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Chapter 3

Cytosolic Calcium Homeostasis in Neurons — Control Systems, Modulation by Reactive Oxygen and Nitrogen Species, and Space and Time Fluctuations

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Additional information is available at the end of the chapter

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1. Introduction

Cytosolic calcium plays a major and central role in neuronal activity and functions both in brain and in peripheral nervous systems, and its sustained alteration is a critical event that leads to neuronal death. On these grounds, it is not surprising that a sustained alteration of intracellular calcium homeostasis in neurons is a point of convergence of the cellular mechanisms underlying many neurodegenerative processes in the brain. Indeed, this has been shown to be the case for the brain's neurodegenerative diseases of higher incidence to humans, like Alzheimer's and Parkinson's, or in the acute neurodegeneration observed in amyotrophic lateral sclerosis, and also for major brain insults, such as excitotoxicity in trauma and ischemia-reperfusion, inflammation and neurotoxicity by drugs and environmental chemicals.

Sustained deregulation of cytosolic calcium concentration have been reported in neuronal apoptosis and necrosis, the two major cellular death pathways involved in brain neurodegeneration. It has been experimentally demonstrated and confirmed by many investigations using cell cultures that a sustained rise of cytosolic calcium concentration in the neuronal soma within the range 0.5-1 μM elicits a rapid necrotic neuronal death, mediated by calcium-dependent proteases activation, like calpains. On the other hand, long-term sustained cytosolic calcium concentrations below 60-70 nM in the neuronal soma promote the slow development of apoptotic neuronal death of neurons in culture [1,2]. Since the central role of calcium in neurotransmitter secretion and neuronal plasticity is also well known, the basal steady state cytosolic calcium concentration in the neuronal soma can be considered as a bioenergetics marker of neuronal activity and survival. We shall then present the major calcium transport

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systems that control the cytosolic calcium homeostasis in the wider space within neurons, i.e. in the neuronal soma. Owing to the large subcellular regionalization of neuronal processes essential for the normal activity of neurons and especially in neuronal signal transduction pathways, we shall also place a particular emphasis in the subcellular compartmentation of these calcium transport systems.

Noteworthy, neurodegenerative processes in the brain also share another common metabolic deviation, namely, that neurons are also exposed to an enhanced oxidative stress in the brain. Using different types of neuronal cultures, many investigators have shown during the last 15 years that the cellular oxidative stress produced by reactive oxygen species (ROS) and reactive nitrogen species (RNS) and a sustained alteration of the intracellular calcium homeostasis are metabolic deregulations usually observed during the early stages of the development of the process of neuronal death and before the cell viability loss induced entry in the irreversible steps characterized by the activation of proteases. In addition, it has been proposed that alterations of the intracellular calcium homeostasis of glial cells can also contribute to inflammation and damage in the brain in neurodegenerative processes [3]. Therefore, a better knowledge of the major molecular pathways contributing to induce the oxidative stress in the brain and the deregulation of intracellular calcium homeostasis in neurons should be expected to lead to the development of novel and more efficient therapies against brain neurodegeneration.

The fact that the most relevant calcium transport systems for the fine tuning of cytosolic calcium homeostasis in neurons have been shown to be molecular targets for ROS/RNS generated in neurodegenerative insults and diseases will be analyzed next in this context. As most of neurotoxic ROS/RNS species react with many intracellular molecules and these species are short-lived within the cells, the extent of chemical modification of each calcium transport system by ROS/RNS is strongly dependent on its relative proximity to the ROS/RNS source. In functional terms, it has been shown by many studies that ROS/RNS can elicit estimulation or inhibition of key proteins of calcium signalling pathways in neurons, and that these effects are strongly dependent on the specific protein, on the ROS/RNS concentration in the micro-environment and on the accummulated dose of ROS/RNS (time of exposure). Therefore, clustering of these systems within subcellular microdomains plays a major role in cross-modulation between calcium and ROS/RNS intracellular signalling, and this point will be specifically addressed thereafter in this chapter.

Furthermore, the accummulated experimental evidences pointing out that there is an intimate cross-talk between calcium and ROS/RNS intracellular signalling pathways are now ovel-whelming, including the modulation of ROS/RNS sources by calcium in neurons and the redox modulation of calcium transport systems. Both, calcium and ROS/RNS intracellular signalling show a clear pattern of local and focalized transients of intracellular concentration (peaks). Therefore, clustering of calcium transport systems responsible of the rise of cytosolic calcium and ROS/RNS sources within the same subcellular microcompartments will generate over-lapping focalized points of high concentration of calcium and ROS/RNS. In addition, this clustering will produce transient and highly focalized cytosolic calcium concentration peaks near the calcium entry points and associated calcium concentration waves owing to the rapid

diffusion coefficient of calcium ions. Thus, we shall discuss the space and time fluctuations of cytosolic calcium concentrations that are known to be produced by the activity of calcium transport systems more relevant for the control of cytosolic calcium homeostasis in the neuronal soma. Finally, the last section of this chapter is focussed in the most relevant calcium buffering systems expressed in neurons and their modulation by oxidative stress, since calcium buffering systems of the neuronal cytosol play a major role to attenuate the local gradients of calcium concentration.

2. Neuronal cytosolic calcium homeostasis is attained by functional coupling between different types of calcium transport systems

A highly efficient spatial and temporal coupling between the activity of transport systems producing calcium entry to the cytosol and those extruding calcium out of the cytosol is a basic bioenergetics need for brain neurons, as they establish many functional synapses and have to maintain and rapidly restore cytosolic calcium in the neuronal soma within the narrow concentration window that allows for neuron survival. Extensive experimental studies carried out during last thirty years have settled the major molecular actors that allow neurons to achieve this goal, see for example the reviews [4-7], and these are schematically presented in the diagram of the Figure 1. Thus, the control of cytosolic calcium homeostasis in neurons is primarily the result of the activity of transport systems at the plasma membrane acting in concert, with the help of calcium transport systems located in intracellular stores, mainly in the endoplasmic reticulum and mitochondria. The concentration gradient of calcium ions across the neuronal plasma membrane in the brain is by far larger than the concentration gradients of other ions involved in the control of neuronal excitability, like potassium, sodium and chloride. In addition, cytosolic calcium binding proteins provide the neurons with buffering capacity to attenuate the peak height of free cytosolic calcium concentration spikes after focal neuronal stimulation by some neurotransmitters or after high frequency repetitive neuronal stimulation [8].

In primary cultures of cerebellar granule neurons, calcium entry through L-type voltage-operated calcium channel (L-VOCC) accounts for more than 75% of the increase of the steady-state cytosolic calcium in the neuronal soma after partial depolarization of the plasma membrane upon raising the extracellular potassium concentration from 5 to 25 mM [9]. The particular relevance of this observation for neuronal survival is highlighted by the fact that the apoptosis of these neurons induced by low potassium (5 mM) in the extracellular medium can be blocked simply by raising the extracellular potassium concentration up to 25 mM [1,10].

Many other experimental data accumulated along the last two decades point out that the transport systems more potent to elicit a fast and sustained increase of cytosolic calcium in neurons are located at the plasma membrane, i.e. ionotropic receptors and VOCC. These calcium transport systems are activated by extracellular stimuli, neurotransmitters or neuromodulators, either directly or indirectly through plasma membrane depolarization. Let us recall here, for example, that high frequency stimulation of neurons by application of electrical

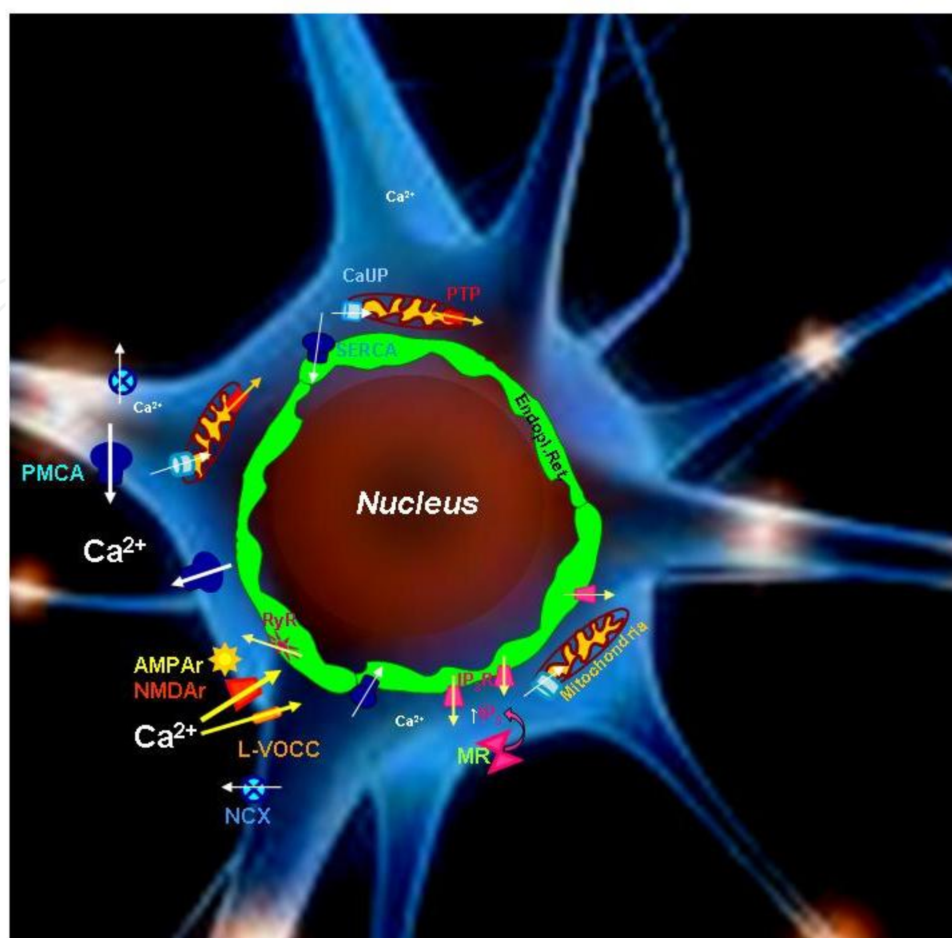

Figure 1. Diagrammatic image illustrating the major calcium transport systems controlling the concentration of cytosolic calcium in the neuronal soma. Yellow and white arrows indicate cytosolic calcium entry and extrusion transport systems, respectively. The thickness of the arrow indicates the relative relevance. Abbreviations: Endopl.Ret., endoplasmic reticulum (green space); NMDAr, NMDA receptor; AMPAr, AMPA receptor; L-VOCC, L-type voltage- operated calcium channel; PMCA, plasma membrane calcium pump; NCX, sodium-calcium exchanger; MR, metabotropic receptor; RyR, ryanodine receptor; IP_3R, IP_3 receptor; SERCA, endoplasmic reticulum calcium pump; PTP, mitochondrial permeability transition pore; CaUP, mitochondrial calcium uniporter.

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depolarizing pulses or of the appropriate neurotransmitter (chemical stimulation) can lead to neuronal tetanic activity. Because of their focalized distribution pattern in the neurons and also because of the large differences in the intensity of calcium currents across activated ionotropic receptors and VOCC, significant calcium concentration gradients between different cytosolic regions of the neuron during normal neuronal activity are expected to develop at least transiently. In contrast, the rise of IP_3 following activation of phospholipase C after stimulation of members of the large family of G-coupled neurotransmitter receptors [5], also located at the plasma membrane, promoted calcium release from intracellular stores displaying calcium spikes of smaller intensity and a more widespread increase of calcium concentration within the cytosol.

Taking into consideration the large number of different chemical molecules that promote neuronal stimulation within the brain and the high frequency of the stimulation events, it is

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wonderful for its simplicity that the concerted action of only a few calcium transport systems can maintain neurons functional and alive for so many years during human lifetime. Why such a simple design? As the evolution selects the living structures and organisms that optimize the use of metabolic energy [11] and the bioenergetics costs for building a complex structural design is always higher than the costs for building simpler structures, probably the answer is: to optimize the use of the metabolic energy in neurons. Let us recall here that the large needs of metabolic energy to continuously restore the electric potential of the plasma membrane of active brain neurons, which is essential for their proper biological functions, would not allow them to develop a safe system for the control of cytosolic calcium homeostasis of a high-bioenergetics cost of maintenance and repair. Noteworthy, maximal energy optimization within the cells can be attained when the coupling between molecules involved in energy transduction makes use of the information or entropic energy stored in subcellular structures, i.e. minimizing stochastic collisional events that dissipate a large amount of energy, and this seems to be the case. For example, the subcellular distribution of the calcium transport systems in neurons enables them to use cytosolic calcium for highly polarized, rapid and specific synaptic responses, and also for more slowly developing adaptative responses, like long term post-synaptic potentiation or depression [4,5]. Furthermore, the different levels of expression of ionotropic and metabotropic receptors in distinct types of neurons allows for differential selectivity and sensitivity in calcium modulation of neuronal threshold excitability, thereby linking regionalization of neuronal responses within the brain structures with the major neurotransmitter pathways.

2.1. The calcium entry systems of the neuronal plasma membrane

All neurons express different types of functional VOCC. On the basis of their unitary conductance, on their rate of inactivation and their subcellular location the most relevant for neuronal calcium homeostasis are the L-VOCC. The L-VOCC unitary conductance has been reported to be in the range of 20-25 pS, while reported unitary conductances for N-, P/Q- and R-type range between 10 and 20 pS, and L-VOCC inactivation kinetics is slower than that of the other VOCC types [12-17]. In addition, L-VOCC are polarised in the neuronal soma and at the conical neck leading to neurite extensions [18], whereas N-, P/Q and R-types of VOCC are largely enriched in the presynaptic plasma membranes and its activation serves largely to elicit neurotransmitter release at the synapses [14,19-21]. On these grounds, taking also into consideration the rate of kinetics inactivation of the P/Q-VOCC, these channels should afford a contribution to the cytosolic calcium homeostasis of the neuronal soma much lower than that of L-VOCC but higher than that of N-, R- and T-types of VOCC. Indeed, using specific channels blockers we have experimentally assessed that the sum of the contributions of non-L-VOCC calcium channels to the cytosolic calcium homeostasis of the neuronal soma of primary cultures of cerebellar granule neurons in a standard Locke's medium with 25 mM K^+ is lower than 20%, while the L-VOCC contribution is 80% or higher (*unpublished results*).

L-VOCC, which are expressed in all neurons, are by far the most relevant calcium channels not only for the tuning of steady-state cytosolic calcium homeostasis in neurons (see above), but also for the overall threshold neuronal excitability, see [22-24]. The L-VOCC family, also

known as Ca_V1, has four subtypes: $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ and $Ca_V1.4$ [25]. $Ca_V1.2$ and $Ca_V1.3$ are expressed in neurons, cardiac and endocrine cells, while $Ca_V1.1$ and $Ca_V1.4$ are specific of skeletal muscle and retina, respectively [26]. In brain, near 80% of L-VOCC belongs to the $Ca_V1.2$ subtype and 10-25% to the subtype $Ca_V1.3$ [27]. It has been reported that inactivation of the gene encoding for $Ca_V1.2$ in the hippocampus and neocortex of mouse ($Ca_V1.2$HKO) leads to a selective loss of N-methyl-D-aspartate (NMDA) receptors-independent long-term potentiation [28]. The activity of these calcium channels is modulated not only by the plasma membrane potential but it is also dependent upon their phosphorylation by protein kinases. Meanwhile the activation of different isoforms of protein kinase C (PKC) has been reported to produce stimulation or inhibition of L-VOCC activity in different cellular types [29], the activation of protein kinase A (PKA) and of calcium/calmodulin-dependent protein kinase II (CaMKII) have been shown to increase the activity of L-VOCC. Moreover, both PKA and CaMKII have been shown to form complexes with L-VOCC subunits. In brain, PKA associates with L-VOCC subunit α1c [30]. L-VOCC subunits α1c and β2 are phosphorylated by PKA [31-34], and this produces an increase of L-VOCC activity. It has been demonstrated that this increase of L-VOCC activity is mediated by phosphorylation of Ser478 and Ser479 of the β-subunit and also by phosphorylation of Ser1928 of the α1c-subunit, as their mutations led to complete elimination of the PKA-induced increase of calcium currents catalyzed by L-VOCC [29,35]. Regarding CaMKII, the amino acids sequence near Thr498 of the L-VOCC subunit β2a shows a high homology with the self-inhibitory domain of the CaMKII and with the binding domain of this kinase in the NR2B subunit of NMDA receptors [36]. Indeed, it has been shown the co-localization within neurons of the L-VOCC ($Ca_V1.2$ type) and CaMKII [37] and also of the L-VOCC subunit β2a with CaMKII, and this has led to the suggestion that the L-VOCC subunit β2a can act as an associated protein of CaMKII *in vivo* [36]. Phosphorylation of L-VOCC by CaMKII takes place not only in Thr498 of the β2a subunit but also in Ser1512 and Ser1570 of the α1 subunit and leads to an increase of the intensity of calcium currents through these channels [36, 38-40]. It has been proposed that the modulation of L-VOCC by CaMKII can be relevant to potentiate the raise of cytosolic calcium concentration in response to hormones and growth factors [41,42]. In contrast, the excessive activation of the L-VOCC ($Ca_V1.3$ type) by CaMKII over-stimulation has been correlated with the loss of dendritic spines in the striatum observed after dopamine depletion in animal models of parkinsonism [43].

The most potent calcium ionotropic receptors present in the neurons of the mammalian brain are L-glutamate receptors of the NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtypes, except those AMPA receptors formed only with GluR2 subunits [44,45], and P_{2X}-purinergic receptors [46]. NMDA and AMPA receptors are present in most fast excitatory synapses in the brain, allowing for neuronal responses in the milliseconds time scale range, and P_{2X}-purinergic receptors display also a widespread distribution in the brain. The more limited distribution in brain of L-glutamate receptors of the kainate subtype, its low ionic selectivity for calcium and the slight calcium currents generated upon its activation compared to those observed upon activation of AMPA and NMDA receptors [47], suggest that they can play at most a secondary role in the tuning of cytosolic calcium homeostasis of a very limited number of brain neurons.

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The L-glutamate receptors are expressed in the vast majority of glutamatergic neurons, and they are present in the major structures of mammalian brain (neocortex, striatum, hippocampus and cerebellum). NMDA, AMPA and kainate receptors are oligomeric integral membrane proteins, being their calcium channel structure predominantly formed by a combination of different, though highly homologous, subunits [48]. Among these receptors, NMDA receptors play an outstanding role in neurosciences, as supported by many experimental evidences in studies of brain development [49], long term post-synaptic potentiation [4] or brain damage after ischemia-reperfusion [44,45]. Three major reasons allows to explain the dominant role of NMDA receptors over AMPA and kainate receptors in the brain: (1) the NMDA single channel conductance is higher than AMPA single channel conductance, 40-50 pS versus ~20 pS [16,50]; (2) their higher affinity for the endogenous agonist L-glutamate, e.g. the EC_{50} for L-glutamate is ~10 μM for NMDA receptors and ~200 μM for AMPA receptors, and (3) the slower desensitization rate of NMDA receptors, e.g. several hundreds of milliseconds for NMDA receptors while it is ~10 milliseconds for AMPA receptors [48]. Nevertheless, the maximal activation of NMDA receptors not only requires the presence of L-glutamate but also co-stimulation by glycine or D-serine in the brain and relief of Mg^{2+} inhibition [48,51]. Both, AMPA and P_{2x} receptors can potentiate NMDA receptor activation in the brain. AMPA receptors co-localization with NMDA receptors allows that plasma membrane depolarization induced by activation of AMPA receptors elicits the relief of Mg^{2+} inhibition of NMDA receptors. Phosphorylation by PKC and CaMKII promotes synaptic incorporation of AMPA receptors during long-term post-synaptic potentiation (LTP), and the latter kinase also enhances the channel conductance of this receptor [52-54]. On the other hand, it has been shown that facilitation of L-glutamate release by P_{2x} activation can lead to a stronger NMDA receptor activation. The calcium channel in the NMDA-receptor structure can be formed by different combinations of subunit 1 (NR1) and one of the isoforms of subunit 2 (NR2A, NR2B, NR2C and NR2D) [55]. The expression of functional NMDA receptors is a relatively slow process during the maturation of neurons [56]. Therefore, in molecular terms there are different isoforms of functional NMDA receptors whose level of expression varies from one type of neurons to another, and also during neuronal maturation. In addition, NMDA receptors are found in synaptic and in extra-synaptic locations [56-58]. As activation of extra-synaptic NMDA receptors can lead to a less focalized increase of cytosolic calcium, the extra-synaptic NMDA receptors are likely to play a role more relevant than synaptic NMDA receptors in the control of cytosolic calcium homeostasis in the neuronal soma. Phosphorylation of NMDA receptors *in vitro* by PKA and by some PKC isoforms increases their activity [59]. The co-stimulation of PKA and PKC elicits the phosphorylation of Ser896 and Ser897 leading to activation of NMDA receptors, while phosphorylation of Ser890 by only PKC leads to a subcellular re-localization of the NR1 subunit of NMDA receptors, which is reverted upon dephosphorylation [60].

2.2. Transport systems that release calcium from intracellular stores

The long-term control of neuronal calcium homeostasis also involves several major calcium transport systems of the subcellular organelles that behave as relevant neuronal intracellular stores, namely, endoplasmic reticulum and mitochondria.

The endoplasmic reticulum Ca^{2+}-ATPase (SERCA) helps to pump calcium out from the cytosol to the endoplasmic reticulum internal space [5], while calcium release from the endoplasmic reticulum involves the activation of IP_3 receptors and/or ryanodine receptors in different neuronal responses and in synaptic plasticity [5,61-63]. Although the neuronal endoplasmic reticulum can accumulate much lower amounts of calcium than the sarcoplasmic reticulum of muscle cells, it is still significant for hippocampal neurons as shown in several studies, see e.g. [4,64]. In these cases, calcium release from the endoplasmic reticulum can sustain a moderate increase of cytosolic calcium, which has been shown to play a significant role in the process of LTP in hippocampal neurons [4,64]. By itself, calcium filling of the endoplasmic reticulum is relevant for neuronal survival to ensure the correct protein folding of many proteins, particularly proteins of the plasma membrane or to be secreted to the extracellular space, as the activity of several endoplasmic reticulum protein-chaperones is dependent on the calcium concentration in the internal space of the endoplasmic reticulum [65]. Depletion of calcium in the endoplasmic reticulum has been shown to elicit the opening of specific calcium channels of the plasma membrane, the store-operated calcium entry (SOCE) [5,66-67]. The presence of SOCE in neurons has been documented during last years [4,68], and its opening elicits a transient increase of cytosolic calcium under neuronal stress conditions to restore the calcium levels of intracellular stores. The inhibition of SERCA by selective inhibitors, thapsigargin or cyclopiazonic acid, is needed to induce the large calcium depletion in the endoplasmic reticulum required for SOCE in experiments with cells in culture. Thus, this process can be seen as a 'rescue call' at the cellular level and operates under conditions of severe energetic depletion of the neurons. Indeed, it is to be recalled here that these channels and in particular the isoforms TRPC-3 and -6 have been also involved in neuronal survival of CGN [69]. More recently, Selvaraj *et al*. [70] have demonstrated that in a mouse neurotoxin-based model of Parkinson's disease, reduced Ca^{2+} influx through transient receptor potential C1 (TRPC1) channels in the plasma membrane of dopaminergic neurons triggers a cell death-inducing endoplasmic reticulum-stress response. These latter results highlighted for the first time the relevance of calcium homeostasis in Parkinson's disease.

In contrast, the large population of neuronal mitochondria can store relatively large amounts of calcium, high enough to elicit a large increase of cytosolic calcium as shown by several studies, see e.g. [71,72]. Nevertheless, the rate of calcium fluxes across the mitochondrial membrane transporters in normal cells is much slower than that measured for the major endoplasmic reticulum calcium transport systems listed above. However, calcium release from mitochondria high enough to promote a large and sustained rise of cytosolic calcium in neurons has been observed only during the development of neuronal cell death, as a consequence of the steady opening of the high permeability mitochondrial transition pore [73]. On these grounds, large calcium release from mitochondria has been proposed to be part of the molecular mechanism that triggers irreversible events in neuronal cell death through calpains activation. On the other hand, the uptake of calcium by mitochondria takes place through a calcium uniporter [71,73], with a rate of uptake in the submicromolar calcium range much lower than the major cytosolic calcium extrusion pathways, namely, PMCA and SERCA in neurons [4,6].

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2.3. The transport systems involved in calcium extrusion from the cytosol

The major plasma membrane calcium extrusion systems, PMCA and Na^{+}/Ca^{2+}-exchanger (NCX) are expressed in all neuronal types. PMCA provides the major extrusion pathway operating in neurons for the maintenance of cytosolic calcium concentrations below the neurotoxic calcium range, i.e. <0.4 μM cytosolic calcium [6,74,75]. As PMCA is active at cytosolic calcium concentrations below 0.4 μM [6,74], neurons must spend a significant amount of metabolic energy (ATP) to maintain cytosolic calcium within the short concentration range which is required for neuronal survival. Therefore, the cytosolic calcium concentration can be considered a key bioenergetics marker of neuronal activity and survival. In contrast, NCX is more potent than the PMCA at cytosolic calcium concentrations ≥0.5 μM [6,76]. On these grounds, NCX can be seen as a safety system to minimise neuronal damage associated with cytosolic calcium ≥0.4 μM, as its activation when cytosolic calcium reaches this range allows neurons to rapidly reset cytosolic calcium to the concentration window that allows neuronal survival, see above. The expression levels of different neuronal isoforms of PMCA undergo significant changes during neuronal maturation [77], and a similar observation has been reported for NCX isoforms [78]. This has been seen as a neuronal adaptative response to the fine set of free cytosolic calcium concentration and control of cytosolic calcium homeostasis, since it has been demonstrated that different PMCA isoforms show different affinity for calcium [79]. On the other hand, although both PMCA and NCX are found in the plasma membrane of the neuronal soma and neuronal dendrites, recent data cast doubt on the current assumption that both PMCA and NCX are homogeneously distributed in the plasma membrane. For example, regulatory effects of actin cytoskeleton have been recently reported on the NCX activity [80], and actin filaments are components of caveolin-rich structures associated with 'lipid rafts' [81].

SERCA, which catalyzes the ATP-dependent calcium uptake by this subcellular organelle, plays only a secondary role as a system for calcium extrusion from the cytosol because in neurons PMCA is a calcium pump more potent than SERCA [4]. The calcium uptake by mitochondria is performed mainly via the Ca^{2+} uniporter driven by the large mitochondrial inner membrane potential [73], although the contribution of an alternate transport system yet ill-defined in molecular terms cannot be excluded under conditions of high frequency of cytosolic calcium peaks [82]. Nevertheless, in neurons the rate of calcium uptake by mitochondria is much slower than the rate of calcium extrusion from the cytosol via the plasma membrane systems, i.e. the PMCA and NCX, and via the SERCA.

3. Compartmentation of calcium transport systems relevant for the control of cytosolic calcium homeostasis in nanodomains of the neuronal plasma membrane and functional implications

Many recent experimental evidences have demonstrated that the calcium transport systems of the neuronal plasma membrane more relevant for the control of cytosolic calcium homeostasis are clustered within focalized nanodomains of a diameter size lower or equal to few

hundreds of nanometers. Lipid rafts of the plasma membrane are dynamic nanodomains of a dimension between 10 and 200 nm [83], which define cellular sub-microdomains of the plasma membrane anchoring caveolins, see e.g. [81], and it has been suggested that caveolin-rich nanodomains associated with neuronal plasma membrane lacking the morphological appearance of "caveola invaginations" can serve to focalize signal transduction in neurons [84]. Indeed, the putative implication of lipid rafts in the regulation of intracellular calcium homeostasis and calcium signalling pathways was already suggested in the 1970´s [85,86], but only during the last decade this hypothesis has been experimentally demonstrated, see [87,88].

Lipid rafts are enriched in cholesterol and sphingolipids [83], including a lipid family particularly enriched in the plasma membrane of neurons: the gangliosides [89], and define nanodomains of the plasma membrane for the anchoring of caveolins, flotillin, actin microfilaments and also an increasingly higher number of palmitoylated or farnesylated proteins, see [81]. The isoform caveolin-1 binds to cholesterol and sphingolipids [90-92], and also promotes the transport of cholesterol from the endoplasmic reticulum to the plasma membrane [93]. These nanodomains are merging as unique platforms for intracellular signalling in neurons, as pointed out in [84,94,95], and their stability is currently rationalized in terms of specific protein/protein or protein/lipid interactions. Noteworthy, as caveolins can act as scaffolding proteins in protein/protein interactions within these nanodomains [96,97], these interactions also bear functional relevance for the protein partners and, therefore, these nanodomains cannot be solely seen as structural elements of the plasma membrane. In this regard, it has been reported that cholesterol depletion with methyl-β-cyclodextrin, a chemical widely used to solubilise lipid rafts, alters the basal current of L-VOCC in foetal mouse skeletal muscle cells and cardiomyocytes [98,99]. Also the calcium-dependent exocytosis in synaptosomes is sensible to the cholesterol content of the plasma membrane [100], and probably one of the best documented functions of caveolins is their implication in the maintenance of intracellular cholesterol homeostasis [101].

Noteworthy, using hippocampal neurons in culture it has been demonstrated the regulation of caveolins expression by L-glutamate [102], and an increased level of caveolins expression has been reported in Alzheimer's disease which has been correlated with the increased level of cellular cholesterol observed in these patients [103]. On the other hand, knockout mice in caveolin-1 have impaired nitric oxide and calcium signalling pathways, displaying severe vascular and pulmonary anomalies and uncontrolled cellular proliferation [104], and caveolins mutations has been associated with muscle disorders and cancer [96]. Moreover, lipid rafts alterations have been reported in a significant number of pathologies [105,106].

The association of the muscle type of L-VOCC with lipid rafts sub-microdomains in cardiomyocytes was established nearly 10 years ago [81,107]. Later, we have demonstrated L-VOCC association with lipid rafts nanodomains in mature primary cultures of cerebellar granule neurons using FRET microscopy imaging [108]. This association of L-VOCC with lipid rafts nanodomains has a major functional relevance for the regulation by protein kinases of the calcium influx through these channels in neurons. First, as noted previously in this chapter within the brain the α1c subunit of L-VOCC forms a complex with PKA [30] and Razani *et al*. [109] have demonstrated the co-localization and direct interaction between the scaffolding

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domain of caveolin-1 and the catalytic subunit of PKA *in vivo* and *in vitro*, respectively. Second, some experimental data have suggested the possibility of direct association of CaMKII with lipid rafts [110], which is consistent with the reported co-localization of Ca_V1.2, the predominant L-VOCC subtype in the brain, and CaMKII [37]. Functional regulation of L-VOCC by lipid rafts is also supported by the modulation of the level of phosphorylation of L-VOCC by cholesterol depletion in cardiomyocytes [99].

Since the two major subtypes of L-VOCC present in the brain, namely Ca_V1.2 and Ca_V1.3, directly interact with many proteins having the PDZ binding domain [111,112], proteins that also bind to the NMDA receptor [113], the association of these receptors with lipid rafts nanodomains is not an unexpected finding. The presence of NMDA receptors in isolated lipid rafts has been shown by different investigators [114-117], and using fluorescence resonance energy transfer (FRET) microscopy imaging their association with lipid rafts nanodomains in mature primary cultures of cerebellar granule neurons has been demonstrated in a recent work of our laboratory [117]. The critical role of proteins with PDZ domains in the association of NMDA receptors with neuronal lipid rafts has been experimentally demonstrated using genetically modified mice, as mutations in the NR2A and NR2B subunits which impair their interaction with PDZ domains led to a reduction of NMDA receptors association with lipid rafts [118]. It has been suggested that the clustering of NMDA receptors in lipid rafts-associated sub-microdomains can potentiate the activation of these receptors, thereby serving as a molecular mechanism for potentiation of the synaptic efficiency in neuronal connections [116,117]. Because AMPA receptor clustering near NMDA receptors plays a key role for NMDA receptor activation and LTP induction, it is of special neurophysiological relevance to note here that the association of AMPA receptors with molecular components of the lipid rafts of neuronal plasma membranes has also been experimentally demonstrated [114,119,120].

The association with lipid rafts of the major systems of the neuronal plasma membrane for extrusion of calcium from the cytosol, PMCA and NCX, has also been experimentally assessed, although to the best of our knowledge only in the case of PMCA this has been reported with neuronal plasma membranes at the time this chapter was written. PMCA association with lipid rafts has been shown using preparations of synaptic plasma membranes [121] and also in primary cultures of rat cortical and hippocampal neurons [122]. Earlier, it was shown that the C-terminal domain of the PMCA interacts with proteins with PDZ domains [123]. Moreover, Jiang *et al.* [122] showed that disruption of lipid rafts domains by chronic depletion of cholesterol elicited a marked decrease of PMCA activity, suggesting that PMCA associated with lipid rafts is more active than PMCA bound to non-raft domains. NCX has been shown to be associated with lipid rafts in the smooth muscle of coronary arteries [124], it has also been shown to be present in membrane fractions of vascular endothelial cells enriched in the lipid rafts markers caveolin-1 and e-NOS [125] and the direct interaction of cardiac NCX with caveolin-3 has been demonstrated by co-precipitation [126].

On these grounds, lipid rafts nanodomains of the neuronal plasma membrane can be seen as microchip-like structures for the fine coupling and control of systems playing a major role in the maintenance of a cytosolic calcium homeostasis within the range that allows for survival and normal functionality of neurons. Because of the relevance of oxidative stress in neurode-

generation it is of utmost importance to note that two enzymatic sources of ROS/RNS have been shown to be also associated with these lipid rafts nanodomains in the neuronal plasma membrane, namely, neuronal nitric oxide synthase (nNOS) and cytochrome b_5 reductase (Cb_5R). Sato *et al.* [127] showed that two domains of the nNOS, the oxygenase and the reductase domains, interact with the scaffolding domain of caveolin-1. More recently, using FRET microscopy imaging our group has shown that nNOS is associated with lipid rafts nanodomains enriched in NMDA receptors and L-VOCC in mature cultures of primary cerebellar granule neurons [117]. Since nitric oxide play a very important role in neuromodulation, this association bears a special relevance as protein/protein interactions regulate the enzyme activity of nNOS as well as define anchoring points for the subcellular location of this protein [127,128]. Indeed, it has been shown that the interaction of nNOS with caveolin-3 in skeletal muscle modulates the catalytic activity of NOS [128]. In addition, previous works of our laboratory have shown that the Cb_5R, whose deregulation at the onset of neuronal apoptosis generates a burst of superoxide anion that stimulates the entry in the irreversible phase characterized by caspases activation [10,129-131], is also associated with lipid rafts nanodomains enriched in L-VOCC and NMDA receptors in mature cultures of primary cerebellar granule neurons [108,130,131]. Moreover, the association with these lipid rafts nanodomains of a source of nitric oxide (nNOS) and of a source of superoxide anion (Cb_5R) point out that these nanodomains may play also a major role in the focalized generation of the harmful oxidant peroxynitrite in the plasma membrane when the neurons are exposed to sustained cellular stress conditions. Let us recall here also that some mitochondria, a widely accepted major ROS-producing subcellular compartment, are also close to the plasma membrane in many neuronal types, because the cell nucleus occupies a large volume of the neuronal soma.

This protein clustering associated with lipid rafts nanodomains of the neuronal plasma membrane is summarized in the Table 1, where proteins of the cytoskeleton typically associated with lipid rafts are also included. Noteworthy, ROS significantly alter the actin polymerisation/depolymerisation dynamics, reviewed in [132]. Because actin microfilaments are part of the structural protein network of proteins associated with lipid rafts nanodomains, ROS are expected to produce a significant distortion of this protein network, like nNOS which has been shown to associate with the neuronal cytoskeleton in synaptic terminals [133]. Indeed, regulatory effects of actin cytoskeleton have been reported on NMDA receptors activation [134], on the distribution of L-type calcium channels in myocytes [135], and on the activity of NCX [80].

Structural elements	Calcium transport systems	ROS/RNS sources	Regulatory kinases
Cholesterol, Caveolins,	L-VOCC,	nNOS and Cb$_5$R	PKA and CaMKII
Sphingolipids, Flotillin,	NMDA and AMPA receptors,		
Actin microfilaments,	PMCA and NCX		
PDZ-binding proteins			

Table 1 Molecules associated with lipid rafts in the neuronal plasma membrane of special relevance for cytosolic calcium homeostasis and ROS/RNS-calcium signalling cross-modulation.

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In spite of the well known relevance of L-glutamate AMPA and NMDA receptors clustering in LTP, the regulation of incorporation and dissociation of proteins in nanodomains or sub-microdomains associated with lipid rafts is still poorly understood and, thus, it is a pending issue. It is to be noted also that knowledge of the time scale range of the clustering dynamics of proteins within these nanodomains is a basic need to properly understand their formation and plasticity, and this is particularly relevant to reach firm conclusions regarding their role as structural or adaptive elements in rapid and slow neuronal responses.

In conclusion, a close spatial location of these calcium transport proteins in the neuronal plasma membrane can also afford a fast and fine tuning of cytosolic calcium concentrations. Moreover, as major redox centers producing ROS are also tighly associated with lipid rafts nanodomains, this compartmentation allows also to rationalize on simple grounds the intimate cross-talk between ROS and calcium signalling in neurons, as well as between oxidative stress and sustained cytosolic calcium deregulation, reviewed in [136,137].

4. Sustained alteration of cytosolic calcium homeostasis in neuronal death

Neuronal survival is extremely dependent of the fine tuning of cytosolic calcium homeostasis, because cytosolic calcium concentration has to be maintained within a relatively narrow window for neuronal survival [1], for example, between 70 and 200 nM for cerebellar granule neurons in culture [9,138]. An overwhelming amount of experimental data reported by many investigators from different countries show that sustained deviations of cytosolic calcium concentration out of this narrow window lead to neuronal cell death. Besides rapid necrotic neuronal death induced by sustained cytosolic calcium concentration higher than 0.4 μM for periods in the minutes time scale range [1,9,44,45,138,139], it has also been shown that apoptotic neuronal death can be induced when cytosolic calcium concentration remains very low for longer periods of time, in the hours time scale range [1,2]. As the extracellular free calcium concentration is approximately 1 mM, this implies that neurons need to sustain a large calcium gradient across their plasma membranes. Owing to the large number of synaptic connections established by neurons in the brain, these cells need to spend a large amount of metabolic energy to maintain their cytosolic calcium homeostasis, because during synaptic activity calcium entry is activated through VOCC and some ionotropic receptors, mainly NMDA receptors. In addition, many neuronal processes are extremely dependent upon cytosolic calcium concentration, such as neurotransmitter secretion and synaptic plasticity [140], neurite growth and sprouting [141] and signalling pathways which mediate the metabolic neuronal responses to a large number of relevant extracellular stimuli [4,5]. Therefore, the cytosolic calcium concentration should be considered a major bioenergetic marker for neuronal activity and survival.

The increase of oxidative stress in brain is a biochemical marker associated with neurodegenerative insults, like ischemia-reperfusion or inflammation, or neurodegenerative diseases of high prevalence and relevance to humans, for example, Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and Huntington's diseases. Many studies have shown that cellular oxidative stress is caused by an imbalance between endogenous antioxidant defences and ROS produc-

tion in favour of the latter, which results in an excessive exposure of cells to harmful ROS/RNS. On the other hand, it is well established now that the calcium transport systems most relevant for the cytosolic calcium homeostasis in neurons are molecular targets for ROS/RNS and that their chemical modification by these reactive species lead to their functional impairment. Indeed, oxidative chemical modifications of these calcium transport systems have been reported to take place *in vivo*. Moreover, many experimental studies reported during last 10 years led to the conclusion that ROS produce a sustained deregulation of cytosolic calcium homeostasis in neurons. For example, neuronal death mediated by calpains activation can be taken as a biological marker of a sustained rise of cytosolic calcium concentration [142-144]. Another examples are provided by the central role of L-VOCC and ROS in the apoptosis induced by low extracellular potassium concentration [2,9,10,129,131,145], and also by L-glutamate excitoxicity-induced neuronal death [44,45,146]. Thus, sustained alterations of neuronal cytosolic calcium are expected to be a convergent cellular mechanism in brain neurodegeneration. Consistent with this hypothesis, alterations of neuronal calcium homeostasis and brain oxidative stress have been reported in the case for the brain neurodegenerative diseases of higher incidence to humans, like Alzheimer's [147,148] and Parkinson's [149,150], or in the acute neurodegeneration observed in amyotrophic lateral sclerosis [146,151], and also for major brain insults, such as excitotoxicity in trauma and ischemia-reperfusion [44,45], inflammation [152,153] and neurotoxicity by drugs and environmental chemicals [139,154].

Most ROS/RNS that are produced in cellular oxidative stress in mammalian tissues have been demonstrated to be strongly neurotoxic to neurons *in vitro*. This is a relatively large list of ROS/RNS, and we shall concentrate in this chapter in those most studied as agents in brain neurodegeneration, namely, superoxide anion, H_2O_2, hydroxyl radicals, lipid hydroperoxides, and nitric oxide-derived ROS, mainly peroxynitrite and nitrogen dioxide. Because of the calcium dependence of the activity of nNOS, the main enzymatic system responsible for the production of nitric oxide in neurons [133], RNS should be expected to play a particularly relevant role as intracellular biomarkers of the level of coordination or deregulation of calcium and ROS signalling pathways in neurons. However, it is still a matter of debate whether *in vivo* all of these ROS/RNS can reach concentrations high enough to act as causal agents or merely as agents that potentiate or accelerate the rate of an ongoing neuronal death process in the brain. Moreover, the analysis and dissection of the chemical reaction pathways of each one of this ROS/RNS is further complicated by the fact that *in vivo* they generate radicalic chain chemical reactions. Therefore, it is critical to identify the major subcellular primary sources of these ROS/RNS in different neurons and in different degenerative processes in the brain, and this is an issue yet to be settled in many cases, as during lasts years the experimental evidences have pointed out that the relative relevance of different ROS/RNS seems to be largely dependent on the neurodegenerative disease or brain insult.

5. Modulation by ROS/RNS of calcium transport systems relevant for the control of neuronal cytosolic calcium homeostasis

ROS and RNS producing oxidative stress to neurons can be generated by neuronal and also by non-neuronal cells, like microglia or endothelial cells of the brain blood vessels. It is to be

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noted that oxidative stress-induced brain degeneration is a relatively slow process, in most neurodegenerative diseases developing in periods of time of years and in acute brain ischemia-reperfusion in a time range from minutes to several days, depending upon the intensity of the oxidative stress insult. Thus, in a brain suffering oxidative stress neurons are exposed for relatively large time periods to either extracellularly and/or intracellularly generated ROS/RNS. Because the extracellular liquid bathing the brain and stem neurons is poorer in antioxidants than the blood, due to the low permeability and high selectivity of the blood-brain barrier, the extracellular antioxidant protection in the brain is notably lower than that of other organs and tissues in mammals. Under these environmental conditions the plasma membrane of neurons, where major calcium transport systems controlling the cytosolic calcium homeostais are located, is particularly sensitive to the oxidative stress generated in the brain by vicinal neuronal and non-neuronal cells. The major ROS/RNS reported to play a significant role in the enhanced brain oxidative stress associated with neurodegenerative diseases and insults like ischemia-reperfusion and inflammation can be split into three major groups: (i) primary biochemical ROS/RNS, i.e. chemical species directly generated by some enzymes or proteins during brain activity in normal or pathophysiological conditions, (ii) secondary biochemical ROS/RNS, chemical species derived by rapid reaction between the primary biochemical ROS/RNS or by systems involved in their detoxification, and (iii) radicalic chain ROS/RNS, chemical radicals involved in the initiation of radical reaction chains or that are largely generated within radical reaction chains.

Superoxide anion is a primary biochemical ROS that plays a key role in the generation of many of the more harmful ROS and RNS detected in the oxidative stress-induced degeneration of the brain. Superoxide anion can be produced by neuronal and non-neuronal cells within the brain. Because of the relatively low permeability to superoxide anion of lipid bilayers [155], extracellular superoxide anion must be largely generated by redox centres of the plasma membrane of neuronal and non-neuronal cells. In glial, macrophages and endothelial cells there are NADPH oxidases of the NOX family, which are under the control of transcriptional antioxidant-responsive elements (ARE), reviewed in [156]. In contrast, we found that in the plasma membrane of neurons the NADH-dependent production of superoxide anion associated with their NADH oxidase activity was nearly ten-fold higher than their NADPH activity [157,158]. Indeed, an overshot of superoxide anion production at the plasma membrane is an early event in the apoptosis of cerebellar granule neurons induced by extracellular K^+ deprivation [10,131], an overshot that we have found to be largely catalyzed by deregulation of cytochrome b_5 reductase associated with plasma membrane lipid rafts sub-microdomains [130,131]. Mitochondria is now widely accepted as the major source of intracellular superoxide anion in oxidative stress-induced neuronal death in cultures *in vitro*, particularly by complexes I and III of the mitochondrial respiratory chain [159]. In addition, non-mitochondrial enzymes that use oxygen as substrate can also become a source of intracellular superoxide anion in neurons, such as the conversion of xanthine dehydrogenase into xanthine oxidase either by direct oxidation and/or by proteolytic activation during oxidative stress-induced neuronal death [160].

Nitric oxide is the major primary biochemical RNS produced in oxidative stress-induced brain degeneration, and although not harmful by itself, its reaction with superoxide anion yields peroxynitrite (a secondary biochemical ROS/RNS), probably the most neurotoxic ROS/RNS generated during oxidative stress-mediated brain neurodegeneration, see e.g. [161,162]. The reaction between nitric oxide and superoxide anion is very fast, such that it is considered a diffusion-controlled chemical reaction due to the very high value of the bimolecular rate constant, (4-7) 10^9 M^{-1} s^{-1} [163]. Peroxynitrite, in spite of its short lifetime within the cells [162], has been shown to be a very harmful ROS/RNS involved in the brain damage produced by ischemia-reperfusion [161], by inflammation and spinal cord injury [164,165] and also in neurodegenerative diseases and aging [166,167]. Peroxynitrite can elicit functional damage of biomolecules and subcellular structures acting either as a potent oxidant ($E^{0'}$ = 1.2-1.4 V) or through the generation of harmful radicals such as hydroxyl and nitrogen dioxide free radicals, reviewed in [162,168]. Due to this, peroxynitrite can produce oxidation of protein cysteines to disulfide bonds, sulfenic and sulfinic acids eventually leading to sulfonic acids, oxidation of protein methionines, nitration of protein tyrosines and lipids, lipid peroxidation, coenzyme Q oxidation, and DNA and RNA oxidation. Because the activation of neuronal nitric oxide synthase requires an increase of cytosolic calcium, peroxynitrite is one of the more harmful ROS/RNS produced in the oxidative stress accompanied by sustained alterations of the neuronal cytosolic calcium homeostasis. Indeed, this has been shown to be the case for the excitotoxic neuronal death elicited by L-glutamate through activation of NMDA receptors [166,169].

ROS/RNS initiating lipid oxidation and peroxidation, i.e. self-accelerating chemical radical chains, are the other group of ROS/RNS playing a major role in brain damage by oxidative stress. Among them, H_2O_2 has required a large attention because is one of the major products generated under conditions that elicit over-production of superoxide anion, as it is a product of superoxide dismutase activity. In addition, intracellular traces of metal ions such as Fe^{3+} or Cu^{2+} can catalyse Fenton-like reactions in neurons, generating hydroxyl radical from superoxide and H_2O_2 [170]. Hydroxyl radical is one of the most potent cytotoxic oxygen radicals, which can attack a large variety of important biomolecules, from small biomolecules such as coenzyme Q or α-tocopherol [171] up to large biomolecules like proteins, RNA and DNA [170,172]. Since hydroxyl radical can be also generated from peroxynitrite decomposition (see above), it turns out that it is a converging point between the oxidative stress pathways involving ROS and RNS derived from nitric oxide. The involvement of hydroxyl radical in oxidative stress-induced neuronal damage has been suggested, for example, in the pathophysiological case of spinal cord trauma [173], amyotrophic lateral sclerosis [174] and Parkinson's disease [170,175].

Lipid ROS are a family of harmful ROS detected in oxidative stress-mediated brain degeneration that also catalyze chemical radical reaction chains. They can be produced as primary biochemical ROS by cyclooxygenases (COX) and lipoxygenases in some brain oxidative stress insults, such as ischemia-reperfusion [176], or Parkinson's disease [177]. Indeed, inhibitors of the neuronal COX-2 isoform have been reported to attenuate brain damage after ischemia-reperfusion [176]. Moreover, the oxidation of dopamine by the microglial COX-1 isoform and

also by COX-2 isoform in the dopaminergic neurons of the substantia nigra has been involved in the pathogenesis of Parkinson's disease [177]. It is to be noted though that lipid ROS are also generated during hydroxyl radical- and hydrogen peroxide-induced lipid oxidation and peroxidation, respectively [170]. In addition, lipid oxidation and peroxidation also release the aldehydes malondialdehyde and 4-hydroxynonenal, which have been shown to be highly neurotoxic compounds [178,179]. Because of the self-propagating properties of lipid radical chains once they are initiated, and also due to the high toxicity for neurons of lipid breakdown compounds released, the possibility of cell rescue after the threshold antioxidant barrier against lipid oxidation/peroxidation is surpassed can be considered negligible. The extent of lipid oxidation marking the 'point of no return' for neurons survival has not been firmly established yet, but it is likely to be at most only a few per cent of the total lipids [180]. On these grounds, an enhanced lipid oxidation should be expected to be a late and largely irreversible step in neuronal death. This view is consistent with the many reports showing that largely damaged brain areas after an ischemia-reperfusion insult display a marked increase of lipid peroxidation.

5.1. Modulation by ROS/RNS of the major calcium entry systems of the neuronal plasma membrane

5.1.1. Voltage-operated calcium channels

As indicated above in the section 2 of this chapter, the L-type are the most relevant VOCC in the fine tuning of the steady state level of cytosolic calcium concentration in the neuronal soma and, thus, in the fine tuning of threshold neuronal excitability [22-24]. L-type VOCC as a primary target for ROS in brain is also supported by the hypoxic up-regulation of these channels, which is mediated by Alzheimer's amyloid peptides [181]. L-VOCC contain two vicinal cysteines at positions 271 and 272 which are involved in their interaction with syntaxin 1A, thereby playing a major role in their regional localization in plasma membrane microdomains [182]. In addition, three cysteines are located in the calcium-pore region (Cys^{330}, Cys^{1383} and Cys^{1396}) [183]. Therefore, L-VOCC contains redox centres that have been shown to react with ROS/RNS in other proteins, for example, in NMDA-receptors (see below).

Studies with neurons in culture have provided ample experimental evidences of direct modulation of L-VOCC by the major ROS/RNS involved in brain ischemia-reperfusion, inflammation and/or neurodegeneration. The L-VOCC antagonist nifedipine has been reported to protect CNS neurons against hydrogen peroxide-induced death, which is mediated by a sustained increase of cytosolic calcium, pointing out activation of L-type VOCC by H_2O_2 [184]. H_2O_2 was shown later to activate recombinant calcium channel α_{1C} subunit stably expressed in HEK 293 cells [185]. In addition, nitric oxide has been reported to induce activation of L-VOCC in hippocampal neurons by plasma membrane depolarization [186] or to inhibit calcium channel gating via activation of cGMP-dependent protein kinases [187]. In contrast, exposure to peroxynitrite has been reported to produce decrease of calcium influx through L-VOCC at low submicromolar doses in rat cerebellar granule neurons in culture and increase of calcium influx through L-VOCC at higher micromolar doses in rat cerebellar granule

neurons in culture [9] and in mouse cerebral cortical neurons [188]. Hydroxyl radicals, a radical produced during the decomposition of peroxynitrite, have been reported to suppress the calcium influx through L-VOCC in mouse cortical neurons [189]. Consistent with these results, dihydropyridine L-VOCC blockers afford protection against neuronal death induced by exposure of neurons *in vitro* to the peroxynitrite-releasing agent SIN-1 [9,190]. Because of the short lifetime and high reactivity of these radicals this is likely to be due to direct chemical modification of L-VOCC, although it is to be noted that this chemical modification is yet unknown. In addition, it has been reported that eicosanoids and ROS generated during arachidonic acid oxidative metabolism also activate L-VOCC [191], and that the lipid peroxidation product 4-hydroxynonenal causes opening of the L-VOCC, resulting in an increase of cytosolic calcium and neuronal death which is prevented by the L-VOCC blocker nimodipine [192]. Direct redox modulation of L-VOCC is further supported by its activation by hydrogen sulphide [138]. Further studies are needed to reach firm conclusions regarding the molecular mechanisms of modulation of different neuronal L-VOCC subtypes by ROS.

Only very scarce experimental studies have been done on the putative modulation of N-, P/Q- and R-type VOCC by ROS/RNS, despite the fact that N- and R-type of calcium channels are blocked by heavy metals such as Pb^{2+} and Hg^{2+} that are likely to interact with thiols [193]. $Ca_V2.2$ (N-type) channel gating is inhibited by nitric oxide via cGMP-dependent protein kinase, as it is also the Ca_V1 (L-type) channel [187]. Also, the lipid peroxidation product 4-hydroxynonenal increased the calcium influx through L-type and other ill-defined types of VOCC [178].

5.1.2. NMDA and other ionotropic receptors with calcium channel activity

It is well known the relevant role of NMDA-receptor mediated excitotoxic neuronal death in ischemia-reperfusion brain injury, see [44,45], in multiple chemical sensitivity in brain [194], in neuronal glutathione depletion [195] and in hydrogen sulfide-induced neuronal death [138,196]. Therefore, it is not surprising that the redox modulation of the NMDA-receptor is by far the most studied within the group of ionotropic receptors. The redox modulatory site of the NMDA-receptor consists of thiols groups that are vicinal in the three-dimensional structure and may form disulfide bonds under the cellular oxidative stress conditions induced by ROS [197], and it acts as a gain control for current flux through the NMDA-receptor [197,198]. Moreover, a significant number of NMDA-receptor cysteines are in the domains of this receptor facing the extracellular space, including at least one pair of vicinal thiols [199]. Thus, this receptor can also play a major role in the rapid neuronal adaptation to changes of the redox potential in the extracellular fluids within the brain, and the different types of NMDA-receptors display a redox response that is dependent on the type of NR2 forming the channels [55]. The differential redox-sensitivity of NMDA receptors isoforms led to the discovery of two redox modulatory centres within the NMDA-receptor structure, one formed by Cys744 and Cys798 on the subunit NR1 and a second one on the subunit NR2A [200,201]. Whereas the redox centre of the subunit NR1 plays a major role in the redox modulation of NR1/NR2C- and NR1/NR2B-containing receptors, the redox centre of subunit NR2A is sufficient for the expression of redox sensitivity in NR1/NR2A-containing receptors [201]. Redox active compounds modulate NMDA-receptors such that reduction of NMDA-receptor

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increases NMDA-receptor activity and their oxidation leads to a decrease of NMDA-receptor activity [202].

Because of the high physiological relevance of nitric oxide and of NMDA receptors in the brain, the modulation of NMDA receptors by nitric oxide is of particular relevance. Nitric oxide inhibition of NMDA-receptor response in cortical neurons in culture has been rationalized in terms of NO-induced disulfide bonds between vicinal thiols of the NMDA-receptor, and was proposed to afford neuronal protection against L-glutamate excitotoxicity [203]. Indeed, it has also been reported that thiol-reducing agents such as dithiothreitol increase the open dwell-time and opening frequency of NR1/NR2A channels [55,201]. Consistent with these findings, it has been reported that the novel neuromodulator hydrogen sulphide potentiates NMDA-receptor response in hippocampal neurons [204] and in cerebellar granule neurons [138], and that over-stimulation of NMDA-receptors by hydrogen sulphide can lead to excitotoxic neuronal death [138,196]. Glutamate-induced excitotoxic neuronal death has been shown to mediate brain injury after a transient focal cerebral ischemia episode [44,45]. Inhibitors of the H_2S-producing enzymes cystathionine β-synthase and cystathionine γ-lyase reduced the infarct volume in a dose-dependent manner, while administration of sodium hydrosulfide significantly increased the infarct volume after a transient focal cerebral ischemia insult [205]. Exposure of neurons to peroxynitrite also leads to activation of calcium entry through NMDA-receptors [166,169]. This effect of peroxynitrite has been rationalized in terms of the rise of L-glutamate concentration within the synaptic cleft, either due to potentiation by nitric oxide and/or peroxynitrite of L-glutamate secretion in synaptic terminals [194,206] or of inhibition of L-glutamate transporters catalyzing its re-uptake [207].

Besides the major role of NMDA-receptors on the neuronal damage elicited by ROS and/or oxidative stress, AMPA receptors have been also involved in the neurotoxicity of ROS. It has been reported that the increase of cytosolic calcium associated with the influx of Ca^{2+} through the ionotropic AMPA-receptors can stimulate nNOS leading to an enhanced production of nitric oxide within L-glutamatergic neurons [208]. Moreover, antagonists of AMPA/kainate-receptors have been reported to prevent the loss of cell viability induced by the peroxynitrite-releasing agent SIN-1 in mixed cortical cell cultures containing both neurons and astrocytes [209]. AMPA-receptors contain a disulfide bond between cysteines 260 and 315 in the ligand binding domain of receptor subunit GluRD, which has been proposed to act as a redox centre implicated in direct redox modulation of these receptors [210]. Nevertheless, the redox modulation of AMPA-receptors is a topic that will require further studies to develop an integrative view of its modulation by the different ROS that has been implicated in brain damage.

Finally, the response of the purinergic ionotropic P2X-receptors has been shown to be altered by acute hypoxia, an effect that has been proposed to be mediated by ROS because H_2O_2 attenuated the effect of hypoxia on homomeric P2X2 whole-cell currents, which are reversibly reduced to 38% of control by H_2O_2 [211]. Yet, studies regarding the putative modulation of P2X-receptors by other ROS are a pending issue.

5.2. Modulation by ROS/RNS of the transport systems that release calcium from intracellular stores

5.2.1. Endoplasmic reticulum

ROS/RNS have been shown to elicit a potent stimulation of calcium release from the endoplasmic reticulum, through activation of IP_3 and ryanodine receptors.

Superoxide anion and H_2O_2 have been reported to induce calcium release from the endoplasmic reticulum of neurons through activation of IP_3 receptors [212,213]. Oxidized glutathione and the alkyl mercury compound thimerosal, a thiol specific agent, increase the affinity of IP_3 receptors for IP_3, thereby sensitizing this receptor to basal IP_3 level in the cell and promoting calcium release from the endoplasmic reticulum to the cytosol [214,215]. Cysteine clusters highly reactive against ROS have been recently identified in the IP_3 receptors [216]. IP_3 receptors are inhibited by interaction with luminal endoplasmic reticulum proteins through luminal-facing domains of the receptor containing reduced cysteines, and oxidation of these cysteines weakens these interactions leading to IP_3 receptor activation [63,217]. Moreover, nitric oxide-induced increase of IP_3 binding to the IP_3 receptor in hypoxic brain has been proposed to mediate IP_3 receptors activation in calcium-dependent neuronal apoptotic death induced by hypoxia [218].

Although all ryanodine receptor isoforms are expressed in the brain, the isoform 2 is the most heavily expressed [219, 220]. Many studies have addressed the redox modulation of the ryanodine receptors in myocytes and in neurons, reviewed in [62,64,221]. Nitric oxide activates the skeletal and cardiac ryanodine receptors [222,223]. The activation of the ryanodine receptor by nitric oxide has been shown to be due to the presence of highly reactive cysteines of the receptor, which are S-nitrosylated upon exposure to nitric oxide [222-224]. The cysteines that are S-nitrosylated upon *in vitro* exposure to nitric oxide have been identified [225]. However, *in vivo* the extent of S-nitrosylation of ryanodine receptor cysteines is highly modulated by the physiological oxygen tension, leading to the concept that ryanodine receptors can operate as a coupled redox sensor for oxygen and nitric oxide [226,227]. *In vitro* studies have shown that these cysteines of the ryanodine receptor are highly sensitive to oxidative stress and are likely to mediate the redox ryanodine receptor response to another ROS, as they are also prone to reversible S-glutathionylation or oxidation to disulfide bonds [225]. The ryanodine receptors are also activated by hydroxyl radical, H_2O_2, the disulfide bond-forming agent diamide and also by oxidized glutathione [221,224,228]. Overall, oxidizing conditions favor the opening of the ryanodine receptor calcium channel, and on these grounds it has been proposed that activation of these calcium channels are also involved in the pathology of brain ischemia-reperfusion [229] and Alzheimer´s disease [230]. Noteworthy, a moderate and sustained stimulation of the ryanodine receptors in the hippocampus has been involved in the sustained increase of cytosolic calcium needed for the induction of the long-term postsynaptic potentiation associated with memory formation [231,232].

Calcium accumulation within the luminal space of the endoplasmic reticulum is performed by Ca^{2+}-ATPases (SERCA), whose activity is inhibited by exposure to H_2O_2, superoxide anion and peroxynitrite [233-236], the major ROS produced in brain insults such as ischemia-

reperfusion or inflammation and in neurodegeneration. Despite that the isoforms of SERCA most sensitive to ROS, i.e. SERCA2 isoforms, are expressed in brain, the relevance of the impairment of their activity to alterations of neuronal cytosolic calcium homeostasis has yet to be conclusively demonstrated, probably because in neurons the PMCA is a calcium pump more potent than SERCA for calcium extrusion from the cytosol.

The apparently higher susceptibility to ROS/RNS of the calcium release systems of the endoplasmic reticulum, ryanodine and IP_3 receptors, should lead under oxidative stress conditions to at least a partial depletion of the calcium concentration within the luminal space of this subcellular compartment, see for example [237]. It should be noted, though, that in neurons the amount of calcium stored in the endoplasmic reticulum is small compared with the amount of calcium entering through plasma membrane calcium channels and ionotropic receptors. However, in most severe cases the depletion of calcium can elicit the opening of plasma membrane SOCE, see section 2.2 of this chapter. Thus, the relevance of calcium release from the endoplasmic reticulum or of inhibition of the SERCA to the observed alterations by ROS/RNS of cytosolic calcium homeostasis will strongly depend on the differential expression of SOCE isoforms in different type of neurons. On the other hand, the depletion of calcium of the endoplasmic reticulum may lead to a dysfunctional endoplasmic reticulum by itself, because of the relevance of the endoplasmic calcium concentration for the correct folding and processing of membrane and secretory proteins [65, 238]. On these grounds, these authors have proposed that ROS/RNS-induced endoplasmic reticulum dysfunction can be a mechanism underlying slow-developing cell injury in ischemia-reperfusion, epileptic seizures and degenerative diseases of the brain like Alzheimer's and Parkinson's diseases. In addition, it has been recently shown that mutations in presenilin-1 and -2 observed in nearly 40% of familial Alzheimer's disease lead to calcium release from the endoplasmic reticulum [239]. Moreover, presenilins by themselves can form calcium leak channels in the endoplasmic reticulum whose properties are altered in mutant presenilins linked to Alzheimer's disease [240].

5.2.2. Mitochondria

A key role has been proposed for mitochondrial dysfunctions in the onset or development of neuronal death in the brain mediated by the enhanced oxidative stress observed in relevant neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis and Friedreich's ataxia, and in harmful brain insults like ischemia-reperfusion and glutamate excitotoxicity, reviewed in [166,241,242]. Mitochondrial calcium overload is observed in excitotoxic conditions that produce a sustained increase of neuronal cytosolic calcium or high frequency repetitive cytosolic calcium peaks [73]. ROS/RNS have been shown to promote opening of the permeability transition pore of mitochondria and this effect of ROS/RNS is enhanced by mitochondrial calcium overload [71,73,166]. Opening of the permeability transition pore leads to a significant calcium release from mitochondria which contributes to foster excitotoxic neuronal death [71, 243], and also is an important factor in necrotic cell death following ischemia-reperfusion [73] or in neurons exposed to transient hypoglycemia [244]. Consistently, calcium-dependent mitochondrial dysfunction by peroxynitrite has been

demonstrated to elicit necrotic cell death via activation of calpains [245]. In addition, opening of this pore has also been shown to mediate the neuronal apoptosis elicited by 3-nitropropionic acid, an agent which has been used to mimic in model rodents the brain neurodegeneration observed in Huntington's disease [246]. Despite that most of studies concerning ROS-stimulated release of calcium from mitochondria point out a major role of the permeability transition pore, it should be recalled that the inner membrane Na^+/Ca^{2+}-exchanger, i.e. the other major mitochondrial calcium release system [71], is also sensitive to oxidative stress. It has been reported that oxidative stress mediated by H_2O_2 modulates this exchanger and can lead to activation of caspase 3-dependent apoptosis due to mitochondrial Na^+ overload [247].

The permeability transition pore opening induced by ROS/RNS is mediated by oxidation of critical thiols of proteins forming the pore, as it can be elicited by a relatively large number of oxidizing agents such as diamide, dithiopyridine, singlet oxygen, diazoxide, nitric oxide, S-nitrosothiols and selenium [221]. The adenine nucleotide transporter of the inner mitochondrial membrane and the voltage-dependent anion channel of the outer mitochondrial membrane have been proposed to be part of the molecular structure of the permeability transition pore, and both proteins have shown to be modulated by oxidative stress and exposure of mitochondria to chemically defined ROS, such that oxidation of thiols of the adenine nucleotide transporter facing to the mitochondrial matrix have been shown to elicit the opening of the permeability transition pore, reviewed in [73].

5.3. Modulation by ROS/RNS of the transport systems involved in calcium extrusion from the cytosol

Much of the interest on modulation of PMCA and Na^+/Ca^{2+}-exchanger of neurons by ROS is based on the reported decrease of these activities in synaptic plasma membranes in aging, and the possibility that this could lead to a sustained increase of the steady state cytosolic calcium in aged animals with respect to young animals [248,249].

5.3.1. PMCA

It has been shown that incubation of brain synaptic plasma membranes with Fe^{2+}/EDTA, H_2O_2, peroxyl radicals generated by azo-initiators and peroxynitrite resulted in a significant loss of PMCA activity [250-253]. Inhibition of purified PMCA by H_2O_2 has been proposed to be due to oxidation of two cysteines of this protein [253]. Also, lipid peroxidation and the lipid peroxidation product 4-hydroxynonenal have been shown to inhibit the PMCA actvity [254]. In the case of incubation with peroxynitrite, the loss of Ca^{2+}-ATPase activity was paralleled by decrease of ATP-dependent calcium uptake activity and by a significant increase of tyrosine nitration of the PMCA [252]. However, it is to be noted that all these studies were carried out *in vitro* with purified plasma membranes in an altered environment with respect to the normal redox cytosolic environment in living neurons, and this has to be taken into account since endogenous antioxidant levels of reduced glutathione has been shown to largely attenuate the inhibition of PMCA by peroxynitrite [252]. In addition, the concentrations of H_2O_2 and peroxynitrite producing approximately 50% inhibition of the PMCA in these studies, higher than 100 μM in both cases, were much higher than those reported to be attained in brain after

transient focal ischemia or inflammation. For the case of neuronal exposure to peroxynitrite fluxes mimicking those attained in inflammation or ischemia-reperfusion, in a previous work of our laboratory it was shown that in cerebellar granule neurons in culture the PMCA is significantly inhibited in less than 2 hours exposure to micromolar concentrations of peroxynitrite, although it was also noted that the PMCA has nearly ten-fold lower sensitivity to peroxynitrite than L-VOCC [9].

$Na^{+}/Ca^{2+}-exchanger\ (NCX)$

The NCX has been reported to be less sensitive to inhibition by the peroxyl radical azo initiator 2,2'- azobis (2-amidinopropane) dihydrochloride (AAPH) and peroxynitrite than the PMCA [251], and also to be insensitive to inhibition by up to 700 μM of H_2O_2 [255]. The Na^+/Ca^{2+}-exchanger activity of synaptic brain plasma membranes and in transfected CHO-K1 cells has been reported to be inhibited by exposure to AAPH and also to peroxynitrite [255], although it must be noted that peroxynitrite only afforded a partial inhibition of the exchanger caused by decrease of its affinity for calcium without a significant change of the V_{max}. The inhibition induced by both oxidants correlated with the formation of higher molecular weight aggregates of the Na^+/Ca^{2+}-exchanger, and in addition AAPH also caused fragmentation of the exchanger protein.

In contrast, in cardiac muscle myocytes, hypoxia inhibits the Na^+/Ca^{2+}-exchanger and ROS are required for its rapid reactivation upon reoxygenation [256]. This is consistent with the earlier demonstration in ventricular myocytes of stimulation of the Na^+/Ca^{2+}-exchanger by H_2O_2 and superoxide anion [257]. Owing to the different pattern of Na^+/Ca^{2+}-exchanger isoforms expression in brain cells and cardiac myocytes, more experimental studies are needed to reach solid conclusions regarding the effects of oxidative stress on the activity of this exchanger in different neuronal types and also in the glial cells of the brain.

6. Space and time fluctuations of cytosolic calcium in the neuronal soma

As indicated previously in this chapter, protein compartmentation within microdomains allows for a more efficient and rapid functional coupling between influx and efflux calcium transport systems, and this is particularly relevant for neuronal activity, as neurons have to deliver fast responses to many repetitive and simultaneous extracellular stimuli coming from different neighbour cells. Studies on calcium signalling in neurons have played a pioneer role to demonstrate the outstanding role of subcellular compartmentation in the control of neuronal activity, see for example [5]. As analyzed in more detail in the section 3 of this chapter more recently reported experimental data point out that the calcium transport systems of the plasma membrane more relevant for the control of cytosolic calcium homeostasis in neurons are associated with lipid rafts sub-microdomains or nanodomains. This is an emerging scenario that opens new perspectives for the rationalization of the modulation of cytosolic calcium peaks amplitude and also of the rate of attenuation of calcium local gradients in neurons, as both parameters are strongly dependent on the spatial proximity between systems controlling calcium entry and extrusion from the cytosol. For example, the rationalization of the transient

calcium gradients observed between different regions of the neuronal cytosol can be done on simple grounds taking into account a polarised or focalized distribution of the major calcium transport systems of the neuronal plasma membrane. Note that, as indicated before in this chapter, sustained cytosolic calcium concentrations higher than 0.4 μM are strongly cytotoxic to neurons, but it is a need for neurons to reach these concentrations in the environment of cytosolic proteins and enzymes having EC_{50} values for calcium between 0.4 and 1 μM. Indeed, some of these proteins play a key role in neuronal plasticity and functional responses critical for proper brain development and function, like calmodulin, nNOS, GAP-43 and CaMK, to cite only a few of well-established examples.

The calcium concentration reaches values in the micromolar range upon activation of L-VOCC and NMDA receptors in small volume elements close to the cytosolic side of their calcium channel structures [258], see also the Figure 2a. This generates a calcium concentration wave that diffuses within the cytosolic space, because the protein cytosolic buffering systems are not fast enough to trap all incoming calcium ions through these calcium channels [259, 260]. Due to the rapid diffusion of calcium ions in the aqueous space of the cytoplasm, ~300 $\mu m^2 s^{-1}$, the calcium entry through the high conductance L-VOCC and NMDA receptors channels will rapidly raise the calcium concentration to the micromolar range within the associated lipid rafts nanodomains. As these nanodomains have sizes lower than 200 nm, it can be derived that in less than 1 microsecond the incoming calcium ions will diffuse within the whole space of the nanodomain, i.e. in the time scale range characteristic for fast conformational relaxation in proteins. Thus, this clustering serves to built up a very efficient molecular switch for signal transduction in calcium signalling pathways within neurons, with a time response as fast as the rapid conformational relaxations elicited by regulatory direct protein/protein interactions. However, nanodomains can be seen as multi-port exit molecular devices that can serve to many uni-port exit molecular devices, through regulatory direct protein/protein interactions. Therefore, the localized calcium rise within these nanodomains not only serves to guarantee the maximal possible activation of proteins or enzymes with EC_{50} values ≥0.4 micromolar, such as those listed above, but also to elicit rapid integrative cellular responses. We shall next briefly analyze several integrative responses of relevance for the rapid and fine control of cytosolic calcium homeostasis in neurons elicited by the localized calcium rise within the nanodomains associated with lipid rafts.

The association of CaMKII with L-VOCC subunit β2a and with NMDA receptors subunit NR2B, mentioned in the section 2.1 of this chapter, implies that this protein is present in neuronal nanodomains associated with lipid rafts. A direct consequence of the steep calcium concentration gradient generated by calcium entry through L-VOCC and NMDA receptors is the stronger selective activation of the pool of CaMKII that lies in their vicinity over other CaMKII pools present in neurons. Thus, this will selectively potentiate phosphorylation of CaMKII substrates present in lipid rafts associated nanodomains. Regarding the cytosolic calcium homeostasis in neurons, the more relevant effect is the activation of L-VOCC upon phosphorylation by CaMKII, as this potentiates the increase of the local gradient of calcium concentration within these nanodomains, leading to a longer lasting increase of the concentration of cytosolic calcium with the concomitant increase in neuronal secretory activity and

excitability (Figure 2b). Indeed, it has been shown that L-VOCC plays a relevant physiological role in NMDA receptors-independent long-term potentiation [28]. The activation and synaptic clustering of AMPA receptors upon phosphorylation by CaMKII has been shown to potentiate NMDA receptors activation in the induction of LTP [53]. Noteworthy, L-VOCC blockers like nifedipine and nimodipine and AMPA antagonists/inhibitors have been shown to have anti-epileptic therapeutic effects, pointing out that overstimulation of L-VOCC and/or AMPA underlies, at least, some types of epileptic seizures.

The high concentration of calcium attained within the nanodomains associated with lipid rafts allows for a stronger and faster selective stimulation of the pool of nNOS localized therein. Because of the rapid diffusion coefficient of nitric oxide, these nanodomains can be seen as the most relevant plasma membrane points for focalized nitric oxide generation in neurons and, therefore, define the sub-microcompartments of neurons where higher transient concentrations of nitric oxide are attained upon nNOS stimulation. This fact and the vicinal location of nNOS and NMDA receptors within these nanodomains, i.e. separated by a distance lower than 40 nm [117], makes of NMDA receptors a major cellular target for the chemical reactivity of released nitric oxide. As the calcium currents through NMDA receptors are inhibited by exposure of these receptors to nitric oxide, see the section 5.1 of this chapter, the co-localization of nNOS and NMDA receptors within these nanodomains serves to potentiate a feedback retroinhibition mechanism for the attenuation of excessive NMDA receptors activity which would lead to neuronal excitotoxicity [117], i.e. these nanodomains can be also seen as a molecular microchip-like structure designed for neuronal protection against the harmful consequences of overstimulation by L-glutamate (Figure 2c). On these grounds, the reported stimulation of L-VOCC by nitric oxide, see the section 5.1 of this chapter, can be rationalized as a molecular compensatory mechanism for the fine tuning of NMDA receptor activity, as it will lead to an increase of L-glutamate secretion near these nanodomains and this should avoid excessive depression of NMDA receptor activity in the neuron.

The latter point already highlights a major role of the nanodomains associated with lipid rafts in the intimate cross-talk between calcium and nitric oxide signalling for the normal physiological activity of neurons, but also points out that excessive calcium entry through L-VOCC or NMDA receptors should rapidly lead to unusually large peaks of nitric oxide generation in these nanodomains. As indicated above in this chapter, it is well established now that the sustained rise of intracellular calcium and/or nitric oxide can induce neuronal death and are common features in brain degeneration. Many experimental evidences accumulated up to date reveal that in some cases the induction of oxidative stress in brain neurodegeneration takes place before a sustained cytosolic calcium homeostasis deregulation can be observed. For example, in the case of inflammation of a brain area induced either by a traumatic shock injury or cerebral stroke the neurons are exposed to a ROS/RNS overshot largely generated by vicinal glial and vascular endothelial cells. The major sources for the overshot of ROS/RNS observed in this inflammation episode are the increase of iNOS expression, which produces a nitric oxide overshot, and activation of plasma membrane NADPH oxidases, which produces a superoxide anion overshot. Therefore, within the brain area affected by inflammation neurons suffer a long-lasting exposure to an extracellular microenvironment where the simultaneous presence

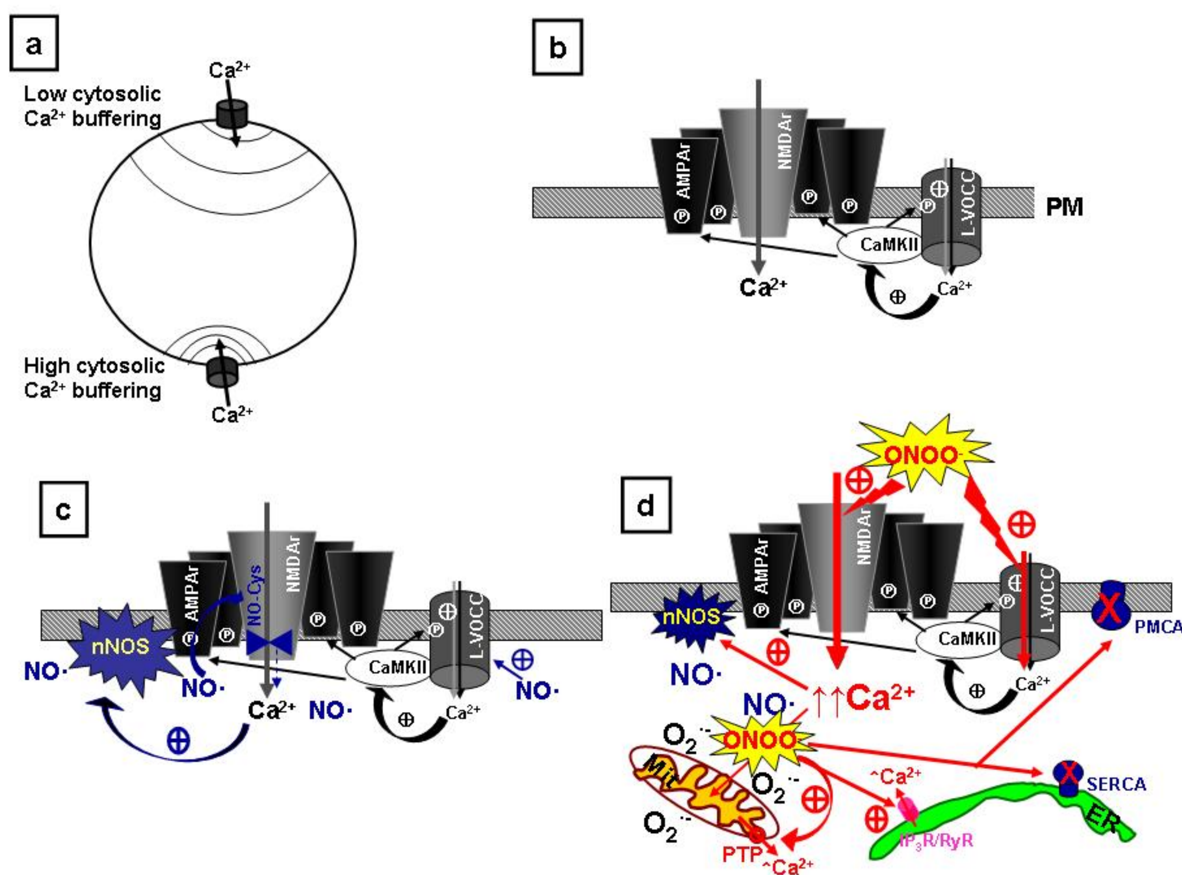

Figure 2. Functional implications of the association of calcium transport systems and ROS/RNS-sources in the neuronal plasma membrane. (a) Generation of transients of micromolar calcium concentrations within nano- or sub-micro-volume elements. The size attained by these volume elements is strongly dependent on the intensity of the total calcium inward current through the calcium transport systems clusters within lipid rafts-associated nanodomains and on the cytosolic calcium buffering capacity (see the text). (b) Faster and long-lasting potentiation of NMDA receptors (NMDAr). Calcium entry through L-VOCC triggers the activation of associated CaMKII, which elicits (i) a feedback activation of L-VOCC potentiating calcium entry and (ii) a recruitment of activated AMPA receptors (AMPAr). (c) Potentiation of NO -mediated protection against L-glutamate excitotoxicity. The co-localization of nNOS allows to reach higher NO concentrations near NMDA receptors and L-VOCC potentiating its effects on these calcium transport systems. (d) Peroxynitrite-induced sustained cytosolic calcium deregulation. A dramatic consequence of an unbalanced overstimulation of calcium transport systems that raise the cytosolic calcium concentration. Other abbreviations used in this figure: PM, plasma membrane; Mit, mitochondria; ER, endoplasmic reticulum; $ONOO^-$, peroxynitrite;, protein phosphorylation; ⊕, stimulation; ⋈ and X, inhibition or blockade.

of high nitric oxide and superoxide anion concentrations generate significant amounts of peroxynitrite, see e.g. [162,169]. In this oxidative scenario, the calcium-entry transport systems more relevant for the control of neuronal cytosolic calcium homeostasis associated with lipid rafts nanodomains, NMDA and AMPA receptors and L-VOCC, are strongly activated by peroxynitrite, see the section 5.1 of this chapter. This produces a large peak of calcium concentration within these nanodomains, large enough to elicit a large increase of cytosolic calcium and a strong stimulation of nNOS, leading to an intracellular burst of nitric oxide, and stimulation of the neuronal metabolic activity and associated intracellular superoxide anion

generation, oxidative conditions that generate intracellular peroxynitrite. In turn, these intracellular oxidative conditions produce the release of calcium from endoplasmic reticulum and mitochondria (see the section 5.2 of this chapter), contributing to a further and more widespread rise of cytosolic calcium concentration, and partial inactivation of the extrusion systems of the neuronal plasma membrane, PMCA and NCX (see the section 5.3 of this chapter). These latter effects lead to impairment of the ability of neurons to restore the low cytosolic calcium concentration needed for their normal function, and as a result lead to a long-lasting rise of cytosolic calcium concentration which can eventually reach the level that elicits a rapid necrotic death. Thus, impairment of the calcium transport systems of nanodomains associated with lipid rafts results in generation of an intracellular ROS/RNS oxidative stress that amplifies the oxidative stress suffered by exposure of neurons to a combined ROS/RNS extracellular oxidative stress (Figure 2d). Indeed, many experimental studies have shown that pharmacological compounds that inhibit the calcium currents through NMDA and AMPA receptors and L-VOCC behave as protection agents against neuronal death in inflammatory brain insults.

Experimental evidences have pointed out that there is a large mesh/network of lipid rafts-associated nanodomains in the plasma membrane of the soma of primary cultures of cerebellar granule neurons, where they are particularly enriched in neuron/neuron contact areas [130], and microscopy images have also shown a distribution map that closely overlap with the distribution map of flavoproteins bound to the plasma membrane [130,261], consistent with the association of the flavoproteins nNOS and cytochrome b_5 reductase with these nanodomains. Because of the strong impairment of the activity of calcium transport systems present in these nanodomains by many ROS/RNS that can be generated in the neuronal cytoplasm under a variety of cellular stress conditions, it should be expected that even exposure of neurons to a relatively mild oxidative stress should elicit a partial failure of the control of calcium homeostasis within these neurons. Owing to the large intracellular space occupied by nuclei in these neurons, partial failure in the control of cytosolic calcium homeostasis should elicit significant fluctuations of the cytosolic calcium concentration even in the absence of neuronal stimulation. The occurrence of basal endogenous oscillations of the cytosolic calcium concentration have been reported in *in vitro* cultures of different types of neurons, see for example [186,262-264]. We have recorded synchronized fluctuations of the cytosolic calcium concentration in primary cultures of rat cerebellar granule neurons, of an average amplitude of ±0.15 units of the ratio 340/380 in cells loaded with fura-2, by simply increasing the intensity of UV-irradiation in the epifluorescence microscope [Marques-da-Silva D and Gutierrez-Merino C, *unpublished results*], conditions that promote an increase of H_2O_2 production by cellular flavoproteins. The implication of nanodomains associated with lipid rafts in the generation of these cytosolic calcium fluctuations is unravelled by their attenuation by specific inhibitors or blockers of the calcium transport systems associated with these nanodomains. In this particular case the calcium entry through L-VOCC plays a major role in the modulation of the amplitude of the UV-induced fluctuations of cytosolic calcium concentrations. However, it is to be noted that other calcium transport systems associated with lipid rafts can also play a major role under different experimental conditions, as it has been shown that NCX interac-

tions with another proteins bound to lipid rafts can elicit cytosolic calcium oscillations in oocytes [265].

6.1. The role and relevance of cytosolic calcium buffering systems

The activation of a channel with a typical conductance of 2.6 pS, like that of some calcium channels, can generate a calcium diffusion sub-microcompartment where the calcium concentration is higher than 1 μM, but the effective dimensions of this volume element is largely dependent upon the calcium buffering capacity of the microenvironment, increasing from only several nanometers with a millimolar calcium buffering capacity up to 82 nm in presence of a calcium buffering capacity equal to that afforded by 0.1 mM fura-2 [258]. The higher the conductance of the calcium channel, the higher the effective dimension of this sub-microcompartment. Using the equations derived in [260], for channels with unitary calcium conductances in the range of 20 to 40 pS, i.e. that of L-VOCC and NMDA receptors (section 2.1 of this chapter), effective dimensions of a sub-microcompartment with calcium concentrations higher than 1 μM can extend to several hundreds of nanometers taking into account that only micromolar concentrations of calcium buffering systems are present in the neuronal cytosol (Figure 2a). Because of the high neurotoxicity of cytosolic calcium concentrations in the micromolar range, a decrease of the calcium buffering capacity of the cytosol shall increase the propensity for rapid degeneration of neurons.

On these grounds, it can be easily understood that the role of the cytosolic calcium buffering in neurons has attracted considerable interest, not only because of the abundance of calcium-binding proteins in the nervous system but also because of the specificity of their regional distribution in the brain. It is also relevant herein to note that an altered expression of the major calcium-binding proteins has been noticed in damaged brain regions of patients suffering from acute insults, such as stroke or epileptic seizures, and from chronic human neurodegenerative disorders which develop with an enhanced oxidative stress in the brain, such as Alzheimer's, Huntington's, Parkinson's and Pick's diseases [266]. Several of the major calcium-buffering proteins present in the brain have been reported to show altered expression levels in degenerating brain regions, namely, parvalbumin, calbindin-D28K and S100, all of them members of the EF-hand calcium binding proteins like the calcium-binding protein calmodulin ubiquitously expressed in all mammalian cells. Furthermore, it has been proposed that the lack of calcium buffering proteins parvalbumin and calbindin-D28K may be considered one of the factors that render human motor neurons particularly vulnerable to calcium toxicity following glutamate receptor activation in amyotrophic lateral sclerosis [267]. Consistently, it has been reported that parvalbumin overexpression delays disease onset in a transgenic model of familial amyotrophic lateral sclerosis [268], a devastating and oxidative stress-mediated neurodegenerative disease of the brain.

Because of the high relevance of calmodulin as a multifunctional modulator of cellular calcium homeostasis and also of cellular calcium signalling pathways [4-6], this is the calcium binding protein of the EF-hand family whose functional and structural alterations by ROS have been more extensively studied [269]. In this regard, calmodulin-dependent proteins particularly relevant for the control of calcium homeostasis in neurons are the calcium transport systems

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PMCA and IP_3-receptors, see above. In addition, calmodulin modulates signalling pathways controlling neuronal activity and synaptic plasticity like the protein phosphatase calcineurin, CaMK and IQ motif-containing proteins, such as myosins, Ras exchange proteins and GAP-43 among others [141,270,271]. Noteworthy, the activity of calcineurin has been reported to be decreased in sporadic and familial amyotrophic lateral sclerosis [272]. Calmodulin has been shown to become more oxidized in aged animals [269], pointing out that the physiological oxidative stress developed in the tissues is enough to lead to a sustained chemical modification of this protein. *In vitro* calmodulin suffers chemical oxidative modifications upon exposure to either H_2O_2 or peroxynitrite, for a review on this topic see [269]. Two vicinal methionine residues close to the carboxyl-terminus of calmodulin, Met-144 and Met145, are oxidized to methionine sulfoxide in aged tissues and also by H_2O_2 and more efficiently by peroxynitrite. Calmodulin oxidation leads to inhibition of the target proteins by non-productive association and stabilization of their inactive state. This has been experimentally demonstrated for the PMCA [273-275]. The oxidation of these methionines is reversible *in vivo*, as methionine sulfoxide reductases can efficiently reduce them back to methionine, restoring normal calmodulin function [269]. The fact that in aged tissues this oxidation is not fully reverted indicates a functional loss of this recycling process during aging. Thus, oxidation of calmodulin leads to a transient inactivation of neuronal PMCA. On these grounds, the fact that high levels of expression of calcium binding proteins are observed in neurons expressing nNOS [276-279] can be seen as a protective mechanism to attenuate long-lasting calcium transients in these neurons, which could eventually elicit cell death through calpains activation.

The widespread expression of calmodulin in the brain, its high level of expression in neurons relative to other cell types and its pleiotropic cellular functions confer a high relevance to the oxidative modifications of this protein by ROS/RNS. Regarding specifically the calcium transport systems associated with lipid rafts nanodomains, a loss of functional calmodulin leads to a marked decrease of the CaMK activity and this, in turn, leads to a decrease of the activity of the calcium entry systems L-VOCC and AMPA and NMDA receptors. As a result, the calcium concentration within these sub-microcompartments will be lowered up to levels closer to those found in the overall cytosol. Although the PMCA will also be inhibited, this inhibition by itself cannot compensate a large decrease of the inward calcium currents for two major reasons: (i) in neurons PMCA is also stimulated by phosphatidylserine and in these cells calmodulin stimulation is weak relative to other cell types [280], and (ii) the higher potency for transport across the open calcium channels of L-VOCC and NMDA receptors with respect to that of PMCA. Therefore, these nanodomains can eventually enter in a latent state regarding calcium and nitric oxide signalling in neurons. A simple and rational hypothesis merges from this conclusion, namely, that this could be a molecular mechanism underlying the observed loss of neuronal threshold excitability in aging and brain neurodegeneration. Owing to its putative relevance for the search of new therapeutic drugs and treatments for slow-developing neurodegenerative processes, this hypothesis deserve to be experimentally assessed in future studies.

7. Concluding remarks

The organization of the major calcium transport systems controlling the cytosolic calcium homeostasis within nanodomains of the neuronal plasma membrane associated with lipid rafts is opening new perspectives for regulation and deregulation of calcium signalling in neurons. In addition to the relevance of this fact for the efficient neuronal function in brain associative structures, like the concerted activity in neuronal circuits and LTP, the co-localization of ROS/RNS enzyme sources within these nanodomains is of particular relevance for neurodegenerative insults and diseases. The basic reason for this conclusion is that the calcium transport systems playing a major role in cytosolic calcium homeostasis and calcium-mediated neuronal activity are highly sensitive to modulation by ROS/RNS, and that oxidative stress is a common feature observed during the development of brain damage elicited in the most frequent brain insults and neurodegenerative diseases of high prevalence in humans. Yet, the actual knowledge of the molecular structure and plasticity of these nanodomains is still very limited, both in terms of their molecular composition in different types of neurons and of the factors controlling its formation and structural organization. Moreover, the molecular mechanisms leading to deregulation of the ROS/RNS enzyme sources associated with these nanodomains remain to be established, as well as the structural changes induced in these nanodomains by exposure to the different ROS/RNS that are generated in neurodegenerative insults and diseases. Because of the central role of cytosolic calcium in the control of neuronal activity, plasticity and survival it can be foreseen that these nanodomains will become a relevant pharmacological target in the search for alternate and novel therapies aiming to prevent or slowdown neurodegenerative processes in the brain.

Abbreviations used in the text

AAPH, 2,2'- azobis (2-amidinopropane) dihydrochloride; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; CaMKII, calcium/calmodulin-dependent protein kinase, isoform II; Cb_5R, cytochrome b_5 reductase; cGMP, 3′,5′-cyclic guanosine monophosphate; CNS, central nervous system; COX, cyclooxygenase; EDTA, ethylenediamine-tetraacetic acid; FRET, fluorescence resonance energy transfer; LTP, long-term post-synaptic potentiation; NCX, sodium-calcium exchanger; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric oxide synthase; NOX, ROS-generating NADPH oxidases; PKA, protein kinase A; PKC, protein kinase C; PMCA, plasma membrane calcium pump; ROS, reactive oxygen species; RNS, reactive nitrogen species; SIN-1, 3-morpholinosydnonimine; SOCE, store-operated calcium entry; UV, ultraviolet; VOCC, voltage-operated caclium channels (L-VOCC, L-type VOCC; N-VOCC, N-type VOCC; etc).

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