Chapter 1
General introduction
Preface

“Milch macht müde Männer munter” (english: milk cheers up tired chaps, dutch: melk is goed voor elk) is a slogan from an old commercial created by the German milk industry in the 1950s that was thought to increase the sales quantity of milk by highlighting its healthy ingredients, especially calcium (Ca^{2+}). Although it turned out that milk is not an indispensable food, it has now become clear that Ca^{2+} indeed plays a central role in our body including the brain.

The primary objective of this work is to study how neuronal Ca^{2+}-activated potassium (K^{+}) channels (K_{Ca}) regulate Ca^{2+} homeostasis in the context of neurotoxicity triggered by (oxidative) stress and mitochondrial dysfunction. In this general introduction, I will focus on the importance of Ca^{2+} on physiological and pathological processes in neurons. After presenting principal pathways regulating neuronal Ca^{2+} homeostasis, K_{Ca} channels and their relevance in health and disease conditions will be discussed. Subsequently, intracellular Ca^{2+} homeostasis will be described with particular emphasis on energy metabolism, mitochondrial respiration and the interplay between the endoplasmic reticulum and mitochondria.

1. Neuronal Ca^{2+} homeostasis

Key physiological processes in neurons such as action potential formation and propagation are regulated through neurotransmitter release and dynamic ion flux (Ca^{2+}, sodium (Na^{+}), K^{+}). Among these ions, Ca^{2+} builds the driving force for the initiation and propagation of neuronal signaling events\(^1,2\). For instance, in presynaptic compartments, action potentials are generated through opening of voltage-dependent Ca^{2+} channels, thereby initiating the release of neurotransmitters into the synaptic cleft. Furthermore, the maintenance of a proper membrane potential is sustained by the activation and deactivation of K^{+} and Na^{+} channels, and to a great extent also by Ca^{2+} channels (see figure 1). Owing to the fact that Ca^{2+} acts as a second messenger molecule in multiple downstream signaling pathways, it is critical to control and maintain intracellular Ca^{2+} ([Ca^{2+}]) homeostasis.

**Neurotransmitter receptors**

Neurotransmitters are released from presynaptic membranes into the synaptic cleft, and bind to the corresponding receptors on postsynaptic membranes. Depending on the type of neurotransmitter receptor, the membrane is either hyperpolarized due to chloride (Cl^{-}) extrusion (inhibitory effect) or depolarized due to Na^{+} release (excitatory effect). Some neurotransmitter receptors such as those for glutamate, dopamine, acetylcholine (ACh) and \(\gamma\)-aminobutyric acid (GABA), also modulate the [Ca^{2+}], concentration following ligand-gated activation. The impact of neurotransmitter receptors on [Ca^{2+}], will be described below using glutamate, dopamine and GABA as examples.
In excitatory synapses, the action potential is propagated and neuronal excitability is enhanced through the release of glutamate, dopamine or ACh. Among these, glutamate appears to be the most prominent neurotransmitter as it binds to metabotropic or ionotropic glutamate receptors throughout the central nervous system. Ionotropic glutamate receptors such as N-methyl-D-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR) or kainate receptors (KAR) bind glutamate, thereby allowing the flux of cations. NMDAR are usually occupied and therefore blocked by bound divalent Mg²⁺ and Zn²⁺ ions³–⁸. Membrane depolarization activates NMDAR by releasing the Mg²⁺/Zn²⁺ blockade, allowing influx of Na⁺ and Ca²⁺ into the synapse⁹,¹⁰. The increase in [Ca²⁺], activates subsequent signaling mechanisms leading to propagation of the signal. Uncontrolled NMDAR activation through glutamate binding or other NMDAR agonists amplifies Ca²⁺ influx and induces neuronal cell death, a process known as excitotoxicity¹¹,¹². In cultured neurons, excitotoxicity also promotes mitochondrial damage through mitochondrial Ca²⁺ ([Ca²⁺]ₘ) overload, thereby depolarizing the mitochondrial membrane and stimulating the generation of toxic reactive oxygen species (ROS) in the mitochondria¹³–¹⁵. NMDAR inhibition by the antagonist MK-801 prevented neuronal cell death and restored [Ca²⁺], homeostasis in different cell death models in vitro and in vivo¹⁶–¹⁹. Therefore, the therapeutic potential of NMDAR antagonists was exploited in different neurodegenerative diseases, and the NMDAR inhibitor memantine is used for the symptomatic treatment of patients with Alzheimer’s disease (AD) or Parkinson’s disease (PD)²⁰. Similar to NMDAR, KAR activation was also associated with neurodegeneration, and administration of kainate to neuronal cultures is an established model to study epilepsy in vitro.

Apart from glutamate, dopamine acting on dopaminergic (DA) neurons also alters neuronal Ca²⁺ homeostasis. DA neurons are expressed in the striatum, substantia nigra, cortex and hippocampus, they control movement and motor function, and their expression is highly associated with the pathology of PD²¹–²⁴. Dopamine receptors belong to the G protein-coupled receptors that are divided into D₁-like (D₁ and D₅) and D₂-like (D₂₂) receptors. They are differentially expressed in the DA neurons of different brain regions and have distinct effects on adenylyl cyclases²⁵–²⁸. Upon coupling to G₄ or G₁ proteins, these receptors can activate adenylyl cyclases to generate cyclic adenosine monophosphate (cAMP) which in turn activates its main target cAMP-dependent protein kinase A (PKA). PKA subsequently phosphorylates, and thereby activates different targets including cyclic nucleotide-gated Ca²⁺ channels at the plasma membrane²⁹–³⁴. For instance, D₁ receptor activation in striatal slices induced cAMP-dependent Ca²⁺ influx and the consequent release of γ-aminobutyric acid (GABA) to inhibit neuronal activity³⁵. In mouse striatopallidal neurons, the activation of D₂ receptors strongly reduced glutamate release and subsequent Ca²⁺ influx³⁶.
Dopamine receptors also crosstalk with NMDAR, as dopamine D₄ receptor activation in the murine prefrontal cortex reduced NMDAR currents as well as NMDAR expression at the plasma membrane. In line with these findings, low dose dopamine treatment prevented delayed Ca²⁺ deregulation and excitotoxic cell death of DA neurons in response to glutamate treatment. Loss of DA neurons is associated with the progression of PD, and supplementation with dopamine receptor agonists or Levodopa is a strategy to treat symptoms of PD.

The most important inhibitory neurotransmitter is GABA which binds to corresponding GABA receptors and induces Cl⁻ influx, thereby further lowering the membrane potential and inhibiting neuronal firing. The class of GABA receptors includes the sub-types fast responding, ionotropic, ligand-gated (GABAₐ) receptors and the slow responding, metabotropic, G protein-coupled (GABAₐ) receptors. Pharmacologically, GABAₐ receptors can be targeted by picrotoxin and bicuculine. GABAₐ receptor activation in pyramidal neurons prevented their excitation and contributed to the regulation of neuronal Ca²⁺ homeostasis. During retinal neurogenesis, application of GABA activated GABAₐ receptors and increased Ca²⁺ influx through L-type Ca²⁺ channels (LTCC). This LTCC-mediated increase in Ca²⁺ enhanced the sensitivity of cerebellar Purkinje cells to GABA. Accordingly, inhibitors of LTCC also blocked GABAₐ receptors in vitro. Furthermore, in globus pallidus neurons, both GABA receptor subtypes are expressed and their modulation altered Ca²⁺ currents. This effect was blocked by inhibitors of both, LTCC and N-type Ca²⁺ channels (NTCC), respectively.

Ca²⁺ channels
 Voltage-gated Ca²⁺ channels (VGCC)
 Voltage-gated Ca²⁺ channels (VGCC) are activated in response to depolarization of the membrane. LTCC and NTCC are expressed in different neuronal cell types where they contribute to synaptic plasticity and the regulation of neuronal firing. Due to their function, LTCC are frequently associated with the onset or the predisposition to certain brain diseases such as affective disorders or PD. The different LTCC isoforms (Ca₂⁺_1.1-1.4) are distinguished based on the pore-forming α-subunit and are differentially expressed in the central nervous system where they predominantly localize to presynaptic membranes. Mechanistically, LTCC-mediated Ca²⁺ influx activates Ca²⁺-dependent downstream processes such as gene transcription through binding to Ca²⁺-sensitive calmodulin, and are subject to phosphorylation by calmodulin kinase II (CaMKII) or PKA, and dephosphorylation by calcineurin. NTCC (Ca₂⁺_2.2) are sensitive to inhibition by ω-conotoxin, and are involved in synaptic transmission.
Figure 1. Plasma membrane channels involved in regulating neuronal Ca\textsuperscript{2+} homeostasis.
Neuronal Ca\textsuperscript{2+} homeostasis underlies multiple lines of control. Ca\textsuperscript{2+} uptake, and therefore elevations in intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]), are controlled by neurotransmitter channels. N-methyl-D-aspartate (NMDAR), \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate (KAR) receptors are mixed ion channels activated by extracellular glutamate. Dopamine receptors (DR) and \(\gamma\)-aminobutyric acid receptors (GABAR) may activate intracellular signaling pathways to facilitate Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels (VGCC). Neuronal nicotinic acetylcholine receptors (nAChR) increase the activity of phospholipase C to produce inositol-1,4,5-triphosphate (IP\textsubscript{3}) which induces IP\textsubscript{3} receptor-dependent Ca\textsuperscript{2+} release from the endoplasmatic reticulum (ER). ER-Ca\textsuperscript{2+} release also activates Ca\textsuperscript{2+}-release activated Ca\textsuperscript{2+} channels (CRAC) to drive Ca\textsuperscript{2+} import. The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger regulates Ca\textsuperscript{2+} efflux, and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (K\textsubscript{Ca}) are activated by increases in [Ca\textsuperscript{2+}]. Full line: direct effect of channel activation at the plasma membrane on intracellular Ca\textsuperscript{2+}, dashed line: indirect effect following activation of pathways activated by intracellular Ca\textsuperscript{2+}.

Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCX/NCKX)
Ca\textsuperscript{2+} flux across neuronal membranes is further regulated by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers, which can be K\textsuperscript{+} independent (NCX) or K\textsuperscript{+} dependent (NCKX). Under physiological conditions, NCX/NCKX are driven by the electrochemical gradient of Na\textsuperscript{+}, and thus extrude Ca\textsuperscript{2+} into the extracellular space, thereby influencing synaptic plasticity and memory formation\textsuperscript{61–63}. Upon depolarization of the membrane or opening of Na\textsuperscript{+}-gated channels, the Ca\textsuperscript{2+} exchanging mode of NCX/NCKX is reversed, and Ca\textsuperscript{2+} influx is initiated\textsuperscript{64,65}. Animal models of NCX/NCKX knockout have shown that these exchangers are relevant for Ca\textsuperscript{2+} clearance from neuronal cells. Ncx2 knockout mice showed a delay in Ca\textsuperscript{2+} clearance from hippocampal neurons and improved cognitive functions\textsuperscript{62}, and Ncxk2 knockout rats showed enhanced [Ca\textsuperscript{2+}]\textsubscript{i} elevations and an increased infarct size in a model of cerebral ischemia\textsuperscript{66}.

Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} (CRAC) channels
The mechanisms of Ca\textsuperscript{2+} influx from the extracellular space into the cell described above, are early events which contribute to neuronal cell death. Notably, Ca\textsuperscript{2+} influx
also promotes neuronal damage at later stages as a response to the replenishment of intracellular Ca^{2+} stores via store-operated Ca^{2+} entry (SOCE). The endoplasmic reticulum (ER) is the biggest intracellular Ca^{2+} store in neurons (discussed in part 3). In response to activation of ER-resident Ca^{2+} channels and subsequent release of Ca^{2+} into the cytosol, Ca^{2+} release-activated Ca^{2+} (CRAC) channels at the plasma membrane are activated to enhance Ca^{2+} influx in order to refill the depleted intracellular stores.

Stromal interaction molecules (STIM1, STIM2), situated in the ER membrane, sense changes in ER-Ca^{2+} ([Ca^{2+}]_{ER}) and subsequently translocate to and activate CRAC channels, such as Ca^{2+} release-activated Ca^{2+} modulator 1 (ORAI1), to induce Ca^{2+} influx. In neurons, the interaction between STIM2 and ORAI1 depends on [Ca^{2+}]_{i}, while the interaction between STIM1 and ORAI1 depends on [Ca^{2+}]_{ER} release. STIM1 is capable of sensing changes in [Ca^{2+}]_{ER} through an EF-hand Ca^{2+} binding domain (CaBD) in the ER lumen. STIM1-mediated plasma membrane localization was inhibited in response to SOCE-mediated [Ca^{2+}]_{i} elevation to prevent detrimental Ca^{2+} overload. SOCE is critical for neuronal differentiation during development and for synaptic plasticity. Stim1 knockdown impaired neuronal differentiation, and Orai1 knockdown reduced [Ca^{2+}]_{ER} load leading to defective SOCE in both conditions. Further, inhibition of SOCE through genetic depletion of ORAI1 prevented neuronal damage in a model of oxidative stress-induced cell death. In line with these findings, upregulation of the immediate early gene Homer1a prevented late Ca^{2+} dysregulation in the same model through disrupting the interaction of STIM1 and ORAI1 required to induce SOCE.

Besides ORAI1, also transient receptor potential channels (TRPC) were identified as mediators of SOCE through binding Ca^{2+} in their pore domain. In a model of Huntington’s disease in mice, huntington fibrils enhanced late Ca^{2+} entry, and this was attenuated by knockdown of TRPC1 suggesting an involvement of these channels in SOCE.

**Ca^{2+}-activated K^{+} channels**

A great impact on neuronal excitability is also elicited by Ca^{2+}-activated K^{+} (K_{Ca}) channels, belonging to a large family of K^{+} channels that were first identified in nervous tissues. K_{Ca} channels are expressed in a variety of cell types, including neuronal and cardiac cells, where they are involved in membrane potential regulation. In neurons, K_{Ca} channels mediate after-hyperpolarization, decrease neuronal excitability, and regulate synaptic plasticity. K_{Ca} channels are classified, based on their Ca^{2+} conductance, into large conductance (BK; 100-300pS), intermediate conductance (IK; 25-100pS) and small conductance (SK; 2-25pS) K_{Ca} channels (see table 1). Furthermore, they differ in their pharmacological properties and their dependence on Ca^{2+} for channel activation.
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detailed review in chapter 2). While BK channel activation is mediated by voltage changes and the presence of Ca\(^{2+}\) reduces the voltage range required for activation, SK and IK channel activation fully depends on the binding of Ca\(^{2+}\) to C-terminally bound calmodulin\(^{85-87}\). A common feature of all K\(_{Ca}\) channels is that pharmacological activation of the channels in pathological conditions is associated with protective effects that lead to preservation of cell viability\(^{88-90}\).

Most studies investigated the function of BK channels and SK channels, while the number of studies on IK channels is limited. BK channel activation is an established strategy to protect against neuronal and cardiac dysfunction. For instance, in organotypic hippocampal slice cultures and cultured primary cortical neurons, activation of BK channels by NS1619 or NS11021, respectively, strongly attenuated excitotoxic neuronal cell death\(^{91,92}\). NS11021 also protected cardiomyocytes against O\(_2\) deprivation and ischemia/reperfusion injury in vitro\(^{93,94}\). So far, studies on the IK channel subtype mainly focused on the generation of isotype-selective pharmacological compounds to modulate IK channel function, but only few studies actually investigated their effects in disease models. For instance, the pharmacological activator SKA-121, having a higher specificity to IK than SK channels, reduced blood pressure and heart rate in hypertensive rats suggesting a protective potential in cardiac diseases\(^{95}\).

In addition to plasma membrane expression, all K\(_{Ca}\) channel groups were identified in the mitochondria in different cell types where they contribute to mitochondrial function\(^{96-98}\). Mitochondrial isoforms of the BK channel (mitoBK) subtype influence the mitochondrial membrane potential (\(\Delta\Psi\)\(_m\)), and upon activation, increase K\(^+\) flux from the cytosol into the mitochondrial matrix\(^{99,100}\), an effect that contributes to the neuro- and cardioprotection mediated by mitoBK channel activation. The function of SK channels expressed in the inner mitochondrial membrane (mitoSK), compared to that of plasma membrane SK channels, is incompletely understood. Thus, the role of mitoSK channels in cell death mechanisms warrants further studies.

<table>
<thead>
<tr>
<th>K(_{Ca}) channel</th>
<th>Abbreviation</th>
<th>Conductance [pS]</th>
<th>Mode of activation</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large conductance</td>
<td>BK</td>
<td>100-300</td>
<td>Voltage, Ca(^{2+})</td>
<td>PM, IMM</td>
</tr>
<tr>
<td>Intermediate conductance</td>
<td>IK</td>
<td>25-100</td>
<td>Ca(^{2+})</td>
<td>PM</td>
</tr>
<tr>
<td>Small conductance</td>
<td>SK</td>
<td>2-25</td>
<td>Ca(^{2+})</td>
<td>PM, ER, IMM</td>
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*PM: plasma membrane, ER: endoplasmic reticulum, IMM: inner mitochondrial membrane
Physiological SK channel function in the brain

The small conductance type of \( K_{Ca} \) channels (SK) is subdivided into three isoforms (SK1-3) and shows a differential expression pattern in the central nervous system\(^{101} \). At neuronal plasma membranes, SK channels are situated in close proximity to NMDA receptors and LTCC\(^{102} \), and become activated upon NMDA receptor-mediated \( Ca^{2+} \) influx. Elevations in \([Ca^{2+}]_i\) increase the opening probability of these channels and initiate \( K^+ \) efflux which leads to hyperpolarization of the membrane and reduction of the excitatory postsynaptic potential\(^{103–105} \). Furthermore, SK channel activation modulates neuronal spike frequency and duration\(^{106,107} \). In addition to \( K^+ \) efflux, SK channel activation attenuates \([Ca^{2+}]_i\) accumulation which creates a local current that re-establishes NMDA receptor blockade by Mg\(^{2+}\), thereby delaying the initiation of subsequent action potentials. Thus, the function of SK channels in regulating \([Ca^{2+}]_i\) homeostasis is crucial as, for instance, prolonged NMDA receptor-mediated or ischemia-induced \([Ca^{2+}]_i\) influx promotes cell death, thus SK channel opening can prevent neuronal cell death\(^{108–110} \).

SK channels in the hippocampus and the cortex have a strong impact on the regulation of synaptic plasticity, thus affecting learning and memory formation. \( Ca^{2+} \) influx and subsequent neuronal excitation are key mechanisms to enhance synaptic plasticity and to induce long-term potentiation (LTP) for memory formation. SK channel inhibition in mice by the bee venom toxin apamin enhanced NMDAR-dependent neuronal excitation in the hippocampus, inducing LTP and resulting in improved performance in Morris Water Maze tests and object-recognition tests\(^{111–113} \). SK channel expression at the plasma membrane was found to correlate with LTP induction and spatial learning, as PKA-mediated SK channel internalization was increased during learning\(^{114} \). In another study, activation of muscarinic M1 receptors by LH-77-28-1 induced hippocampal LTP by inhibiting SK channels, thereby relieving the negative feedback of SK channels on NMDA receptors\(^{115} \). Accordingly, enhanced SK2 channel expression in the dentate gyrus was accompanied by a post-training memory deficit in SK2 channel transduced animals\(^{116} \).

SK channels and neurodegeneration

Due to their differential expression pattern in the brain and involvement in \([Ca^{2+}]_i\) homeostasis, SK channels have been linked to brain injuries and neurodegenerative diseases.

During ischemia, the \( O_2 \) supply of the brain is significantly reduced, leading to oxidative stress and neuronal cell death. Ischemic brain damage at early stages is initiated in the infarct core region and then progresses to surrounding areas (penumbra). In models of oxidative stress and ER stress in hippocampal-derived immortalized cells \textit{in vitro}, SK channel activation protected against cell...
death\textsuperscript{97,117,118}. Furthermore, the SK channel agonist NS309 reduced the infarct size in a model of cerebral ischemia \textit{in vivo}\textsuperscript{89}.

AD is a neurodegenerative disease mainly affecting elderly people that develops due to the generation of neurotoxic amyloid beta (Aβ) plaques and neurofibrillary tangles that induce neuronal cell death. Interestingly, due to the effect of NMDA receptors on synaptic plasticity, these receptors have been associated with AD progression. For instance, in human postmortem brains, NMDA receptor expression in specific hippocampal regions correlated with the predisposition for developing Aβ plaques\textsuperscript{119}, and in rat hippocampal slices, Aβ oligomers caused spine loss by enhancing NMDA receptor activation\textsuperscript{120}. Stimulation of NMDA receptors using glutamate \textit{in vitro} is an established model to study neurodegenerative diseases, and SK channel activation was shown to prevent glutamate-induced neuronal cell death by attenuating NMDA receptor-mediated Ca\textsuperscript{2+} influx and [Ca\textsuperscript{2+}]\textsubscript{i} dysregulation\textsuperscript{89}.

PD is a neurodegenerative disorder characterized by loss of DA neurons in the substantia nigra which are involved in motor coordination and memory formation. Interestingly, SK channels regulate motor activity, as mice expressing the so-called \textit{Kcnq2} \textit{frissonant} deletion mutant show locomotor deficits and tremor similar to PD patients\textsuperscript{121}. In mice, SK channel activation blocked the hyperactive phenotype induced by the methylphenidate\textsuperscript{122}. In an \textit{in vitro} model of PD, cell death of DA neurons was induced by the mitochondrial complex I inhibitor rotenone, and SK channel activation preserved the dendritic network and cell viability\textsuperscript{123}. In another \textit{in vitro} PD model induced by 6-hydroxydopamine (6-OHDA), SK channel opening by 1-EBIO normalized the irregular firing pattern of DA neurons, and preserved cell viability\textsuperscript{124}. In line with these findings, glutamate-mediated stimulation of AMPAR in DA neurons reduced SK channel currents\textsuperscript{125} further suggesting a link between SK channel activity and survival of DA neurons which is relevant for PD treatment.

Epilepsy is a neurological disorder characterized by spontaneous seizures particularly in the temporal lobe causing hippocampal sclerosis and granule cell dispersion\textsuperscript{126–128}. \textit{In vitro}, temporal lobe epilepsy (TLE) is mimicked by the treatment with either pilocarpine to enhance cholinergic signaling, kainate to induce KAR-mediated Ca\textsuperscript{2+} influx, or Mg\textsuperscript{2+} deprivation leading to uncontrolled NMDAR stimulation and Ca\textsuperscript{2+} dysregulation\textsuperscript{129–132}. \textit{In vivo}, TLE can be induced either by kainate injections into the amygdala, intrahippocampal infusion with 4-aminopyridine (4-AP) or by stimulation of perforant pathway (PP) neurons which project from the hippocampal formation to the entorhinal cortex\textsuperscript{133–135}. Only a few studies have investigated the protective potential of SK channel activation in the treatment of epilepsy to date. NS309 treatment in a 4-AP-induced model of epilepsy prevented the epileptic discharge of hippocampal neurons\textsuperscript{136}. Similarly, enhanced SK2 channel expression after viral SK2 plasmid delivery to the dentate gyrus in rats alleviated kainate-induced lesions in the CA3 region \textit{in vivo}, and
reduced granule cell excitability in hippocampal slices in vitro\textsuperscript{116}. In contrast, SK channel inhibition by UCL1684 in an epilepsy model induced by pilocarpine enhanced neuronal spiking in hippocampal slices\textsuperscript{137}, and SK channel blockage by apamin potentiated epileptiform neuronal activities after Mg\textsuperscript{2+} starvation\textsuperscript{138}, suggesting that SK channel activation may be a therapeutic option in for the prevention of epileptic seizures.

2. The power generating organelle: the mitochondrion

Ca\textsuperscript{2+} signaling, and especially disturbances in [Ca\textsuperscript{2+}], will ultimately affect intracellular structures such as mitochondria. Mitochondria are small organelles with a unique composition and function. They are composed of a permeable outer membrane (OMM) and a non-permeable inner membrane (IMM) which is folded into cristae. Metabolic processes occur in the mitochondrial matrix, and the IMM is pivotal for electron flow-dependent mitochondrial respiration.

Mitochondrial dynamics

Mitochondria undergo dynamic structural changes, such as fission and fusion, allowing an adaptation to changes in energy demands, and facilitating the clearance of damaged organelles. The establishment of high-resolution fluorescence microscopy in the 1990s provided the starting point for detailed analyses of mitochondrial dynamics in physiological and pathological conditions\textsuperscript{139}.

Mitochondrial fusion

Mitochondria are capable of fusing with adjacent mitochondria to increase the area for electron flow and adenosine triphosphate (ATP) generation. This is a two-step process involving the fusion of the OMM and the IMM. Fusion of the IMM was sensitive to inhibition of glycolysis by 2-deoxyglucose and dissipation of the IMM potential by ionophores, while OMM fusion depended on the hydrolysis of guanosine triphosphate (GTP) and subsequent activation of mitofusins 1 and 2 (MFN1, MFN2)\textsuperscript{140,141}. Electron cryo-tomography has shown that upon hydrolysis and binding of GTP, MFN1 and MFN2 residing in the OMM heterodimerize to form ring-like structures around contact points between two adjacent mitochondria, thereby fusing the OMM\textsuperscript{142,143}.

Enhancing mitochondrial fusion to prevent mitochondrial fragmentation has been explored as a strategy to prevent cell death involving mitochondrial dysfunction. In cerebellar granule cells, overexpression of MFN2 provided protection against oxidative stress by attenuating mitochondrial fission and cristae reorganization that would facilitate cytochrome c release\textsuperscript{144}. MFN2 was also linked to mitochondrial metabolism and energy production, and a lack of MFN2 expression resulted in reduced respiration, energy generation and mitochondrial biogenesis\textsuperscript{145}. Optic atrophy 1 (OPA1), a GTPase residing in the IMM, dimerizes to drive IMM fusion
which depends on the presence of MFN1 in the OMM\textsuperscript{146}. Depolarization of the OMM induced degradation of OPA1 and subsequent mitochondrial fragmentation, and mitochondrial fusion was restored by overexpression of OPA1\textsuperscript{147}. Furthermore, OPA1 activity is involved in organizing mitochondrial cristae structures and in sequestering cytochrome c within the cristae\textsuperscript{148,149}.

**Mitochondrial fission**

Mitochondrial fission is initiated by Fission 1 (FIS1) and the GTPase dynamin-related protein 1 (DRP1). FIS1 serves as an adapter molecule on the OMM which counteracts OPA1, and FIS1 knockdown prevented apoptotic cell death\textsuperscript{150}. Upon phosphorylation, DRP1 translocates from the cytoplasm to the mitochondria and induces OMM fission by creating ring-like structures around constriction points\textsuperscript{151}. DRP1 translocation is initiated by phosphorylation of Ser616 by extracellular signal related kinase 2 (ERK2) or PKA-mediated DRP1 phosphorylation at Ser656\textsuperscript{152,153}. Further, DRP1 was deactivated by calcineurin-mediated dephosphorylation of Ser656, and also by activation of LTCC, Ca\textsuperscript{2+} ionophore treatment and Ca\textsuperscript{2+} release from the ER\textsuperscript{153}. DRP1-mediated mitochondrial fission prevented [Ca\textsuperscript{2+}]\textsubscript{m} uptake and impaired mitochondrial function under basal conditions\textsuperscript{154}. Further, overexpression of a dominant-negative DRP1 mutant to suppress endogenous DRP1 activity during apoptosis prevented mitochondrial fragmentation, cytochrome c release and mitochondrial swelling\textsuperscript{155}. Thus, the mitochondrial morphology is a key determinant of cell fate. Once these organelles are damaged, the “point of no return” is passed and cell death is inevitable.

**Energy generation in the cell**

Energy is generated in several catabolic, multistep processes involving the breakdown of large macromolecules into smaller molecules. These metabolic pathways occur in the cytoplasm and in the mitochondria, and mitochondria-related processes are sensitive to changes in O\textsubscript{2} availability and mitochondrial integrity.

**Glycolysis**

Glycolysis represents the first step in energy production (Figure 2A), and is independent of the O\textsubscript{2} level in the cell. In a process involving ten reactions, the hexa-carbon sugar molecule glucose is broken down into pyruvate. Some of the steps involved in glycolysis are rate-limiting. For instance, the phosphorylation of glucose depends on hexokinase-mediated glucose import which is feedback-inhibited by increasing concentrations of the product glucose-6-phosphate\textsuperscript{156}. If sufficient ATP is present, glycolysis is halted. The net result of glycolysis is 2 pyruvates, 2 ATP, 2 NADH+H\textsuperscript{+} and 2 molecules of water (H\textsubscript{2}O). In order to facilitate continuous energy production, the reduced NADH+H\textsuperscript{+} has to be recycled which requires the presence of an electron acceptor. Under normoxic conditions, the final electron acceptor is O\textsubscript{2} being converted to H\textsubscript{2}O
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(aerobic respiration). Under hypoxic conditions, pyruvate is metabolized into lactate (anaerobic respiration) to produce energy, a process that is favored in cancer cells and known as the Warburg effect. This phenomenon primarily occurs in the center of solid cancers where the $O_2$ availability is limited. Due to the solidity of the tumor, the core region faces hypoxic conditions and maintains its ability to survive due to this metabolic switch. The outer areas are mostly impermeable to pharmacological compounds, thereby rendering resistance to pharmacological intervention. On the molecular level, the metabolic shift from aerobic respiration to glycolysis has been associated with decreased or defective expression of mitochondrial complex I and complex IV (see part 2).

Citric acid cycle
The main function of the citric acid cycle (CAC) (Figure 2B) in the mitochondrial matrix is the regeneration of $NAD^+$ into $NADH+H^+$. Pyruvate produced by glycolysis is catabolized into acetyl-CoA by pyruvate dehydrogenase which is subsequently converted into different carboxylic acid intermediates such as citrate, succinate, fumarate and malate by corresponding dehydrogenases. In the final step, malate is used to produce oxaloacetate which can be converted back into acetyl-CoA upon decarboxylation by pyruvate decarboxylase. Importantly, $[Ca^{2+}]_\text{m}$ uptake is essential to preserve mitochondrial metabolism. The activity of pyruvate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase and oxaloacetate dehydrogenase requires the presence of sufficient $[Ca^{2+}]_\text{m}$, and limited $[Ca^{2+}]_\text{m}$ availability reduces the citric acid cycle-dependent production of substrates used for respiration. Thus, $[Ca^{2+}]_\text{m}$ uptake enhances the conversion of pyruvate into substrates that are used for subsequent mitochondrial respiration in order to generate ATP, and concomitantly supports $NAD^+$ regeneration as an electron donor.

Mitochondrial respiration
When $O_2$ is available, aerobic respiration in the mitochondria is enhanced (Figure 2C). The interconversion of $NAD^+$ to $NADH+H^+$ is used to transfer electrons across the electron transport chain (ETC) located in the IMM to facilitate further redox reactions. The ETC is generated by four complexes (complex I-IV) establishing the electrochemical proton motif force to drive the oxidative phosphorylation (OXPHOS) of ADP to ATP. NADH dehydrogenase (complex I) is a multiprotein complex responsible for the conversion of $NADH+H^+$ into $NAD^+$. NAD binds to complex I, and two electrons are transferred onto different iron-sulfur (Fe-S) clusters. During this process flavin mononucleotide (FMN) is reduced to FMNH$_2$, and four $H^+$ are transferred from the matrix into the intermembrane space. Succinate dehydrogenase (complex II) converts succinate into fumarate and transfers electrons from flavin adenine

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dinucleotide (FAD) in the mitochondrial matrix through Fe-S clusters. The cytochrome bc₁ complex (complex III) consisting of one cytochrome c (CytC) and two cytochrome b (CytB) subunits, oxidizes ubiquinol and reduces CytC in a two-step process wherein four $H^+$ are generated. CytC oxidase (complex IV) depends on the presence of $H^+$ and O₂, and ultimately establishes the proton motif force by both, the CytC-oxidation driven electron transfer through Fe-S clusters to convert into H₂O, and the direct transfer of $H^+$ into the intermembrane space. The high $H^+$ gradient is used by ATP synthase (complex V). Complex V comprises the membrane integral $F_0$ portion which acts as an $H^+$ channel, and the matrix $F_1$ portion which catalyzes the oxidation of NAD⁺ and ubiquinol to generate ATP.

Figure 2. Energy generation in the cell: glycolysis, citric acid cycle and mitochondrial respiration.

In order to preserve cell survival, the energetic demands of a cell are covered by enhancing glycolysis and/or mitochondrial metabolism and respiration, depending on the availability of O₂. (A) When glucose is taken up into cells, the hexose ring is broken down into smaller molecules in a multistep process involving the intermediates glyceraldehyde-3-phosphate (G3P) and phosphoenolpyruvate (PEP) from which pyruvate and ATP are generated. Pyruvate is transported into mitochondria where it is hydrolyzed to acetyl-CoA and enters the citric acid cycle to drive respiration, and to produce ATP. (B) Acetyl-CoA is converted into different substrates (citrate, isocitrate, succinate, fumarate, malate, oxaloacetate) wherein different electron donors are liberated (Coenzyme Q₁₀, NADH⁺H⁺). Some of the enzymes involved in substrate catabolism are Ca²⁺ dependent. (C) The electron transport chain is established by four mitochondrial complexes that facilitate redox reactions of electron carriers or acceptors, thereby liberating $H^+$ into the intermembrane space. $H^+$ from complexes I, II and III are the driving force for complex V to produce ATP. Due to their ability to perform redox reactions, complexes I-III and V are sources of reactive oxygen species (ROS).

CytC and coenzyme Q₁₀ (Q₁₀; ubiquinone) in the mitochondrial matrix are essential co-factors that frequently undergo redox reactions to maintain mitochondrial respiration. Q₁₀ drives the regeneration of NAD⁺ from complex I and
is reduced to ubiquinol upon the generation of FMNH\textsubscript{2}. Ubiquinol generated at complex II is oxidized in complex III, and again at later stages in complex V. In hepatocytes, chromium toxicity led to Q10 deficiency and mitochondrial damage\textsuperscript{162}, and in fibroblasts a decrease in Q10 levels correlated with an increase in ROS due to the lack of electron acceptance\textsuperscript{163}. Due to its antioxidant properties, the therapeutic potential of Q10 is investigated to increase mitochondrial function and to prevent cell death in conditions of oxidative stress\textsuperscript{164,165}. CytC is a critical co-factor for complex IV function, and triggering apoptosis diminished the mitochondrial CytC pool leading to attenuated respiration, diminished Ca\textsuperscript{2+} retention and cell death\textsuperscript{166,167}.

**Respiration-induced formation of ROS**

ROS, such as hydroxyl radicals (‘OH), superoxides (‘O\textsubscript{2}−) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), are short-lived toxic free radicals generated from non-toxic precursor molecules, and are frequently produced in the cell as part of the physiological redox homeostasis. The most prominent ROS precursors are superoxide anions. These are converted to hydroxyl radicals in an iron-dependent reaction, or react to O\textsubscript{2} and H\textsubscript{2}O. In addition to superoxide anions and hydroxyl radicals, peroxides also play an important role in cell death induction (discussed in part 4). ROS can be produced in the cytosol, for instance by peroxidation of lipids of the plasma membrane, yet the largest pool of ROS is generated by electron leakage from the ETC\textsuperscript{168,169}. Complexes I, II, III and V generate superoxide anions which accumulate in the mitochondrial matrix. Complex III also facilitates the reverse transfer of superoxide anions from the mitochondrial matrix into the cytosol\textsuperscript{170}. Controlled ROS production, within the cells’ redox balance, are also required for some signaling pathways and cellular maintenance. As long as the endogenous antioxidant defense is intact, the redox balance is maintained and ROS are used in signaling pathways or transformed into non-reactive molecules. However, when physiological levels of ROS are exceeded, cell death is inevitable.

Interfering with a functional ETC or blocking individual mitochondrial complexes causes either cell cycle arrest or detrimental ROS formation which promotes cell death. For instance, challenging HeLa cells, lacking a functional ETC, with ethidium bromide initiated genotoxic stress and led to cell cycle arrest\textsuperscript{171}. The complex I inhibitor rotenone induced neuronal cell death through enhancing the formation of mitochondrial ROS, subsequent mPTP opening and CytC release\textsuperscript{172–174}. Mild inhibition of complex II by 3-nitroproprionic acid in neurons induced a slight increase in ROS accompanied by a drop in ATP levels, without altering mitochondrial morphology\textsuperscript{175}, and full blockage of complex II through malonate or lonidamine induced cell death through excessive ROS formation\textsuperscript{176,177}. Similarly, antimycin A which inhibits complex III promoted cell cycle arrest and apoptosis\textsuperscript{178,179}. Complex IV is antagonized by potassium cyanide, and feedback
inhibits complexes II and III\textsuperscript{180}. Complex V inhibition by oligomycin is less effective in inducing cell death under standard culturing conditions\textsuperscript{181}. Interestingly, in cancer cells oligomycin induced a metabolic shift towards glycolysis to compensate for the lack of OXPHOS suggesting that oligomycin could become toxic when glucose is eliminated from the culture medium\textsuperscript{182}.

ROS production can also act as an adaptive mechanism to prevent the spreading of cell death in response to a subsequent stress, a process termed mitohormesis\textsuperscript{183–185}. Mitohormesis is characterized by temporally elevated ROS levels through endogenous pathways which prevents subsequent insults, and thereby may facilitate longevity\textsuperscript{186}.

**Mitochondrial ion homeostasis**

Given the sensitivity of the mitochondrial respiratory chain to changes in proton or free radical levels, an adequately balanced ion flux is essential in maintaining mitochondrial respiration and ATP production. Imbalances or dysregulation of ion homeostasis can cause $\Delta \Psi_m$ dissipation, mitochondrial swelling and cell death. In the mitochondria, $K^+$ and $Ca^{2+}$ fluxes are especially important for preserving the $\Delta \Psi_m$ (see figure 3).

**Mitochondrial $K^+$ homeostasis**

$K^+$ homeostasis in mitochondria is mainly regulated by the IMM-resident ATP-dependent $K^+$ channels (mK\textsubscript{ATP}), the mitochondrial $K^+/H^+$ exchanger (mKHE) and $Ca^{2+}$-activated $K^+$ channels (see part 1) expressed in the IMM (mitoK\textsubscript{Ca}). Imbalances in mitochondrial matrix $K^+$ ($[K^+]_m$) can cause $\Delta \Psi_m$ dissipation, and subsequent mitochondrial rupture\textsuperscript{187,188}.

The mK\textsubscript{ATP} channel facilitates $[K^+]_m$ uptake, and its activation depends on intact mitochondrial respiration\textsuperscript{176,189–191}. Apoptosis in differentiated neurons is preceded by $\Delta \Psi_m$ depolarization and ROS formation, which was prevented by pharmacological inhibition of mK\textsubscript{ATP} channels\textsuperscript{192}. The mKHE mediates $K^+$ influx into the mitochondrial matrix in exchange for $H^+$, thereby contributing to the proton gradient across the IMM\textsuperscript{193,194}.

Mitochondrial K\textsubscript{Ca} channels of the BK channel (mitoBK) type have been extensively studied in cardiomyocytes and neurons. Pharmacological mitoBK channel activation enhances $[K^+]_m$ efflux leading to $\Delta \Psi_m$ depolarization through inhibition of mitochondrial complexes\textsuperscript{100,195} and ROS formation\textsuperscript{196}. Interestingly, mitoBK channels preserved cell viability in various models of cardiac and neuronal toxicity indicating an involvement of mitoBK channel-induced ROS production and $\Delta \Psi_m$ depolarization, thereby underlying the molecular mechanisms of mitohormesis\textsuperscript{86,93,197,198}. The mitochondrial IK channel (mitoIK) subtype was identified in mitochondria derived from melanoma cells where it led to $\Delta \Psi_m$ hyperpolarization, and pharmacological mitoIK channel inhibition decreased
mitochondrial respiration in a subset of pancreatic ductal adenocarcinoma
cells\textsuperscript{199,200}. Mitochondrial SK (mitoSK) channels have been identified in cardiomyocytes and
neurons\textsuperscript{97,201}. It is well established that SK channel activation confers
neuroprotection by enhancing mitochondrial resilience to cell death, however, the
impact of mitoSK channels during SK channel-mediated protection on cell viability
and mitochondrial function are largely unknown. Recently, in a study using
immortalized neuronal HT22 cells, the pharmacological SK channel activator
CyPPA slightly induced $\Delta \Psi_m$ depolarization and ROS formation indicating that
neuroprotective effects of mitoSK channel activation might also involve
mitohormesis\textsuperscript{117}.
General introduction

Figure 3. Ca\(^{2+}\) and K\(^{+}\) channels in mitochondria and the ER.

(A) On the outer mitochondrial membrane (OMM), mitochondrial Ca\(^{2+}\) uptake is facilitated by voltage-dependent anion channels (VDAC) that become permeable for different ions including Ca\(^{2+}\). Ca\(^{2+}\) uptake across the inner mitochondrial membrane (IMM) into the mitochondrial matrix is directly regulated by the mitochondrial calcium uniporter (MCU), and supported by the activity of mitochondrial ryanodine receptors (mRyR), the mitochondrial Na\(^{+}/Ca\(^{2+}\) exchanger (mNCX) and the H\(^{+}/Ca\(^{2+}\) exchanger (HCX). In addition, Ca\(^{2+}\) influx is influenced by the activity of big (BK\(_{Ca}\)) and small (SK\(_{Ca}\)) conductance Ca\(^{2+}\)-activated K\(^{+}\) channels. BK\(_{Ca}\), IK\(_{Ca}\) and SK\(_{Ca}\) channels together with the mitochondrial ATP-dependent K\(^{+}\) pump (K\(_{ATP}\)) and the mitochondrial K\(^{+}/H^{+}\) exchanger (KHE) regulate K\(^{+}\) flux into mitochondria. (B) In the endoplasmic reticulum (ER) membrane, Ca\(^{2+}\) release occurs through RyR and inositol-1,4,5-triphosphate receptors (IP\(_3\)R), and Ca\(^{2+}\) retention is regulated by the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA). The activity of Ca\(^{2+}\) channels in the ER membrane is strongly influenced by KHE and SK\(_{Ca}\) channels which control K\(^{+}\) uptake into the ER lumen.

Mitochondrial Ca\(^{2+}\) homeostasis

Transient oscillations in [Ca\(^{2+}\)]\(_{m}\) are buffered by temporal increases in mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{m}\)) uptake, leading to transient enhancement of mitochondrial metabolism\(^{202,203}\). Thus, [Ca\(^{2+}\)]\(_{m}\) uptake is critical for mitochondrial function and is subject to strict regulation.

The first step in [Ca\(^{2+}\)]\(_{m}\) uptake is through the non-selective voltage-dependent anion channel (VDAC) located in the OMM\(^{204,205}\). VDAC-induced Ca\(^{2+}\) entry into the mitochondrial matrix is mediated by the mitochondrial calcium uniporter (MCU)\(^{206-208}\). Additionally, the reverse mode Na\(^{+}/Ca\(^{2+}\) exchanger (mNCX), the H\(^{+}/Ca\(^{2+}\) exchanger (HCX; LETM1) and the mitochondrial isoform of the ER-resident ryanodine receptor (mRyR) contribute to [Ca\(^{2+}\)]\(_{m}\) regulation\(^{209-214}\). Continuous accumulation of [Ca\(^{2+}\)]\(_{m}\) in the mitochondrial matrix is detrimental and promotes cell death\(^{215,216}\). Excessive [Ca\(^{2+}\)]\(_{m}\) influx results in dissipation of ΔΨ\(_{m}\), collapse of the metabolic machinery, and in opening of the mitochondrial permeability transition pore (mPTP), mitochondrial swelling and cell death\(^{217-219}\).
Inhibition of $[Ca^{2+}]_m$ uptake by Nelfinavir or the MCU inhibitor Ruthenium Red successfully preserved mitochondrial integrity and protected against cell death in HT22 cells exposed to oxygen-glucose deprivation both, in vitro and in an in vivo model of hypoxia ischemia/reperfusion in neonatal mice. Genetic downregulation of MCU expression by RNA interference was protective against NMDAR excitotoxicity in primary neurons. Antagonizing mRyR by dantrolene or mNCX by CGP37157 also attenuated $[Ca^{2+}]_m$ dysregulation in striatal and cortical neurons, respectively. SK channels at the neuronal plasma membrane provide protection in different cell death models by inhibiting $[Ca^{2+}]_i$ influx and $[Ca^{2+}]_m$ dysregulation, yet the effect of mitoSK channels on $[Ca^{2+}]_m$ homeostasis has not been elucidated.

**Mitochondrial matrix swelling, mitochondrial permeability transition pore and cell death**

A reduction in cellular antioxidants such as glutathione (GSH) can induce mitochondrial matrix swelling, and ultimately lead to mitochondrial disruption through mPTP opening. Matrix swelling induced by $Ca^{2+}$ or ROS leads to CytC release and caspase activation. However, $Ca^{2+}$-mediated swelling induced ROS-dependent dissipation of the $\Delta \Psi_m$ through enhanced $[Ca^{2+}]_m$ uptake, yet a strong increase in ROS led to matrix swelling and attenuated NADPH production by inhibiting citrate synthase and citrate dehydrogenase activity.

Mitochondrial ROS, at non-detrimental levels, are generated through peroxidation of cardiolipin at the IMM which leads to dissociation of CytC from cardiolipin and subsequent VDAC1-dependent release of CytC into the cytosol. Similarly, $Ca^{2+}$-induced matrix swelling and CytC release at low $Ca^{2+}$ concentrations did not induce mPTP opening or mitochondrial damage in brain mitochondria, and maintained the activity of ATP synthase. In contrast, liver mitochondria were vulnerable to mitochondrial swelling and sensitive to $Ca^{2+}$-induced CytC release. $[Ca^{2+}]_m$ elevations were shown to enhance cardiolipin-mediated ROS accumulation and to induce mPTP opening, thereby accelerating CytC release and caspase activation. In addition, mitochondrial ROS accumulation in astrocytes potentiated $[Ca^{2+}]_{ER}$ release and mPTP opening leading to $[Ca^{2+}]_m$ dysregulation and cell death.

**3. The endoplasmic reticulum (ER)**

$Ca^{2+}$ homeostasis in the endoplasmic reticulum (ER) is considerably influenced by changes in $[Ca^{2+}]$. The ER is closely associated with the nuclear envelope and is formed by an endomembrane system (cisternae), the site of protein synthesis, protein folding and subsequent protein secretion into vesicles that target the Golgi apparatus. ER dysfunction leads to defective protein folding, and accumulation of such misfolded proteins in the ER lumen initiates the unfolded protein response.
General introduction

(UPR)$^{235}$. The UPR is a tightly controlled molecular pathway that initially increases the expression of molecular chaperones to compensate for the misfolding and to induce ubiquitin-mediated proteasomal degradation of misfolded proteins to restore ER function$^{236}$. However, prolonged activation of the UPR induces ER stress and caspase-dependent cell death$^{237}$.

$\text{Ca}^{2+}$ signaling in the ER

The ER constitutes the major source of $\text{Ca}^{2+}$ ([Ca$^{2+}]_\text{ER}$) within the cell, and [Ca$^{2+}]_\text{ER}$ homeostasis is maintained by the activity of inositol-1,4,5-triphosphate receptors (IP$_3$R), RyR, the sarco-/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA), KHE and KCa channels.

The IP$_3$R is the main [Ca$^{2+}]_\text{ER}$ release channel, and is activated by binding of its ligand IP$_3$. IP$_3$ is generated through G-protein coupled receptor signaling at the plasma membrane which activates phospholipase C to hydrolyze phosphatidylinositol-biphosphate (PIP$_2$) into IP$_3$ and diacylglycerol$^{239,240}$. Low [Ca$^{2+}]_\text{ER}$ levels also activate IP$_3$R-dependent [Ca$^{2+}]_\text{ER}$ release while high [Ca$^{2+}]_\text{ER}$ levels block IP$_3$R signaling$^{241–244}$. Pharmacological inhibition of the IP$_3$R with 2-aminoethoxydiphenyl borate (2-ABP) or xestospongin C attenuated [Ca$^{2+}]_\text{ER}$ release and subsequent Ca$^{2+}$ signaling$^{245,246}$. Ca$^{2+}$-regulated RyR in the ER membrane also modulate neuronal [Ca$^{2+}]_\text{ER}$ homeostasis as they facilitate Ca$^{2+}$-induced Ca$^{2+}$ release from the ER$^{247,248}$, and therefore activate store-operated Ca$^{2+}$ entry (SOCE) (see part 4).

SERCA pumps coordinate [Ca$^{2+}]_\text{ER}$ re-uptake and are regulated by the movement of counterions to evoke an electroneutral state in the ER lumen$^{249}$. Irreversible inhibition of SERCA activity by thapsigargin led to complete [Ca$^{2+}]_\text{ER}$ depletion which culminated in ER stress and cell death$^{250,251}$. SERCA-mediated [Ca$^{2+}]_\text{ER}$ uptake depends on proton flux mediated by SK channels (ER-SK) and KHE in the ER. Recently, it was shown that SK channel-mediated K$^+$ flux drives KHE-mediated H$^+$ entry, thereby allowing [Ca$^{2+}]_\text{ER}$ uptake through SERCA$^{118,252}$. Additionally, pharmacological ER-SK channel activation attenuated thapsigargin-induced [Ca$^{2+}]_\text{ER}$ release and protected against cell death induced by brefeldin A which initiated prolonged UPR$^{118}$.

ER-mitochondrial crosstalk and Ca$^{2+}$ signaling

The communication and association of organelles is essential for the exchange of proteins, metabolites, lipids or Ca$^{2+}$ to maintain intracellular signaling$^{253–258}$. ER and mitochondria are physically linked at a local and dynamic interface, termed mitochondria-associated ER membrane (MAM) wherein signaling between the organelles occurs$^{254}$. This physical connection is mainly formed upon the interaction of ER-bound IP$_3$R and OMM-bound VDAC being facilitated by the heat shock protein 70 family member glucose-regulated protein 75 (GRP75) at multiple sites in
the MAM\textsuperscript{259–263}. Additionally, ER/SR and mitochondria can be tethered by heterocomplexes formed between MFN1 and MFN2\textsuperscript{264}, and Mfn2 deficiency impaired [Ca\textsuperscript{2+}]	extsubscript{m} uptake and mitochondrial bioenergetics\textsuperscript{265}. At the MAM interface the distance between ER and mitochondria is temporally reduced to ~10-25nm\textsuperscript{266}, and contact sites with greater distance function to refill the ER following Ca\textsuperscript{2+} depletion\textsuperscript{267}. The Ca\textsuperscript{2+} microdomains that are generated by IP\textsubscript{3}R are propagated through the MAM interface\textsuperscript{268}, and are sufficient to activate MCU-dependent [Ca\textsuperscript{2+}]	extsubscript{m} uptake and to stimulate oxidative phosphorylation and ATP generation\textsuperscript{269}. Interestingly, ER stress inducing ER dysfunction, [Ca\textsuperscript{2+}]	extsubscript{ER} depletion and cell death, is accompanied by an increase in ER-mitochondrial coupling in early phases after stress induction. This early increase in MAM formation led to a rise in [Ca\textsuperscript{2+}]	extsubscript{m}, and therefore resulted in increased OXPHOS and ATP production preceding the detrimental reduction of respiration at later stages of ER stress\textsuperscript{270}. In contrast, interfering with MAM formation directly reduced mitochondrial respiration and energy metabolism through an impairment of Ca\textsuperscript{2+} transfer\textsuperscript{271,272}.

**ER-mitochondrial coupling and cell death**

ER-mitochondrial coupling is essential to maintain cellular bioenergetics\textsuperscript{270}. However, when control mechanisms are disrupted, MAM formation is detrimental. For instance, ER stress and prolonged exogenous stimulation of IP\textsubscript{3}R to release Ca\textsuperscript{2+} from the ER caused [Ca\textsuperscript{2+}]	extsubscript{m} accumulation, mPTP opening and cell death\textsuperscript{273}. Furthermore, stimulating IP\textsubscript{3}R-dependent [Ca\textsuperscript{2+}]	extsubscript{ER} release by palmitate, CytC or MFN2 overexpression enhanced [Ca\textsuperscript{2+}]	extsubscript{m} overload and potentiated apoptosis induction\textsuperscript{274–276}. In addition, overexpression of VDAC which increased the Ca\textsuperscript{2+} permeability of the OMM amplified Ca\textsuperscript{2+} signaling through the MAM interface, and increased the susceptibility to apoptosis\textsuperscript{277}. Oxidative stress also affects MAM-dependent signaling. Oxidative stress in hepatocytes induced by xanthine/xanthine oxidase treatment enhanced IP\textsubscript{3}R-dependent [Ca\textsuperscript{2+}]	extsubscript{ER} release and mitochondrial [Ca\textsuperscript{2+}]	extsubscript{m} dysregulation\textsuperscript{278}, and TNF\textalpha treatment of smooth muscle cells induced ROS formation and ER stress resulting in disrupted MAM formation and reduced [Ca\textsuperscript{2+}]	extsubscript{m} uptake\textsuperscript{279}.

**4. Neuronal cell death mechanisms induced by glutamate**

Neurodegeneration and the underlying molecular mechanisms can be studied using different model systems in vitro and in vivo. Neuronal cell death initiated by glutamate is a well-defined cell death paradigm wherein glutamate stimulates NMDAR, thereby inducing excessive neuronal firing, oxidative stress, activation of caspases and ultimately cell death. However, glutamate in cells lacking NMDAR can induce caspase-independent cell death which is mediated by oxidative stress and mitochondrial cell death signaling (see figure 4).
Figure 4. Glutamate-induced neuronal cell death: excitotoxicity versus oxytosis.

In general, glutamate exposure results in cell death of neurons, yet the molecular pathways involved in glutamate toxicity may be distinct depending on the neuronal cell type. (Left part) In primary cortical neurons (PCN) where N-methyl-D-aspartate receptors (NMDAR) are expressed, glutamate binds to these receptors, thereby relieving their blockage and triggering Ca\(^{2+}\) influx into the cells (excitotoxicity). An increase in intracellular Ca\(^{2+}\) facilitates PKA-dependent phosphorylation to activate and open other Ca\(^{2+}\) channels, thereby enhancing the influx of Ca\(^{2+}\) into the cytosol. NADPH oxidases (NOX) generate reactive oxygen species (ROS) and neuronal nitric oxide synthase (nNOS) converts L-arginine into nitric oxide (NO) and citrulline, wherein reactive nitrogen species (RNS) are produced. This leads to ROS accumulation in the mitochondria. Further, Ca\(^{2+}\) from the endoplasmic reticulum (ER) is released and propagated to the mitochondria. ER-Ca\(^{2+}\) release activates Ca\(^{2+}\) channel in the plasma membrane to refill the depleted stores (store-operated Ca\(^{2+}\) entry, SOCE). Ultimately, mitochondrial damage leads to the release of cytochrome C (CytC) into the cytosol which executes cell death. (Right part) In immortalized hippocampal neurons (HT22 cells) which do not express NMDAR, glutamate toxicity induces cell death by a different mechanism (oxytosis). Glutamate blocks the Cys/Glu antiporter (X\(_{\text{ct}}\)) in the plasma membrane leading to loss of glutathione (GSH) and glutathione peroxidase 4 (GPX4) activity. This enhances the oxidation of lipids by 12/15-lipoxygenases (12/15LOX), wherein ROS are produced. 12/15LOX activation promotes Ca\(^{2+}\) influx, and triggers the translocation of pro-apoptotic proteins (BID and BAX) to the mitochondria, thereby inducing permeabilization of the mitochondrial membrane leading to further ROS production. ER-Ca\(^{2+}\) release results in accumulation of Ca\(^{2+}\) in the mitochondria and in the cytosol, and is facilitated through the interaction of stromal interaction molecule (STIM) and ORAI to induce SOCE. Mitochondrial Ca\(^{2+}\) influx and ROS formation impair mitochondrial respiration and therefore potentiate mitochondrial damage. In addition, dynamin-related protein 1 (DRP1) accumulates at the mitochondria and induces mitochondrial fission. Mitochondrial damage ultimately leads to the liberation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus where it induces DNA damage and cell death. Full line: effect of glutamate toxicity, dashed line: indirect effect of glutamate on Ca\(^{2+}\) influx.
Excitotoxicity

In excitable neurons, glutamate stimulates NMDAR leading to Ca^{2+} influx. This prolongs neuronal excitation and induces subsequent excitotoxic cell death mediated by activation of the caspase cascade. Similarly, enhanced synaptic expression of NMDAR also promotes excitotoxicity. Excitotoxicity-induced [Ca^{2+}]_i dysregulation leads to mitochondrial damage through enhancing [Ca^{2+}]_m influx which depolarizes the mitochondrial membrane and stimulates the generation of mitochondrial ROS. Excessive NMDAR activation further induced the generation of soluble ROS largely depending on the activity of NADPH oxidases (NOX), which contribute to excitotoxic cell death in vitro and in vivo, and NOX inhibition by apocynin and by compounds interfering with the formation of NOX complexes prevented cell death. In addition, free radicals are generated from the activation of neuronal nitric oxide (NO) synthase which, in conditions of enhanced NMDAR activation, favors the production of citrulline, NO, and NO-derived peroxynitrite (RNS) from L-arginine. [Ca^{2+}]_i dysregulation, ROS formation, RNS generation and mitochondrial damage ultimately cooperation in the execution of neuronal cell death by both, necrotic and CytC-dependent apoptotic signaling pathways. Pharmacological NMDAR antagonism successfully prevented neuronal decline by inhibiting NMDAR-mediated Ca^{2+} influx at early stages, and is used for the therapeutic intervention against neurodegenerative diseases.

Oxytosis

In immature neurons and in immortalized hippocampal HT22 cells, glutamate induces oxidative stress and subsequent mitochondrial dysfunction leading to cell death independent of NMDAR (oxytosis). Oxytosis is distinct from glutamate-induced excitotoxicity and the classical programmed apoptosis, wherein cell death is executed independent of the cellular cascade triggering CytC release from the mitochondria and caspase activation. Furthermore, the subcellular localization of the tumor suppressor p53 which is critical for apoptosis induction, is dispensable during oxytosis. Glutamate initiates cell death directly at the plasma membrane by perturbing the import and export of glutamate and cystine, respectively. High extracellular glutamate blocks the extrusion of glutamate and the import of cystine along the Cys/Glu antiporter (X_{CT})^*, thereby limiting the availability of cystine to be incorporated into GSH which is composed of glutamate, cystine and glycine. GSH aids in maintaining a physiological redox balance due to its ability to become reduced (GSH) or oxidized (GSSG). Increasing either GSH component might help to restore normal GSH levels, as shown for N-acetylcysteine (NAC) in red blood cells and neurons. The supplementation of GSH has also emerged as a strategy to block oxidative stress at early stages in this lethal pathway.
As a consequence of reduced GSH levels, the activity of glutathione peroxidase 4 (GPX4) is reduced. GPX4 is a key enzyme involved in maintaining the redox balance within cells, and GPX4 deficiency in mice introduced a cell death pathway strongly resembling oxytosis \(^{302}\). Loss of GSH and GPX4 enhanced the activity of 12/15-lipoxygenases (12/15-LOX), thereby establishing pathological levels of free radicals and inducing Ca\(^{2+}\) influx into the cytosol \(^{302,303}\). These early elevations in ROS can be prevented by treatment with radical scavengers such as Trolox or NAC which also blocked neuronal cell death \(^{299}\). In addition, CytC oxidases (COX) contribute to ROS generation, however, COX are dispensable during oxytosis \(^{299}\).

The generation of lipid peroxides facilitates the translocation of pro-apoptotic proteins to the mitochondria where they alter the mitochondrial morphology and impair mitochondrial function. Among the pro-apoptotic proteins translocating to the mitochondria, the BH3-interacting domain death agonist (BID) has a central role. Upon induction of apoptosis, BID is transactivated and translocates to the OMM where it interacts with BAX \(^{304,305}\). This induced oligomerization of BAX and facilitated the BAX-VDAC1 interaction, thereby inducing mitochondrial damage through enhanced OMM permeation \(^{306}\). In addition, BID-mediated mitochondrial damage accelerated ROS formation and mitochondrial fission downstream of 12/15-LOX activation, thereby further conferring damage \(^{299,307}\). Pharmacological inhibition and CRISPR/Cas9-mediated knockout of Bid provided full protection against glutamate-induced cell death in HT22 cells \(^{308}\).

Oxytosis induces the phosphorylation-dependent activation and mitochondrial accumulation of DRP1 \(^{309}\). Mitochondrial DRP1 promotes mitochondrial fission, and pharmacological inhibition of DRP1 by mDivi compounds successfully prevented cell death and mitochondrial damage \(^{310}\). In the same study, these neuroprotective effects of DRP1 inhibition were further confirmed in an \textit{in vivo} model of transient focal ischemia. Upon activation of DRP1, OPA1 released into the cytosol may prevent mitochondrial fusion as a potential repair mechanism, thereby leading to cell death \(^{311}\).

The initial [Ca\(^{2+}\)]\(_{i}\) elevation induced by activation of 12/15-LOX is further propagated into massive [Ca\(^{2+}\)]\(_{m}\) uptake in response to OMM permeation. [Ca\(^{2+}\)]\(_{m}\) overload, in concert with ROS accumulation due to enhanced cardiolipin peroxidation \(^{227}\), leads to breakdown of mitochondrial metabolism and the respiratory chain, and results in loss of ATP synthase activity. Mitochondrial damage represents the “point of no return” at which mitochondrial breakdown and cell death become inevitable.

Downstream of mitochondrial damage, additional mechanisms contribute to the execution of cell death. For instance, Ca\(^{2+}\) influx induced by SOCE is a late event during glutamate-induced oxytosis in HT22 cells. In glutamate-resistant HT22 cells, ORAI1 expression was downregulated leading to a reduction of SOCE indicating that ORAI1 is essential for SOCE in these cells \(^{75}\). Along with ORAI1, TRPC1 is involved in the late Ca\(^{2+}\) entry in HT22 cells following glutamate exposure as...
genetic downregulation or pharmacological inhibition of TRPC1 blocked cell death\(^\text{312}\). The ability of mitochondria to buffer rising levels of cytosolic Ca\(^{2+}\) also reflects on the induction and termination of SOCE, as mitochondria facilitate the activation of this process and control the extent and duration of the resulting Ca\(^{2+}\) influx\(^\text{313}\).

Together with mitochondrial dysfunction and SOCE, cell death pathways are facilitated by translocation of the apoptosis-inducing factor (AIF) from the mitochondria to the nucleus where it induces chromatin condensation and DNA damage. In HT22 cells, genetic ablation of AIF and inhibition of the AIF-cyclophilin A interaction prevented cell death\(^\text{314,315}\).

Different strategies have been exploited in the past years to protect neuronal cells from glutamate-induced cell death through mitochondrial conservation. These were mainly related to signaling components i) upstream of mitochondrial damage that sequester ROS and restore glutathione levels, ii) to components that directly induce mitochondrial damage through translocation to the OMM such as BID or DRP1, and iii) components downstream of mitochondria which are liberated from the mitochondria, including AIF or CytC, to execute cell death. However, many aspects such as mitochondrial Ca\(^{2+}\) or K\(^{+}\) channels, or the crosstalk between mitochondria and the ER to facilitate Ca\(^{2+}\) transfer also affect mitochondrial integrity and, therefore, represent potential neuroprotective targets.
**Scope of the thesis**

Under physiological conditions, small conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK) channels play a key role in synaptic plasticity and in regulating neuronal firing activities. Owing to their ability to induce after-hyperpolarization by which neuronal excitability is decreased and NMDAR-mediated Ca\(^{2+}\) influx is attenuated, SK channels provide protection in different models of increased neuronal excitability and neuronal cell death.

The identification of intracellular isoforms of SK channels raises the question of what the function of these intracellularly expressed SK channels is at the level of the mitochondria, and what impact these channels might have on SK channel-mediated protection. In *chapter 2*, I review and discuss current studies on the molecular mechanisms underlying neuroprotection mediated by SK channels expressed at the plasma membrane, the ER (ER-SK) and the IMM (mitoSK) revealing the requirement of studies delineating the impact of intracellularly expressed SK channels.

SK channel function has been studied extensively in various models of neurodegeneration *in vitro* and *in vivo* where their pharmacological activation prevented neuronal hyperexcitability. However, SK channel activation in the context of enhanced neuronal firing, for instance as in epilepsy, has not been fully elucidated. Only few studies reported a protective role in experimental models of epilepsy, and *in vivo* evidence for a role of SK channels in epileptogenesis is largely missing. In *chapter 3*, I investigate SK channel expression in different *in vitro* and *in vivo* models of epilepsy and assess functional consequences of epilepsy induction on mitochondrial performance, a critical parameter for preserving cell survival. As epileptogenesis and seizure duration was shown to involve the post-transcriptional regulation of protein expression, I also exploit the potential involvement of microRNA-dependent regulation of SK channel protein expression.

In *chapter 4*, I investigate the role of mitoSK2 channels in a model of oxidative stress (oxytosis), as cell death was triggered by glutamate primarily through mitochondrial dysfunction. To this end, I performed a detailed analysis of mitochondrial function, regarding mitochondrial respiration and Ca\(^{2+}\) uptake, to delineate mitoSK channel function under physiological and pathophysiological conditions, thereby exploiting the therapeutic potential of SK channel activation for enhancing mitochondrial resilience in conditions of oxidative stress and neuronal decline. This study proposes that mitoSK channels mediate neuroprotection through attenuating mitochondrial Ca\(^{2+}\) overload and reducing mitochondrial respiration.

The dysregulation of Ca\(^{2+}\) signaling across the plasma membrane and subsequent intracellular Ca\(^{2+}\) accumulation is involved in the development and progression of neurodegenerative diseases. However, Ca\(^{2+}\) signaling inside the cell is more complex as multiple organelles contribute to Ca\(^{2+}\) homeostasis. The ER and
mitochondria both constitute large Ca$^{2+}$ sources, and the maintenance of Ca$^{2+}$ homeostasis at these intracellular sites is critical for the metabolic activity and for cell survival. In **chapter 5**, I investigate how modulation of ER-mitochondrial connectivity affected the sensitivity of neuronal cells to glutamate-induced oxidative stress through altering the expression and function of GRP75, the molecular chaperone involved in physically connecting ER and mitochondria. In **chapter 6**, I investigate consequences of ER-mitochondrial connections on mitochondrial bioenergetics using an inducible system to link ER and mitochondria. SK channels control Ca$^{2+}$ handling at the level of the ER and at the level of the mitochondria (118 and **chapter 4**). Thus, SK channels located at either organelle might contribute to Ca$^{2+}$ signaling at the ER-mitochondrial interface. In **chapter 6**, I elucidate the role of SK channels at ER-mitochondrial contact points and investigate their neuroprotective potential in conditions of enhanced ER-mitochondrial coupling, and thus increased ER-mitochondrial Ca$^{2+}$ transfer. Finally, in **chapter 7**, the main findings of this work are placed into context and compared to the current literature. This chapter covers open questions and provides suggestions on future directions for each chapter.
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