The Puzzling Role of Neuron-Specific PMCA Isoforms in the Aging Process

Tomasz Boczek 1,2, Tomasz Radzik 1, Bozena Ferenc 1 and Ludmila Zylinska 1,*

1 Department of Molecular Neurochemistry, Medical University, 92-215 Lodz, Poland; tomasz.boczek@umed.lodz.pl (T.B.); tomasz.radzik@umed.lodz.pl (T.R.); bozena.ferenc@umed.lodz.pl (B.F.)
2 Department of Ophthalmology, Stanford University School of Medicine, Palo Alto, CA 94304, USA

* Correspondence: ludmila.zylinska@umed.lodz.pl; Tel.: +48-42-272-5680

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Abstract: The aging process is a physiological phenomenon associated with progressive changes in metabolism, genes expression, and cellular resistance to stress. In neurons, one of the hallmarks of senescence is a disturbance of calcium homeostasis that may have far-reaching detrimental consequences on neuronal physiology and function. Among several proteins involved in calcium handling, plasma membrane Ca\(^{2+}\)-ATPase (PMCA) is the most sensitive calcium detector controlling calcium homeostasis. PMCA exists in four main isoforms and PMCA2 and PMCA3 are highly expressed in the brain. The overall effects of impaired calcium extrusion due to age-dependent decline of PMCA function seem to accumulate with age, increasing the susceptibility to neurotoxic insults. To analyze the PMCA role in neuronal cells, we have developed stable transfected differentiated PC12 lines with down-regulated PMCA2 or PMCA3 isoforms to mimic age-related changes. The resting Ca\(^{2+}\) increased in both PMCA-deficient lines affecting the expression of several Ca\(^{2+}\)-associated proteins, i.e., sarco/endoplasmic Ca\(^{2+}\)-ATPase (SERCA), calmodulin, calcineurin, GAP43, CCR5, IP\(_3\)Rs, and certain types of voltage-gated Ca\(^{2+}\) channels (VGCCs). Functional studies also demonstrated profound changes in intracellular pH regulation and mitochondrial metabolism. Moreover, modification of PMCA membrane composition triggered some adaptive processes to counterbalance calcium overload, but the reduction of PMCA2 appeared to be more detrimental to the cells than PMCA3.

Keywords: plasma membrane Ca\(^{2+}\)-ATPase; isoforms; PC12 cells; bioenergetics; Ca\(^{2+}\) signaling; aging

1. Introduction

The aging process is a physiological phenomenon affecting all living organisms and is associated with perpetual changes in metabolic control, the expression and stability of genes, and cellular resistance to stress. Aging itself is not a disease, but can predispose to many diseases. This process is particularly noticeable in the central nervous system (CNS), as mature neurons have no or very limited capacity for renewal. The most difficult in studies on brain aging is to separate the effects from causes, because they frequently overlap [1]. The evident hallmarks of many age-related events are calcium dyshomeostasis, mitochondrial dysfunction, oxidative stress, and impaired proteostasis [2–5]. Senescence in neurons is related to protracted elevations in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_c\)) levels and prolonged Ca\(^{2+}\) signaling following excitatory stimulation [6–8]. Physiologically, intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is regulated by balancing calcium influx through voltage-gated Ca\(^{2+}\) channels (VGCC), receptor-operated calcium channels (ROCC), and store-operated calcium entry (SOCE), with active efflux by plasma membrane Ca\(^{2+}\)-ATPase (PMCA), sodium-calcium exchanger (NCX), and reuptake into the endoplasmic reticulum by sarco/endoplasmic Ca\(^{2+}\)-ATPase (SERCA) [9–11].

Due to high buffering capacity of mitochondria, they are also an important component for maintaining calcium homeostasis and regulation of intracellular Ca\(^{2+}\) signaling [12,13]. The key role of
mitochondria is production of ATP through oxidative phosphorylation, and the major energy source
in the brain is glucose. The energy produced in mitochondria is vital to meet neuronal high basal
energy requirements, including maintenance of the membrane potential [14]. It is well-documented
that energy metabolism decreases during aging due to overproduction of reactive oxygen (ROS) and
nitrogen species (RNS), as well as deterioration of protective and repair systems [15]. In oxidative
stress conditions, overproduction of ROS is expected to profoundly alter cellular metabolism and its
regulation [16]. According to the free radical theory of aging, which is currently the most accepted one to
explain age-associated degeneration, the products of nucleic acids, proteins, lipids, and carbohydrates
oxidation are all implicated in the aging process [17]. This theory proposed by Harman [18] implicates
mitochondrial membrane potential loss and concomitant decrease in ATP production as a part of a
negative feedback loop toward age-associated deregulation of \([\text{Ca}^{2+}]_i\).

The brain is especially vulnerable to oxidative damage but due to its high complexity, it is difficult
to define precisely all the factors that trigger the aging process. It is now well established that deficits in
ATP supply, which is critical for reestablishment of ion balance by the plasma membrane \(\text{Ca}^{2+}\)-ATPase,
can produce diverse defects in brain function. This posits disturbed calcium homeostasis in the center
of several age-associated brain pathologies and should be considered as an important risk factor for
the development of neurodegenerative diseases.

2. Plasma Membrane \(\text{Ca}^{2+}\)-ATPase in Neuronal Cells

Despite the discovery of hundreds of proteins capable of calcium buffering, one of the main roles
in controlling intracellular \(\text{Ca}^{2+}\) concentration is attributed to the plasma membrane \(\text{Ca}^{2+}\)-ATPase, the
most sensitive cellular calcium detector. There are four main isoforms of PMCA (PMCA1–PMCA4),
that are similar in structure, despite low homology at the amino acid level [19,20]. Depending on the cell
and tissue type, there may be nearly 30 different PMCA variants created by alternative splicing of the
original transcripts, which generates enzymes of different structural and biochemical properties, such
as \(\text{Ca}^{2+}\) affinity, velocity of \(\text{Ca}^{2+}\) transport, regulatory mechanisms, or ability to interact with different
signaling and regulatory proteins [21–23]. PMCA1 and PMCA4 are thought to be the constituent forms
and are expressed in large numbers of cells [19,24].

The presence of PMCA2 and PMCA3 is limited almost exclusively to the excitable cells, and
because of their prominent expression in the brain, they are referred to as neuro-specific [19,24–26].
PMCA2 and PMCA3 possess a high basal activity and belong to so-called “fast” isoforms, whereas
PMCA1 and PMCA4 are much slower. PMCA, by interacting locally with a number of protein partners,
can be incorporated into intracellular signaling pathways [27]. It suggests that PMCA may play a
role that is far beyond its classically attributed function of a calcium transporter. In the brain, the
expression profile of PMCAs changes significantly during development, confirming the unique role of
each isoform [26,28,29]. Even though PMCA is primarily localized at multisynaptic endings and in a
close proximity to neurotransmitter release sites (active zone) of the presynaptic terminals, particular
PMCA isoforms are distributed in the brain in a region-dependent manner. It is now well documented
that PMCA activity and the amount of decline with age, and concomitant elevated calcium can lead to
apoptosis or necrosis, depending on the extent of metabolic stress and cytosolic \(\text{Ca}^{2+}\) overload [2,30–32].
Although the half-life of PMCA in the whole brain homogenate was estimated to be \(12 \pm 1 \text{ days}\),
there is no available data for different PMCA isoforms and variants turnover rates [33]. Moreover,
accumulation of age-dependent modifications of PMCA protein structure, despite the relatively long
half-life, may alter the enzyme efficiency in \(\text{Ca}^{2+}\) extrusion [34–36].

Interesting data related to neurospecific PMCAs function were obtained after discovering that
some mutations in PMCA2 are linked to hereditary deafness [37,38] and mutation of PMCA3 was
found in cerebellar ataxia [39], but in each case, the total efficiency of \(\text{Ca}^{2+}\) extrusion was reduced. It
may suggest that impaired PMCA2 or PMCA3 function cannot be simply substituted for other isoforms.
Using several models, including the adult and aged mouse brain, Alzheimer’s disease-affected human
brain, SH-SY5Y human neuroblastoma cells, as well as purified synaptosomal PMCA from pig
cerebrum, Mata’s group discovered that PMCA activity was inhibited by tau protein and amyloid-β peptides, and PMCA4b appeared to be the most sensitive target for that inhibition [40–43]. Moreover, CaM exhibited a protective action against functional impairment of PMCA by tau and Aβ peptide. Thus, although PMCA2 was shown to be more resistant to amyloid-β inhibition, its downregulation in the aging brain, together with lowered PMCA4 activity and the CaM amount, could be responsible for progressive development of AD symptoms.

One of the widely used in vitro model of sympathetic neurons are differentiated PC12 cells. This line was isolated from the pheochromocytoma tumor of rat adrenal glands that have an embryonic origin from the neural crest, which gives PC12 cells unique ability to acquire a number of features characteristic for mature neurons [44]. Upon treatment with nerve growth factor (NGF), they cease proliferation, start to extend neurite-like protrusions, express functional neuronal membrane channel, develop action potentials, and respond to acetylcholine treatment. Similar effects can be triggered by dibutyryl cyclic adenosine-3’, 5’-monophosphate (db-cAMP) [45,46]. Whereas in undifferentiated PC12 cells, all main PMCA isoforms have been detected, with PMCA4b constituting a major one, the differentiation process stimulates the expression of additional splice variants (i.e. 1c, 2a, 2c, 4a) although with unknown physiological function [47,48].

To evaluate the possible contribution of neuro-specific PMCA isoforms to the aging process, we developed PC12 lines deficient in PMCA2 (PC12_2) or PMCA3 (PC12_3). Our extensive functional and molecular analyses gave new insights into downstream cellular processes that can be modified by altered PMCA composition.

3. Differentiated PC12 Cells as a Model of Aging Neuron

The knockdown of PMCA isoforms was done using eukaryotic vectors containing antisense sequences designed to either PMCA2 or PMCA3, and in stably transfected PC12 lines the protein level of each isoform was reduced by almost 50% [49]. Staining of PMCA-deficient cells with the marker protein of growth cones showed the absence of a central structure surrounded by F-actin-rich filopodia and lamellipodia and strong condensation of this protein in neuronal terminals, suggesting that growth cones can be in the retraction phase. Reduced growing potency and neurite retraction are characteristic for aged neurons and frequently observed in response to injury or disease [50,51].

A direct functional consequence of a partial loss of each isoform was a moderate, but sustained, increase in resting Ca\(^{2+}\) concentration by 30–40 nM. In addition, PMCA2 deficiency increased the percentage of cells in the late apoptotic phase, which was detected by AnnexinV/propidium iodide assay and confirmed by the observed DNA fragmentation. Similar effects were reported for rat primary neurons and human SH-SY5Y neuroblastoma cells transfected with PMCA2 siRNA [52]. Beside higher basal Ca\(^{2+}\) level and impaired Ca\(^{2+}\) clearance following stimulation, these authors also demonstrated more intensive cell death upon exposure to excitotoxic concentrations of agents rising intracellular Ca\(^{2+}\). The correlation between PMCA2 down-regulation and disturbances of cell function, including the augmented cell death, was reported in neurons, which strongly implicated a protective PMCA2 role [53–57].

Although reduction of “fast” PMCA isoforms affected functionality of the cells, it did not change the total amount of PMCA protein which could be due to up-regulation of PMCA1 isoform in both PMCA-deficient lines, and PMCA4 that increased only in response to PMCA3 silencing [49]. Nevertheless, elevated cytoplasmic Ca\(^{2+}\) concentration indicated that these adaptive changes were insufficient to fully substitute even for the partial loss of PMCA2 or PMCA3 function. However, in the PC12_3 line, compensatory up-regulation of PMCA4 seemed to counteract Ca\(^{2+}\)-dependent induction of apoptosis more effectively, suggesting that PMCA3 deficit could be less detrimental for intracellular calcium homeostasis.

Another protective mechanism aimed against Ca\(^{2+}\) cytotoxic effects was up-regulation of SERCA2 and SERCA3 isoforms in both PMCA-deficient lines [49]. It correlated with higher accumulation of Ca\(^{2+}\) in the endoplasmic reticulum. SERCA family includes three members (SERCA1–3), and two
splice variants of SERCA2. SERCA2b is ubiquitously expressed, while SERCA2a and SERCA3 exhibit more discrete localization [58,59]. Since SERCA’s half-life (10–14 days) has been suggested to be similar to PMCA [2], and energetic cost of calcium transport by SERCA is lower than that of PMCA (2Ca$^{2+}$/ATP vs. 1Ca$^{2+}$/ATP, respectively) [60], this compensatory effect observed in PC12 cell lines could provide long-term multifaceted protection against calcium cytotoxicity. However, the molecular mechanism(s) of the interplay between these calcium pumps remains a mystery.

Further analysis of the effects of reduced PMCA2 or PMCA3 expression on the kinetics of Ca$^{2+}$ extrusion showed a significantly increased influx and longer half time ($t_{1/2}$) of Ca$^{2+}$ clearance after KCl-induced depolarization [49]. In excitatory cells, membrane potential-dependent [Ca$^{2+}$]c raises are generated mainly by the voltage-gated Ca$^{2+}$ channels (VGCCs), of which four types: N, L, P/Q, and T are present in PC12 cells [61,62]. The profiling of VGCC expression showed elevated transcript level of P/Q and L types in both PMCA-deficient lines and the T-type solely in PMCA2-deficient cells. Functional analysis with specific pharmacological inhibitors revealed that higher expression of these VGCCs corresponded to their increased contribution to the total Ca$^{2+}$ load induced by KCl.

It has been widely documented that during aging, ion channel dysfunctions participate in the generation of ionic dyshomeostasis, alteration of membrane potential as well as signal transduction pathways, which can eventually modify cellular physiology [63]. Earlier studies have showed that the aging-related increase in Ca$^{2+}$-mediated responses depended on greater activity of L-VGCC [64,65]. Moreover, a growing body of evidence indicated an increase in the expression and function of L-type Ca$^{2+}$ channel with aging [66–69], and higher L-VGCC density in the hippocampus was positively correlated with cognitive impairment in aged animals [62]. An interesting observation was an up-regulation of T-type VGCC in response to PMCA2 depletion and its increased contribution to Ca$^{2+}$ influx. Physiologically, T-type channels regulate neuronal excitability, differentiation, growth, and proliferation [70]. An important link was shown between T-type calcium channels and long-term potentiation (LTP) [71]. Downregulation of the T-type VGCC was demonstrated in the mouse model of Alzheimer’s disease [72]. It was also reported that a closing time in some populations of these channels is significantly longer than the other VGCC [72]. Therefore, one can hypothesise that together with a reduced Ca$^{2+}$ extrusion, elevated presence of T-type channels in PMCA2-deficient line might contribute to sustained increase in [Ca$^{2+}$].

Based on this data, it can be assumed that higher amplitude of [Ca$^{2+}$]c rises during depolarization reflects the presence of more functional calcium channels in the plasma membrane. Activation of individual channels and coupling their functions with downstream cellular signaling pathways, can induce a different response e.g., by modulation of specific genes expression. In addition, changed VGCC profile in response to manipulations in PMCA expression strongly suggests a transcriptional link between these two opposing systems of Ca$^{2+}$ transport.

The changes in main components of calcium handling systems described above revealed some differences in the cell response, which seemed to be driven by the presence of particular PMCA isoform (Table 1). Interestingly, knockdown of PMCA2 reproduced a wider spectrum of morphological and molecular abnormalities observed during physiological brain aging.
Table 1. Summary of differences between PMCA2 and PMCA3 down regulation in PC12 cells.

| Protein or mRNA | PC12_2  | PC12_3 |
|-----------------|---------|--------|
| PMCA1           | increased | increased |
| PMCA2           | decreased | unchanged |
| PMCA3           | unchanged | decreased |
| PMCA4           | unchanged | increased |
| Total PMCA      | unchanged | unchanged |
| SERCA2          | increased | increased |
| SERCA3          | increased | increased |
| CaM             | decreased | decreased |
| CaN             | increased | increased |
| L-type VGCC     | increased | increased |
| D/Q-type VGCC   | increased | increased |
| T-type VGCC     | increased | unchanged |
| CCR1            | increased | unchanged |
| CCR3            | unchanged | increased |
| CCR5            | increased | increased |
| IP$_3$R1        | decreased | decreased |
| IP$_3$R2        | decreased | decreased |
| IP$_3$R3        | increased | increased |
| Total IP$_3$R   | unchanged | increased |

4. The Interplay between PMCA, Calcium, and Mitochondrial Function during Aging

Mitochondria are central hubs in neuronal pathology integrating energy production, Ca$^{2+}$ homeostasis, cell signaling, and controlled cell death [73]. Preservation of these functions is especially important in the brain as neurons contain a large number of mitochondria to fulfill energy needs for synaptic processes. The decrease in mitochondrial functionality in the brain, especially in the hippocampus, is associated with the normal aging process and progressive loss of synaptic function [74]. Changes in mitochondrial activity during aging have also been associated with increased mitochondrial ROS production, causing cellular damage of mitochondrial and nuclear DNA and advancing age-related diseases [75].

One of the most important regulators of mitochondrial function in aging is the second messenger Ca$^{2+}$. Buffering of [Ca$^{2+}$]$_i$ by mitochondria increases when cells age, as was demonstrated in peripheral sympathetic neurons and intestinal smooth muscle cells [76,77]. The regulation of [Ca$^{2+}$]$_i$ by mitochondria seems to be especially relevant in neuronal cells, in which local Ca$^{2+}$ uptake by neighboring mitochondria regulates the duration of cytosolic Ca$^{2+}$ fluxes generated by the influx through plasma membrane calcium channels [78]. The possibility that changes in intracellular Ca$^{2+}$ homeostasis might explain mitochondrial impairments in senescent cells arise from calcium hypothesis of neuronal aging [79]. According to this hypothesis, a change in any of the Ca$^{2+}$ transporting systems over a long time period should affect [Ca$^{2+}$]$_i$. To confirm it, several research groups showed elevated resting [Ca$^{2+}$]$_i$, and reductions in the amplitude of stimulation-induced [Ca$^{2+}$]$_i$ signal in aged neurons [80,81]. A similar effect on Ca$^{2+}$ homeostasis was observed by us in response to PMCA2 or PMCA3 knockdown, pointing out a slower calcium clearance following Ca$^{2+}$ loads, which is a common hallmark of aging cells. In PMCA-deficient cells, higher resting [Ca$^{2+}$]$_i$ may directly reflect a partial loss of calcium clearing potency and sensitize mitochondria to buffer cytosolic calcium transients even in a condition of moderate increases in [Ca$^{2+}$]$_i$.

It is expected that accumulation of Ca$^{2+}$ by mitochondria will stimulate Ca$^{2+}$-dependent dehydrogenases of the TCA cycle, providing reducing equivalents to boost the activity of respiratory chain and thereby, ATP production via oxidative phosphorylation (OXPHOS) [82]. The ATP supply is then needed for restoration of ion gradient across the plasma membrane by PMCA or Ca$^{2+}$ transport to the ER by the SERCA pump. However, in the case of mitochondrial overwhelming with Ca$^{2+}$, permeability of the inner mitochondrial membrane increases dramatically, resulting in the dissipation
of mitochondrial membrane potential, loss of mitochondrial respiration, and finally, initiation of cell death [83,84]. Large dissipation of mitochondrial gradient was observed in PMCA2-deficient cells during massive Ca\(^{2+}\) entry, an event that was blocked by cyclosporine A or bongkrekic acid, suggesting the involvement of mitochondrial permeability transition pore (mPTP). The mPTP is a nonselective channel located in the inner mitochondrial membrane that allows the release of excessive Ca\(^{2+}\) accumulated in mitochondria. However, overload of the mitochondrial matrix with Ca\(^{2+}\) or ROS may trigger mPTP prolonged opening and liberation of small molecules and pro-apoptotic factors, finally causing membrane potential loss, reduction in ATP level, and eventually, cell death [85,86]. Similar to the sequence of events demonstrated in PMCA2-deficient cells during depolarization, Scorrano and coworkers showed that in vitro depolarization induced mPTP opening when mitochondria were suitably loaded with Ca\(^{2+}\) [87]. The explanation of this phenomenon is likely to involve the enhancement of ROS formation, what can be suggested in these cells by a higher level of GSH as an adaptive response to free radicals overload [88].

An enhanced susceptibility to mPTP opening and the loss in PMCA activity in synaptic membrane are both reported in the aged brain [89]. In contrast to the catastrophic nature of the mPTP opening, the mitochondrial membrane was able to repolarize in PMCA2-deficient cells upon stimulus withdrawal, pointing out the ability of the electron transport chain (ETC) to rebuild the proton gradient and restore membrane potential. Because even partial loss of PMCA2 led to higher percentage of apoptotic cells [49], it is plausible that only partial or brief opening of mPTP was sufficient to drive cell death. Recent comparative studies showed a decreased threshold for Ca\(^{2+}\) concentration that induced mPTP opening in mitochondria isolated from old rats [90]. Therefore, it is possible that PMCA2 loss and concomitant changes in the cytosolic/mitochondrial calcium circuit can increase the probability of mPTP opening at lower rises in [Ca\(^{2+}\)]. Whether mPTP is involved in the physiological aging process is still under debate, but the latest data demonstrated reduced mitochondrial calcium buffering capacity and increased sensitivity to mPTP formation in putamen of aged monkeys [91].

Neurons rely almost exclusively on the mitochondrial OXPHOS system to generate ATP and the primary role of mitochondrial calcium ([Ca\(^{2+}\)]\(_{m}\)) is to boost energy production. During respiration, electrons from reducing equivalents (NADH and FADH\(_2\)) are transferred through ETC complexes (complex I-V) and H\(^+\) are pumped out to intermembrane space, creating an electrical (\(\Delta\psi\)) and pH (\(\Delta\text{pH}\)) gradients, both thermodynamically equivalent to drive ATP synthesis. Formation of \(\Delta\psi\) is fundamental for Ca\(^{2+}\) uptake to mitochondria and adequate mitochondrial function, mainly for ATP synthesis by the F\(_{0}\)F\(_{1}\) ATP synthase [92]. Mitochondria also buffer cytosolic Ca\(^{2+}\) elevations caused by the influx through plasma membrane Ca\(^{2+}\) channels [93] or by depletion of the internal Ca\(^{2+}\) stores [94]. The ER together with the mitochondria located in regions so called mitochondria-associated ER membranes (MAMs) form highly sophisticated toolkits enabling Ca\(^{2+}\) trafficking between these organelles [95].

It has been demonstrated that PMCA2 and PMCA4 are tethered to specialized calcium microdomains located at the plasma membrane through the interaction with postsynaptic density protein 95 (PSD-95) [96]. PMCA/PSD-95 complexes further recruit N-methyl-D-aspartate (NMDA) receptor subunits R1 and R2A to form multiprotein aggregates [97]. By bringing PMCA to a close proximity of Ca\(^{2+}\) entry sites, formation of such structures can allow for rapid and effective response to local Ca\(^{2+}\) increases. Disrupted NMDA/PSD-95 interaction, observed in animal models of several neurological diseases [98,99], is expected to result in receptor hypersensitivity and excitotoxicity, ultimately leading to cell death. Many important studies suggest that Ca\(^{2+}\) uptake by the mitochondrial uniporter (mtCU) plays an essential role in NMDA receptor activity-driven excitotoxicity [100–102]. NMDA receptor stimulation was found to increase mitochondrial membrane depolarization in neurons overexpressing mtCU, whereas knockdown of endogenous mtCU reduced NMDA-induced increases in mitochondrial Ca\(^{2+}\), making cells resistant to excitotoxicity [103]. In the scenario of NMDA receptor-induced and Ca\(^{2+}\)-mediated mitochondrial swelling referred to as “mitochondrial aging”, progressive loss of respiratory control is in fact due to abrupt increase in mPTP conductance. Increased
permeability of mitochondrial membrane initiated at the level of individual mitochondria is spread across increasing fraction of the mitochondrial population (for comprehensive review see [104]), and usually leads to a massive cell death. Such mode of neuronal death triggered by overstimulation of NMDA receptors is associated with cerebral ischemia and neurodegenerative diseases [105–107].

The mtCU complex consists of pore-forming subunit (MCU) and several regulatory subunits (MICU1, MICU2, MICU3, MCUb, and EMRE) [108–110]. Mitochondrial calcium uptake 1 and 2 (MICU1 and MICU2) form a central semiautonomous assembly [111] to establish a threshold for MCU opening, keeping it closed at nanomolar [Ca$^{2+}$]c, thus protecting mitochondria from Ca$^{2+}$ overload [112]. Elevations of [Ca$^{2+}$]c above 2–3 µM are expected to trigger channel opening and promote cooperative increase in Ca$^{2+}$ uptake as described in earlier studies [113,114]. The recent studies on MICU1−/− mouse showed that in purified mitochondria, the MICU1 absence augmented calcium uptake at low [Ca$^{2+}$]c, but inhibited the uptake rate when [Ca$^{2+}$]c increased [115]. Young MICU1−/+ mice had underdeveloped cerebellum, abnormal persistence of the outer granular layer at postnatal day 12, and altered arborization of Purkinje cells. Interestingly, surviving MICU1−/− mice appeared to improve over time and previous histological abnormalities seen in cerebellum were resolved [115]. In addition, the differences in resting calcium, ATP level, and muscle lactate were no longer visible, although MICU1−/− mice did continue to have neurological and muscular defects. The authors suggested that such gradual improvement is due to age-related functional remodeling of mitochondria, which has been recently demonstrated in mouse with reduced MCU activity [116]. In the case of MICU1 deletion, this remodeling appears to involve alterations in the expression ratio of MCU to EMRE.

EMRE is important for Ca$^{2+}$ uptake and bridges the calcium-sensing role of MICU1 and MICU2 with the calcium-conducting function of MCU [109]. It seems to act as scaffold for the correct stoichiometric assembly of the complex and in its absence, uniporter current is lost despite proper MCU expression and oligomerization. Liu and colleagues [115] observed reduced EMRE expression in older MICU1−/− mice, which correlated with phenotypic improvement. They were, however, unable to obtain MICU1−/−EMRE−/− mice due to compromised survival. Nonetheless, deletion of one allele of EMRE on the background of MICU1−/− resulted in reduced calcium uptake at both low and high extra-mitochondrial calcium levels. Consistent with that, matrix Ca$^{2+}$ level in brain mitochondria was similar between wild type and MICU1−/− EMRE−/− mice. This genetic background also produced age-appropriate cerebellar morphology but when compared to MICU1−/−, MICU1−/− EMRE−/+ mice exhibited phenotypic and behavioral improvement. In neurons, non-assembled EMRE is degraded by m-AAA protease, which ensures sufficient assembly of gatekeeper subunits with MCU [117]. Very recently, it has been demonstrated that accumulation of constitutively active MCU-EMRE channels lacking gatekeeper subunits due to loss in m-AAA protease led to mitochondrial Ca$^{2+}$ overload, mPTP opening, and subsequent neuronal death [117].

The function of other mitochondrial uniporter subunits is currently under intensive investigation. When looking at the tissue-specific MCU complex composition, MICU3 is predominantly expressed in the central nervous systems, although its low levels were also detected in skeletal muscle [118,119]. It can form heterodimers with MICU1 but not MICU2, but the function is opposite to MICU2, as its expression promotes mitochondrial Ca$^{2+}$ accumulation at high [Ca$^{2+}$]c. Therefore, MICU3 is considered to act as a highly potent cooperative MCU activator with no gatekeeping function. Patron and colleagues [120] have recently performed the extended analysis of MICU3 function in neurons. They found that MICU1/MICU2 dimers ensured low Ca$^{2+}$ cycling in the steady-state conditions whereas MICU1/MICU3 dimers activated MCU-dependent Ca$^{2+}$ uptake even in the low [Ca$^{2+}$]c. MICU3, when silenced, was also the only MICU isoform that effectively decreased mitochondrial Ca$^{2+}$ transients evoked by synaptic activity [120].

In principle, aged neurons undergo considerable changes in Ca$^{2+}$ store content, mtCU expression, and ER-mitochondria domains [121]. Increased mtCU over time is thought to respond to higher Ca$^{2+}$ release from the ER, which is in line with the observation of increased mitochondrial-ER coupling in
aging in the face of mitochondrial depolarization [121]. In view of that, silencing of MICU3 could potentially slow down age-associated mitochondrial deficits by decreasing the amplitude of \([\text{Ca}^{2+}]_c\) rises, as shown by Patron et al. [120], while MICU2 knockdown is expected to exert the opposite effect.

Overall, the effect of higher \([\text{Ca}^{2+}]_m\) is expected to coordinate upregulation of OXPHOS machinery resulting in faster respiration and ATP production (Figure 1).

![Figure 1. Differences in mitochondrial function in PMCA-deficient PC12 cells. Higher resting \([\text{Ca}^{2+}]_i\) that reflected a partial loss of calcium clearing potency might sensitize mitochondria to buffer cytosolic calcium transients. Because PMCA operates as a 1Ca\(^{2+} / 1H^+\) antiporter, the extrusion of Ca\(^{2+}\) introduces large quantities of H\(^+\) that are readily transported to mitochondria. Due to alkalinization of mitochondrial matrix in steady-state conditions and because pH\(_{\text{cyto}}\) was higher, but still lower than pH\(_{\text{mito}}\), in the cells increased pH gradient which could drive a higher rate of ATP synthesis, more significantly in PC12_2 cells. The loss of fast acting PMCA2 could stimulate other ATP-dependent Ca\(^{2+}\) extruding proteins to restore \([\text{Ca}^{2+}]_c\), thereby increasing cellular demand for ATP. Also, in this line, OXPHOS rather than anaerobic glycolysis was a main energy supplying pathway. Interestingly, knockdown of PMCA3 isoform did not change the rate of OXPHOS. However, enhanced lactate release and greater sensitivity of the ATP level to glucose withdrawal in the presence of pyruvate suggests the reliance on anaerobic glycolysis, so the local synthesis of ATP may be sufficient to provide an adequate amount of energy. Other details are in the text. PMCA - plasma membrane Ca\(^{2+}\)-ATPase.

The recent report [122] showed that the basal rate of mitochondrial respiration was increased in aged endothelial cells, indicating enhanced mitochondrial contribution to energy balance in steady-state conditions and age-related increase in ER-mitochondrial Ca\(^{2+}\) transfer. In our study, a positive correlation between oxygen consumption and the ATP level was observed in PMCA2-deficient cells which indicates higher energy demands. Indeed, the experiments with KCN and oligomycin showed that OXPHOS rather than anaerobic glycolysis is a main energy supplying pathway in these cells. This was further supported by higher basal glucose consumption and lower NAD(P)H level accompanied by increased activity of proton-pumping ETC complexes (I, III, and IV). These findings are in agreement with the boosting effect of Ca\(^{2+}\) on mitochondrial metabolism and suggest that the rate of OXPHOS in PMCA2-deficient cells may be controlled at the activity of ETC proteins. Interestingly, knockdown of PMCA3 isoform did not change the rate of OXPHOS. However, enhanced lactate release and greater sensitivity of the ATP level to glucose withdrawal in the presence of pyruvate suggests the reliance on anaerobic glycolysis. This brings up an intriguing question about preferential fueling of PMCA isoforms by different energy-generating pathways. It is tempting to hypothesize that the loss of fast acting PMCA2 will stimulate other ATP-dependent Ca\(^{2+}\) extruding proteins to restore \([\text{Ca}^{2+}]_i\), thereby increasing cellular demand for ATP. When PMCA3 was depleted, \([\text{Ca}^{2+}]_c\) rise was only
moderate, so the local synthesis of ATP by glycolysis may be sufficient to provide adequate amount of energy (Figure 1).

Whatever the explanation might be, increased \([\text{Ca}^{2+}]\), as a result of PMCA2 or PMCA3 knockdown seems to be central for understanding the specific coupling between PMCA2 and mitochondrial metabolism of aging neurons. Our results of increased mitochondrial activity, coexisting with higher \(\text{Ca}^{2+}\) accumulation in the ER, as described in a previous chapter, are consistent with the recent hypothesis of enhanced ER-mitochondrial \(\text{Ca}^{2+}\) influx during aging and are further supported by in vitro studies based on rat hippocampal neurons [121]. Although the exact mechanism is not known, the mild ER stress due to increase in ER loading with \(\text{Ca}^{2+}\) observed in PMCA-deficient cells, which yet does not induce massive cell death, can eventually cause slow \(\text{Ca}^{2+}\) leak and activation of proteins involved in ER-mitochondrial tethering. This crosstalk might help to sustain elevated mitochondrial ATP production, creating a positive feedback loop temporarily prolonging ER function during aging. Based on the biphasic model of mitochondrial aging reported for primates and rodents, increased respiration and activity of the ETC complexes in PMCA2-deficient cells may reflect the early stage in which \(\text{Ca}^{2+}\)-dependent increased mitochondrial activity boosts the generation of harmful ROS. As the aging progresses, ROS accumulation combined with a loss of scavenging mechanisms negatively affect the activity of ETC, leading to severe mitochondrial dysfunction [123], which has been widely reported as a hallmark of ageing. Strong experimental data seem to support this hypothesis, as increased expression of mitochondrial genes of complexes I, III, IV, and V was reported in 18-month old mice in the hippocampus, medial prefrontal cortex, and striatum [124], whereas later in aging, the expression of ETC proteins as well as the activity of complexes I and IV decreased in substantia nigra, hippocampal dentate gyrus, frontal cortex, and cerebellum [125,126].

The fundamental question in respect to higher mitochondrial activity in PMCA2-deficient cells is how the driving force for ATP synthesis is generated. The findings of Poburko et al. [127] showed that bursts of proton accumulation in the cytosol generated by the activity of PMCA were readily transmitted to mitochondria. A similar relationship between \(\text{pH}_{\text{mito}}\) and \(\text{pH}_{\text{cyto}}\) was previously demonstrated in MDCK cells and was attributed to the activity of mitochondrial proton antiporters [128]. The \(\text{pH}_{\text{mito}}\) recordings suggested that rapid equilibration of mitochondrial matrix pH to cytosolic pH changes is accounted by \(\text{P}_{\text{i}}/\text{H}^{+}\) symport and \(\text{K}^{+}/\text{H}^{+}\) exchanger. However, the data regarding the effect of agonist-induced \([\text{Ca}^{2+}]_{i}\) elevations on mitochondrial pH are conflicting, as both acidification and alkalization of matrix were reported [129–131]. In their excellent study, Poburko et al. provided several lines of evidence that PMCA was the source of \(\text{H}^{+}\) during \([\text{Ca}^{2+}]_{i}\)-dependent mitochondrial acidification. First, the acidification matched the kinetic property expected for \(\text{Ca}^{2+}/\text{H}^{+}\) antiporter coupling \(\text{Ca}^{2+}\) extrusion to the proportional load of \(\text{H}^{+}\); second, the acidification was attributed to \(\text{Ca}^{2+}\) fluxes at the plasma membrane but not to \(\text{Ca}^{2+}\) release from intracellular stores; third, alkaline extracellular pH or \(\text{La}^{3+}\), both known to inhibit PMCA activity, prevented cytosolic acidification. The transmission of acid generated by PMCA to mitochondria may constitute a mechanism, protecting cells from calcium excess, as low \(\text{pH}_{\text{mito}}\) is known to inhibit \(\text{Ca}^{2+}\)-dependent mPTP opening and reduce ROS generation [132,133].

Our concurrent measurements of \(\text{pH}_{\text{cyto}}\) and \(\text{pH}_{\text{mito}}\) showed, however, alkalization of mitochondria matrix in steady-state conditions in response to PMCA2 or PMCA3 knockdown (7.78 ± 0.01, 7.62 ± 0.01, respectively, vs. 7.53 ± 0.02 in control). Because \(\text{pH}_{\text{cyto}}\) was also higher in PMCA-deficient cells, but still lower than \(\text{pH}_{\text{mito}}\), which is consistent with chemiosmotic coupling hypothesis, pH gradient across the inner mitochondrial membrane (\(\Delta\text{pH} = \text{pH}_{\text{mito}} - \text{pH}_{\text{cyto}}\)) was increased in these cells. Based on the PMCA/pH relationship, we anticipate that knockdown of neuro-specific PMCA isoforms, which are considered as fast reacting, dramatically limits the number of \(\text{H}^{+}\) entering cytosol leading to \(\text{pH}_{\text{mito}}\) increase. In these circumstances, the rise in \(\text{pH}_{\text{mito}}\) could result from compensation of \(\text{Ca}^{2+}\) charge by moving \(\text{H}^{+}\) down the ETC complexes. It is therefore attractive to propose that increased \([\text{Ca}^{2+}]_{i}\) observed in PMCA2-deficient cells, and hence higher \([\text{Ca}^{2+}]_{\text{Im}}\), is a main trigger for increased proton motive force to drive higher rate of ATP
synthesis. To support this, application of FCCP which collapses the inner mitochondrial membrane potential, evoked significant rise in \([\text{Ca}^{2+}]_m\) in PMCA-deficient lines suggesting higher \(\Delta \Psi_m\)-dependent mitochondrial Ca\(^{2+}\) loading, especially when PMCA2 was knocked down. The phenomenon of higher magnitude of \([\text{Ca}^{2+}]_c\) rises in response to FCCP was also demonstrated by Behringer and colleagues in old microvascular endothelium, suggesting greater capacity of mitochondria for Ca\(^{2+}\) during aging [134]. However, respiration-dependent Ca\(^{2+}\) entry is expected to lower \(\Delta \Psi_m\), as clearly observed in isolated mitochondria exposed to supraphysiological levels of extramitochondrial Ca\(^{2+}\) [135]. Under physiological conditions, Ca\(^{2+}\) uptake would not always alter \(\Delta \Psi_m\), which was demonstrated in isolated cardiomyocytes or it can provoke short-lasting \(\Delta \Psi_m\) decreases that would not inhibit ATP synthesis [136]. Transient and small fluctuations in \(\Delta \Psi_m\) in response to cytosolic Ca\(^{2+}\) peaks were indeed observed in other systems [137], suggesting plausible cell-specific effect of Ca\(^{2+}\) on \(\Delta \Psi_m\)-driven ATP synthesis. In line with that, Jouaville and colleagues [138] in their key paper suggested that mitochondrial ATP production can be differentially modulated in response to different Ca\(^{2+}\) signals generated by various external stimuli. In HeLa cells, that are glycolytic similar to PC12 cells, they demonstrated large increases in \([\text{Ca}^{2+}]_c\) and [ATP]\(_m\) and concomitant stable rise in \(\Delta \Psi_m\) upon histamine stimulation. This suggested that increased [ATP]\(_m\) reflected long-lasting elevation of the mitochondrial energy state. Similarly, increase in both \(\Delta \Psi_m\) and \(\Delta \phi\) components of the proton motive force was seen after stimulation with vasopressin [139], which is known to produce cytosolic [Ca\(^{2+}\)]\(_c\) transients that are relayed to mitochondria. The authors suggested that respiratory chain activity may be regulated by a long-lasting mitochondrial signal generated by \([\text{Ca}^{2+}]_m\) transients and thus, partially by \([\text{Ca}^{2+}]_c\) levels, which is coherent with our data from PMCA2-depleted line. These findings also fit the mitochondrial memory mechanism proposed by Jouaville et al. [138] stating that long-term priming of mitochondrial activity persists longer than the rise in \([\text{Ca}^{2+}]_m\). This allows mitochondria to meet the energy needs without the risk of organelle Ca\(^{2+}\) overload. Although, the mechanistic explanation of such “memory” is unknown, it plausibly involves the changes in \(\Delta \Psi_m\), as observed by us and/or Ca\(^{2+}\)-dependent activation of Fo/F1 ATP synthase. Indeed, several in vitro and in vivo studies demonstrated that the rate of ATP synthesis can be controlled directly at the level of Fo/F1 ATP synthase, independently from the respiratory rate and \(\Delta \Psi_m\) [140,141]. Fo/F1 ATP synthase can bind Ca\(^{2+}\) directly [142] but its activity can be also regulated by phosphorylation of the \(\gamma\)-subunit, which in turn is driven by mitochondrial Ca\(^{2+}\) [143]. Moreover, a recent study has shown that S100A1 protein binds Fo/F1 ATP synthase in Ca\(^{2+}\)-dependent fashion, increasing cellular capacity for ATP synthesis [144], thus providing another way for \(\Delta \Psi_m\)-independent regulation of energy generation.

Contrary, none of the PMCA-deficient lines showed a correlation between \([\text{Ca}^{2+}]_c\) response to FCCP and mitochondrial membrane hyperpolarization. Instead, FCCP produced massive loss of \(\Delta \Psi_m\) and slight hyperpolarization of plasma membrane suggesting that the plasma membrane potential is not created by proton pump and the protonophore equilibrates pH across the plasma membrane by carrying H\(^+\) from cytoplasm to the extracellular milieu. It has been demonstrated that lack of protons in mitochondrial matrix under the excess of electron donors strongly promotes ROS generation [135]. Therefore, taking into account higher \(\text{pH}_{\text{mito}}\) in PMCA-deficient cells, increased accumulation of Ca\(^{2+}\) by mitochondria consisted with the observation of over-activation of ETC complexes, our results point to a specific coupling between PMCA2 generated calcium fluxes and metabolic activity and may mirror specific cellular alterations observed in early aging.

5. PMCA and Ca\(^{2+}\)-Regulated Proteins—CaM, GAP43, CaN in Ageing

The main function of PMCA is to maintain intracellular Ca\(^{2+}\) homeostasis, but it is now well documented that efficiency of this control is modulated by several independent regulatory mechanisms [145]. Among them, activation of PMCA by calmodulin (CaM) is the most prominent one, and PMCA is the only calcium pump directly regulated by CaM [146]. Both “fast” PMCA isoforms with a high basal activity exhibit 5 to 10-fold higher affinity for CaM compared to PMCA1 and PMCA4, but are weakly stimulated by CaM [20,147,148]. In rats, CaM is encoded by three non-allelic genes—\(\text{Calm1},\)
Calm2, and Calm3—all ultimately producing the identical CaM protein. The total CaM amount varies among tissues but the highest concentration (over 30 µM) can be detected in the brain [149]. Because CaM can regulate nearly 300 proteins, there is a high competition rate for CaM binding which is dictated by the affinity of its particular protein partners [150]. Such competition is thought to regulate frequently contradictory processes that are concurrently activated in response to [Ca$^{2+}$]; rises. The prevalence in activation of certain CaM downstream signaling pathways may therefore sensitize neurons to aging-related physiological and pathological stimuli. Like PMCA, CaM shows functional defects in the aged brain, mainly due to intensive oxidation of multiple methionines [2]. Oxidatively modified CaM can still bind PMCA, but cannot stimulate pump activity. Moreover, it also prevents PMCA activation by unoxidized CaM. Although CaM half-life is approximately 18 ± 2 hours, these global structural CaM alterations provide a possible mechanism for the loss of proper calcium regulation during aging [32,151].

Early studies on PC12 cells suggested the inversed relationship between CaM level and neurite elongation in response to a differentiation signal [152]. In our studies, we observed decreased CaM immunoreactivity in both PMCA-deficient lines and proposed that the underlying mechanism involved differential regulation of CaM gene expression [153]. Interestingly, the expression of Calm1 and Calm2 was downregulated in both modified lines but PMCA2 depletion additionally reduced the expression of Calm3. Since PMCA2 and PMCA3 have high basal activity, but are less sensitive to CaM stimulation, limited CaM availability could result in decreased activation of other PMCA isoforms. As a consequence, impaired total PMCA extruding activity can lead to permanent rise in [Ca$^{2+}$], more significantly in the PC12_2 line.

An additional physiological mechanism that can affect CaM function is a formation of complex with growth-associated protein 43 (GAP43, neuromodulin), which is also a marker of differentiating neurons [154,155]. A unique function of this multifunctional presynaptic protein is CaM binding, thereby it can control CaM availability in the cell. Especially high levels of GAP43 were reported at specific sites such as neuronal growth cones, where GAP43 may play a role of abundant CaM reservoir [156]. Since GAP43 half-life is about 2–3 days, this period may be sufficient for reprogramming of CaM-dependent signaling pathways in the cell. Critical for this process is the relationship between binding of CaM and protein kinase C (PKC)-mediated phosphorylation of GAP43. Dephosphorylated GAP43 binds CaM at low Ca$^{2+}$ concentration, while phosphorylation by PKC at Ser 41 liberates CaM due to significant reduction in GAP43 affinity. Dephosphorylation by e.g., calcineurin (CaN), reverses this process and decreases available free CaM [156]. This mechanism directly controls the functional CaM level in the cell, and indirectly all Ca$^{2+}$/CaM-dependent processes.

In neurons, GAP-43 is distributed throughout all cellular compartments with the highest density found in axon terminals and is implicated in synapse formation and neurite branching [154,157,158]. It has been proposed that the level of GAP-43 might determine the progress of neurogenesis, particularly in the hippocampus and associate cortex in the adult human brain [159]. Physiologically, GAP43 is linked with synaptic plasticity, regulation of neurotransmitter release, learning, and mnemonic function [156]. Behavioral studies demonstrated that reduced GAP-43 level led to some deficits, while increased GAP-43 level enhanced behavioral performance [160]. A study on rats indicated the contribution of this protein to the general age-dependent decline in brain plasticity [161]. During aging, less GAP43 mRNA was detected in the hippocampus, and the phosphorylation level decreased by nearly 50% [162]. Another study on rats reported a lowered GAP43 immunoreactivity in the dentate gyrus, cingulate cortex, and olfactory bulb [163–165]. In humans, GAP43 expression predominates during the first decade of life and remains nearly unchanged during further life [166]. However, in Alzheimer’s disease (AD) reduction of neuronal GAP-43 mRNA and GAP-43 protein has been linked to memory dysfunction [167,168].

Our studies showed increased GAP43 mRNA and protein level in both PMCA-deficient lines [169]. Interestingly, we detected a higher amount of dephosphorylated GAP43 protein, while its phosphorylation level (P-GAP43) was significantly decreased. Higher GAP43/P-GAP43 ratio indicates
enhanced binding of CaM by dephosphorylated form of GAP43. Indeed, in PMCA-deficient lines, a much stronger fluorescent signal from GAP43 and calmodulin-specific antibodies was detected suggesting a more intensive formation of the GAP43/CaM complex which was further confirmed by co-immunoprecipitation. Both lowered CaM presence detected in these lines and stronger formation of CaM/GAP43 complex may additionally decrease CaM regulatory potency.

Further studies using cyclosporine A, an inhibitor of serine-threonine phosphatase—calcineurin (CaN), showed that formation of CaM/GAP43 is controlled by CaN activity [169]. CaN is the only reported phosphatase completely dependent on Ca\(^{2+}\)/CaM, and it constitutes slightly more than 1% of all brain proteins [170]. In neurons, CaN can be detected in cytoplasm, but also in nearly all subcellular structures i.e., endoplasmic reticulum, Golgi apparatus, nucleus, synaptic vesicles, microsomes, outer mitochondrial membrane, and plasma membrane [30]. Distribution of CaN in the cell appears to be an important factor regulating its local activity. CaN controls numerous and diverse cellular processes mainly via activation of NFAT transcription factor (nuclear factor of activated T cells). Higher activity of calcineurin was observed in aging, as well as in rat and mice models of AD [171,172], and likewise other Ca\(^{2+}\)/CaM-regulated enzymes, it was linked to an increase in [Ca\(^{2+}\)]. Some data suggested the relationship between CaN/NFAT-dependent regulation of PMCA and brain aging, along with the onset of neurodegenerative diseases [40,42,173].

To elucidate whether altered composition of PMCA isoforms could affect the expression and activity of CaN in PC12 cells, molecular and biochemical analyses were performed [169]. Using the real-time PCR technique, we detected up-regulation of CaN mRNA by approximately 60% and 85% in PMCA2 or PMCA3-deficient lines, respectively. These changes were also seen at the protein level and gave rise to higher CaN activity. Albeit, there was no direct interaction between GAP43 and PMCA, diminished calcium pumping activity considerably affected CaN and CaM functions.

One of the physiologically important CaN actions is an orchestration of a sequence of signaling events leading to dephosphorylation of conserved phosphoserine residues in the transcription factor NFAT, its translocation to the nucleus, and subsequent transcriptional activation of NFAT-regulated target genes [170]. However, besides the role of CaN in transcriptional signaling, increasing the number of studies reported the interaction of CaN with other substrates, targeting proteins and regulators of CaN activity. Among others, CaN was shown to interact directly with PMCA2 and PMCA4 isoforms, which resulted in the inhibition of CaN-regulated processes, including activation of NFAT [174–177]. On the other hand, the expression of PMCA4b in neurons was shown to be controlled by CaN [178]. In our model of differentiated PC12 cells, we detected strong interaction between PMCA2 and CaN. However, high-resolution confocal imaging supported by precipitation of PMCA/CaN immunocomplexes, demonstrated lower degree of colocalization and significantly decreased CaN binding to PMCA in PC12_2 cells. The same set of experiments showed lack of detectable changes in the ability of PMCA2/CaM complex formation between control and PC12_3 cells. Interestingly, in both PMCA-deficient lines, lower PMCA4/CaN immunoreactivity was present, suggesting that PMCA depletion may increase a pool of potentially active CaN. In this context, fast acting PMCA2 and PMCA3 isoforms may be seen as regulators of local CaN availability and function. Control of local CaN activity is beneficial for cell survival in the condition of calcium overload and together with reported protective role of PMCA4 [145,146,148] may be of paramount importance for effective anti-apoptotic protection in PMCA3-deficient line.

Taken together, changes in PC12 cell lines initiated by moderate, but prolonged increase in [Ca\(^{2+}\)], due to specific PMCA depletion produced distinct cell responses, which clearly depended on PMCA isoforms composition and isoform-specific regulatory mechanisms. When induced changes are adaptive, the cells can effectively counteract Ca\(^{2+}\)-dependent destructive processes and retain the ability to survive. However, these protective actions in PMCA2 down-regulated cells were found to be much less efficient. One can assume that not only changes in global [Ca\(^{2+}\)] concentration, but also spatially and temporally limited modifications in transmission of calcium signal played a decisive role
in the cell response, and the detrimental effects over time were not sufficiently counterbalanced by these adaptive mechanisms.

6. PMCA-Deficient PC12 Cells as a Tool for Modeling Functional Adaptive Strategies in Neurons during Aging

As described above, aberrant PMCA-mediated Ca\(^{2+}\) clearance was associated with perturbation in mechanisms responsible for maintenance of calcium homeostasis. A summary of the detected changes in the expression of analyzed components in PC12_ and PC12_3 are presented in Table 1. As profound changes in CaM expression and availability demonstrated in PMCA-deficient cells could significantly alter CaN activation and CaN-downstream processes, we turned our attention toward a potential cross talk between PMCA, CaM, and CaN in shaping of the Ca\(^{2+}\) signal. One of the first basic question we asked was the role of the functional interaction between these proteins in the differential regulation of CaM transcripts and whether this may create a feedback loop for PMCA function.

Looking for a mechanism(s) that could underlie the diminished expression of CaM genes, a microarray screening of fundamental transcription factors was performed [153]. Among hundreds of analyzed genes, only the expression of Nfatc2, whose activity is associated with Ca\(^{2+}\), increased in both PC12-deficient lines, which led us to the hypothesis that NFATc2 may function as a transcriptional regulator of calmodulin genes. The NFAT family is composed of five proteins, of which NFATc1, NFATc2, NFATc3, and NFATc4 are activated by Ca\(^{2+}\) and their action is controlled by calcineurin [179,180]. In steady-state conditions, NFAT resides in the cytosol in the phosphorylated state, but increased \([\text{Ca}^{2+}]_c\) activates CaN, which dephosphorylates NFAT causing its translocation to the nucleus, where it becomes transcriptionally active. A return of \([\text{Ca}^{2+}]_c\) to the resting level initiates re-phosphorylation and rapid relocation of NFAT back to the cytosol. Our results showed that moderately increased basal \([\text{Ca}^{2+}]_c\) in PMCA-deficient lines sufficiently induced its nuclear translocation and DNA binding activity. Moreover, we found that a sequence for NFATc2 binding is located in the promoter region of Calm2 and Calm3 genes, but not in Calm1. Chromatin immunoprecipitation assay revealed higher promoter occupancy by NFATc2 in PMCA-modified cell lines resulting in diminished transcription of both CaM genes. In addition, overexpression of the constitutively active form of CaN increased NFATc2 nuclear accumulation and its promoter activity, which potentiated Calm2 and Calm3 repression. This strongly indicated the NFATc2 repressive role toward CaM gene expression. Further experiments with NFATc2 silencing, using selective siRNA, showed a partial rescue of the expression of Calm2 in both lines and Calm3 in PC12_2 cells, and confirmed that the activation of the CaN/NFAT pathway may repress CaM genes, but to various extent in each of the PMCA-deficient lines.

The differences in PMCA isoform ratio could affect the regulation of the downstream events including CaN/NFAT-dependent regulation of Calm2 and Calm3 genes. It was reported that CaN interacts with PMCA2 and PMCA4 which resulted in inhibition of its phosphatase activity [175]. In line with it, lowered PMCA2 amount could be partially responsible for diverse cell response. The second important player and limiting factor was the amount of CaM available for binding. This could profoundly interfere with CaN/NFAT activation in both PC12-deficient lines, further suggesting the existence of the feedback mechanism by which CaM could affect its own expression. This specific regulation seems to be a direct consequence of selective PMCA isoform silencing, because no similar effect was observed in the control PC12 cells. Finally, lowered CaM level may have potential consequences on Ca\(^{2+}\) extrusion by PMCA, as was reported in senescence neurons [30,32,181]. In addition, since aging was shown to be associated with excessive Ca\(^{2+}\) influx through L-type VGCC, which is inactivated by Ca\(^{2+}\)/CaM complex and directly modulated by CaN [64,182,183], reduced CaM level may thus potentiate calcium influx and inhibit CaN activity as well. In the context of neuronal aging, these results shed new light on molecular basis of neurodegenerative diseases and demonstrated several lines of cellular protection from the negative effects of Ca\(^{2+}\) overload. In addition to the membrane components, the maintenance of calcium homeostasis is coupled with the multifunctional endoplasmic reticulum, which contains several Ca\(^{2+}\) sensitive transporters, including
IP$_3$ receptors are intracellular ubiquitously expressed Ca$^{2+}$ channels that exist in three main isoforms: IP$_3$R-1, IP$_3$R-2, and IP$_3$R-3. In the central nervous system, the presence of all isoforms, with the predominance of IP$_3$R-1, was detected, although their subcellular compartmentalization varied in different brain regions [185–187]. In the rat brain, IP$_3$R-1 was found in high amounts in Purkinje neurons in cerebellum and was localized to dendrites, dendritic spines, cell bodies, axons, and axonal terminals [188,189]. In the hippocampus, IP$_3$R-1 is mostly expressed in the CA1 region, with substantially less expression in CA3 and only moderate levels in the granule cells of the dentate gyrus [185]. A particular role of IP$_3$Rs in the hippocampus is related to learning and memory abilities, and changes in the IP$_3$R isoform composition during aging may have an impact on increased deficits in these processes [190]. In other type of neurons, a high level of IP$_3$R-1 was found in cell bodies and proximal dendrites. IP$_3$R-2 was mostly detected in glia, whereas IP$_3$R-3 was predominantly expressed in neuronal terminals in limbic and basal forebrain regions [191]. The expression of particular receptors during aging is differentially regulated, also in a brain region-specific manner [192,193]. Moreover, IP$_3$Rs are dynamically regulated by the formation of homo- or heterotetrameric complexes, thus their relative expression together with other components will determine the final cell response [194,195]. It has been proposed that the level of IP$_3$ receptors declines progressively during aging. However, due to the oxidative modifications that are found to increase IP$_3$R function in the brain, IP$_3$ downstream signaling may not be compromised which is thought to represent a compensation for an altered redox state [186]. Thus, diverse processing of each IP$_3$R isoform may activate various cell signaling pathways that can initiate the adaptive response or lead to cell death [187,193]. Since IP$_3$R-mediated Ca$^{2+}$ release from the ER and mitochondrial Ca$^{2+}$ homeostasis are physiologically coupled, their improper cooperation may significantly affect cell viability [196].

Release of Ca$^{2+}$ from the ER by IP$_3$ receptors requires binding of both, IP$_3$—arising from stimulation of phospholipase C (PLC), and Ca$^{2+}$. All isoforms exhibit specific characteristics: IP$_3$R-1 possesses low Ca$^{2+}$ affinity and moderate affinity for IP$_3$, IP$_3$R-2 represents the isoform with the highest affinity for both Ca$^{2+}$ and IP$_3$, and IP$_3$R-3 is the most sensitive for modulation by Ca$^{2+}$, but displays the lowest IP$_3$ affinity [194,195,197]. Interestingly, cytosolic Ca$^{2+}$ regulates IP$_3$Rs in a biphasic way: low Ca$^{2+}$ level stimulates the receptors, but the concentration above 300 nM inhibits their transport activity [197]. Our analysis showed that PC12 cells, like the majority of available cell lines, express all three main IP$_3$Rs with IP$_3$R-3 being the most prominent subtype [198], although IP$_3$R-1 and IP$_3$R-2 were also detected [193]. In PMCA-deficient cells IP$_3$R-1 and IP$_3$R-2 expression decreased, whereas IP$_3$R-3 was higher (by 40%) than in the control, matching the changes at the protein level. Because this relationship occurred at the transcriptional level, our data could suggest the existence of Ca$^{2+}$-dependent negative feedback loop for IP$_3$R-1 and IP$_3$R-2 expression. Western blot analysis using an antibody recognizing all IP$_3$R isoforms revealed higher total protein level only in the PC12_3 line, pointing out a switch between IP$_3$Rs ratio in response to PMCA depletion. Such changes, together with higher presence of PMCA4 and highly active PMCA2, may represent an additional cellular defense mechanism aimed in response to PMCA3 depletion to overcome potentially harmful consequences of calcium excitotoxicity. PMCA4 seems to be central for this defense as it can bind plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP$_2$), protecting it from hydrolysis by PLC, potentially decreasing IP$_3$ production and, subsequently, Ca$^{2+}$ release from the ER [199]. Some of the adaptive mechanisms revealed in our studies could be relevant to the physiological events occurring during aging, but long-lasting Ca$^{2+}$
Dysregulation of calcium homeostasis may promote a variety of potentially detrimental consequences including proteolytic processes triggering apoptosis and/or necrosis.

The wide spectrum of these processes can be propagated by pro-inflammatory chemokines [200] such as CCL5, which induces phospholipase C-mediated liberation of Ca^{2+} from the ER by IP_3-gated channels. CCL5 can mobilize cytosolic Ca^{2+} after binding to three receptors—CCR1, CCR3, and CCR5, which are cell surface-associated, immune-regulatory G protein-coupled receptors (GPCRs) [201,202]. CCR5 activity has been implicated in normal cell development, but up-regulation of CCR5 was observed in a number of neurological disorders and models of CNS injury [203,204]. Over-activation of CCR5 with subsequent rise in cytosolic Ca^{2+} is known to affect chemotaxis, secretion, and gene expression and could lead to inflammatory and degenerative processes in the CNS [205]. Moreover, higher CCR5 expression in T cells during aging was demonstrated in humans and rodents at both mRNA and protein levels [206–208]. It should be noted that higher CCR5 activity may intensify not only CCL5-dependent events, but also the signaling induced by other known CCR5 ligands such as CCL3 and CCL4, of which expression is stimulated in the aging brain [209,210].

All three receptors were detected in our experimental model cells, but only CCR5 was localized in the plasma membrane which is required for CCL5 action. Interestingly, in each of the PMCA down-regulated PC12 lines, CCR5 expression increased by nearly 50%. Functional assays in Ca^{2+}-free medium showed that binding of CCL5 to its membrane receptor mobilized Ca^{2+} from the ER more intensively in PMCA-deficient lines but the highest amplitude of [Ca^{2+}]_c rises was detected in PC12_2 cells. In addition, there were significant differences between lines with respect to the rate of Ca^{2+} clearance and the duration of the prolonged calcium signal upon chemokine stimulation. The return of Ca^{2+} to its new steady-state level was 3 times longer in PC12_2 and about 2 times longer in PC12_3 than in control cells. The differences in kinetic parameters could reflect actual rate of Ca^{2+} release from the ER and its extrusion to extracellular milieu. It is worth to emphasize that PMCA3-deficient cells still possess very active PMCA2, but also higher protein level of PMCA4, thus could operate more effectively to control [Ca^{2+}]_c. Despite that, the efficiency of this adaptation is still lower than in control PC12 cells with a full set of PMCA isoforms.

Structural and functional changes in PMCA-deficient PC12 cell lines were initiated by moderately increased cytosolic Ca^{2+}, but downstream long-time effects seemed to be specifically linked to the function of particular isoforms. Some of the induced adaptive mechanisms were sufficient for protection of cell viability as observed in PMCA3-deficient cells (Figure 2) in comparison to less effective compensatory changes aimed in response to PMCA2 silencing (Figure 3). This underlines the unique role(s) of PMCA2 and PMCA3 in neuronal cells and indicates that long-lasting calcium dyshomeostasis due to diminished PMCA activity might be central to the aging process and variety of neuropathologies.
Figure 2. Functional changes in PMCA3-deficient PC12 cells. PMCA3 silencing increased \([\text{Ca}^{2+}]_i\), although PMCA1 and PMCA4 were up-regulated as compensatory effect, but it did not change the total amount of PMCA protein. Increased expression of L- and P/Q types of voltage-gated Ca\(^{2+}\) channels (VGCCs) could also raise \([\text{Ca}^{2+}]_i\). The observed up-regulation of CaN affected transcriptional activity of NFATc2, which repressed CaM genes and finally decreased available CaM. Since CaN was also shown to interact directly with PMCA2 and PMCA4 isoforms, the noticed lower PMCA4/CaN complex formation (not shown) could further increase a pool of potentially active CaN. The amount of CCR5 increased in PC12_3 cells, which is linked to IP_3-mediated Ca\(^{2+}\) release from endoplasmic reticulum (ER). The altered ratio of IP_3Rs expression with the enlarged total quantity, could further increase \([\text{Ca}^{2+}]_i\). However, PMCA4 was shown to bind plasma membrane PIP_2, potentially decreasing IP_3 production and, subsequently, Ca\(^{2+}\) release from the ER. All these changes, together with higher presence of PMCA4 and highly active PMCA2, may overcome potentially harmful consequences of calcium excitotoxicity. Green up-arrows reflect the increase and red down-arrows reflect the decrease of the amount or activity. Other details are in the text.

Figure 3. Functional changes in PMCA2-deficient PC12 cells. Down-regulation of PMCA2 increased \([\text{Ca}^{2+}]_i\) due to lowered calcium extrusion potency, despite compensatory up-regulation of PMCA1 and unchanged total PMCA protein level. Elevated expression of VGCC types, particularly T-type that exhibits slow deactivation rate, might contribute to the Ca\(^{2+}\) influx. Up-regulated CaN affected transcriptional
activity of NFATc2, which repressed CaM genes and lowered CaM amount. Limited CaM availability could weaken its stimulatory effect on PMCA. The amount of CCR5 increased in PC12_2, also with subsequent IP3-mediated Ca2+ release from ER. Altered ratio of IP3Rs expression with higher in the presence of IP3R-3, the most sensitive for modulation by Ca2+, but with the lowest IP3 affinity, could further increase [Ca2+]i. PMCA2 depletion induced some adaptive changes to counteract Ca2+-dependent destructive processes, but because of the enlarged percentage of apoptotic cells, these protective actions appeared to be much less efficient. Green up-arrows reflect the increase and red down-arrows reflect the decrease of the amount or activity. Other details are in the text.

7. Conclusions

Aging, as a physiological process, involves the perpetual adaptation to varying internal and external conditions by generation of the complex protective mechanisms. This new adaptive state enables the cells to continuously make short-term adjustments for optimal functioning, but because of the multifactorial nature of aging, the efficiency of these adaptations depends on the intensity of senescent signaling [211,212]. Calcium dyshomeostasis is a factor with especially strong destructive potential. Less efficient Ca2+ extruding systems and aberrant regulation of downstream effectors could disturb the function of mitochondria and endoplasmic reticulum leading to cell death, as was widely documented in aging cells [122,213].

Studies performed on our PC12 cell model of senescent neurons revealed an apparent contribution of PMCA2 and PMCA3 isoforms to the aging process. A controlled deficiency of PMCA2 or PMCA3 and resulting permanent increase in intracellular Ca2+, allowed to track unique functions that each of these isoforms may potentially perform in aging. The most important findings demonstrate that the altered composition of PMCA isoforms is linked to:

- intracellular pH regulation and changes in the mitochondrial bioenergetic processes,
- adaptive functional changes in the VGCC and in Ca2+ transport systems to the ER,
- regulation of CaM availability by Ca2+/CaN-dependent complex formation with GAP43,
- reduced CaM level and repressive regulation of Calm2 and Calm3 genes by NFATc2,
- altered expression of CCL5-sensitive receptors—CCR1, CCR3, and CCR5,
- altered expression of IP3 receptors.

Comparison between PC12_2 and PC12_3 lines clearly indicates that down regulation of PMCA2, apart from several adaptive or compensatory processes, triggers more potentially damaging events that are also observed during neuronal aging, including higher sensitivity to inflammation. A declining potency to maintain cellular homeostasis as a potential contributor to age-dependent neuronal senescence markedly increases the risk for neurodegenerative processes. Although differentiated PC12 cells can only partially mimic the physiological processes in the functional neuron, the changes observed in our model might represent the age-related mechanisms common in functionally different cell types.

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