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Zinc: new clues to diverse roles in brain ischemia

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Abstract

Cerebral ischemia is a leading cause of morbidity and mortality, reflecting the extraordinary sensitivity of the brain to a brief loss of blood flow. A significant goal has been to identify neuronal injury pathways that are selectively activated following stroke and may be amenable to drug therapy. An important advance was made close to a quarter century ago, when Ca^{2+} overload was implicated as a critical link between glutamate excitotoxicity and ischemic neurodegeneration. However, early hope for effective therapies faded to frustration, as glutamate-targeted trials repeatedly failed to demonstrate efficacy in humans. In a review in 2000 in this journal, we described new evidence linking a related cation, zinc (Zn^{2+}), to neuronal injury, emphasizing sources and mechanisms of Zn^{2+} toxicity. The current review highlights progress over the last decade, emphasizing mechanisms through which Zn^{2+} ions, from multiple sources, participate together with Ca^{2+} in different stages of ischemic injury cascades.

Zn^{2+} in the nervous system

Zn^{2+} is an essential cofactor for many enzymes and transcription factors, and its levels in all cells are tightly regulated by the combined actions of transporter proteins, channels and intracellular binding proteins. Additionally, it appears to play roles in neuronal synaptic transmission, as high concentrations of Zn^{2+} are accumulated in many synaptic vesicles within the central nervous system (CNS) (due to the activity of a selective Zn^{2+} transporter ZnT3 [1]) and there is evidence for its release in response to presynaptic activation [2–4]. Observations that forebrain ischemia resulted in a loss of synaptic Zn^{2+} fluorescence and its appearance in injured neurons, and that selective Zn^{2+} removal with an extracellular chelator was neuroprotective, led to suggestions that synaptically released Zn^{2+} was a novel contributor to ischemic neurodegeneration (as we previously reviewed, see [5]).

It is now well recognized (and was already becoming apparent in 2000), that significant amounts of injurious Zn^{2+} can be released from binding sites within postsynaptic neurons, an important constituent of which are the highly abundant metallothionein (MT) proteins (the primary neuronal isoform being MTIII) [6]. MT's are plentiful proteins containing multiple cysteine residues that can bind up to 7 Zn^{2+} ions, and which can sequester or liberate significant amounts of Zn^{2+} , depending on factors including cellular pH, and the

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redox state of the cell [7]. Considerable progress has been made over the past decade in understanding intracellular Zn^{2+} regulation and mechanisms through which its mobilization can be linked to ischemic injury. Recent studies have also helped to clarify distinct contributions of Ca^{2+} and Zn^{2+} in the induction of ischemic injury pathways, and have elucidated a number of pathways, acting in different time frames after the ischemic event, through which Zn^{2+} may contribute to neurodegeneration. It is our hope that the emerging understanding of the multiple, interacting mechanisms through which Zn^{2+} may affect ischemic injury, reviewed below, will yield new and effective therapeutics for stroke.

Potential Sources of Zn^{2+} following ischemia

Influx from the extracellular space

In recent years, microdialysis studies have confirmed extracellular Zn^{2+} accumulation in both focal and global ischemia models [8, 9]. Understanding the sources of extracellular Zn^{2+} accumulation could be valuable for targeting Zn^{2+} in ischemia, and in future studies it will be useful to test for synaptic vs other sources of ischemic Zn^{2+} accumulations using animals that lack the vesicular Zn^{2+} transporter, ZnT3 . These animals are viable, lack synaptic Zn^{2+} within the CNS, and have been used to study roles of Zn^{2+} in seizure-induced neurodegeneration [10–12]. It is likely that some accumulation occurs via mechanisms similar to those previously suggested for glutamate, including progressive depletion of presynaptic ATP levels, depolarization and vesicular fusion [13]. Recent work also raises the possibility that a significant contributor to Zn^{2+} release could be repetitive spreading depolarizations that occur in the hours and days following stroke, in animal models and human subjects [14]. Waves of synaptic Zn^{2+} release accompany spreading depolarizations in a brain slice model of ischemia [15], and such events might release substantial amounts of Zn^{2+} into the injured brain. The identity and regulation of presynaptic Zn^{2+} transporter proteins are not yet defined, and the possibility of non-vesicular release via reverse transport analogous to glutamate release during ischemic disruption of presynaptic ion gradients [16] is currently unknown.

Zn^{2+} influx from the extracellular space is well-demonstrated to induce injury in cultured neurons, often after entry through channels and transporters that were originally defined as Ca^{2+} -permeable [5]. A subtype of AMPA-type ionotropic glutamate receptors continues to be of particular importance in this regard. AMPA receptors that lack a GluR2 subunit in their tetrameric structures are permeable to both Ca^{2+} and Zn^{2+} , and they may mediate significant Zn^{2+} increases during initial ischemic challenges [17, 18]. In addition, delayed downregulation of GluR2 subunit expression appears to be one mechanism through which Zn^{2+} contributes to delayed neurodegeneration. Extracellular Zn^{2+} chelation with CaEDTA (ethylenediaminetetraacetic acid calcium) close to the time of transient global ischemia prevented GluR2 downregulation, suggesting that Zn^{2+} may activate the signaling pathway that leads to the GluR2 downregulation [19]. This appears to be mediated in part via upregulation of a transcriptional repressor, REST (repressor element-1 silencing transcription factor)/NRSF (neuron-restrictive silencing factor) [20]. Interestingly, delayed application of either Zn^{2+} chelators or GluR2-lacking AMPA receptor blockers (2–3 days after the ischemic episode) attenuated both intracellular Zn^{2+} accumulation and delayed cell death, suggesting that Zn^{2+} flux through the newly inserted channels may contribute to the delayed neurodegeneration [19, 21]. Other potential routes of Zn^{2+} entry that may contribute to deleterious intracellular Zn^{2+} accumulation are indicated in Figure 1, including Zrt/Irt -like protein (Zip)-1 and -3 channels that have been recently implicated in seizure induced injury [22]. In contrast to observations with global ischemia, the situation with focal ischemia is not as clear-cut, because CaEDTA can provide a substantial improvement in stroke outcome measured at early time points, but protection did not appear to be sustained [23].

Furthermore, one study actually shows a significant increase in infarct volume following CaEDTA in middle cerebral artery occlusion studies [24].

One reason for the complex and divergent effects of Zn^{2+} chelation in focal ischemia models could be due to overlapping deleterious and beneficial effects of synaptic Zn^{2+} . Zn^{2+} inhibits NMDA channels through multiple mechanisms [25], and also potentially inhibits acid-sensing ion channels; cation channels that have been strongly implicated in ischemic injury [26, 27]. Thus, in contrast to intracellular accumulation, extracellular actions of Zn^{2+} , following accumulation during ischemia may serve to limit neuronal injury.

Liberation from intracellular stores during ischemia

As mentioned above, MT are highly abundant Zn^{2+} buffering proteins which are capable of binding large quantities of Zn^{2+} [7]. Oxidative stress and acidosis, both of which occur prominently in ischemia, can induce Zn^{2+} release from MT, resulting in substantial increases in intracellular Zn^{2+} . Such intracellular release was well demonstrated in cultured neurons [4, 28, 29] and became a candidate mechanism to explain Zn^{2+} accumulation observed in hippocampal pyramidal neurons in ZnT3 knockout animals [11, 30]. Consistent with this hypothesis, knockout of MTIII was capable of reducing seizure-induced postsynaptic Zn^{2+} increases [12]. Further highlighting the important role of cellular redox state in Zn^{2+} mobilization, knockout of a glutamate/cysteine transporter resulted in increased neuronal Zn^{2+} levels following ischemia, apparently due to reductions in glutathione levels [31].

Zn^{2+} -MT interactions may have divergent effects on neuronal viability, in some ways analogous to the complex effects of extracellular Zn^{2+} accumulation discussed above. That is, in addition to providing an important source of toxic Zn^{2+} release, under some circumstances, MTIII appears able to induce protective effects by binding excess Zn^{2+} that can accumulate after transmembrane flux [32]. Recent work adds further complexity by demonstrating that Zn^{2+} itself can induce protein kinase C (PKC)-dependent MT phosphorylation, resulting in an amplification of acute Zn^{2+} increases during *in vitro* ischemia [33].

Mobilization of intracellular Zn^{2+} could also be particularly important during reperfusion injury, in which restoration of blood flow after a period of ischemia paradoxically accelerates aspects of the neurodegenerative processes. Although direct contributions of Zn^{2+} to reperfusion injury have not yet been examined, it seems likely that Zn^{2+} could contribute, as an oxidative burst would likely trigger enhanced intracellular Zn^{2+} mobilization, thereby enhancing Zn^{2+} -triggered injury processes. From the considerations above, it may be difficult to predict whether the balance of MT effects is beneficial or deleterious in different ischemia models. There has only been a single report of the effect of MTIII-KO animals in focal ischemia, which found knockouts to have more severe injury [34], perhaps because they cannot sequester Zn^{2+} released from other sources.

In addition to MT, Zn^{2+} release from other intracellular compartments might make significant contributions to intracellular accumulation. Like Ca^{2+} , Zn^{2+} can accumulate in the mitochondrial matrix, and as discussed below, this accumulation could be a critical contributor to its toxic effects. However it is also possible that efflux of Zn^{2+} from mitochondria could contribute directly to cytosolic Zn^{2+} increases, through pathways that include depolarization and opening of the mitochondrial permeability transition pore (mPTP) [29, 35]. Demonstration that distinct Zn^{2+} transporters are present on other intracellular compartments raises the possibility that other stores could also contribute [36]. Determining the relative contributions of MT, mitochondria, and possibly other intracellular stores is an interesting area for future work that may yield new interventions to limit pathologic Zn^{2+} elevations in the post-ischemic period.

Relationship between Ca^{2+} and Zn^{2+} in the induction of ischemic neurodegeneration

There has been considerable discussion of the relative contributions of Zn^{2+} and Ca^{2+} to ischemic injury, resulting in part from the sensitivity of Ca^{2+} indicators to Zn^{2+} (see Box 1). However, recent studies simultaneously tracking changes in these two ions suggest that they each contribute distinct and important effects to neuronal injury.

BOX 1

Relationship between Ca^{2+} and Zn^{2+} in ischemia studies

There are multiple reasons why it has been difficult to distinguish between the roles of Ca^{2+} and Zn^{2+} in ischemic injury:

1. **Many well-established Ca^{2+} influx pathways are also capable of mediating flux of Zn^{2+} .** These include voltage-dependent channels, sodium calcium exchangers and some ionotropic glutamate receptors (most notably GluR-2 lacking AMPA/KA receptors). Effects of inhibitors of these pathways have usually been taken to implicate Ca^{2+} , but there can be occasions where endogenous Zn^{2+} contributes significantly to membrane flux [39].
2. **Agents commonly used as Ca^{2+} chelators are well-recognized to bind Zn^{2+} , often with affinities significantly higher than for Ca^{2+} .** Zn^{2+} effects can be blocked by " Ca^{2+} " chelators such as EDTA. A derivative issue is that, because many Ca^{2+} indicators (e.g. Fura-2) are based on these chelators, Zn^{2+} binding can cause significant changes in fluorescence that could potentially be misinterpreted as Ca^{2+} signals. This issue has long been recognized and exploited for Zn^{2+} studies [41, 42], and recently discussed again in the context of ischemia [4, 43].
3. **Zn^{2+} can be found as a contaminant in many experimental recording conditions.** Zn^{2+} is not normally added to recording solutions, but nonetheless can be present in sufficient amounts as a contaminant [44], which leads to significant accumulation during ischemic depolarizations.

Progress on differentiating between these signals has been made with the following approaches:

1. **Selective chelation of Zn^{2+} is possible.** Chelators that effectively bind Zn^{2+} , but do not significantly influence physiological Ca^{2+} concentrations are available. The membrane-permeable analog TPEN is widely used, and extracellular chelation is commonly achieved by using EDTA that is pre-bound with Ca^{2+} . Zn^{2+} binding to CaEDTA is relatively slow because it requires displacement of Ca^{2+} . CaEDTA or chelex treatment effectively removes contaminating Zn^{2+} from recording solutions.
2. **Selective discrimination of Zn^{2+} and Ca^{2+} indicator signals is possible.** Zn^{2+} sensitive indicators that lack significant Ca^{2+} responsiveness (such as Newport Green, FluoZin-3, and the Zinpyr indicators) have been widely used for ischemia studies [4, 45], but Ca^{2+} indicators that lack Zn^{2+} sensitivity are not generally available. However it is possible to use combinations of low affinity " Ca^{2+} " indicators and high affinity Zn^{2+} indicators to separate signals in intact neurons [39, 40, 46], where absolute levels of Ca^{2+} are far higher than those of Zn^{2+} . Future development and implementation of ratiometric Zn^{2+} indicators, and Ca^{2+} indicators which lack Zn^{2+} binding should also be very useful.

Recent work suggests that aberrant Zn^{2+} accumulation can serve as an upstream contributor to deregulation of Ca^{2+} to toxic levels within neurons. In studies of hippocampal neurons in brain slices, it was shown that metabolic competence of dendritic segments was responsible for maintaining Ca^{2+} at physiological levels [37]. It is possible that intracellular Zn^{2+} accumulation initiates Ca^{2+} overload by decreasing ATP availability (see below) in these compartments, contributing to the initiation of injury [38]. As noted above, waves of spreading depolarization have emerged as a contributor to post-ischemic injury in a variety of models. Sustained Ca^{2+} increases following spreading depolarizations are likely to be essential for acute injury, but in *in vitro* models, Zn^{2+} accumulation can be important for triggering these events [39]. Further recent studies examined the relationship between Ca^{2+} and Zn^{2+} in acute neurodegeneration, in a hippocampal slice oxygen and glucose deprivation (OGD) ischemia model [40]. Zn^{2+} increases were observed prior to toxic Ca^{2+} overload, and preventing Zn^{2+} accumulation delayed the onset of irrecoverable Ca^{2+} increases. In all of these models, loss of Ca^{2+} homeostasis and sustained Ca^{2+} overload were most directly linked to the ultimate loss of membrane integrity and death, but Zn^{2+} increases appeared to contribute significantly to triggering the degenerative cascades.

Influences of Zn^{2+} on cellular metabolism following ischemia

Multiple and potent effects of Zn^{2+} on mitochondrial function

In 2000 we highlighted emerging clues to potent effects of Zn^{2+} on mitochondrial function, which likely has relevance to ischemia. Insights over the past decade have made it progressively clear that Zn^{2+} /mitochondrial interactions do indeed occur in, and appear to contribute to ischemic injury.

Pathological Zn^{2+} accumulation can affect mitochondrial function in various ways. Zn^{2+} induces swelling and release of reactive oxygen species (ROS) from mitochondria in isolation or in cultured neurons [5, 47, 48]. Although Ca^{2+} can induce similar pathologic responses in mitochondria, Zn^{2+} may do so with considerably greater potency [48]. However, not all studies find equally potent Zn^{2+} -induced mPTP opening [49], possibly reflecting organ differences (liver vs brain) or other factors, and uncertainty remains regarding both the amount and mechanisms of ROS generation that occurs with physiologically relevant Zn^{2+} exposures.

Regarding sites of Zn^{2+} effects on mitochondria, they appear to be complex and multiple [29]. Zn^{2+} inhibits electron transport in isolated mitochondria [50], and appears to irreversibly block critical mitochondrial enzymes, an effect which appears to be linked to activation of the mPTP [51]. Ischemia-induced injury could result from inhibition of mitochondrial ATP production, stimulation of ROS production/release, or release of pro-apoptotic factors such as cytochrome C or apoptosis-inducing factor (AIF) [48] in response to mPTP activation, although it is noted that many mechanistic studies have involved addition of exogenous Zn^{2+} at levels that may be non-physiological.

However, several studies do support contributions of endogenous Zn^{2+} to mitochondrial dysfunction in models of ischemia. Zn^{2+} chelation at the onset of ischemia attenuated mitochondrial cytochrome C release and downstream caspase 3 activation [19]. Also, ischemia was found to induce Zn^{2+} dependent opening of large, multi-conductance channels through the mitochondrial outer membrane [52]. In addition, in hippocampal slices subjected to OGD, endogenous Zn^{2+} was found to accumulate in mitochondria shortly after onset of OGD, and to contribute to an irrecoverable loss of the mitochondrial membrane potential [40].

Cytosolic targets linked to inhibition of metabolism

Zn^{2+} appears to have other metabolic effects largely independent of direct effects on mitochondria, and understanding of these pathways has markedly increased over the past decade. In cultured neurons, Zn^{2+} has been found to trigger activation of PKC, resulting in induction and activation of NADPH-oxidase, and also to induce nitric oxide synthase (NOS), together resulting in generation of superoxide, nitric oxide and peroxynitrite [53, 54].

Free radical-induced break of DNA strands results in activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), which catalyzes attachment of ADP ribose units from NAD^+ to various nuclear proteins [55], resulting in NAD^+ depletion, glycolytic block and secondary ATP depletion. In relation to Zn^{2+} neurotoxicity, Zn^{2+} causes PARP activation and NAD^+ depletion, and blockers of NADPH-oxidase, NOS or PARP attenuated delayed Zn^{2+} induced injury to cultured neurons [53]. PARP activation leads to release of AIF from mitochondria, which then translocates to the nucleus and triggers caspase-independent DNA fragmentation and cell death [56]. Whereas some studies suggested that PAR (the polymer product of PARP) can directly induce AIF release from mitochondria [57], a distinct mechanism was provided by the finding that the block of glycolysis and consequent mitochondrial inhibition induced by NAD^+ depletion alone accounted for release of AIF and cell death [58]. Interestingly, pyruvate is highly protective against Zn^{2+} neurotoxicity (as well as ischemic injury) [59, 60], perhaps because it can bypass the glycolytic block and promote regeneration of NAD^+ via lactate dehydrogenase [58, 60]. Other mechanisms likely also contribute to Zn^{2+} dependent NAD^+ depletion. Sirtuins are NAD^+ catabolic enzymes, activators of which enhanced NAD^+ loss and Zn^{2+} neurotoxicity, and antagonists of which were protective [61].

Zn^{2+} -dependent PARP cleavage (rather than activation) may play a role in *ischemic preconditioning*, a phenomenon wherein a sublethal episode of ischemia triggers changes in the tissue that result in decreased susceptibility to subsequent ischemia. A recent study suggested that Zn^{2+} dependent activation of caspase-3 could be responsible for the PARP cleavage, such that there would be less PARP contributing to injury upon subsequent toxic exposure [62].

Whereas it is clear that Zn^{2+} can have powerful effects on neuronal metabolism, it is evident that Zn^{2+} might also have important effects on astrocytic ATP production. For example, ATP reductions caused by Zn^{2+} in turn lead to impaired astrocytic glutamate uptake, an effect that could significantly increase excitotoxic components of ischemic episodes [63]. Like the effects described above for neurons, astrocytic ATP depletion appeared to be due to PARP activation. Zn^{2+} also plays a role in the activation of microglia, the resident immune cells of the CNS, contributing to late stages of injury after an ischemic episode. These microglial effects also appear to involve Zn^{2+} activation of PARP [64].

MAP kinases and transcriptional regulation in Zn^{2+} dependent neurodegeneration

It is increasingly clear that Zn^{2+} has powerful effects on a multitude of cell signaling cascades. These effects are complex, and depending upon conditions, the net result may either be protective or injurious. Several studies suggest that Zn^{2+} can mediate neuronal injury via activation of members of the mitogen-activated protein (MAP) kinase family, particularly P38 and extracellular signal-regulated kinases (ERK). MAP kinases play crucial roles in the regulation of cellular survival, proliferation and death, and are strongly activated by various stress conditions, including ischemia [65].

In neuronal cultures, intracellular Zn^{2+} mobilization (likely upon release from MTs or other redox sensitive binding sites, as discussed above) results in p38 MAP kinase activation, with consequent phosphorylation and membrane insertion of Kv2.1 K^+ channels, increased K^+ currents, caspase cleavage and cell death [28, 66–68]. Although ERK1/2 activation has most often been associated with cell survival and differentiation, multiple studies have found (particularly with prolonged activation) that it can trigger cell death [69]. ERK1/2 activation has been most often (but not exclusively) reported to contribute to Zn^{2+} -induced neuronal cell death after brief exposures of neurons to extracellular Zn^{2+} [70, 71]. In this scenario, inhibition of phosphatases by Zn^{2+} may be responsible for activation of the kinase, which then results in effects including mitochondrial dysfunction and activation of transcription factors like Egr-1 or Elk-1, leading to caspase independent cell death. In another paradigm, Zn^{2+} -regulated transcriptional changes, rather than causing injury, may contribute to preconditioning effects, with the result that Zn^{2+} handling is improved upon a subsequent ischemic challenge [33].

Concluding remarks

The above discussions highlight new insights, largely from the past decade, into ways in which Zn^{2+} may contribute to different stages and components of the ischemic injury cascade (see Figure 2). Although still a relatively new field, the picture is coming into sharper focus, with clear evidence for distinct roles of Zn^{2+} at different sites of action and likely different time frames after ischemic insults.

With brief sublethal ischemia, extracellular Zn^{2+} accumulation may be protective, by preventing overactivation of NMDA receptors. In addition, subtoxic intracellular Zn^{2+} accumulation may trigger preconditioning effects, thereby diminishing susceptibility to subsequent ischemia. With longer durations of ischemia, toxic intracellular Zn^{2+} accumulation can lead to prominent metabolic consequences. Importantly, these events may be amplified, as Zn^{2+} triggered ROS generation (produced by mitochondria, NADPH-oxidase, and other sources) can trigger further intracellular Zn^{2+} mobilization.

With very large Zn^{2+} loads, mitochondria may be important early targets, contributing to metabolic failure underlying Ca^{2+} deregulation and rapid cell death. However, if reperfusion occurs prior to cell death, many other factors will come into play. Repetitive spreading depressions and a reperfusion-associated oxidative burst may conspire to increase neuronal Zn^{2+} loads and enhance injury. In addition, there could be feed forward interactions accentuating Zn^{2+} dependent metabolic disruption, as ROS may induce delayed NADPH oxidase activation [72], and NADPH-oxidase may enhance mitochondrial ROS release [73], both acting to amplify Zn^{2+} mobilization.

If neurons do not succumb to these subacute events, more delayed processes involving activation of MAP kinase pathways or alterations in gene expression (resulting in events including the upregulation of Zn^{2+} -permeable, GluR2-lacking AMPA channels) may come into play in determining neuronal survival. Despite the considerable advances over the past decade, this field is still in its infancy. New targets of Zn^{2+} are being elucidated increasingly frequently, and some of these targets will no doubt modulate ischemic neurodegeneration (either positively or negatively). For example, Zn^{2+} has been recently found to activate an extracellular G-protein linked receptor linked to intracellular Ca^{2+} mobilization [74], to increase tropomyosin-related kinase B (TrkB) receptor-mediated neurotrophin signaling via multiple mechanisms [75, 76], to inhibit the $K(+)/Cl(-)$ co-transporter 2 (KCC2) [77] and to contribute to activation of the lysosomal/autophagy system [78]. Specific tests of the involvement of these, and other, novel Zn^{2+} mechanisms in global and focal ischemia models will likely

reveal new targets for effective therapeutic intervention, not only in acute ischemia, but in distinct stages after reperfusion before degeneration becomes irreversible.

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Figure 1. Zn^{2+} accumulation in the ischemic brain

Two main categories of Zn^{2+} mobilization have been linked to ischemic injury: 1) influx from the extracellular space, and 2) liberation from intracellular pools. These main pathways are shown, together with the possible impact of Zn^{2+} accumulation on Ca^{2+} homeostasis. During ischemia, significant extracellular accumulation may result from inappropriate fusion of Zn^{2+} -containing synaptic vesicles, either due to presynaptic metabolic failure or propagation of spreading depolarization events. Postsynaptic accumulation can occur via ion channels or Zn^{2+} -selective transporters, including reversal of transporters due to postsynaptic Na^+ accumulation. Intracellular Zn^{2+} pools include Zn^{2+} binding proteins (particularly MTIII) and organelles such as mitochondria which can sequester Zn^{2+} . Oxidative stress, and pH decreases which accompany ischemia may liberate substantial amounts of Zn^{2+} from these sources. Zn^{2+} accumulation appears to contribute to deregulation of Ca^{2+} homeostasis: Zn^{2+} can disrupt both glycolytic and mitochondrial ATP production, which in turn contributes to both excessive Ca^{2+} influx, and impaired Ca^{2+} extrusion, contributing to acute cell death.

Figure 2. Zn^{2+} injures neurons via multiple pathways acting over different time frames
 It is clear that the magnitude of the early Zn^{2+} load is crucial to determining the cell's fate. With the largest loads, Zn^{2+} likely contributes to catastrophic mitochondrial failure, loss of Ca^{2+} homeostasis and ROS release, resulting in acute necrosis. If a neuron survives an acute ischemic episode, multiple other mechanisms may come into play. Oxidative stress resulting from mitochondrial disruption, or NADPH-oxidase activation can damage nuclear DNA, resulting in PARP activation. PARP activation results in PAR accumulation and NAD $^{+}$ depletion, which can result in metabolic/mitochondrial inhibition. Consequent release of apoptotic mediators AIF and cytochrome C from mitochondria can lead to nuclear DNA cleavage and apoptosis, resulting in modestly delayed neuronal injury (probably after hours). If a neuron is not killed by the above mechanisms, activation of P38 and/or ERK1/2 MAP kinases can contribute to slower apoptotic and non-apoptotic injury pathways (hours to days), or Zn^{2+} -dependent activation of the transcriptional repressor REST can result in downregulation of GluR2, resulting in increased numbers of Ca^{2+} and Zn^{2+} permeable AMPA channels and contributing to delayed neurodegeneration (up to several days after the initial challenge). Finally, low Zn^{2+} rises, which would not in themselves cause injury, might contribute to the induction of repetitive spreading depolarizations, which put a large metabolic burden on neurons and may promote and extend injury. Repetitive spreading depolarizations may also provide a significant source of Zn^{2+} release and accumulation for hours or days following stroke. Conversely, moderate Zn^{2+} loads may activate preconditioning pathways, leaving the tissue with a decreased susceptibility to a subsequent ischemic episode. Further clarification of different pathways activated in distinct time frames after ischemia could suggest multiple targeted therapeutic interventions, some of which may be effective well after the initial ischemic episode.