mere end-stage epiphenomenon in any of the conditions that are associated with acute ROS stress. In this respect it is remarkable that the relative contributions of some basic metabolic pathways to neuronal ATP generation and antioxidant potential remain controversial. For example, the relative contribution of neuronal glycolysis and the glial lactate shuttle to ATP generation in neurons at rest and after depolarization are not clearly established (17, 73, 84). The same is true for NADPH production through the pentose phosphate cycle (which competes with glycolysis for glucose-6-phosphate) (10). Obtaining a detailed and quantitative understanding of the relative contribution of each of these pathways to a neuron's energy and redox balance may lead to a rational approach of, for example, dietary interventions that aim at supplying optimal substrates for the brain in physiological and pathological conditions. One such intervention, the ketogenic diet for epilepsy, is a reminder that such approaches are possible in principle, although the underlying mechanisms are poorly understood at the molecular level (59).

It should be kept in mind that outcomes of clinical trials using antioxidant strategies in stroke or cardiac ischemia have been consistently negative so far (22, 72, 83, 94). Reperfusion using the "clot-busting" drug TPA is an approved therapy for both conditions that is likely to increase ROS production. This may indicate that reperfusion-induced ROS generation reaches critical levels at approximately 6 and 3 hours after cardiac and cerebral ischemia, respectively. If this is the case, then it can be assumed that the highest potential of antioxidant therapies in these conditions lies in improving outcome of reperfusion therapy and extending its time window. However, the currently existing clinical trial data do not support this theory either (22, 83). A major challenge for any antioxidant therapy lies in the short half-life of ROS. Thus, in order to be effective, ROS scavengers must be lipid permeable to access ROS sources such as complex I of the ETC. Also, they need to be present at high concentrations because the most common lipid-permeable antioxidant compounds such as vitamin E are not catalysts of ROS conversion, but instead are scavengers that are consumed by free radicals and must therefore be replaced or converted into their reduced form. However, the latter process is typically impaired in situations like ischemia or reperfusion. However, if antioxidants are not efficient enough in scavenging ROS, then the question is, why do they improve outcomes in animal models of stroke and cardiac ischemia (including larger sized animals)? The animal models used may not adequately reflect the human pathological conditions and they certainly do not adequately predict outcomes of clinical trials. It may also be that preserving high ATP levels is a more powerful intervention than reducing ROS levels in human stroke or myocardial ischemia. Supporting such an interpretation is the fact that those therapies that are proven effective treatments in these conditions (reperfusion in both conditions as well as nitrates and ACE inhibitors in myocardial infarction [71, 100]) clearly will affect ATP supply or demand, but have no direct connection to ROS generation or scavenging. Conversely, those studies that looked specifically at ROS inhibitors or at reducing reperfusion injury had negative or mixed results (22).

ROS and mPTP

ROS are strongly implicated in sensitizing the mPTP (44, 54, 102), and ROS have been implicated in physiological cell signaling, rather than just cell damage (32, 39, 86). However, our understanding of the role of ROS in gating the mPTP and regulating Ca^{2+} homeostasis in physiological conditions is still rudimentary. One main reason is that our tools to study both the mPTP and ROS in intact cells (versus isolated mitochondria) are quite limited. From our current knowledge, one might predict that ROS generation would increase in the late phase after a Ca^{2+} challenge at a time point when $[Ca^{2+}]_{cyt}$ has dropped, but $[Ca^{2+}]_{mit}$ is still high, and O_2 consumption and mitochondrial membrane potential are high. The high mitochondrial membrane potential would cause an increase in the rate of $O_2^{\cdot-}$ production that would then cause the transient opening of the mPTP in order to let Ca^{2+} leave the

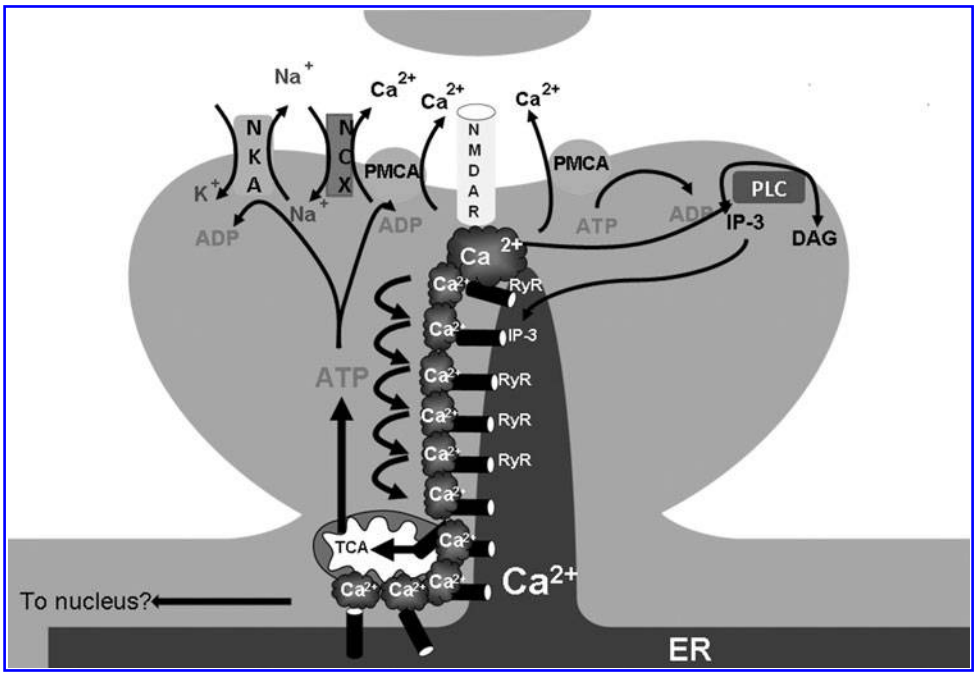

FIG. 3. Ca^{2+}-induced Ca^{2+} release (CICR). A postsynaptic spine is depicted. In CICR, Ca^{2+} enters the cytosol through channels in the plasma membrane. The increased $[Ca^{2+}]_i$ will cause the opening of nearby ryanodine receptors (RyR) in the ER membrane. Additionally, increased $[Ca^{2+}]_i$ levels result in increased IP_3 levels through phospholipase C (PLC) activation. Opening of RyRs and IP_3 receptors causes "hotspots" of $[Ca^{2+}]_i$ that may cause further opening of ER Ca^{2+} channels and also promote mitochondrial Ca^{2+} uptake.

mitochondria in a situation where ATP availability is high. At the same time the mPTP-induced reduction in mitochondrial membrane potential would ensure that $O_2^{\cdot-}$ generation will decrease again, thus preventing excessive build-up of ROS. Cyclophilin D is a good candidate protein to have a modulating function in this model. This autoregulatory scenario for ROS-induced mPTP opening in physiological conditions, however attractive it may seem, is not supported by recent findings with a new mitochondrially-targeted $O_2^{\cdot-}$-sensitive form of yellow fluorescent protein (YFP) (90). In the latter study, mitochondrial ROS production was found to occur in stochastic bursts that were independent of $[Ca^{2+}]_{mit}$. Also the $O_2^{\cdot-}$ bursts (flashes) were caused by mPTP opening, rather than facilitating it and they were inhibited by complex I inhibitor rotenone. At the moment, these findings are not easily reconciled with all of our existing knowledge on mitochondrial ROS generation. They are a timely reminder that more sophisticated tools for the study of mitochondria *in situ* are needed and that the results of such *in situ* studies may well change the current models of mitochondrial function.

Ca^{2+} Homeostasis and the ER

The ER is a Ca^{2+} store within the cell with a typical intraluminal Ca^{2+} ($[Ca^{2+}]_{ER}$) of about 0.5 m*M*. Ca^{2+} is pumped into the ER by SERCA (subtype 1-3). The IP_3-Rs (subtype 1–3), activated by IP_3) and RyRs (subtype 1–3, activated by Ca^{2+}) are the main channels through which Ca^{2+} leaves the ER. In addition, a small amount of Ca^{2+} leaves the ER also in the absence of any ligand-activated Ca^{2+} efflux (as evidenced by the increase in $[Ca^{2+}]_{cyt}$ after SERCA inhibition) through the so-called leak channel. Recently, the presenilin 1 and 2 proteins were found to have ER leak channel properties (88). However, other reports found that presenilin 1 is not a leak channel, but rather enhances the outflow of Ca^{2+} from the ER through IP_3-R (16) and RyR (15). These ER Ca^{2+}-regulating functions of the presenilin protein were reported to be independent of its function as protease in the γ-secretase complex.

Stromal interaction molecules 1 and 2 (Stim1 and 2) have been identified recently as ER calcium sensors (101) that, by binding to the plasma membrane channels Orai (subtypes 1–3), induce storage-operated Ca^{2+} entry (SOCE) (24, 74, 98); these proteins mediate the signal of low $[Ca^{2+}]_{ER}$ to Orai at the plasma membrane and induce the opening of Orai and thus induce Ca^{2+} influx from the plasma membrane. The name Orai was inspired by Greek mythology (24), where Orai are the keepers of heaven's gate. Synonyms are Ca^{2+} release-activated Ca^{2+} channel or Ca^{2+} release-activated Ca^{2+} modulator (CRACC or CRACM, respectively). Although the latter names better describe the protein function, it appears that the name Orai has been adopted by the majority of researchers and therefore will be used in this review.

Although the number of proteins involved in Ca^{2+} homeostasis in the ER is still relatively manageable, their elaborate kinetics and regulatory mechanisms make the regulation of Ca^{2+} homeostasis in the ER very complex. Nevertheless, there are two main aspects to ER Ca^{2+} homeostasis, namely, "calcium-induced calcium release" (CICR) and "storage-operated calcium entry" (SOCE). CICR is seen where a comparatively small increase in $[Ca^{2+}]_{cyt}$ induces the opening of RyRs (RyR1 can also be activated by depolarizing membrane potential in skeletal muscle cells and possibly also in neurons), which will then release more Ca^{2+} from the ER. If the increase in $[Ca^{2+}]_{ER}$ is accompanied by an increase in IP_3 (typically via activation of phospholipase C) then IP_3-Rs will also release Ca^{2+} from the ER (Fig. 3). SOCE describes a situation where the decrease in $[Ca^{2+}]_{ER}$ (typically caused by opening of RyR or IP_3-R) is sensed by Stim1, which will then bind and facilitate the opening of Orai at the plasma membrane, resulting in a further increase in $[Ca^{2+}]_{cyt}$. It is important to note that Stim is a true $[Ca^{2+}]_{ER}$ sensor because the stimulus for SOCE is a decrease in $[Ca^{2+}]_{ER}$ rather than an increase in $[Ca^{2+}]_{cyt}$. However, among the transient receptor potential channels (TRPCs), some are sensitive to $[Ca^{2+}]_{cyt}$ and so can mediate increased Ca^{2+} influx from the plasma membrane in response to increased $[Ca^{2+}]_{cyt.}$ But only if a

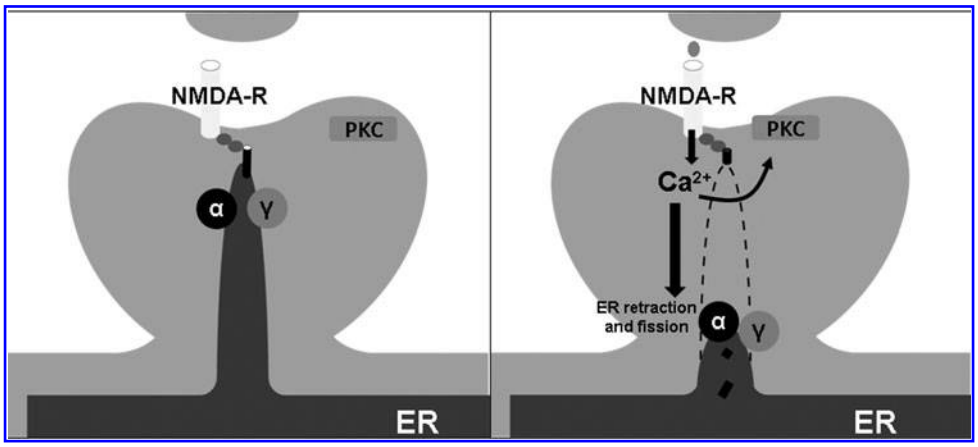

FIG. 4. ER trafficking and Y-secretase. The ER can extend into synaptic spines. Upon activation of NMDA receptors the ER will retract and undergo fission events. Blockade of α- or Y-secretase prevents NMDA-induced retraction of ER membranes from synaptic spines. This suggests that certain, yet unidentified, single transmembrane-domain ER proteins that normally anchor the ER in the synaptic spine are substrates for α- and Y-secretase.

reduction of $[Ca^{2+}]_{ER}$ initiates the Ca^{2+} influx is it called SOCE. Additionally, interactions between TRPCs and Stim1 have also been described (13, 99). It is apparent that all these mechanisms amplify a cytosolic Ca^{2+} signal, but there are also multiple feedback mechanisms that exist to prevent Ca^{2+} overload. Indeed, the different RyR, IP3-R and SERCA isoforms have different kinetics and are differently regulated by $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{ER}$. For example: IP3-R1 is activated by 300–400 n*M* $[Ca^{2+}]_{cyt}$, but is inactivated by higher or lower concentrations; IP3-R2 and 3 are not inactivated by higher $[Ca^{2+}]_{cyt}$ concentrations; nanomolar concentrations of ryanodine typically activate RyRs and micromolar concentrations block RyR opening; and SERCAs are activated by low $[Ca^{2+}]_{ER}$ and the different SERCAs have different kinetics. Accordingly, the precise RyR, IP_3-R and SERCA isoform composition, and the subcellular localization of the ER, are important in shaping the magnitude and kinetics of a cell's CICR and SOCE responses.

The characteristic kinetics and feedback mechanisms of RyR and IP_3-R subtypes have been reviewed previously (89), and will not be detailed here. The ability to act as an amplifier for $[Ca^{2+}]_{cyt}$ elevation makes the ER an organelle that is, at least in theory, capable of generating Ca^{2+} transients in the absence of significant plasma membrane depolarization. Such Ca^{2+} transients have been described experimentally in neurons (18), but generally in neurons, ER Ca^{2+} release follows the membrane potential, as Ca^{2+} transients without plasma membrane changes may dissociate a neuron's Ca^{2+} homeostasis from synaptic input. Because CICR and SOCE have the potential for reciprocal stimulation, it is theoretically possible that such Ca^{2+} transients migrate along the ER and they may even continue through discontinuous ER membranes all the way to the nuclear envelope, where they may increase nuclear Ca^{2+} concentration (89). Obviously this is of great importance in nonexcitable cells (*e.g.*, immune cells), as in these cells Ca^{2+} influx through VGCC is limited. It may also be important in neurons, because nuclear rises in $[Ca^{2+}]$ (but not $[Ca^{2+}]_{cyt}$) are essential for the phosphorylation and activation of the cAMP response element binding protein (CREB) transcription factor. Phospho-CREB will increase transcription of the brain-derived neurotrophic factor (*BDNF*) gene, among other genes. The importance of sustained CREB activity is illustrated by the neurodegenerative phenotype of old heterozygous CREB knockout mice (61). However, it is unclear whether in neurons, in addition to depolarizations associated with axon potential generation, CICR also contributes to relevant increases in nuclear $[Ca^{2+}]$ through Ca^{2+} transients in the absence of such depolarizations. Importantly, translational responses in a neuron can be restricted to a synaptic region. Therefore, in neurons a more obvious role for CICR lies in fine tuning the Ca^{2+} response to synaptic input (33, 89). This also includes the fine tuning of nuclear envelope Ca^{2+} release after full depolarization (33). The precise role of SOCE remains somewhat unclear (see Ref. 11 for a discussion on this latter issue). It has been shown that CICR modulates $[Ca^{2+}]_{cyt}$ after potassium chloride-induced depolarization. Specifically, ER Ca^{2+} release accelerates the rise and slightly increases the amplitude in $[Ca^{2+}]_{cyt}$ and in turn ER Ca^{2+} uptake accelerates the decline in $[Ca^{2+}]_{cyt}$. Such a kinetic profile with steep rise and decline contributes to a Ca^{2+} response that has all-or-nothing characteristics and thus will help to improve the signal to noise ratio in Ca^{2+} signaling.

At the synapse, another mechanism exists that shapes the Ca^{2+} signaling response in an all-or-nothing fashion; it is mediated by calpacitin family proteins, which includes neurogranin, growth associated protein 43, and pep 19 (also known as pcp4) (27, 28). These proteins scavenge calmodulin and prevent calmodulin from binding and activating other proteins such as Ca^{2+}/calmodulin-dependent kinase. Calpacitins are Ca^{2+}-binding proteins, but with a high Kd. Thus, only when $[Ca^{2+}]_{cyt}$ reaches high (micromolar) concentrations will these proteins bind Ca^{2+} and through a conformational change release calmodulin to activate Ca^{2+}/calmodulin dependent kinase and other binding partners. This sudden and massive release of calmodulin upon reaching a critical $[Ca^{2+}]_{cyt}$ led to the name calpacitin, in analogy to capacitors in electricity. Obviously, if CICR modulates $[Ca^{2+}]_{cyt}$ rises to have steep rise and decline kinetics it will further contribute to a clearly defined Ca^{2+} signal that is limited in duration and distribution within the neuron, thus enhancing the calpacitin-mediated Ca^{2+} response so that the neuron can appropriately translate synaptic activity to the biochemical apparatus. If aging or pathological processes lead to an alteration of CICR it will impact activity-dependent Ca^{2+} signaling in a neurons and thus change the adaptive response of the neuron to its activity status with potentially harmful consequences.

Another important aspect of ER Ca^{2+} release is that it helps to create micro-domains of high $[Ca^{2+}]_{cyt}$ near RyRs and IP_3-Rs. These micro-domains have been shown to be important for mitochondrial Ca^{2+} uptake, because the close opposition

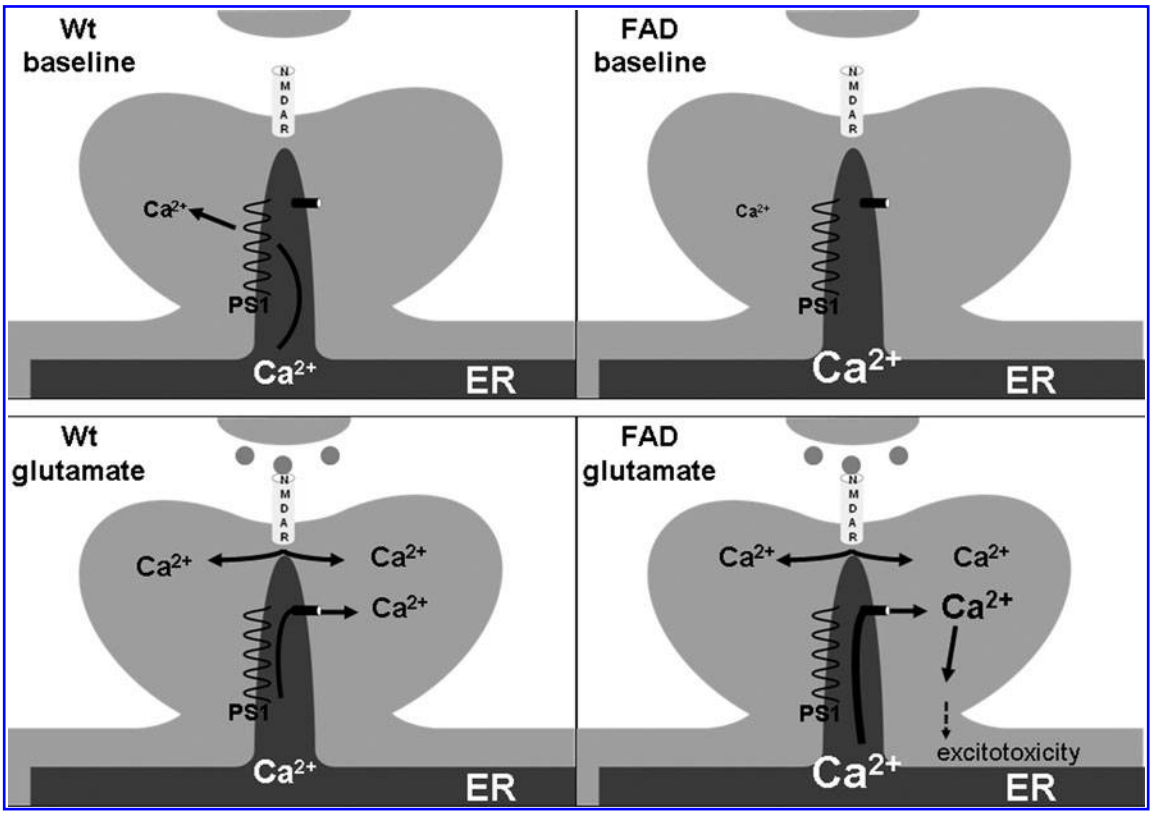

FIG. 5. Presenilin as an ER leak channel. In this model, uncleaved presenilin 1 (PS1) acts as a Ca^{2+} leak channel in the ER membrane. PS1 mutations causing familial Alzheimer's disease (FAD) result in a loss of function of the ER leak channel properties of PS1. This results in increased $[Ca^{2+}]_{ER}$ and increased CICR. Increased CICR can lead to $[Ca^{2+}]_i$ levels that promote excitotoxicity and neuronal cell death.

of ER calcium release sources and mitochondrial Ca^{2+} uniporter ensure that the uniporter becomes activated through high local $[Ca^{2+}]_{cyt}$ that exceeds the average $[Ca^{2+}]_{cyt}$ (77). The high uptake of Ca^{2+} into the mitochondrial matrix will induce increased production of ATP and NADH and thus ensure sufficient levels reducing equivalents and high energy phosphates. For the physical connection between ER and mitochondria (tethering), the protein mitofusin 2 that is expressed on both organelles is required (62) It is conceivable that in neurons CICR from the ER helps to transmit the synaptic input signal to mitochondria that may reside somewhat more distant from a synaptic spine and thus in the absence of CICR may not be exposed to the high micro-domain $[Ca^{2+}]_{cyt}$ that is required for increased ATP generation (Fig. 3). If this is true, then ER trafficking is important in order to make the appropriate connections between ER, synaptic spine, and mitochondria. Similarly, mitochondrial motility is important. Mitochondrial motility has been shown to be controlled by the GTPases Miro 1 and 2, the adaptor protein Milton, and their interaction with kinesin in a Ca^{2+}-dependent manner (81, 91). Notably, the molecular determinants of ER trafficking are not very well understood. ER membranes have been shown to reside in synaptic spines and upon NMDA receptor activation undergo rapid fission and fusion events (52). Also trafficking of ER patches in synaptic spines has been shown to be dependent on PKC, metalloproteinases, and gamma secretase activity (67). Taken together, these studies suggest that neuronal activity and endoproteolytic cleavage of ER resident transmembrane proteins by a sheddase (*e.g.*, α-secretase) and γ-secretase are important in determining the subcellular localization of ER membranes (Fig. 4). The relevant substrates for α- and γ-secretase in these paradigms have not been established and it is of interest to see if either established (*e.g.*, APP, ErbB, neuregulin) or yet undiscovered substrates (STIM1 or 2?) are involved. Thus, presenilins, apart from being implicated as ER leak channel proteins and modulators of IP_3-R gating, via their protease activity in the γ-secretase complex, also regulate ER trafficking, thus being major players in ER Ca^{2+} homeostasis regulation in addition to their well-established role in Alzheimer's disease (AD). Obviously, this suggests a connection between AD and ER Ca^{2+} homeostasis.

The uncleaved presenilin 1 and 2 holoproteins, when recombinantly expressed and added to artificial lipid bilayer membranes, were found to have Ca^{2+} channel properties (88). Importantly, this property was lost in PS1 with mutations linked to AD. From these studies, PS 1 and 2 were implicated as the ER leak channels and the AD-linked mutations were found to increase $[Ca^{2+}]_{ER}$ and CICR from ER. The latter aspect was thought to contribute to neuronal vulnerability in excitotoxicity in AD, a finding that had been observed much earlier in neuronal cell lines expressing mutated PS1 (34, 63). This study implicates that in absence of the ER leak channel the remaining SERCA activity (which is negatively correlated with $[Ca^{2+}]_{ER}$) will continue to pump Ca^{2+} into the ER. Upon opening of RyR or IP3-R, the increased driving force will then

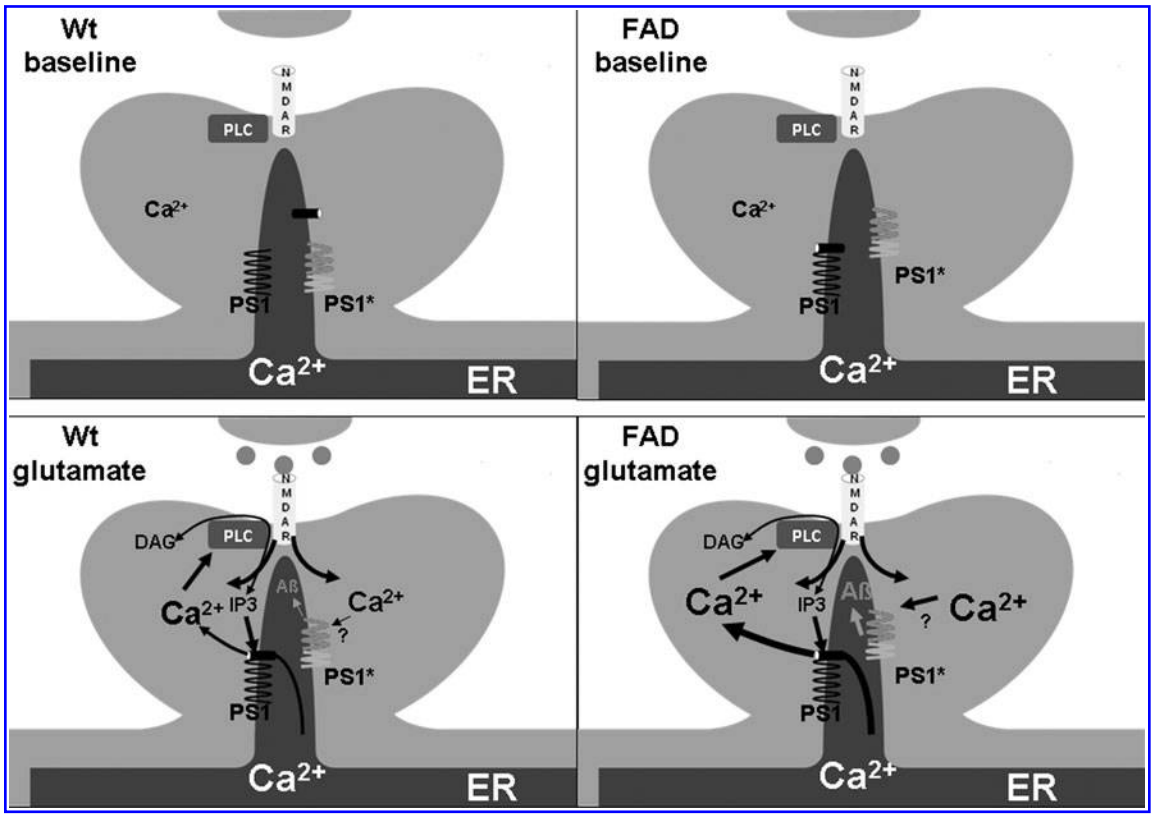

FIG. 6. Presenilin as an IP_3R modulator. In this model, Presenilin 1 (PS1) modulates the gating of the IP_3R in the ER. Wild-type PS1 will slightly prolong the time that the IP_3R is in the open confirmation in situation of CICR. PS1 mutations causing familial Alzheimer's disease (FAD) cause PS1 to "lock" the IP_3R in the open conformation and also increase the flux of Ca^{2+} through the IP_3R, resulting in increased $[Ca^{2+}]_i$ in a situation of CICR. Depolarization and CICR also lead to increased activity of auto cleaved PS1 in the γ-secretase complex.

result in increased $[Ca^{2+}]_{cyt}$ (Fig. 5). In a second publication (16), ER membranes from nuclear envelopes of PS1 infected cells were tested electrophysiologically. Here PS1 was not found to have leak channels properties, but to modulate IP_3-R gating. Specifically, wild-type PS1 was found to slightly increase the duration of the IP_3-R open status, but not to increase the frequency of opening, whereas AD PS1 mutations increased both the channel open probability and the duration of the open time, resulting in IP_3-R being "locked" in the open conformation (Fig. 6). Accordingly, IP_3-mediated increases in $[Ca^{2+}]_{cyt}$ were amplified in cells expressing mutant PS1, in AD patient fibroblasts (29), and in neurons in a mouse model that expresses mutant presenilin 1 (87). In both publications, the effect on ER Ca^{2+} homeostasis was mediated by the uncleaved PS1 holoprotein and thus independent of γ-secretase activity (which requires PS1 endoproteolytic cleavage). However, in the second publication mutant PS1-mediated enhancement of IP_3-R gating resulted in increased γ-secretase activity and increased β-amyloid (Aβ) production (Fig. 6). This raises the possibility that, apart from directly affecting the proteolytic activity of γ-secretase (5), AD PS1 mutations may also act indirectly through enhanced ER Ca^{2+} release.

It is unclear if IP_3-R-mediated ER Ca^{2+} release enhances endoproteolytic cleavage of PS1 or enhances activity of pre-existing γ-secretase complexes. While the literature so far is unclear about the mechanism, it unambiguously concludes that mutant PS1 causes increased CICR responses. Apart from contributing to direct cell death through excitotoxicity, such an alteration may affect synaptic plasticity in more subtle ways. For example, it was found that in mutant PS1 knock-in mice LTP was not impaired, but the effect of cholinergic transmission was reversed (*i.e.*, instead of enhancing LTP, cholinesterase inhibitors caused LTP reduction in these mice). It was found that NMDA receptor currents in these mice were reduced but could be restored to normal levels by intraneuronal Ca^{2+} chelation (92). These results suggest that increased ER Ca^{2+} release in PS1 knock-in mice results in (a compensatory) inactivation/desensitization of NMDA receptors. Similar results were found in neurons of so-called triple transgenic mice (that in addition to the PS1 mutant knock-in express APP and tau protein mutations) (92). Interestingly, Aβ also has been found to inhibit glutamatergic synaptic transmission (through NMDA receptor stimulation and possibly desensitization). Thus, PS1 mutations may cause a reduction of NMDA receptor synaptic transmission by two different mechanisms that may contribute to the cognitive deficits seen in patients with such mutations.

If CICR in patients with PS1 mutations contributes to Aβ production and/or cell death, then apart from Aβ reduction, inhibition of CICR may be a therapeutic option in such cases. Importantly, in humans PS1 mutations are strongly associated with intractable epileptic seizures (much more so than sporadic AD), indicating that these mutations have a profound effect on overall network excitability.

While the connection between ER Ca^{2+} release and AD is obvious in familial cases with PS1 mutations, it is less clear in sporadic AD. The data from PS1 knock-in mice suggest a connection between ER Ca^{2+} release and strength of NMDA currents. If this connection is reciprocal, then it is conceivable that a decline in NMDA-mediated currents during aging will result in a compensatory increase in ER-mediated CICR, which could then cause increased Aβ production through γ-secretase, resulting in further NMDA current reduction by Aβ.

The dual function of presenilin in ER Ca^{2+} homeostasis and its established role in APP processing reveals a link that connects the Ca^{2+} theory of aging with the Aβ theory of AD. Given the connection between ER and mitochondria and the fact that Ca^{2+} cycling through the ER is dependent on ATP, it is also apparent how ROS-mediated mitochondrial alterations may impact ER Ca^{2+} homeostasis. Again, it should be emphasized that while mitochondrial dysfunction, ROS stress, and impaired Ca^{2+} homeostasis feed each other and ultimately will lead to neuronal cell death, in early stages of aging or cognitive impairment, neuronal cell death is unlikely to play a substantive role. On the other hand, it is in the early stages of aging and neurodegenerative disorders that the therapeutic potential is highest. In these early stages the effect of ROS and mitochondrial dysfunction is likely to result in compensatory changes of neuronal transmission that adjust for reduced ATP generation and Ca^{2+} buffering capacity. A precise understanding of these compensatory adjustments is likely to provide us insight into altered synaptic processing and network activity. This may lead to the discovery of new strategies to prevent cognitive decline during aging and in AD.

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Abbreviations Used

$A\beta$ = amyloid beta
AD = Alzheimer's disease
ADP = adenosine-diphosphate
AMP = adenosine-monophosphate
APP = amyloid beta precursor protein
ATP = adenosine-triphosphate
BDNF = brain-derived neurotrophic factor
Ca^{2+} = calcium
$[Ca^{2+}]_{cy}$ = cytosolic calcium concentration
$[Ca^{2+}]_{mit}$ = mitochondrial calcium concentration
cAMP = cyclic AMP
CICR = calcium-induced calcium release
CREB = cAMP response element binding protein
ER = endoplasmic reticulum
ETC = electron transport chain
IP_3-R = inositol-triphosphate receptor
K^+ = potassium
mPTP = mitochondrial permeability transition pore
Na^+ = sodium
NAD^+/NADH = nicotinamide adenine dinucleotide (oxidized/reduced form)
$NADP^+$/NADPH = nicotinamide adenine dinucleotide phosphate (oxidized/reduced)
NCX = sodium calcium exchanger
NMDA = N-methyl-D-aspartate
O_2 = oxygen
PMCA = plasma membrane calcium ATPase
PS1 = presenilin 1
ROOR = peroxide, not further specified
ROS = reactive oxygen species
RyR = ryanodine receptor
SERCA = sarcoplasmic and endoplasmic reticulum ATPase
SOCE = storage operated calcium entry
SOD = superoxide dismutase
Stim1/2 = stromal interaction molecule 1/2
TCA = tricarboxylic acid cylce
VGCC = voltage gated calcium channels
V_{max} = maximal enzymatic reaction velocity
YFP = yellow fluorescent protein

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