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Mitochondrial Zn^{2+} Accumulation: A Potential Trigger of Hippocampal Ischemic Injury

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Abstract

Ischemic stroke is a major cause of death and disabilities worldwide, and it has been long hoped that improved understanding of relevant injury mechanisms would yield targeted neuroprotective therapies. While Ca^{2+} overload during ischemia-induced glutamate excitotoxicity has been identified as a major contributor, failures of glutamate targeted therapies to achieve desired clinical efficacy have dampened early hopes for the development of new treatments. However, additional studies examining possible contributions of Zn^{2+}, a highly prevalent cation in the brain, have provided new insights that may help to rekindle the enthusiasm. In this review, we discuss both old and new findings yielding clues as to sources of the Zn^{2+} that accumulates in many forebrain neurons after ischemia, and mechanisms through which it mediates injury. Specifically, we highlight the growing evidence of important Zn^{2+} effects on mitochondria in promoting neuronal injury. A key focus has been to examine Zn^{2+} contributions to the degeneration of highly susceptible hippocampal pyramidal neurons. Recent studies provide evidence of differences in sources of Zn^{2+} and its interactions with mitochondria in CA1 versus CA3 neurons that may pertain to their differential vulnerabilities in disease. We propose that Zn^{2+}-induced mitochondrial dysfunction is a critical and potentially targetable early event in the ischemic neuronal injury cascade, providing opportunities for the development of novel neuroprotective strategies to be delivered after transient ischemia.

Keywords

calcium; cell death; excitotoxicity; ischemia; mitochondria; reactive oxygen species (ROS); zinc

Ischemic Stroke: The Role of Ca^{2+}

Ischemic stroke is a leading cause of disability and death worldwide, reflecting the extreme sensitivity of brain to even brief (several minutes) disruption of blood flow. Despite

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extensive efforts to understand the basis of this unique vulnerability with the aim of developing neuroprotective interventions, attempts to date have failed, with the maintenance and prompt restoration of perfusion being the only presently available therapeutic approach.

Considerable evidence implicates a role for "excitotoxicity" (neuronal damage triggered by excessive release of the excitatory neurotransmitter glutamate) occurring in conditions including ischemia, prolonged seizures and trauma. Excitotoxic mechanisms have been extensively investigated, and a critical early finding was that brief strong activation of highly Ca^{2+} permeable *N*-methyl-D-aspartate (NMDA) type glutamate receptors (NMDAR) results in delayed Ca^{2+}-dependent neurodegeneration (Choi 1987; Choi and others 1988). After the brief exposure, intracellular Ca^{2+} levels recover for a period of time before undergoing a sharp and sustained rise (termed "Ca^{2+} deregulation") that is strongly correlated with cell death (Randall and Thayer 1992).

It is also apparent that oxidative mechanisms contribute to the neuronal injury, induced after production of reactive oxygen species (ROS; including superoxide and nitric oxide) (Lafon-Cazal and others 1993; Sattler and others 1999).

Mitochondria have been implicated as important targets of Ca^{2+} effects. Ca^{2+} enters mitochondria through a specific channel (the mitochondrial Ca^{2+} uniporter, MCU), and under normal circumstances, physiological mitochondrial Ca^{2+} rises help to regulate mitochondrial metabolic function by matching ATP production to need (Nicholls and Budd 2000). Mitochondria are also important buffers of large cytosolic Ca^{2+} loads (Wang and Thayer 1996; White and Reynolds 1997). However, with excess accumulation, Ca^{2+} can disrupt mitochondrial function, with effects including increased superoxide production (Dugan and others 1995; Reynolds and Hastings 1995) and opening of a large conductance inner membrane channel (the mitochondrial permeability transition pore; mPTP), that can lead to mitochondrial swelling and the release of cytochrome C and other pro-apoptotic peptides (Nicholls and Budd 2000). Recent studies have also demonstrated the importance of another distinct mechanism of excitotoxic superoxide generation, via Ca^{2+}-dependent activation of the superoxide-generating cytosolic enzyme NADPH oxidase (NOX) (Brennan and others 2009; Clausen and others 2013), and it is likely that depending on conditions both sources can contribute.

However, despite considerable early hope and some promising results in animals, use of NMDAR antagonists (to prevent Ca^{2+}-mediated injury and deregulation) have yielded little benefit in human studies (Hoyte and others 2004; Ikonomidou and Turski 2002), necessitating a further search for new targets yielding better efficacy.

Zn^{2+}: A Distinct Ionic Contributor to Brain Injury

Zn^{2+} is a critical and highly prevalent cation in all tissues. It is particularly prevalent in brain, which has an overall Zn^{2+} content estimated to be 100 to 200 μM and is especially high in certain limbic and forebrain regions, including hippocampus, amygdala, and cortex (Frederickson 1989). Despite the high total Zn^{2+}, virtually all of it is bound or sequestered; while precise measurements are difficult (as it can bind numerous ligands with a wide range of affinities), it is agreed that free intracellular Zn^{2+} levels are subnanomolar (Colvin and others 2010; Maret 2015). Reflecting its importance in all tissues, there are two families of transporters (with >20 variants identified to date) dedicated to movement of Zn^{2+} between compartments, with the Zrt-, Irt-like protein (ZIP) family moving Zn^{2+} into cytosol, and the Zn^{2+} transporter (ZnT) family moving Zn^{2+} from cytosol out of the cell or into subcellular compartments (Kambe and others 2014). In neurons, most (~ 90%) of the Zn^{2+} is bound to or associated with proteins, and it is an integral component of numerous enzymes, transcription factors and structural proteins (Frederickson 1989).

Synaptic Zn^{2+}: A Modulator of Neurotransmission and Contributor to Injury

A distinct and critical pool of brain Zn^{2+} is that which is sequestered within presynaptic vesicles of some excitatory neurons. This pool of free or loosely bound Zn^{2+} is visualized by histochemical procedures like Timm's silver sulfide staining or labeling with Zn^{2+}-sensitive fluorescent dyes and is often referred to as chelatable or "histochemically reactive" Zn^{2+} (Frederickson 1989; Frederickson and others 1992). This Zn^{2+} has a distinctive distribution, generally corresponding with areas of greatest total Zn^{2+}; high levels are found in hippocampus (particularly the dentate granule cells and their "mossy fiber" projections, accounting for the distinctive appearance of hippocampus after Timm's staining; see Fig. 1A), as well as in cortex and amygdala. In these neurons, the Zn^{2+} appears to be loaded into vesicles at millimolar concentrations by the vesicular Zn^{2+} transporter, ZnT3 (Cole and others 1999). It is further evident that this Zn^{2+} is co-released with glutamate on stimulation (Assaf and Chung 1984; Howell and others 1984; Sloviter 1985), and peak levels at synapses may reach into the 100 μM range with strong activation (Ueno and others 2002; Vogt and others 2000), constituting about a 10,000-fold increase over physiologic resting level of extracellular Zn^{2+} (Frederickson and others 2006).

The identification of populations of forebrain excitatory neurons containing substantial quantities of presynaptic vesicular Zn^{2+} begs understanding of the actions and effects of synaptically released Zn^{2+}. While much is not known, Zn^{2+} has complex effects on extracellular receptors, antagonizing NMDAR currents via both voltage-dependent and -independent mechanisms; electro-physiological studies have demonstrated Zn^{2+} release from mossy fibers to provide tonic inhibition of NMDAR on CA3 pyramidal neurons (Vogt and others 2000). In addition, Zn^{2+} has effects on GABA and glycinergic receptors, as well as on a Zn^{2+} sensing G-protein linked metabotropic receptor, and synaptic Zn^{2+} likely has roles in forms of synaptic plasticity (Sensi and others 2011).

Observations that ischemia, prolonged seizures and brain trauma resulted in loss of chelatable Zn^{2+} labeling in presynaptic pools (most evident in the mossy fibers) (see Fig. 1B), and its appearance in somata of injured neurons led to the suggestion that synaptic Zn^{2+} release and its translocation through channels into postsynaptic neurons contributed to their degeneration in these conditions (Frederickson and others 1989; Suh and others 2000; Tonder and others 1990). Indeed, this idea was markedly strengthened by observations that application of an extra-cellular Zn^{2+} chelator decreased both the postsynaptic Zn^{2+} accumulation and subsequent neurodegeneration (Calderone and others 2004; Koh and others 1996; Yin and others 2002).

Paralleling observations of neuronal Zn^{2+} accumulation after seizures or ischemia in vivo, studies in neuronal culture models documented the potent toxic effects of Zn^{2+} and sought to examine its mechanisms. One early aim was to identify the routes through which synaptically released Zn^{2+} can enter postsynaptic neurons to trigger injury. These studies found Zn^{2+} to permeate three distinct channels through which Ca^{2+} also permeates: (1) NMDAR (Koh and Choi 1994), (2) L-type voltage-gated Ca^{2+} channels (VGCC) (Freund and Reddig 1994; Kerchner and others 2000; Weiss and others 1993), and (3) atypical Ca^{2+} permeable AMPA type glutamate receptors ("Ca-AMPAR"); whereas most AMPA receptors are Ca^{2+} impermeable, these lack the GluA2 subunit in their tetrameric structure, and are only present in substantial numbers on small subpopulations of neurons. We found these Ca-AMPAR to be highly Zn^{2+} permeable (Jia and others 2002; Yin and Weiss 1995). However, direct comparison of these routes indicated substantial differences in their Zn^{2+} permeabilities, and corresponding differences in the potency with which Zn^{2+} entry through each of them triggers injury. Consistent with its effective antagonism of NMDAR currents, very little Zn^{2+} permeates NMDARs. Ubiquitously expressed VGCC showed an intermediate permeability, and the selectively expressed Ca-AMPAR had the greatest Zn^{2+} permeability (Sensi and others 1999).

While brief moderate Zn^{2+} exposures to depolarized neurons resulted in sufficient Zn^{2+} entry through VGCC to trigger extensive degeneration over the subsequent day (Weiss and others 1993), several considerations led us to believe that entry through Ca-AMPAR might be of particular importance. First, despite their selective expression (in contrast to the VGCC, they are only present in large numbers on ~13% of neurons in cortical cultures and preferentially found in dendrites of some pyramidal neurons) (Lerma and others 1994; Ogoshi and Weiss 2003; Sensi and others 1999; Yin and others 1994; Yin and others 1999), they permit substantially greater rates of Zn^{2+} entry, and, when present, are concentrated at post-synaptic membranes where the highest levels of extracellular Zn^{2+} are likely achieved. Furthermore, early Zn^{2+} accumulation has been found to trigger a delayed increase in numbers of Ca-AMPAR in many forebrain neurons 2 to 3 days after transient ischemia (due to decreased expression of GluA2), a factor that likely contributes to delayed neurodegeneration (Calderone and others 2004; Gorter and others 1997). Indeed, supporting the significance of this route, a Ca-AMPAR antagonist attenuated Zn^{2+} accumulation and injury both in a slice model of acute ischemia (Yin and others 2002), and when delivered late after transient global ischemia in vivo (Noh and others 2005). However, this does not mean VGCC are unimportant. Although VGCC are not concentrated specifically at synapses, entry through this route would likely occur under pathologic conditions in which extracellular Zn^{2+} accumulation is accompanied by widespread neuronal depolarization. Also, VGCC activity increases with age (Thibault and Landfield 1996), possibly increasing the contribution of this route in aging populations most at risk of brain ischemia.

The generation of ZnT3 knockout mice, which are entirely lacking in chelatable presynaptic Zn^{2+} (Cole and others 1999), provided a valuable tool to test the presumption that presynaptic Zn^{2+} release and its translocation into postsynaptic neurons accounted for the injurious postsynaptic Zn^{2+} accumulation. Consistent with this idea, when ZnT3 knockouts were tested in a prolonged kainate seizure model, the knockouts showed modestly decreased Zn^{2+} accumulation and injury in CA3 pyramidal neurons (which are innervated by the very densely Zn^{2+} containing mossy fibers). Surprisingly, however, Zn^{2+} accumulation and injury were markedly *increased* in CA1 pyramidal neurons of the knockouts, indicating an additional source of Zn^{2+} that did not depend on synaptic release and translocation (Lee and others 2000).

Zn^{2+} Binding Proteins: Buffers of Zn^{2+} Loads or Sources of Non-Synaptic Zn^{2+} Accumulation (or Both)?

Metallothioneins (MT, I-IV) are cysteine-rich peptides with multiple Zn^{2+} binding sites that play critical roles in buffering Zn^{2+} within cells (MT-III being the predominant neuronal isoform), making them likely candidate sources for the non-synaptic neuronal Zn^{2+} accumulation (Maret 1995). Zn^{2+} binding to MTs is highly sensitive to environmental conditions, with metabolic aberrations associated with pathological conditions (specifically oxidative stress and acidosis) destabilizing binding, resulting in release of free Zn^{2+} into cytosol (Jiang and others 2000; Maret 1995). A seminal observation that simple application of a disulfide oxidant to cultured neurons was capable of causing cytosolic Zn^{2+} rises that could trigger delayed neurodegeneration provided the first *proof of principle* that simple mobilization of Zn^{2+} from intracellular buffers could result in neurodegeneration (Aizenman and others 2000). A subsequent study overexpressing MT-III found that depending on conditions it could have divergent effects, either buffering excess Zn^{2+} that enters the cell (and thereby diminishing its toxic effects), or providing a source of injurious Zn^{2+} mobilization, under conditions of oxidative stress (Malaiyandi and others 2004).

Indeed, use of MT-III knockout mice (as well as double MT-III/ZnT3 knockouts) helped clarify the respective contributions of synaptic vs MT-III bound Zn^{2+} in the kainate seizure model. In contrast to the increased Zn^{2+} accumulation seen in ZnT3 knockouts in CA1 neurons, Zn^{2+} accumulation and injury in MT-III knockouts were decreased in CA1, consistent with a dominant contribution of mobilization from MT-III. Conversely, these were increased in CA3 of MT-III knockouts, consistent with synaptic “translocation” predominating, with MT-III in CA3 serving a protective role by helping to buffer Zn^{2+} entering the neurons (Lee and others 2003).

Might these differences in sources of injurious Zn^{2+} accumulation be a factor contributing to their differential disease susceptibilities, with CA3 neurons preferentially degenerating after recurrent limbic seizures (associated with repetitive firing of the Zn^{2+} rich mossy fibers) and CA1 neurons undergoing delayed degeneration after transient ischemia (Ben-Ari and others 1980; Sugawara and others 1999)?

Discrimination of Ca^{2+} and Zn^{2+} Reveals Distinct Contributions

Despite the emerging evidence for contributions of Zn^{2+}, there is still much evidence for important Ca^{2+} contributions in excitotoxicity associated conditions, and it is probable that both ions contribute. However, early attempts to discriminate their contributions were confounded by the fact that until relatively recently, there were no available Zn^{2+}-selective indicators. Furthermore, it became apparent that some effects that had been attributed to Ca^{2+} might actually be partly Zn^{2+} mediated, since available Ca^{2+} indicators bound and responded to Zn^{2+} with higher affinity than Ca^{2+} (Cheng and Reynolds 1998), and fluorescence increases detected by a "Ca^{2+} indicator" in a slice model of ischemia (that would previously have been assumed to reflect Ca^{2+} rises) were found to be substantially diminished by selective Zn^{2+} chelation (Stork and Li 2006). The development of Zn^{2+} selective indicators provided a breakthrough in attempts to study Zn^{2+}-specific effects and discriminate them from those of Ca^{2+}. Furthermore, using a high affinity Zn^{2+} indicator in combination with a low affinity Ca^{2+} indicator, it became possible to simultaneously track changes in both ions (Devinney and others 2005). We used this approach to simultaneously track changes in both Zn^{2+} and Ca^{2+} in single pyramidal neurons in hippocampal slices subjected to oxygen glucose deprivation (OGD) (see Fig. 2A). Interestingly, we found that cytosolic Zn^{2+} rises both preceded and contributed to the onset of terminal Ca^{2+} deregulation events, which still occurred but were significantly delayed by the presence of a Zn^{2+} chelator (see Fig. 2B) (Medvedeva and others 2009). This provided new evidence that Zn^{2+} accumulation might be an early event in the ischemic injury cascade, the appropriate targeting of which might provide therapeutic benefit. As discussed further below, clues from this and other early studies suggested that mitochondria might be an important target for these early Zn^{2+} effects (see Fig. 2C).

Mitochondria: A Critical Target of Zn^{2+}

Paralleling studies of Ca^{2+}, studies over several decades have highlighted ways in which Zn^{2+} affects mitochondrial function. Below, we review the evolution of these data, leading up to our proposition that mitochondrial Zn^{2+} accumulation may be an important early step in the ischemic injury cascade of many neurons. Specifically, as it occurs upstream from terminal Ca^{2+} deregulation, its targeting may provide benefits distinct from those provided by attenuation of Ca^{2+} entry (via NMDAR blockade).

Isolated Mitochondria: Evidence of Potent Zn^{2+} Effects

A number of studies dating back more than 50 years have found that Zn^{2+} can enter mitochondria, inducing effects including swelling, and inhibition of respiration with high potency (Brierley 1967; Skulachev and others 1967). Over the subsequent decades, with growing awareness that Zn^{2+} is a pathophysiologically important ion that contributes to neuronal injury, there has been an increasing interest in determining how Zn^{2+} impacts mitochondria. Zn^{2+} was found to enter mitochondria specifically through the MCU (Saris and Niva 1994), and to trigger opening of the mPTP (Wudarczyk and others 1999). Other studies found potent (submicromolar) Zn^{2+} inhibition of the bc1 complex of the electron transport chain and of the tricarboxylic acid cycle α-ketoglutarate dehydrogenase enzyme complex (Brown and others 2000; Link and von Jagow 1995). Highlighting the complexity of Zn^{2+} effects on mitochondria, we found low (submicromolar) exposures to induce loss of mitochondrial membrane potential ($\Delta\Psi_{mito}$), decreased ROS production and increased O_2 consumption (consistent with uncoupling of the electron transport from ATP synthesis), while slightly higher levels increased ROS generation and decreased O_2 consumption (consistent with inhibition of electron transport) (Sensi and others 2003). A subsequent study reported Zn^{2+}, after entry through the MCU, to induce irreversible inhibition of major thiol oxidoreductase enzymes involved in energy production and antioxidant defense, an effect that appeared to be linked to mPTP opening (Gazaryan and others 2007).

Using isolated brain mitochondria, we found Zn^{2+} (10–100 nM) to potently induce swelling, that appeared to depend on Zn^{2+} entry through the MCU and opening of the mPTP (Jiang and others 2001). We further found that although Zn^{2+} triggered mitochondrial swelling with far greater potency than Ca^{2+}, the effects of these ions were synergistic, with greater swelling when Ca^{2+} was also present (Jiang and others 2001). Indeed, a number of other studies have also suggested that the presence of Ca^{2+} may critically modulate effects of Zn^{2+} on isolated mitochondria. Specifically, Ca^{2+} was found to markedly enhance Zn^{2+} entry through the MCU (Saris and Niva 1994), and Zn^{2+} triggered mPTP opening of de-energized (but not energized) mitochondria was found to be Ca^{2+} dependent (Wudarczyk and others 1999). Interestingly, a relatively recent study exposed purified and substrate attached mitochondria using buffers pretreated to ensure complete elimination of Ca^{2+}, and found Zn^{2+} to have weak depolarizing effects with no evidence of its entry into mitochondria (Devinney and others 2009). Of possible relevance, the MCU and associated regulatory peptides were recently identified and two regulatory peptides (MICU1 and 2), appear to sense Ca^{2+}, inhibiting MCU opening when Ca^{2+} is near resting levels (<100–200 nM) and promoting opening when Ca^{2+} is elevated, thus conferring a sigmoid shaped Ca^{2+} level/conductance relationship to the channel (De Stefani and others 2015; Kamer and Mootha 2015; Marchi and Pinton 2014). Indeed, Ca^{2+} dependence of MCU opening to permit Zn^{2+} entry could help to explain apparent synergism between Ca^{2+} and Zn^{2+} effects on mitochondria.

Thus, it is apparent that Zn^{2+} effects on mitochondria are complex and a better definition of its mechanisms and how its entry is regulated by the MCU are rich areas for further investigation. Yet, the potency of its effects, taken together with the high levels of Zn^{2+} present in neurons, highlight the strong potential for Zn^{2+} to contribute to mitochondrial dysfunction in disease.

Cell Culture Studies: Neuronal Zn^{2+} Entry Results in Mitochondrial Accumulation and Dysfunction Contributing to Cell Death

Culture studies permit investigation of Zn^{2+} effects in the neuronal environment, bringing us a step closer to understanding possible effects in diseases like ischemia. Above, we introduced studies examining routes through which synaptic Zn^{2+} could enter neurons and reported evidence for particularly rapid entry through selectively expressed Ca-AMPAR, with slower entry through VGCC. We subsequently examined effects of this Zn^{2+} entry, and found brief Ca-AMPAR activation, in the presence of 100 to 300 μM Zn^{2+}, to induce rapid loss of $\Delta\Psi_{mito}$ and ROS generation that persisted for at least an hour after the exposure, consistent with the potent neurotoxicity of these exposures. Identical kainate exposures with physiological (1.8 mM) Ca^{2+}, but no Zn^{2+}, triggered smaller and transient episodes of ROS generation. However, if Zn^{2+} and Ca^{2+} were both present during the exposure, the ROS production was significantly greater than with Zn^{2+} alone, again indicating synergistic effects of these ions (Sensi and others 1999; Sensi and others 2000).

In other studies, we induced smaller Zn^{2+} loads, via similar brief Zn^{2+} exposures under depolarizing conditions, to trigger entry through VGCC (rather than Ca-AMPAR). Although still causing considerable delayed neurotoxicity (Weiss and others 1993), these exposures did not cause the acute ROS generation and loss of $\Delta\Psi_{mito}$ seen with rapid entry through Ca-AMPAR (Sensi and others 1999; Sensi and others 2000). This, along with similar findings by others, have led to questions as to the likelihood that mitochondria constitute important targets of Zn^{2+} effects in disease (Pivovarova and others 2014). However, despite the absence of rapid ROS production, these brief episodes of Zn^{2+} entry through VGCC had distinct and long-lasting effects on mitochondria, with low (50–100 μM) exposures resulting in Zn^{2+} accumulation within mitochondria persisting for at least 2 hours after the exposure along with partial loss of $\Delta\Psi_{mito}$ (Sensi and others 2002); similar brief exposures with 300 μM Zn^{2+} (and 1.8 mM Ca^{2+}) triggered mitochondrial swelling, and delayed release of apoptotic mediators (cytochrome C and apoptosis inducing factor) (Jiang and others 2001), possibly consistent with more slowly evolving cell death occurring after these exposures.

Notably, cytosolic Zn^{2+} accumulation results not only from entry of extracellular Zn^{2+}, but also on mobilization from cytosolic pools like MT-III, and studies of the effects of strong cytosolic Zn^{2+} mobilization alone also have found it to induce effects on mitochondria, contributing to loss of $\Delta\Psi_{mito}$ and delayed degeneration (Bossy-Wetzel and others 2004; Sensi and others 2003). In addition, recent studies have highlighted possible contributions of such Zn^{2+} mobilization and consequent mitochondrial dysfunction to the Ca^{2+} dependent excitotoxic injury cascade (Granzotto and Sensi 2015). In pathologic conditions like ischemia or seizures, where synaptic Zn^{2+} release and mobilization from cytosolic buffers both occur, it is likely that both sources contribute to mitochondrial dysfunction. Indeed, in cell culture studies we find evidence for synergistic impact on mitochondria, with even brief and quite low levels of Zn^{2+} entry through VGCC (which alone had little or no acute effect on mitochondria), when combined with disrupted buffering (using DTDP [2,2′-dithiodipyridine], that also by itself had little or no effect), resulting in dramatic potentiation of acute mitochondrial ROS generation and loss of $\Delta\Psi_{mito}$, long lasting inhibition of mitochondrial respiration, and cell death (Clausen and others 2013; Ji and Weiss 2018). Furthermore, although the presence of physiological Ca^{2+} during the brief Zn^{2+} exposure attenuated cytosolic Zn^{2+} loading (due to competition with Zn^{2+} for entry through VGCC), the effects on mitochondrial function and cell death were markedly enhanced, further highlighting the synergistic effects of these two ions. Indeed, the strong correlation between effects of disrupted buffering and presence of Ca^{2+} on mitochondrial function with those on consequent cell death provide further support to the hypothesis that mitochondrial disruption contributes directly to Zn^{2+} triggered neurotoxicity (Ji and Weiss 2018).

Thus, these findings not only indicate the potency with which Zn^{2+} accumulation in neurons can cause mitochondrial dysfunction, they further support the contention that during in vivo ischemia, even low level Zn^{2+} entry from the extracellular space, when combined with impaired intracellular Zn^{2+} buffering and mobilization from intra-cellular pools, has potential to powerfully disrupt mitochondrial function and contribute to subsequent neuronal injury.

Slice and In Vivo Studies Support Contributions of Mitochondrial Zn^{2+} to Ischemic Neuronal Injury

Although the studies discussed above demonstrate that exogenously applied Zn^{2+} can affect mitochondria and contribute to neuronal injury, this does not indicate that endogenous Zn^{2+} actually does so in ischemia. However, recent studies in more pathophysiologically relevant ischemia models provide compelling evidence that mitochondria are indeed important targets of endogenous Zn^{2+} effects. Specifically, in one study, addition of extracellular Zn^{2+} chelators shortly after a transient episode of ischemia reduced the subsequent mitochondrial release of pro-apoptotic peptides (Calderone and others 2004). In another in vivo study, Zn^{2+} was found to accumulate in mitochondria within 1 hour after transient ischemia, contributing to the opening of large, multi-conductance outer membrane channels (Bonanni and others 2006). However, whereas these studies demonstrate that Zn^{2+} contributes to mitochondrial dysfunction after in vivo ischemia, they do not address therapeutically crucial questions including the source and time course of the Zn^{2+} accumulation, and potential avenues for beneficial interventions.

To examine these issues, we have undertaken studies using hippocampal slice OGD models, a paradigm that models aspects of in vivo ischemia while permitting precise control of the microenvironment and detailed measurement of cellular responses. Our early studies in this model (see Fig. 2A and B) found cytosolic Zn^{2+} rises to precede and contribute to the onset of delayed Ca^{2+} deregulation and cell death during prolonged, lethal OGD (Medvedeva and others 2009), with evidence for early Zn^{2+} entry into mitochondria. Subsequent studies provided strong evidence that Zn^{2+} entry specifically through the MCU is a critical early step, triggering mitochondrial dysfunction (including ROS production) that contributes to the occurrence of acute Ca^{2+} deregulation and degeneration of CA1 neurons (Fig. 2C) (Medvedeva and Weiss 2014).

In further studies using this slice OGD model, we have compared the contributions and sources of Zn^{2+} between CA1 and CA3 neurons (Medvedeva and others 2017). First, we found that neuronal Zn^{2+} accumulation contributes to a similar extent in both subdomains, with early Zn^{2+} rises preceding Ca^{2+} deregulation, and Zn^{2+} chelation similarly delaying the onset of the terminal Ca^{2+} deregulation in both regions. However, our studies using ZnT3 and MT-III knockout mice implicated distinct differences in the sources of the Zn^{2+} underlying acute OGD induced injury. Paralleling the differences previously noted after prolonged in vivo seizures (Lee and others 2000; Lee and others 2003), synaptic Zn^{2+} release and its translocation largely through Ca-AMPAR dominated in CA3, and Zn^{2+} mobilization from MT-III dominated in CA1 (see Fig. 3A).

Because most opportunities for intervention are after reperfusion, we examined events occurring after sublethal episodes of OGD (which better model transient in vivo ischemia) and found evidence for substantial difference between CA1 and CA3 mitochondria in their handling of the Zn^{2+} loads. In these studies, we terminated the OGD after the early Zn^{2+} rises had occurred but shortly before the time of the terminal Ca^{2+} deregulation, and in both regions, the cytosolic Zn^{2+} rises gradually recovered, in part due to uptake into mitochondria via the MCU. However, at 1 hour after OGD, there was still considerable Zn^{2+} retained within CA1 mitochondria, whereas in CA3 mitochondrial Zn^{2+} loads recovered far more rapidly (generally within 20 minutes) (see Fig. 3B) (Medvedeva and others 2017). In light of the differential susceptibilities of CA1 versus CA3 neurons in disease, with CA1 neurons undergoing prominent delayed degeneration after transient ischemia, associated with mitochondrial swelling and release of cytochrome C (Sugawara and others 1999), might the persistent Zn^{2+} accumulation within CA1 mitochondria be a trigger of events leading to the delayed degeneration of these neurons? Further elucidation of mitochondrial Zn^{2+} interactions during and after ischemia in hippocampus as well as in other Zn^{2+} rich areas of brain (including cortex) may reveal new therapeutic approaches and time windows for their delivery that may yield improved outcomes.

Neurodegeneration: The Culmination of Cascades of Injury-Promoting Events

Cell death is multistep process, occurring when a sequence of events leads to a state from which the cell cannot recover. As discussed above, ROS production has been strongly implicated as a trigger of the neurodegeneration occurring after excitotoxic Ca^{2+} loading, and downstream events have been identified, including activation of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in DNA repair, which becomes activated in response to ROS induced DNA damage. PARP utilizes NAD^+ as substrate, with strong activity leading to NAD^+ depletion, glycolytic and mitochondrial inhibition, and release of the apoptotic mediator, apoptosis inducing factor (AIF) (Kauppinen and Swanson 2007). Paralleling these studies of Ca^{2+} excitotoxicity, moderate Zn^{2+} exposures have also been found to cause ROS production (in part due to delayed induction of NOX and neuronal nitric oxide synthase) (Kim and Koh 2002; Noh and Koh 2000), resulting in PARP activation, that contributes to the evolving injury (Kim and Koh 2002).

A number of pathways have also been described in which early Zn^{2+} signals can trigger more delayed neurodegeneration. Studies of the delayed neurodegeneration caused by strong intracellular Zn^{2+} mobilization (Aizenman and others 2000) have implicated a distinct pathway, in which activation of p38 MAP kinase results in membrane insertion of Kv2.1 K^+ channels, resulting in K^+ efflux from neurons and consequent apoptosis (McLaughlin and others 2001).

Notably, these mechanisms contributing to delayed degeneration in response to early Zn^{2+} signals represent later steps in cascades, the inciting steps of which are not always apparent. However, in light of our findings that mitochondrial accumulation of endogenous Zn^{2+} under ischemic conditions triggers rapid mitochondrial ROS production (Medvedeva and Weiss 2014), *perhaps mitochondrial ROS constitutes a critical upstream trigger of some of these downstream, neurodegeneration pathways*. Indeed, rapid Zn^{2+} triggered mitochondrial ROS could mediate DNA damage that underlies PARP activation and has been implicated in the activation of p38 MAP kinase occurring upstream from the insertion of Kv2.1 K^+ channels (Bossy-Wetzel and others 2004), raising the possibility that early targeting of mitochondrial Zn^{2+} may have both immediate and delayed therapeutic benefits.

Therapeutic Potential of Targeting Mitochondrial Zn^{2+}: Possible Future Directions

In summary, studies at multiple levels of complexity—ranging from isolated mitochondria and dissociated neurons, to hippocampal slice and in vivo models of ischemia—indicate that Zn^{2+} is likely to contribute to mitochondrial dysfunction, ROS generation, and neurodegeneration in ischemia (and may well do so in prolonged seizures and brain trauma as well). Furthermore, emerging evidence supports the notion that the Zn^{2+} entry into mitochondria is an early event in the ischemic injury cascade (especially in hippocampal CA1), which, as it occurs upstream from onset of terminal Ca^{2+} deregulation, may not be adequately targeted by simply slowing neuronal Ca^{2+} entry (as via NMDAR blockade). We suggest that *Zn^{2+} accumulation in neuronal mitochondria is a targetable early event in the cell death cascade of CA1 and other populations of forebrain neurons*; this idea merits further investigation and examination for therapeutic utility.

With strong and prolonged ischemia, mitochondrial Zn^{2+} loading may result in rapid irreversible mitochondrial disruption and cell death (Medvedeva and others 2009; Medvedeva and Weiss 2014) (see Fig. 2). However, with milder or transient ischemia, mitochondrial Zn^{2+} loading may contribute to the activation of downstream cell death pathways. Optimal interventions might well vary depending on the stage at which they are delivered. We believe that the targeting of specific events in the injury cascade has potential to yield benefit (see Fig. 4).

1. *Early mitochondrial Zn^{2+} accumulation:* At the early stages, Zn^{2+} chelators or MCU blockers might provide benefit by lessening early mitochondrial Zn^{2+} accumulation. Indeed, delayed Zn^{2+} chelation and MCU blockade have each shown beneficial effects in recent in vitro studies (Ji and Weiss 2018; Medvedeva and others 2017; Slepchenko and others 2017). Of note, these interventions could also act to promote injurious Ca^{2+} loading, possibly complicating efforts to use them for therapeutic benefit in vivo. Specifically, while diminishing mitochondrial Zn^{2+} accumulation, Zn^{2+} chelation attenuates physiological antagonism of NMDAR by synaptic Zn^{2+}, thereby increasing neuroexcitation (Cole and others 2000; Dominguez and others 2003; Vogt and others 2000) and MCU blockade during acute stages of ischemia could diminish mitochondrial buffering of cytosolic Ca^{2+} loads (Velasco and Tapia 2000), both effects that could exacerbate early injurious cytosolic Ca^{2+} loading and hasten Ca^{2+} deregulation. For this reason, in acute stages of ischemia, these agents could show greatest benefit when combined with maneuvers (such as NMDAR blockade) to abrogate rapid Ca^{2+} loading (Medvedeva and Weiss 2014).

2. *Mitochondrial ROS generation:* Antioxidants may provide benefit at slightly later stages, in two ways: (a) by diminishing oxidative Zn^{2+} mobilization from buffers (thereby helping to prevent delayed oxidative feedforward amplification of Zn^{2+} triggered mitochondrial disruption) and (b) by decreasing oxidative tissue damage and activation of oxidant triggered downstream pathways (including PARP and p38 MAP kinase).

3. *Opening of the mPTP:* Mitochondrial Zn^{2+} loading may also act upstream to more delayed apoptotic forms of injury, with Zn^{2+} triggered mPTP opening (occurring up to several hours after the Zn^{2+} load) resulting in mitochondrial disruption and release of apoptotic mediators like (cytochrome C and AIF), effects against which mPTP blockers (like cyclosporine A) might provide benefit.

4. *Downstream injury pathways:* As noted above, Zn^{2+} signals have been found to contribute to delayed insertion of new ion channels that promote delayed neurodegeneration. Targeting of these channels (specifically Kv2.1 channels and Ca-AMPAR) may yield benefit from hours to several days after the episode (Aizenman and others 2000; McLaughlin and others 2001; Noh and others 2005; Yeh and others 2017).

In summary, accumulating evidence supports the notion that early mitochondrial Zn^{2+} accumulation after ischemia contributes to mitochondrial dysfunction and may well be a critical triggering event for a number of neurodegenerative cascades. The targeting of these Zn^{2+} triggered events in the post ischemic period has been largely unexplored, yet has potential to yield substantial benefit, and merits further study.

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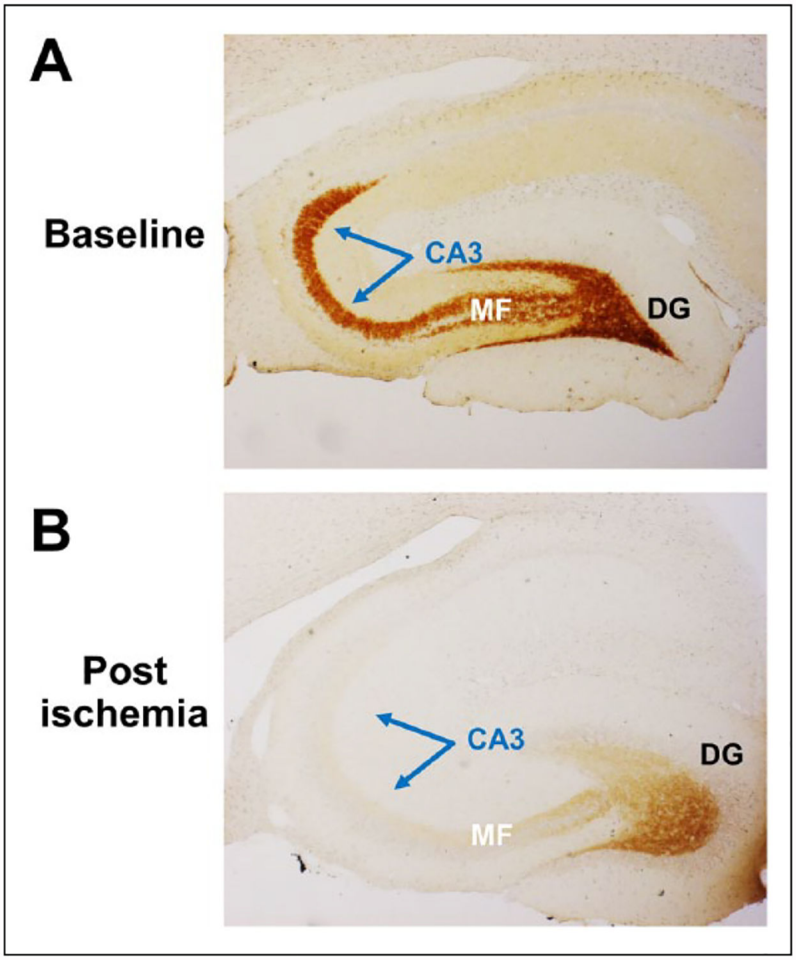

Figure 1.

Synaptic Zn^{2+} is released after ischemia. The mossy fiber pathway (MF) from dentate granule (DG) cells to CA3 pyramidal neurons contains high levels of vesicular Zn^{2+}, accounting for the dark labeling of this pathway on Timm's silver sulfide staining (A). Note the loss of synaptic Zn^{2+} labeling after ischemia (B), resulting from release of this synaptic Zn^{2+}.

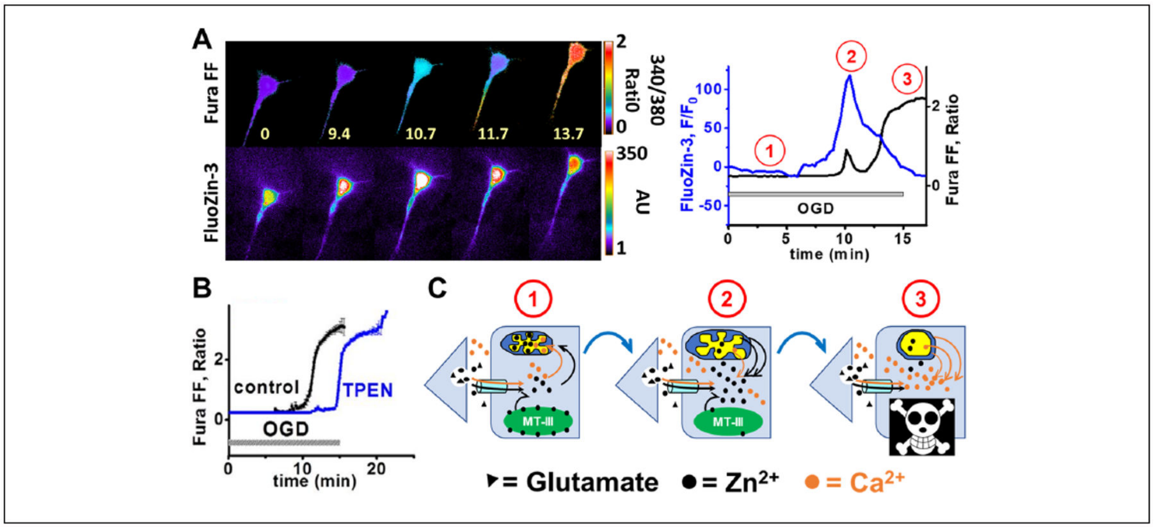

Figure 2.

Zn^{2+} rise precedes and contributes to lethal Ca^{2+} deregulation during prolonged oxygen glucose deprivation. A single CA1 pyramidal neuron in an acute murine hippocampal slice was co-loaded via a patch pipette with the low-affinity Ca^{2+} indicator Fura FF (K_d ~ 5.5 µM) and the high-affinity Zn^{2+} indicator FluoZin-3 (K_d ~ 15 nM), prior to subjecting the slice to prolonged oxygen glucose deprivation (OGD), via perfusion. (A). Zn^{2+} and Ca^{2+} responses in a single CA1 hippocampal pyramidal neuron. (Left) Pseudocolor images. Numbers indicate the duration of the OGD exposure (minutes). Note the early Zn^{2+} rise (FluoZin-3 fluorescence; 9.4 minutes), followed after several minutes by the sharp Ca^{2+} deregulation event (Fura FF fluorescence; 13.7 minutes). (Right) Traces show the time course of the Zn^{2+} and Ca^{2+} rises in the same neuron. Responses in this neuron are representative of published findings (Medvedeva and others 2009). (B). Zn^{2+} contributes to delayed Ca^{2+} deregulation. To validate the role of Zn^{2+} in neuronal injury, hippocampal slices were exposed to OGD alone (control) or in the presence of the Zn^{2+} chelator *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN; 40 µM). Note that TPEN significantly delayed the onset of the terminal Ca^{2+} deregulation. Traces show mean ± SEM of n = 9; from (Medvedeva and others 2017). (C). Schematic of events during lethal OGD. Numbers refer to events occurring at time points indicated on the traces illustrated in (A). (1) Zn^{2+} influx into mitochondria: Zn^{2+} and Ca^{2+} enter postsynaptic neurons through glutamate activated channels. Zn^{2+} is also mobilized from intracellular buffers (largely MT-III) as a result of ischemia-associated oxidative stress and acidosis. The cytosolic Zn^{2+} enters and accumulates in the mitochondria (via the mitochondrial Ca^{2+} uniporter, MCU), contributing to early mitochondrial dysfunction (including reactive oxygen species [ROS] generation and loss of $\Delta\Psi_{mito}$), prior to the sharp cytosolic Zn^{2+} rise. (2) Mitochondrial Zn^{2+} released to cytosol: After a threshold level of Zn^{2+} (and Ca^{2+}) has entered the mitochondria, they undergo a rapid depolarization (loss of $\Delta\Psi_{mito}$), and the Zn^{2+} and Ca^{2+} sequestered within them are released back into the cytosol. At this point, oxidative stress and acidosis prevent Zn^{2+} buffering by MT-III, and the cytosolic Zn^{2+} rises sharply. (3) Ca^{2+} deregulation and cell death: Severe disruption of mitochondrial function and strong ROS production results in loss of ATP, membrane damage, cellular depolarization, and inability to clear or sequester the large Ca^{2+} loads. The sharp cytosolic Ca^{2+} rises also contribute to activation of catabolic enzymes, further accelerating cellular disruption and death. Diagram modified from (Medvedeva and others 2017).

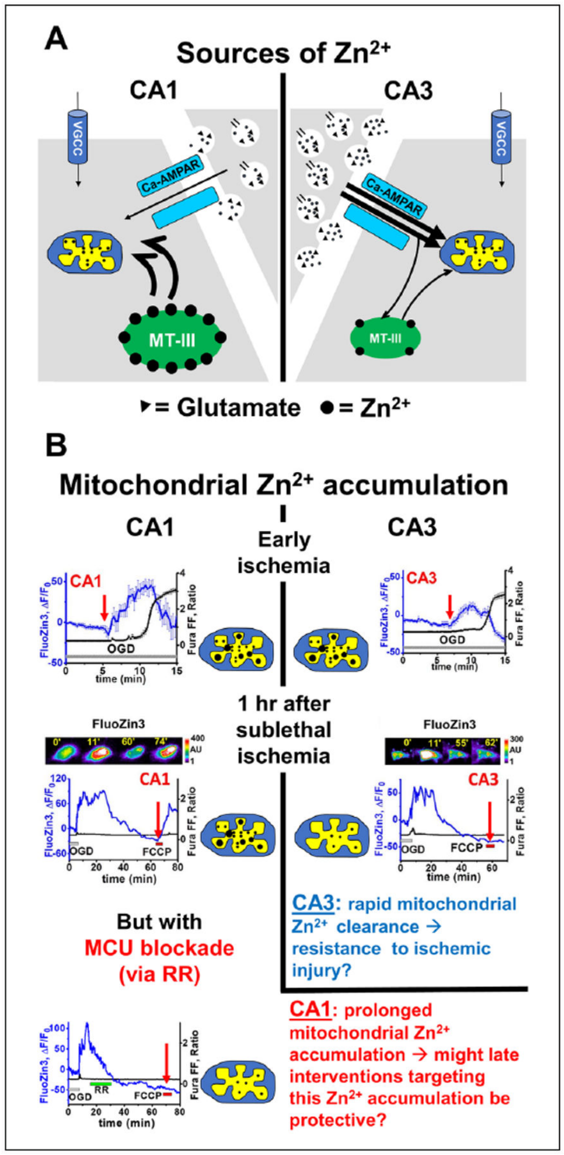

Figure 3.

Differential vulnerability of CA1 versus CA3: Dependence on Zn^{2+} sources and persistence of mitochondrial Zn^{2+} accumulation. (A). Distinct sources of Zn^{2+} contribute to injury in CA1 versus CA3 pyramidal neurons. Early cytosolic Zn^{2+} accumulation contributes to acute oxygen glucose deprivation (OGD)–induced injury in both CA1 and CA3 pyramidal neurons. However, in CA1, the Zn^{2+} largely derives from mobilization from MT-III (left), whereas in CA3, Zn^{2+} translocation through Ca-AMPAR predominates (right) (Medvedeva and others 2017). B). Zn^{2+} enters mitochondria during OGD in both CA1 and CA3, but after sublethal OGD, persists in mitochondria for prolonged periods only in CA1. CA1 and CA3 pyramidal neurons were co-loaded with cytosolic Ca^{2+} and Zn^{2+} indicators, then exposed to either prolonged (lasting until Ca^{2+} deregulation; Top) or sublethal (lasting until cytosolic Zn^{2+} rise; Middle and Bottom) OGD. After sublethal OGD, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP, which induces loss of $\Delta\Psi_{mito}$, releasing mitochondrial Zn^{2+} into the cytosol; 2 μM) and the mitochondrial Ca^{2+} uniporter (MCU) blocker Ruthenium Red (RR; 10 μM) were added as indicated. Mitochondrial diagrams illustrate the anticipated degree of Zn^{2+} accumulation (represented by black dots) at time points indicated by red arrows. Traces and pseudocolor images are reprinted from (Medvedeva and others 2017). (Top) OGD induces rapid mitochondrial Zn^{2+} influx in both CA1 and CA3. During OGD, rapid mitochondrial Zn^{2+} influx occurs early in both CA1 (left) and CA3 (right) pyramidal neurons, contributing to the loss of $\Delta\Psi_{mito}$, release of mitochondrial Zn^{2+} into cytosol, and Ca^{2+} deregulation. Traces show mean ± SEM response of n ≥ 8 neurons. (Middle) Zn^{2+} persists in CA1 mitochondria but is rapidly cleared from CA3 mitochondria after transient OGD. After sublethal OGD, cytosolic Zn^{2+} rises gradually recover in both CA1 and CA3 neurons. To examine the persistence of mitochondrial Zn^{2+} accumulation, FCCP was added as indicated ~1 hour after OGD, to depolarize the mitochondria, releasing sequestered Zn^{2+}. Note the strong response to FCCP in CA1 (left), indicative of prolonged mitochondrial Zn^{2+} sequestration. In contrast, the lack of late FCCP response in CA3 neurons is indicative of the rapidity with which CA3 mitochondria clear Zn^{2+} loads after ischemia (right). Traces and pseudocolor images show responses from representative neurons. (Bottom) Delayed mitochondrial Zn^{2+} uptake depends on entry through the MCU. Note that application of the MCU blocker, RR, to CA1 neurons shortly after OGD, while cytosolic Zn^{2+} was still elevated, blocked mitochondrial Zn^{2+} uptake, and prevented the protracted mitochondrial Zn^{2+} accumulation (as indicated by the lack of FCCP response). Traces show responses of representative neurons.

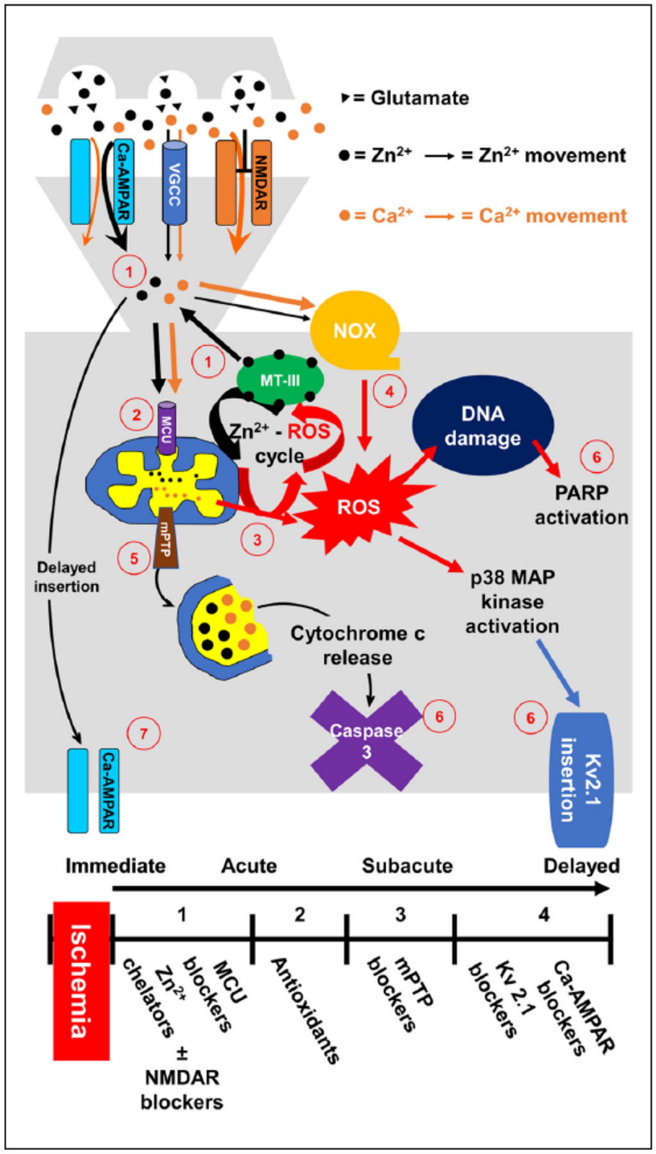

Figure 4.

Zn^{2+}-induced mitochondrial dysfunction is a critical and targetable early contributor to ischemic neuronal injury. During ischemia, Zn^{2+} accumulation in neurons reflects contributions from two primary sources: Zn^{2+} released from presynaptic vesicles that enters postsynaptic neurons (through Ca-AMPAR and voltage gated Ca^{2+} channels [VGCC]), and Zn^{2+} released from MT-III (due to oxidative stress and acidosis) (1). This Zn^{2+} rapidly enters mitochondria through the mitochondrial Ca^{2+} uniporter (MCU) (2). An early consequence of mitochondrial Zn^{2+} accumulation is acute reactive oxygen species (ROS) generation, which can further disrupt cytosolic Zn^{2+} buffering, resulting in more mitochondrial Zn^{2+} entry and consequent dysfunction, thereby initiating a feedforward *Zn^{2+}-ROS* cycle. (3). In addition, Zn^{2+} can induce delayed activation of NOX, producing more ROS, and possibly further amplifying this Zn^{2+}-ROS cycle (4). This protracted Zn^{2+} influx into mitochondria triggers mitochondrial permeability transition pore (mPTP) opening, leading to mitochondrial depolarization, swelling, and cytochrome C release (5). These Zn^{2+} effects on mitochondria (ROS generation and mPTP opening) can activate major downstream events, including direct oxidative damage to proteins and DNA (that can lead to poly(ADP-ribose) polymerase [PARP] activation), activation of the apoptotic pathway via Caspase 3, and activation of p38 MAP (mitogen-activated protein) kinase, promoting the delayed insertionof Kv2.1 K^+ channels (**6**). Furthermore, cytosolic Zn^{2+}, acting through incompletely defined mechanisms, can cause delayed insertion of Ca-AMPAR, further promoting delayed neurodegeneration (**7**). As these steps are temporally discrete, optimal therapeutic strategies will likely target a combination of them at different time points, as highlighted in timeline.