Rethinking the Excitotoxic Ionic Milieu: The Emerging Role of Zn\(^{2+}\) in Ischemic Neuronal Injury

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Abstract: Zn\(^{2+}\) plays an important role in diverse physiological processes, but when released in excess amounts it is potently neurotoxic. In vivo trans-synaptic movement and subsequent post-synaptic accumulation of intracellular Zn\(^{2+}\) contributes to the neuronal injury observed in some forms of cerebral ischemia. Zn\(^{2+}\) may enter neurons through NMDA channels, voltage-sensitive calcium channels, Ca\(^{2+}\)-permeable AMPA/kainate (Ca-A/K) channels, or Zn\(^{2+}\)-sensitive membrane transporters. Furthermore, Zn\(^{2+}\) is also released from intracellular sites such as metallothioneins and mitochondria. The mechanisms by which Zn\(^{2+}\) exerts its potent neurotoxic effects involve many signaling pathways, including mitochondrial and extra-mitochondrial generation of reactive oxygen species (ROS) and disruption of metabolic enzyme activity, ultimately leading to activation of apoptotic and/or necrotic processes.

As is the case with Ca\(^{2+}\), neuronal mitochondria take up Zn\(^{2+}\) as a way of modulating cellular Zn\(^{2+}\) homeostasis. However, excessive mitochondrial Zn\(^{2+}\) sequestration leads to a marked dysfunction of these organelles, characterized by prolonged ROS generation. Intriguingly, in direct comparison to Ca\(^{2+}\), Zn\(^{2+}\) appears to induce these changes with a considerably greater degree of potency. These effects are particularly evident upon large (i.e., micromolar) rises in intracellular Zn\(^{2+}\) concentration ([Zn\(^{2+}\)]\(_{i}\)), and likely hasten necrotic neuronal death. In contrast, sub-micromolar [Zn\(^{2+}\)]\(_{i}\) increases promote release of pro-apoptotic factors, suggesting that different intensities of [Zn\(^{2+}\)]\(_{i}\) load may activate distinct pathways of injury. Finally, Zn\(^{2+}\) homeostasis seems particularly sensitive to the environmental changes observed in ischemia, such as acidosis and oxidative stress, indicating that alterations in [Zn\(^{2+}\)]\(_{i}\) may play a very significant role in the development of ischemic neuronal damage.

INTRODUCTION

Zinc is the second most abundant heavy metal in the human body [1]. The majority (85-95%) of this zinc is tightly bound to intracellular proteins, where it serves as a functional and/or structural component of more than 300 enzymes and proteins involved in DNA transcription and a wealth of other cellular processes. The remainder exists in its free ionic form (Zn\(^{2+}\)), and constitutes a pool of easily identifiable and chelatable cation which may serve a more “active” biological role owing to its greater accessibility.

How this Zn\(^{2+}\) ultimately affects the life and death of neurons has been a subject of great interest in recent years. For instance, a growing number of studies suggest that Zn\(^{2+}\) may act as a very important intra/intercellular signaling messenger [1-3]. A significant amount of Zn\(^{2+}\) can be released from pre-synaptic glutamatergic terminals, and once in the synaptic clef, can modulate the behavior of a variety of ion channels and receptors, thus acting as an “atypical” neurotransmitter. Moreover, Zn\(^{2+}\), like Ca\(^{2+}\), may serve as an important mediator of intracellular signaling.

However, much evidence has been gathered indicating that Zn\(^{2+}\) is also a potent toxic agent which plays a critical role in the neuronal loss observed in a variety of excitotoxic neurological conditions, such as ischemia, epilepsy, and brain trauma [2]. Almost two decades ago, a convergence of in vitro and in vivo studies established Zn\(^{2+}\) as a “killing ion,” and we are now discovering that this cation is, in fact, as injurious as Ca\(^{2+}\), if not more so. In vitro experiments in the mid-1980’s demonstrated that exposure to pathologically relevant (e.g., several hundred micromolar) concentrations of Zn\(^{2+}\) were able to trigger both neuronal and glial injury [4, 5]. At the same time, a number of findings in vivo began to indicate that alterations in cytosolic Zn\(^{2+}\) content might also play a key role in triggering neuronal death. First, direct injection of Zn\(^{2+}\) into the brain was found to promote strong neurotoxicity [6]. More importantly, Zn\(^{2+}\) was also found to be released from pre-synaptic terminals in a Ca\(^{2+}\)-dependent fashion upon sustained synaptic activity [7-9]. Further indictment of Zn\(^{2+}\) as an endogenous neurotoxin was provided through the demonstration that
perforant path stimulation induced a concurrent loss of 
Zn²⁺ in the pre-synaptic mossy fiber terminals and 
neuronal death in hilar interneurons and CA3 
pyramidal cells, the post-synaptic targets of the 
perforant path [10]. Subsequent studies confirmed 
that status epilepticus also induced both Zn²⁺ 
depletion from mossy fiber boutons and injurious 
intracellular Zn²⁺ ([Zn²⁺]ᵢ) accumulation in adjacent 
post-synaptic hilar neurons [11, 12].

This trans-synaptic Zn²⁺ movement, dubbed “Zn²⁺ 
translocation,” was eventually found to play a critical 
role in ischemic injury through the pioneering work of 
the Frederickson group, along with Koh and Choi, in 
the 1990’s [13, 14]. Frederickson and colleagues 
first provided evidence that Zn²⁺ translocation 
occuring upon global ischemia was relevant to 
injury, as the neurons enriched in cytosolic Zn²⁺ were 
revealed to be the same neurons exhibiting cellular 
markers of injury. The case for injurious Zn²⁺ 
translocation was cemented (at least for another few 
years) by the findings of Koh and colleagues, which 
demonstrated that administration of the extracellular 
Zn²⁺ chelator Ca-EDTA before and during transient 
global ischemia (TGI) was able to block [Zn²⁺]ᵢ 
accumulation as well as protect against damage in 
the subpopulation of neurons most vulnerable to 
TGI, the pyramidal neurons of the CA1 hippocampal 
subregion [14].

The past few years have yielded a wealth of 
additional information on the physiology and 
pathophysiology of inter- and intra-cellular Zn²⁺ 
movement, largely due to the combination of more 
sophisticated imaging techniques and increased 
availability of Zn²⁺-sensitive fluorescent probes [15, 
16]. As with any fast-moving field of research, 
however, such an abundance of knowledge has also 
provoked areas of debate. For instance, the 
established concept of Zn²⁺ translocation as a 
necessary step to trigger neuronal injury has been 
recently challenged by the observation that 
transgenic mice lacking pre-synaptic, vesicular Zn²⁺ 
nevertheless undergo [Zn²⁺]ᵢ accumulation and injury in 
some (i.e., CA1) hippocampal regions. Thus, in 
the present review, we summarize the current state of 
knowledge regarding cellular Zn²⁺ physiology and 
discuss the established processes implicating this 
cation in ischemic injury, with particular attention to 
the most recent evidence, controversies, and 
pathogenic hypotheses.

I. ANATOMICAL DISTRIBUTION OF Zn²⁺ IN 
THE NERVOUS SYSTEM

The brain has the highest concentration of zinc of 
any organ in the human body excepting the 
pancreatic beta cells. In addition to being bound to 
proteins as it is elsewhere in the body, Zn²⁺ in the 
central nervous system is found concentrated in the 
synaptic vesicles of excitatory nerve terminals. High 
levels of stainable Zn²⁺ can be found in the 
forebrain, particularly in the cortex, amygdala, 
hippocampus, and olfactory bulb [1]. Low-level Zn²⁺ 
staining has also recently been detected in the 
spinal cord with particularly dense staining in the 
dorsal horn, where Zn²⁺ is thought to be associated with 
the inhibitory GABAergic (rather than glutamatergic) axon terminals [17, 18]. GABAergic 
Zn²⁺-containing neurons have also been very 
recently described in mouse cerebellum [19].

In the forebrain, however, all Zn²⁺-containing 
neurons are glutamatergic—although not all 
glutamatergic neurons contain Zn²⁺. Zn²⁺-containing 
axons are generally corticocortical—corticolimbic, or 
limbic-cortical, and tend to be associational fibers 
rather than distant projections [20]. Among these 
“zincergic” projection systems, the most striking (and 
perhaps most well-known) example is offered by the 
dentate granule cell axons which project to CA3 
pyramidal neurons in the hippocampus, otherwise 
known as the mossy fiber tract (Fig. (1)). The 
presence of vesicular Zn²⁺ is also well established in the 
CA1 subregion of the hippocampus [21-24], with 
a recent study estimating that approximately half of 
the synaptic boutons in the CA1 stratum radiatum contain Zn²⁺ [25]. Discrete patterns of synaptic Zn²⁺ 
have additionally been identified in both 
somatosensory and visual cortex, with some 
evidence that the pattern of distribution in these 
areas may be dynamically modulated by different 
levels of sensory input [26-29].

Most of the data regarding the anatomical 
distribution of Zn²⁺ has been gathered through 
histochemistry, with more recent work utilizing 
fluorescent staining. Zn²⁺ localization in the 
mammalian brain was first reported in the mid-1950’s, 
when it was discovered that the colorimetric Zn²⁺- 
binding reagent dithizone produced a band of vivid, 
bright-red staining when injected in the hippocampus 
[30]. This observation was further refined several 
decades later through modification of the Timm 
silver-enhancing method, originally designed to 
visualize weakly-bound metals. In their landmark 
studies, Haug [31] and Danscher [32-34] used a 
modified Timm’s method to eventually identify a pool 
of weakly-bound (and thus readily-exchangeable) 
Zn²⁺ in the brain that selectively localized to the 
vesicles of pre-synaptic boutons. Their findings were 
later confirmed through the development and use of 
TSQ, a Zn²⁺-selective probe that produces a bright 
fluorescence when in a complex with Zn²⁺ but not 
other Timm-positive metals, such as iron or copper 
[35].

Thus, where Zn²⁺ resides in the brain is generally 
agreed upon; exactly how much Zn²⁺ is present and 
what it is actually doing there, however, are other 
questions entirely. Cytosolic [Zn²⁺]ᵢ in vivo and in 
vitro is thought to be very low [16, 36-38], although a 
definitive calculation remains elusive due to the 
intrinsic limitations of fluorimetric assays and the still 
less-than-satisfactory array of Zn²⁺-sensitive 
fluorescent probes at hand [15, 16, 39]. On the 
other hand, the total amount of extracellular Zn²⁺
Figure 1. Distribution of hippocampal chelatable Zn\(^{2+}\) as identified by ZP1 confocal microscopy.

Live hippocampal slices were stained with the high affinity, Zn\(^{2+}\)-sensitive probe ZP1 and imaged under confocal microscopy. A, 4x image of the whole hippocampus depicts Zn\(^{2+}\)-enriched regions, such as the hilus (H), stratum oriens (SO), and stratum radiatum (SR). B, 40x image of the stratum lucidum (SL). Note the large distinct puncta, likely indicating ZP1 staining of the giant mossy fiber boutons.

([Zn\(^{2+}\)]\(_{e}\)) is estimated to be below 500 nM [1], yet this concentration can dramatically increase in the synaptic cleft following strong synaptic activation. The extent of [Zn\(^{2+}\)]\(_{e}\) rise after sustained activity, however, is presently a matter of some debate. Initially, the Zn\(^{2+}\) concentration of synaptic vesicles was indirectly estimated to be in the millimolar range, with the level of Zn\(^{2+}\) in the synaptic cleft approximating several hundred micromolar following sustained activity [7, 8]. Since then, synaptic Zn\(^{2+}\) release has been re-examined by several different groups using a variety of Zn\(^{2+}\)-sensitive fluorescent probes, resulting in several notably different estimates of [Zn\(^{2+}\)]\(_{e}\) [40-45].

As with [Zn\(^{2+}\)]\(_{i}\), all estimates of activity-triggered changes in [Zn\(^{2+}\)]\(_{e}\) require careful interpretation. For example, one very recent study quite unexpectedly found the peak [Zn\(^{2+}\)]\(_{e}\) obtained following intense synaptic activation to be in the low nanomolar range [45]. The study evaluated the amount of released Zn\(^{2+}\) by measuring changes in the overall epifluorescence of a Zn\(^{2+}\)-sensitive probe in an acute hippocampal slice preparation undergoing synaptic stimulation and, as with previous imaging studies, two potential caveats can be foreseen. First, the intrinsic limitations in temporal and spatial resolution of this method of detection do not allow a definitive evaluation of pathophysiologically relevant changes occurring in microdomains such as the synaptic cleft. Secondly, acute hippocampal slice preparations have been shown to lose much of their synaptic Zn\(^{2+}\) during the dissection process and/or in the early stages of incubation [46]. While this rigorous study offers a fresh perspective by questioning long-held assumptions, it is more likely to be akin to the continuation of a conversation rather than the end of an argument. These findings also underscore the fact that, despite the exponential advances in Zn\(^{2+}\) visualization achieved in the last decade, technical considerations present an ongoing challenge in this field of study.

II. Zn\(^{2+}\) HOMEOSTASIS

As with Ca\(^{2+}\), homeostasis of [Zn\(^{2+}\)]\(_{i}\) levels is controlled by a balance between sequestration, buffering and extrusion. Within the cytosol, sequestration and buffering appear to be largely controlled by a family of low molecular weight, cysteine-rich proteins with multiple binding sites for Zn\(^{2+}\) called metallothioneins (MTs) [47, 48]. Membrane-associated Zn\(^{2+}\) transporters and mitochondria also play important roles in intracellular Zn\(^{2+}\) extrusion, sequestration to intracellular compartments, and buffering.

A. Metallothioneins

The overall relative abundance of zinc in the human body suggests that this cation serves an important role in cell physiology, one in which Zn\(^{2+}\) may act as a dynamic signaling molecule in addition to serving as a passive structural component. As mentioned above, resting [Zn\(^{2+}\)]\(_{i}\) is estimated to be extremely low, prompting the questions of where such "biologically active" Zn\(^{2+}\) might be coming from, and how its distribution might be regulated such that it would be readily available when required for cellular function. If Zn\(^{2+}\) is such an important and potent signaling ion, it stands to reason that well-regulated homeostatic mechanisms are in place to guarantee that the right amount of Zn\(^{2+}\) can be accessed when needed and become sequestered into stores when it is not. One such intracellular Zn\(^{2+}\) store may be provided in the form of MTs, a family of proteins capable of binding multiple Zn\(^{2+}\) ions per peptide and which are sensitive to a variety of
physiological (and pathophysiological) changes in the cytosolic environment, making them attractive candidates for the role of Zn$^{2+}$ “bank.”

MTs, first described nearly 50 years ago by Margoshes and Vallee [49], are ubiquitously expressed throughout the body with varying levels of isoforms in specific tissues, three of which are present in the central nervous system (MT-1, -2, -3). MT-1 and MT-2 are largely expressed in astrocytes and spinal glia with some MT-1 and MT-2 also found in ependymal, meningeal, and endothelial cells, while neurons conspicuously lack these proteins. By contrast, MT-3 is expressed predominantly in neurons and sparingly in glial cells, and is particularly abundant in the hippocampal glutamatergic terminals which are also rich in vesicular Zn$^{2+}$ [48].

All three MT isoforms have a similar structure and are comprised of a single polypeptide chain of 61–68 amino acids, 20 of which are highly conserved cysteine residues [47]. Structural studies show that the physical conformation of MTs exhibits a “dumb-bell” shape with two domains, each binding a cluster of Zn$^{2+}$ ions [50, 51]. In one domain, three Zn$^{2+}$ ions are bound to nine cysteines, whereas the other domain has four Zn$^{2+}$ ions bound to eleven cysteines, whereas the other domain has four Zn$^{2+}$ ions bound to eleven cysteines. Past studies pioneered by Vallee and Maret demonstrated the critical role played by these Zn$^{2+}$/Cys cluster regions in regulating Zn$^{2+}$ binding [52, 53]. Zn$^{2+}$ is bound to MTs with very high affinity ($K_d = 2 \times 10^{-12}$ M$^{-1}$ at pH 7.0) [47], but this binding can be readily modulated by shifts in acid-base equilibrium or, more significantly, by changes in the redox state of the two Zn$^{2+}$/Cys cluster regions [38, 47, 54, 55].

Cellular oxidants such as selenium, glutathione disulfide (GSSG), or disulfides such as 2,2'-dithiodipyridine (DTDP) have been found to promote Zn$^{2+}$ release from MTs, whereas a shift to a more reduced intracellular environment facilitates binding [52, 56, 57]. In particular, changes in the glutathione (GSH) redox state (i.e., the GSH/GSSG ratio) may serve a principal role in regulating Zn$^{2+}$-MT interactions; GSH binds directly to MT and is thought to “activate” the protein in order to facilitate GSSG-mediated Zn$^{2+}$ release [54]. Nitric oxide (NO), an important cellular signaling molecule, also promotes Zn$^{2+}$ release from MTs in vitro and in vivo [58-60] and interacts preferentially with MT-3, the only MT isoform with consensus acid-base sequence motifs for S-nitrosylation in both Zn$^{2+}$/Cys cluster regions [61]. The higher reactivity of MT-3 with NO suggests that this isoform is uniquely positioned to translate NO signaling into Zn$^{2+}$ signaling, and may have significant implications for the role of MT-3 in injury mediated by oxidative or nitrosative stress.

Zn$^{2+}$ binding to MTs may also be indirectly modulated by cellular metabolism, as the GSH/GSSG complex is dependent on the balance between the oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate (NADP$^+$/NADPH), which in turn depends on glucose metabolism by the pentose phosphate pathway [62]. Furthermore, direct binding of ATP to MTs results in several important functional consequences, including a significant change from the “dumb-bell” conformation to a more globular shape, which may play a role in MT sequestration in subcellular compartments [63]. ATP binding also increases the rate of MT reaction with disulfides, enhancing the efficiency of Zn$^{2+}$ transfer from MTs to acceptor molecules [64].

In addition to serving as a primary source of readily accessible Zn$^{2+}$, and being strategically localized for many cytosolic Zn$^{2+}$-dependent enzymatic reactions, emerging data suggest that MTs may also serve a heretofore unappreciated role as modulators of mitochondrial function. In fact, MTs quite surprisingly appear to be able to move into mitochondria despite lacking a specific mitochondrial import sequence, possibly by using pathways similar to those of apocytochrome c or the mitochondrial copper chaperone Cox17 (both of which lack the mitochondrial import sequence) [65]. The conformational changes induced by ATP binding could also favor retention of the peptide in the mitochondrial intermembrane space.

The putative function of mitochondrial MTs remains largely unexplored, but it is intriguing to speculate that these proteins might mediate intramitochondrial Zn$^{2+}$ release and by doing so, also inhibit the respiratory chain [65]. As in the case of cytosolic release, intramitochondrial Zn$^{2+}$ release from MTs may be triggered by changes in acid-base equilibrium, oxidation, or changes in ATP levels. The last two parameters may be especially relevant in mitochondria, given their role in the production of cellular ATP and their constitutive generation of reactive oxygen species (ROS) during mitochondrial respiration.

It is also conceivable that the sensitivity of MTs to changes in cellular redox state and ATP levels might help in fine-tuning mitochondrial activity, or even act as a physiological “brake” in the case of increased respiration. For instance, in the latter scenario, ROS generation would trigger Zn$^{2+}$ release from MTs, resulting in electron transport chain inhibition (see section III B.1, below) and an overall “slowing down” of the rate of respiration. Moreover, excessive ATP production would provide additional negative feedback for ADP-stimulated mitochondrial respiration by acting as an effector for even more Zn$^{2+}$ release from mitochondrial MTs, further promoting Zn$^{2+}$-dependent inhibition of cellular respiration.

B. Membrane-Associated Zn$^{2+}$ Transport Proteins

The proteins directly involved in the active movement of Zn$^{2+}$ across cellular membranes generally belong to two families of transporters: the CDF (Cation Diffusion Facilitator) and ZIP (Zn$^{2+}$-regulated metal transporter, Iron-regulated metal
transporter-like Protein*) families. In humans, those most directly associated with Zn²⁺ transport are the ZnT (Zn²⁺ Transporter) proteins, members of the CDF family which favor Zn²⁺ movement out of the cytosol, either by extrusion or sequestration of the cation into intracellular compartments. In contrast, the ZIP proteins, which to date have been described largely in eukaryotic and plant systems, appear to control Zn²⁺ transport into the cytosol.

To date, seven ZnTs have been identified (ZnT-1-7), and more are likely on the verge of detection, as a very recent report has identified 9 human CDF genes (now also known as SLC30 genes) [66]. ZnT-1 is largely expressed in the brain on neuronal plasma membranes and plays an important role in modulating Zn²⁺ homeostasis, though the exact nature of this role has not been fully elucidated. When first described, overexpression of ZnT-1 was found to confer resistance to toxic exposure of extracellular Zn²⁺ by increasing Zn²⁺ efflux capacity, thus leading to the characterization of ZnT-1 as a Zn²⁺ exporter [67]. Since then, the transporter has also been described to be dynamically up-regulated, possibly by activation of a metal response element in the promoter region of the ZnT-1 gene [68].

Suggesting a likely role for ZnT-1 in the modulation of post-ischemic [Zn²⁺], a recent study indicates that its expression is selectively up-regulated in pyramidal neurons of the CA1 region (the neuronal subpopulation that is particularly vulnerable to injury after global ischemia) 12 hours after ischemic insult. Furthermore, ZnT-1 mRNA was seen to accumulate in cultured hippocampal neurons following exposure to high [Zn²⁺], suggesting that transporter expression might be modulated in response to excess Zn²⁺ and thus act as a “defensive maneuver” against Zn²⁺ toxicity [69]. The notion that ZnT-1 plays a critical role in counteracting Zn²⁺-dependent neuronal injury is further supported by the observation that ZnT-1 expression is greatest in the subpopulation of neurons facing the strongest “zincergic” input. For example, ZnT-1 is robustly expressed in the hippocampal CA3 region, which is the target of the highly Zn²⁺-enriched mossy fiber projection [70].

More recent findings, however, seem to indicate that the net effect of ZnT-1 activity on Zn²⁺ homeostasis could ultimately prove to be quite complex. Surprisingly, recent data from transfected HEK cells suggest that the presence of the transporter facilitates Ca²⁺ influx by direct modulation of voltage-sensitive Ca²⁺ channels1 (VSCC). As VSCC are also Zn²⁺-permeable [36, 37, 71, 72], ZnT-1 might therefore act not only as a route of Zn²⁺ efflux but also as an indirect modulator of Zn²⁺ influx.

ZnT-3 is strongly expressed in brain regions that are rich in histochemically reactive Zn²⁺, such as the entorhinal cortex, the amygdala, and the hippocampus. This transporter is particularly present in the mossy fiber tract and is localized to the membranes of Zn²⁺-containing vesicles in the mossy fiber synaptic boutons. ZnT-3 knockout mice display a conspicuous lack of Zn²⁺ in their hippocampi, and ultrastructural examination demonstrates the absence of Zn²⁺ in their mossy fiber boutons, thus confirming that this transporter is essential for Zn²⁺ influx into synaptic vesicles [73].

The remaining ZnT proteins have variable levels of expression in the brain, and much less is known about their function in general. ZnT-2 expression is limited to small intestine, kidney, placenta, and liver. It is localized on the membranes of acidic vesicles [74] and thought to promote Zn²⁺ uptake from cytosol to an endosomal/lysosomal compartment. ZnT-4 has a ubiquitous pattern of expression in the human body with particular abundance in mammary gland and brain, and available functional studies have highlighted its involvement in the deposition of Zn²⁺ into breast milk. ZnT-1, 2 and -4 mRNA expression have each been demonstrated to be affected by the level of dietary Zn²⁺, illustrating on a systemic level the role these proteins play in Zn²⁺ homeostasis [75].

ZnT-5 is most abundantly expressed in pancreatic beta cells, where it is associated with insulin-containing secretory granules [76]. However, this isoform may serve a more complex homeostatic purpose, as ZnT-5 knockout mice exhibit impaired osteoblast maturation and male-specific sudden death from bradyarrhythmia [77]. ZnT-6 and ZnT-7 have only been characterized very recently; ZnT-6 is present in brain and lung with much lower levels detected in small intestine and kidney, while ZnT-7 is in abundant in liver and small intestine with moderate expression in brain, kidney, spleen, and lung. Both transporters seem to promote Zn²⁺ sequestration in the Golgi apparatus, with ZnT-6 additionally implicated in translocation of Zn²⁺ to vesicular compartments [78, 79]. Why nature has offered so many seemingly redundant or functionally overlapping ZnT proteins, however, is an intriguing question that remains to be explored.

In contrast to our knowledge of ZnT proteins, relatively little is known about the ZIP family of transporters and its human isoforms, which generally possess eight transmembrane domains. Although the precise mechanism(s) of substrate transport have not yet been determined, the ZIP proteins which have been characterized appear to transport metals into the cytoplasm, from either the lumen of cytoplasmic organelles or from extracellular compartments [80]. The human genome encodes for 14 currently-identified human ZIP genes (also known as SLC39 genes; [81]), and three ZIP family members (hZip1, 2, and 4) have been characterized at present. hZip1 may be most prevalent on beta cells, where it is associated with insulin-containing secretory granules [76].

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1 Sekler, I., Zinc signals 2003, personal communication.
C. Mitochondria as Homeostatic Zn²⁺ Buffers

Mitochondria are known to be critically important in buffering cytosolic Ca²⁺, and several lines of evidence support the idea that these organelles also constitute a site of intracellular Zn²⁺ uptake. Zn²⁺ is sequestered by isolated mitochondria [84], possibly via activation of the Ca²⁺ uniporter [85, 86]. Mitochondrial Zn²⁺ uptake has been directly visualized in neurons using Zn²⁺-sensitive mitochondrial fluorophores, and is blocked by treatment with the protonophore FCCP, a maneuver that induces strong mitochondrial depolarization and thus abolishes the driving force for cation entry (Fig. (2A,B)) [38, 87, 88]. Additionally, in neurons, inhibition of mitochondrial Zn²⁺ uptake leads to elevation or prolongation of induced cytosolic [Zn²⁺]i elevations, further implying that these organelles have a high Zn²⁺ uptake capacity and could serve a key role in the clearance of cytosolic Zn²⁺ loads [87]. These induced [Zn²⁺]i elevations are buffered by sequestration of the cation into mitochondria, where it may subsequently be re-released into the cytosol via a Ca²⁺-dependent fashion [89]. Finally, very recent studies indicate that mitochondria do possess a pool of Zn²⁺ under resting conditions that can be released in a Ca²⁺-dependent fashion and/or upon mitochondrial depolarization (Fig. (2C,D)) [38]. While the physiological purpose of this mitochondrial Zn²⁺ is presently unexplored, it is conceivable that these organelles could act as important sources for the cation under physiological and/or pathological conditions.

III. PHYSIOLOGICAL ROLES OF Zn²⁺

A. Zn²⁺ at the Synapse

Although the abundance of Zn²⁺ in the brain was first noted several decades ago, the physiological role of Zn²⁺ in normal synaptic activity remains largely mysterious. Because of its localization in the synaptic vesicles of certain glutamatergic nerve terminals, Zn²⁺, in theory, is uniquely positioned to act as an important modulator of excitatory neurotransmission. Electrophysiological studies (carried out primarily on recombinant receptors) have demonstrated how extracellular Zn²⁺ can inhibit the N-methyl D-aspartate receptor (NMDAR) (Fig. (4)) through both low-affinity, voltage-dependent and high-affinity, voltage-independent mechanisms. In addition, Zn²⁺ potentiates alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor (AMPA)-mediated responses [90-94], possibly by inhibiting receptor desensitization [95]. Given these opposing directions of modulation, one might speculate that the cation could act as a “switch” that shifts the balance of glutamatergic neurotransmission from NMDAR- to AMPAR-mediated responses. The mechanisms underlying Zn²⁺’s extracellular effects have been more extensively investigated for NMDAR rather than AMPAR, however, and more recent studies indicate a rather complex modulation of NMDA receptor activity.

As this review focuses on Zn²⁺’s role in ischemic injury, it is pertinent to consider changes in the extracellular environment during and following ischemia, and how such changes might affect and/or interact with the modulatory effect of the cation. The cellular pathophysiology of ischemia is characterized by excess release of glutamate, development of parenchymal acidosis, and increased production of ROS [96]. Extracellular Zn²⁺, protons, and redox modulation (i.e., oxidation/reduction of extracellular cysteine residues) have each been separately described as allosteric modulators of glutamate receptor activity and of NMDAR in particular. It is therefore worth considering the physiological implication of increased levels of all three modulators co-existing in the ischemic extracellular milieu. With these factors in mind, we first direct the section below toward reviewing several recent findings regarding Zn²⁺ modulation of NMDAR which may be particularly relevant under ischemic conditions.

In addition to ionotropic glutamate receptors, Zn²⁺ also modulates a number of other receptors and ion channels, such as type A γ-aminobutyric acid (GABA_A) receptors, glutamate transporters, and voltage-gated cationic currents, which all participate in the balance between network excitation and inhibition. Although these interactions—particularly those between Zn²⁺ and GABA_A receptors—have largely been examined in the context of epilepsy pathophysiology, they may potentially contribute to overall post-ischemic excitability and will thus be described in brief. Finally, given that network rebuilding or remodeling might be of importance in the functional recovery of the ischemic brain, we will also discuss the emerging role of Zn²⁺ in network plasticity.

1. Modulation at the Receptor Level

a) NMDA

The basis of Zn²⁺ modulation of NMDAR has been greatly elucidated by several recent molecular and structural studies. NMDAR are believed to be heteromeric assemblies of NR1 and NR2 subunits, and the NR2 subunit has four additional subtypes, deemed NR2A-D. While the low-affinity, voltage-dependent inhibition of NMDA currents by Zn²⁺ is a result of channel block, akin to that produced by Mg²⁺ ions, the high-affinity, voltage-independent inhibition is due to a decrease in receptor channel open probability [92, 93, 97, 98]. In addition, while all recombinant NMDAR exhibit similar voltage-dependent block, voltage-independent Zn²⁺ inhibition is primarily determined by the type of NR2 subunit present in the NR1/NR2 subunit assembly [99-102].
Figure 2. Mitochondria can serve as an intracellular site of both Zn\(^{2+}\) sequestration and cytosolic release.

**A, B. Cytosolic Zn\(^{2+}\) rises trigger mitochondrial Zn\(^{2+}\) uptake.**

Cortical neurons loaded with the Zn\(^{2+}\) sensitive mitochondrial probe Rhod-2 were exposed for 10 min to kainate (KA) and Zn\(^{2+}\), and monitored for 20 min before addition of the cell-permeable Zn\(^{2+}\) chelator, TPEN. In (A), confocal images of Rhod-2-loaded cortical neurons are depicted at baseline (1), at the end of the KA/Zn\(^{2+}\) exposure (2), and after addition of the TPEN (3). (4), the imaged field under visible light. Bar = 15 µm. In (B), microfluorimetric images of Rhod-2-loaded cortical neurons are shown at baseline (1), following 10 min exposure to KA/Zn\(^{2+}\) (2), and after addition of TPEN (3). Note the preferential Zn\(^{2+}\) uptake in the mitochondria of Ca-A/K(+) neurons, subsequently identified by KA-stimulated Co\(^{2+}\) uptake (4). Bar = 25 µm. (Modified from [87]).

**Neuronal mitochondria serve as a pool of releasable Zn\(^{2+}\).**

(C) Confocal microscopy of neurons co-loaded with the Zn\(^{2+}\)-sensitive mitochondrial probe RhodZin-3 (red fluorescence; 1) and the mitochondrial marker MitoTracker Green (green fluorescence; 2), shows substantial overlap between these probes (yellow; 3), indicating that these two probes largely target the same intracellular organelles. Bar = 10 µm.

(D) Confocal images of RhodZin-3-loaded mitochondria in intact neurons before (1) and after (2) application of TPEN. The decrement in mitochondrial signal induced by TPEN suggests the presence of an intramitochondrial pool of chelatable Zn\(^{2+}\). Bar = 10 µm. (Modified from [38]).

**Mitochondrial Zn\(^{2+}\) release is Ca\(^{2+}\)-dependent.**

(E) Time course of cytosolic [Zn\(^{2+}\)], rises as indicated by FluoZin-3 fluorescence changes before, during and after exposures to (1) NMDA in a Ca\(^{2+}\) containing (black) or Ca\(^{2+}\) free (red) buffer; (2) the mitochondrial protonophore, FCCP, or (3) FCCP followed by NMDA. Note that NMDA-induced [Zn\(^{2+}\)] increases are dependent upon Ca\(^{2+}\) influx, and that prior exposure to FCCP occludes subsequent NMDA-triggered [Zn\(^{2+}\)] increases, indicating that these two manipulations target a common intracellular Zn\(^{2+}\) pool. (Modified from [38]).
Specifically, receptors containing NR2A subunits have an extremely high sensitivity (IC$_{50}$ ~20 nM) to Zn$^{2+}$, while those containing NR2B, 2C, or 2D subunits have a lower sensitivity to Zn$^{2+}$ inhibition (IC$_{50}$ ~30 μM). Past studies utilizing recombinant NR1/NR2 receptors have localized the site of high-affinity Zn$^{2+}$ binding to the amino-terminal domain of NR2A, and determined that two neighboring histidine residues represent a critical determinant for this voltage-independent Zn$^{2+}$ inhibition [103]. These experiments further suggest that the mechanism of this high-affinity inhibition by Zn$^{2+}$ involves the enhancement of channel sensitivity to proton inhibition, thus implying a convergence of the two mechanisms of pH and Zn$^{2+}$ modulation of NMDAR activity [102-104].

Redox modulation of NMDAR also has two components: a slow and persistent component common to all NMDAR, and a rapid and reversible component specific to those receptors containing the NR2A subunit. Redox modulation occurs as a result of the oxidation or reduction of three pairs of Cys residues: two pairs on NR1 underlying the slower and persistent component, and one pair on NR2A, which controls the rapid component. While disulfide formation between Cys pairs (favored in oxidizing environments) on NR1 results in decreased NMDA-evoked current, greater Zn$^{2+}$ inhibition of NMDA current under oxidizing conditions is observed in NR2A-containing receptors, and vice versa [105]. Thus, while the slow, persistent inhibition of current is a result of the conformational change induced through disulfide bridge formation on NR1, the rapid component of redox modulation is due to an additional potentiation of the voltage-independent Zn$^{2+}$ inhibition described above. The redox-sensitive Cys residues on NR2A do not directly interact with Zn$^{2+}$ under oxidizing conditions, but instead affect a conformational change which both promotes the formation of disulfide bonds and also favors greater inhibition of NMDAR currents by Zn$^{2+}$. The sensitivity of the receptor to Zn$^{2+}$ inhibition may thus be indirectly affected by cellular redox status.

Taken together, these studies highlight the complexity of Zn$^{2+}$'s functional interaction with NMDAR, in that specific amino acid residues can affect high-affinity Zn$^{2+}$ inhibition of NMDAR via either direct (binding of Zn$^{2+}$ to histidine residues of NR2A) or indirect (modulation by oxidation/reduction without specific binding of Zn$^{2+}$ to cysteine residues) means. Furthermore, both the direct and indirect means of Zn$^{2+}$ modulation may additionally interact with other critically relevant allosteric modulators of NMDAR activity (pH and redox). Although it is unclear at present if or how much competition exists in the simultaneous presence of all three modulators, one might predict that the net effect during ischemia would most likely be one of substantially inhibited NMDAR activity.

It may therefore come as a surprise that a recent study suggests that Zn$^{2+}$ may also indirectly increase NMDA current and NMDAR-mediated neuronal toxicity under certain conditions [106]. In these experiments, low micromolar concentrations (50 μM) of extracellular Zn$^{2+}$ were found to promote a delayed increase in NMDA current amplitude in cultured cortical neurons, through selective enhancement of neuronal Src family tyrosine kinase activity and consequent increased NMDAR phosphorylation. Moreover, pre- (but not co-) incubation of neurons with the same concentrations of extracellular Zn$^{2+}$ was found to enhance NMDAR-mediated cell death, suggesting that Zn$^{2+}$ may produce a biphasic effect on NMDAR-mediated neurotoxicity. Thus, it is possible that Zn$^{2+}$ might "prime" the receptor, leading to an eventual potentiation of NMDAR/Ca$^{2+}$-triggered neuronal death when Zn$^{2+}$ is no longer available to exert its direct inhibitory effect [106].

b) GABA

GABA receptors gate heteropentameric chloride channels which are assembled from a large family of receptor subunits, including α (1–6), β (1–4), γ (1–3), δ, ε, θ and ρ (1–2). Zn$^{2+}$ can potently inhibit GABA$_{A}$ receptor function by an allosteric mechanism, but this inhibition is dependent on GABAR subunit composition. Receptors consisting of only α and β subunits are the most sensitive to block by Zn$^{2+}$ (IC$_{50}$ ~1 μM) and the addition of the δ subunit to an αβ assembly enhances Zn$^{2+}$ sensitivity [107]. In contrast, the presence of the γ subunit in an αβ assembly renders the receptor relatively insensitive to Zn$^{2+}$ inhibition [108], along with transforming the mechanism of inhibitory block from noncompetitive to competitive [109].

c) Glutamate Transporters

Excitatory amino acid transporters (EAATs), which may be expressed on either glial cells or neurons, maintain extracellular glutamate concentrations below toxic levels (in the submicromolar range) and regulate the kinetics of glutamate receptor activation by clearing glutamate from the synapse. This glutamate transport by EAATs is coupled to the cotransport of 3 Na$^{+}$ and 1 H$^{+}$ into cells and the counter-transport of 1 K$^{+}$ ion out. In addition, glutamate transport activates an uncoupled anion conductance that does not influence the rate or direction of transport but may have the capacity to influence the excitability of the cell.

Interestingly, recent studies indicate that different EAAT subtypes have differential Zn$^{2+}$ sensitivity. Zn$^{2+}$ is a noncompetitive, voltage-independent inhibitor of the human glial transporter EAAT1 at low micromolar concentrations (IC$_{50}$ = 9.9 +/- 2.3 μM), as well as potentiating the reactive chloride conductance through the transporter [110]. At acidic extracellular pH, which typically favors increased glutamate uptake, both these effects of Zn$^{2+}$ on EAAT1 are abolished, likely due to protonation of histidines which form part of the Zn$^{2+}$ binding site [111]. As the release of glutamate via reverse
operation of glutamate transporters during ischemia has been suggested to contribute to excitotoxicity [112, 113], a pH-dependent block of Zn\(^{2+}\) inhibition during ischemia might conceivably modulate glutamate transporters in a manner resulting in an overall increase in extracellular glutamate levels.

d) Voltage-Gated Ion Channels

Direct modulation of cationic conductances can also modify excitability of neurons and have complex effects on neuronal activity, and in this respect, Zn\(^{2+}\) has been described to block both TTX-insensitive voltage-gated Na\(^{+}\) channels and T-type Ca\(^{2+}\) channels [114, 115]. Micromolar extracellular Zn\(^{2+}\) has also been reported to increase membrane excitability via inhibition of a background cationic current, carried mainly by extracellular Na\(^{+}\) [116].

Zn\(^{2+}\) effects on K\(^{+}\) channels may be of particular note, given the role that potassium plays in ischemic injury (cf. section IVB.2). Zn\(^{2+}\) modulation of the transient outward K\(^{+}\) current I\(_A\) (a Kv4-type channel) shifts the activation and inactivation curves for I\(_A\) in the depolarizing direction, and at higher (millimolar) concentrations significantly slows both activation and deactivation kinetics [117]. The actions of Zn\(^{2+}\) on I\(_A\) are therefore likely to delay repolarization, thus prolonging action potentials and promoting spontaneous activity.

Zn\(^{2+}\) has similar concentration-dependent effects on the gating of diverse delayed rectifier (KV1.1, KV1.5) and inactivating (KV1.4) K\(^{+}\) channels, resulting in a slowing of activation kinetics of these channels with likely delayed repolarization of the neuronal action potential. The similarity in action of Zn\(^{2+}\) across these different types of K\(^{+}\) channels suggests the existence of a common Zn\(^{2+}\) binding domain which influences the voltage sensor upon occupation. Zn\(^{2+}\) modulation of gating of the inactivating human KV1.4 channel may be particularly significant, given the localization of KV1.4 in hippocampal mossy fiber terminals [118].

Finally, Zn\(^{2+}\) has also been reported to potentiate ATP-regulated K\(^{+}\) channels (also known as purinergic or P2X receptors), possibly by increasing the apparent affinity of the receptor for ATP [119, 120]. In the hippocampus, ATP-activated currents are robustly potentiated by micromolar Zn\(^{2+}\) in CA1 neurons [121], yet interestingly, inhibited by Zn\(^{2+}\) in CA3 neurons [122], which may be consistent with differential expression of P2X receptor subunits (for review, see [123]). Extracellular protons may additionally modulate these receptors and enhance Zn\(^{2+}\) potentiation by modulating the affinity of the binding sites for ATP and Zn\(^{2+}\) [120].

2. Effects on Network Excitability

a) A role for Zn\(^{2+}\) in Synaptic Plasticity?

Our knowledge of the impact of endogenously-released Zn\(^{2+}\) on synaptic activity of intact neuronal networks has been advanced by a number of recent studies which have focused on Zn\(^{2+}\)'s action at the mossy fiber (MF)-CA3 synapse in the hippocampus. In addition to being the target of Zn\(^{2+}\)-rich mossy fibers, this synapse has several distinct electrophysiological properties, including robust paired-pulse and frequency facilitation, and long-term potentiation (LTP) which is independent of both NMDAR activation and post-synaptic [Ca\(^{2+}\)]; rise. The presence of uniquely high amounts of pre-synaptic Zn\(^{2+}\) has made the MF-CA3 synapse an appealing system for exploring synaptic Zn\(^{2+}\) physiology.

Thus far, experimental evidence suggests that synaptically-released Zn\(^{2+}\) is able to modulate activity at the MF-CA3 synapse in two general ways. Firstly, as described above, Zn\(^{2+}\) is a potent inhibitor of NMDAR even at low (nanomolar) concentrations, and endogenous Zn\(^{2+}\) appears to tonically occupy the high-affinity, voltage-independent inhibitory site of NMDAR at the MF-CA3 synapse. This was first suggested by the observation that use of the Zn\(^{2+}\) chelator BTC-5N increased the NMDAR response of MF-CA3 synapses while having no effect on neighboring, non-MF synapses in stratum radiatum [124]. Following action potential-driven release, synaptic Zn\(^{2+}\) may additionally block NMDAR at the low-affinity, voltage-dependent site, as pretreatment with 2.5 mM Ca-EDTA was found to block activity-dependent inhibition of NMDAR in the stratum lucidum of CA3 [124]. Zn\(^{2+}\) may further modulate NMDAR not only in the immediate region of release (stratum lucidum), but also nearby in proximal stratum radiatum as a result of Zn\(^{2+}\) spillover, thus resulting in hetero- as well as homo-synaptic modulation of receptor activity [44].

Secondly, recent experimental evidence strongly suggests that intracellular Zn\(^{2+}\) is indeed required for MF-CA3 LTP. Initial experiments in the mocha mouse, a natural mutant lacking Zn\(^{2+}\) in its pre-synaptic MF boutons [125], demonstrated no change in the ability of the MF-CA3 synapse to express facilitation or LTP, and subsequent experiments in wild-type rat brain found that these processes were equally unaffected by extracellular chelation of endogenously-released Zn\(^{2+}\) with 2.5 mM Ca-EDTA [124]. Three caveats may apply to these findings: first, the results in mocha mice do not rule out the possible presence of non-vesicular, releasable pre-synaptic Zn\(^{2+}\) (a concept which will be discussed in greater detail in section IVA.2); second, the amount of Ca-EDTA used was likely insufficient to chelate all the released Zn\(^{2+}\) (see below; [42]); and third, the slower kinetics of Ca-EDTA binding did not allow for reliable, fast chelation of Zn\(^{2+}\) [45].

In light of the above, further insight into the importance of Zn\(^{2+}\) to MF-CA3 LTP was gained through the observation that either depletion of pre-synaptic Zn\(^{2+}\) via chronic dietary deficiency or acute use of membrane-permeable Zn\(^{2+}\) chelators significantly impaired LTP at this synapse (i.e., resulted in less robust potentiation), an effect which was specific to Zn\(^{2+}\)-containing synapses and reversible upon either washout of Zn\(^{2+}\) chelator or...
addition of exogenous Zn\(^{2+}\) immediately following chelation [126]. Of note, membrane-permeable chelation presumably affects both pre- and post-synaptic neurons, such that the preceding findings do not differentiate a pre- or post-synaptic locus of Zn\(^{2+}\) effect, nor do they rule out the possibility of a role for intracellularly released Zn\(^{2+}\).

In fact, an essential role for Zn\(^{2+}\) translocation into CA3 pyramidal neurons was subsequently found to be required for the induction of LTP. In a series of experiments carried out in acute hippocampal slice preparation, effective chelation of synaptic-release Zn\(^{2+}\) (using a minimum of 10 mM Ca-EDTA, as assessed by Zn\(^{2+}\) imaging [42]) blocked both Zn\(^{2+}\) translocation (as revealed by fluorescent imaging) and induction of LTP by high frequency stimulation (HFS). Furthermore, “exogenous” [Zn\(^{2+}\)]\(_i\) loading (through use of a Zn\(^{2+}\)-specific ionophore in conjunction with 100 \(\mu\)M Zn\(^{2+}\)) allowed for LTP induction in the absence of HFS while conversely, HFS-induced LTP was blocked by intracellular Zn\(^{2+}\) chelation, strongly implying that intracellular Zn\(^{2+}\) is required for LTP at this synapse. Finally, LTP could be mimicked in the absence of HFS by addition of exogenous Zn\(^{2+}\) or exogenous glutamate and Zn\(^{2+}\), but not glutamate alone, thereby suggesting that the presence of glutamate in conjunction with Zn\(^{2+}\) is essential for induction of LTP under physiological conditions [42]. Glutamate is likely required (at least in part) because it facilitates Zn\(^{2+}\) entry into postsynaptic neurons (cf. section IVA.1).

Zn\(^{2+}\) effects on network activity in the CA1 region have been much less studied, but an earlier report demonstrated that exogenously-applied Zn\(^{2+}\) (100-300 \(\mu\)M) enhanced AMPAR-mediated responses, while reducing overall excitatory synaptic transmission and inhibiting LTP in CA1 neurons [127]. More recently, the effect of extracellular Zn\(^{2+}\) at the Schaffer collateral-CA1 synapse was re-examined using relatively high (0.3-1 mM) concentrations of Zn\(^{2+}\) [128]. These experiments demonstrated a slow, gradual increase in magnitude of NMDAR-mediated field excitatory post-synaptic potentials (fEPSP) while AMPAR-mediated fEPSPs remained unchanged over the same time period. Interestingly, this Zn\(^{2+}\) exposure increased levels of total and tyrosine-phosphorylated forms of NR2A and NR2B subunits, and the Zn\(^{2+}\)-induced potentiation of NMDAR fEPSPs was completely blocked by specific inhibition of Src family tyrosine kinase activity, thus providing support for the model suggested by Manzerra and colleagues [106]. Surprisingly, however, the use of Zn\(^{2+}\)-chelating agents in stratum lucidum following high frequency stimulation did not appear to have any affect on either excitatory activity or plasticity.

b) Network Effects of Zn\(^{2+}\) and GABA

As the majority of GABAR in adult brain are greatly inhibited by Zn\(^{2+}\), one of the postulated roles of synaptic Zn\(^{2+}\) with respect to inhibitory synaptic transmission is to enhance or promote synchronized GABA release [129-131]. Intriguingly, a very recent study indicates that the mossy fiber varicosities in CA3 show abundant vesicular co-localization of Zn\(^{2+}\) and GABA. In the same study endogenous Zn\(^{2+}\), released from mossy fiber boutons, was found to inhibit IPSCs recorded in CA3 pyramidal neurons. However, because endogenous Zn\(^{2+}\) is released at glutamatergic synapses, the physiological significance of direct Zn\(^{2+}\)-mediated effects on GABA receptors remains uncertain at present. One possibility is that strong Zn\(^{2+}\) release might lead to extrasynaptic spillover resulting in functional modulation of nearby GABA responses, as has been hypothesized to occur in kindling models of epilepsy [132-134]. It may also be possible that direct effects of synaptic Zn\(^{2+}\) on GABAR are involved in normal synaptic physiology in specific regions, as there is emerging evidence that mossy fiber terminals release both glutamate and GABA, as well as Zn\(^{2+}\) and dynorphin [135, 136]. It is possible that Zn\(^{2+}\) associated with the mossy fibers might mainly exert a tonic inhibition of GABA-A receptors.

B. Zn\(^{2+}\) Modulation of Intracellular Signaling and Cellular Metabolism

1. Zn\(^{2+}\) and Mitochondria

Zn\(^{2+}\) is likely to have an important physiological role in modulating mitochondrial function, as this cation has long been known to affect cellular respiration [137-141]. Several recent findings in isolated mitochondria have further clarified its possible sites of action. For instance, Zn\(^{2+}\) has now been shown to interfere with lipoamide dehydrogenase (LADH), a member of the flavin-disulfide oxidoreductase family and one of the most abundant flavoproteins in the mitochondrial matrix. LADH belongs to and is a critical component of the multienzyme NADH-generating complexes \(\alpha\)-ketoglutarate dehydrogenase (KGDHG), pyruvate dehydrogenase, and branched-chain ketoacid dehydrogenase. LADH catalyzes several metabolic reactions, but its principal function is to mediate the reversible transfer of reducing equivalents from dihydrolipoate to NAD\(^+\). The “forward” direction of this reaction, which produces NADH and lipoic acid, has been found to be strongly inhibited by Zn\(^{2+}\) \((K_i < 0.15 \mu\)M; [142]), suggesting that elevated concentrations of the cation inside the mitochondrial matrix could inhibit NADH production by the aforementioned dehydrogenases, which would in turn interrupt the Krebs cycle and mitochondrial energy metabolism.

In addition, Zn\(^{2+}\) has also been described to inhibit glycerol-3-phosphate dehydrogenase (GDPH, IC\(_{50}\) ~ 100 nM; [143]), an enzyme that is not only present in the cytosol but also in the outer side of the inner mitochondrial membrane. Mitochondrial GDPH requires NADH to catalyze the reduction of ubiquinone, and this reaction acts as the primary

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means of ferrying electrons from the cytosol to the mitochondria. Therefore, in theory, Zn$^{2+}$ inhibition of mitochondrial GDPH could lead to a block in the movement of NADPH (produced by glycolysis) from the cytosol to the mitochondria.

Furthermore, recent findings from our group indicate that neuronal mitochondria possess a pool of chelatable Zn$^{2+}$ that might physiologically modulate the function of these organelles [38]. Experiments in isolated mitochondria revealed that Zn$^{2+}$ chelation by addition of the high affinity Zn$^{2+}$ chelator, N,N,N',N-tetrakis(2-pyridylmethyl) ethylenediamine (TREN), results in a decrease in mitochondrial ROS content, raising the intriguing possibility that this pool of intramitochondrial free Zn$^{2+}$ is involved in physiologic regulation of the electron transport chain.

In summary, Zn$^{2+}$ has been shown to exert physiological effects at the cellular and sub-cellular level across a wide range of concentrations. Studies of extracellular Zn$^{2+}$ action on ionotropic receptors and voltage-gated ion channels have demonstrated sensitivity to the cation at nanomolar (NR2A-containing NMDAR), low (1-10) micromolar (GABAA, sensitivity to the cation at nanomolar (NR2A-and voltage-gated ion channels) concentrations, while intracellular targets (LADH, GDPH) may be sensitive to Zn$^{2+}$ on the order of hundreds of nanomolar. In contrast, the studies of Zn$^{2+}$ involvement in synaptic plasticity have heretofore all utilized [Zn$^{2+}$]$^i$ ranging from 100-300 µM; though the actual [Zn$^{2+}$]$^i$ achieved by these exposures is, as alluded to in section I, an issue under continuing investigation.

IV. Zn$^{2+}$ AND MECHANISMS OF ISCHEMIC INJURY

TGI causes the selective and delayed degeneration of certain hippocampal pyramidal neurons, particularly in the CA1 subregion [144]. Considerable evidence supports a role for Zn$^{2+}$ ions in this (and perhaps other types of) ischemic neuronal injury [13, 14, 145]. As previously mentioned, excessive Zn$^{2+}$ exposure and [Zn$^{2+}$]$^i$ rises are potently toxic to neurons in culture [4, 5, 71, 146]. In vivo, [Zn$^{2+}$]$^i$ increases are observed in excitotoxic conditions such as ischemia, epilepsy, and brain trauma [10, 12, 13, 147]. In particular, [Zn$^{2+}$]$^i$ increases precede neuronal degeneration, and application of an extracellular Zn$^{2+}$ chelator has proved neuroprotective in both transient global and focal ischemia [14, 145].

The correlation between post-synaptic [Zn$^{2+}$]$^i$ rises and subsequent neuronal injury poses two principal questions: what is the source of the [Zn$^{2+}$]$^i$ increase, and by what mechanism might it mediate cellular injury? Until recently, these rises were thought to be exclusively the result of Zn$^{2+}$ translocation—i.e., extracellular (and presumably synaptically-released) Zn$^{2+}$ crossing the plasma membrane via some type of ion channel or transporter; the research supporting this translocation model is summarized below. Within the last three years, however, studies making use of transgenic or mutant mice lacking vesicular Zn$^{2+}$ have challenged the assumption that translocation is the only source of increased [Zn$^{2+}$]. In fact, several recent reports suggest that a pool of intracellular Zn$^{2+}$ exists which may significantly contribute to "post-synaptic" [Zn$^{2+}$] rises during ischemia, as will be discussed below.

A. Sources of Post-Synaptic Zn$^{2+}$

1. The "Translocation Model": Routes of Post-Synaptic Zn$^{2+}$ Entry

Given that Zn$^{2+}$ translocation is observed in models of ischemia and epilepsy (both of which are excitotoxic conditions), and that Zn$^{2+}$ is co-released with glutamate at excitatory synapses, it is reasonable to consider that this phenomenon might involve glutamate receptor activation. In order to identify routes of post-synaptic Zn$^{2+}$ entry, it is useful to first consider the permeability of a similar endogenous divalent cation, Ca$^{2+}$.

Ca$^{2+}$ may enter post-synaptic neurons through NMDAR-associated channels and VSCC, both of which are ubiquitously expressed on neurons throughout the brain. However, Ca$^{2+}$ may also flux through Ca$^{2+}$-permeable AMPA/kainate receptor-mediated channels (Ca-A/K channels), an atypical subtype of AMPAR distinctive for its high Ca$^{2+}$ permeability as well as its selectively increased expression in minority subpopulations of forebrain and spinal cord neurons. Ca-A/K channels lack the GluR2 subunit, whose presence in the typical heterotetrameric AMPAR assembly blocks Ca$^{2+}$ entry.

Microfluorimetric and electrophysiological studies of cultured neurons have revealed that Zn$^{2+}$ may enter neurons through any of these three (NMDAR, Ca-A/K, VSCC) routes of Ca$^{2+}$ entry (Fig. (4); 36, 37, 71, 148-152). In addition, Zn$^{2+}$ can serve as a substrate for the Na$^+$/Ca$^{2+}$ exchanger in place of Ca$^{2+}$, which may bring Zn$^{2+}$ into neurons and/or contribute to Zn$^{2+}$ extrusion following intracellular accumulation [36]. More recent evidence suggests the presence of a putative Na$^+$/Zn$^{2+}$ exchanger, likely a separate molecule from the Na$^+/Ca^{2+}$ exchanger [153].

Interestingly, there are significant differences in the relative permeabilities of these channels to Zn$^{2+}$ and Ca$^{2+}$. In vitro studies indicate that while Ca$^{2+}$ entry through either NMDAR-associated channels or Ca-A/K channels results in similarly high (many micromolar) [Ca$^{2+}$]$^i$, Ca-A/K channels have the greatest permeability to Zn$^{2+}$ [148, 149], with VSCC and NMDAR showing intermediate and minimal permeability, respectively. In fact, simultaneous

activation of all three entry routes (NMDAR, AMPAR and VSCC) using the physiological agonist, glutamate, results in preferential $[\text{Zn}^{2+}]$, rises in strongly Ca-A/K channel-expressing $[\text{Ca-A/K}(+)\text{]}$ neurons, lending compelling support to the idea that upon excessive glutamate and $\text{Zn}^{2+}$ co-release in vivo, Ca-A/K channels may act as the main route for injurious $\text{Zn}^{2+}$ influx (Fig. 3A; 150, 156). Moreover, the differential permeability of Ca-A/K channels to $\text{Zn}^{2+}$ may be pathologically relevant in transient global ischemia (TGI), as these channels are both concentrated on post-synaptic membranes where the highest levels of synaptically-released $\text{Zn}^{2+}$ are likely to be achieved, and selectively expressed in subpopulations of neurons such as TGI vulnerable CA1 pyramidal neurons.

In fact, although CA1 pyramidal neurons substantially lack Ca-A/K channels at their soma, they do appear to express some of these receptors in their dendritic tree, where they would likely play important roles in neurotransmission and injury induction [154, 155]. Supporting this hypothesis, selective pharmacological inhibition of Ca-A/K channels has been found to be highly neuroprotective against CA1 pyramidal neuron loss in an in vitro model of global ischemia (Fig. 3B), while NMDAR and VSCC blockade were each found to be only marginally beneficial [156].

Interestingly, not only are these channels present in the dendrites of these most vulnerable neurons, but in the context of TGI, they may also be subject to dynamic, injury-driven up-regulation. In fact, several studies have shown selective decreases in GluR2 subunit expression (and thus increases in the number of functional Ca-A/K channels) in CA1 pyramidal neurons after TGI [157, 158]. Based on these observations, Bennett and Zukin have proposed the "GluR2 hypothesis," which postulates that some forms of neuronal insult selectively trigger an increase in the number of Ca-A/K channels present on the plasma membrane of certain neurons, a phenomenon that likely underlies their selective vulnerability to injury in these conditions [159, 160].

2. Non-Translocation Models: the Emerging Role of Intracellular $\text{Zn}^{2+}$ Release

The conventional idea of a critical role for $\text{Zn}^{2+}$ translocation in triggering neuronal injury has been challenged by recent unexpected observations in ZnT-3 knockout mice (ZnT-3 KO). Despite having virtually no histochemically reactive $\text{Zn}^{2+}$ in their pre-synaptic terminals, the hippocampal neurons of these animals undergo intracellular $\text{Zn}^{2+}$ accumulation and injury following an excitotoxic insult [161]. Even more intriguingly, ZnT-3 KO mice appear to have no significant differences from wild-type mice across a range of electrophysiological and behavioral parameters [162, 163].

These data may seem at first glance to contradict the concept that $\text{Zn}^{2+}$ exerts a critical role in modulating cell physiology, suggesting instead that the cation is simply not as essential a modulator as previously thought. However, as with any experimental evidence gathered from transgenic animals, one should bear in mind that ZnT-3 KO mice may have adapted to the lack of vesicular $\text{Zn}^{2+}$ during gestation by over-expressing other, non-vesicular sources of $\text{Zn}^{2+}$. Thus, an alternative interpretation of these results could be that $\text{Zn}^{2+}$ is actually so vital to cell physiology that multiple systems are in place to regulate $\text{Zn}^{2+}$ homeostasis, such that organisms are able to adapt to the loss of any single system (e.g. vesicular $\text{Zn}^{2+}$) with a powerful compensatory up-regulation of other(s).

For instance, one possible substitute for vesicular $\text{Zn}^{2+}$ release could be release from non-vesicular, presynaptic sites; i.e., $\text{Zn}^{2+}$ mobilized from histochemically "invisible" intracellular stores in the presynaptic terminal, such as mitochondria or MTs, might in theory be released in the synaptic cleft by using plasma membrane systems such as the ZnT-1 transporter or the Na$^{+}$-$\text{Zn}^{2+}$ and Zn$^{2+}$-$\text{H}^{+}$ exchangers. While this specific mechanism is largely speculative, the concept that non-vesicular, presynaptic $\text{Zn}^{2+}$ release occurs is supported by the otherwise puzzling observation that Ca-EDTA is still able to protect against $\text{Zn}^{2+}$ accumulation and injury in both CA1 and CA3 regions in ZnT-3 KO mice [161].

An alternative explanation for the injurious $\text{Zn}^{2+}$ accumulation seen in ZnT-3 KO mice is that some of this $\text{Zn}^{2+}$ originates from one or more sites in the post-synaptic neuron itself. This scenario is not excluded by the neuroprotective effects of Ca-EDTA observed in ZnT-3 KO mice, as it has been shown that extracellular $\text{Zn}^{2+}$ chelators can act as $\text{Zn}^{2+}$ "sponges" which promote removal of the cation from intracellular compartments [164]. The possibility of deleterious $\text{Zn}^{2+}$ release from within the postsynaptic cell may be a more appealing model, given that neurons do possess intracellular sources of the cation such as MTs and mitochondria.

In the case of MTs, a number of recent findings strongly imply that these $\text{Zn}^{2+}$-binding proteins play a part in modulating excitotoxic and ischemic injury, yet the exact nature of this role is not entirely clear. Several factors do suggest that in some instances, MTs serve a protective purpose in neurons. MT-1 and MT-2 mRNA expression has been found to be upregulated within 2 hours of transient focal ischemic insult, and increased expression of MT-1 has been shown to be neuroprotective in focal ischemia [165, 166]. In addition, MT-1 and MT-2 KO mice were observed to develop three times larger infarcts in focal ischemia than wild-type mice, and a separate study found MT-3 KO mice were more sensitive to excitotoxic injury as well [165-167]. In interpreting these data, one should bear in mind that MTs possess intrinsic antioxidant properties which might also contribute to the overall beneficial role exerted by these proteins [168].
Figure 3. Preferential Zn$^{2+}$ accumulation, ROS generation, and injury in highly Ca-A/K channel-expressing neurons.

A. Glutamate receptor activation in the presence of Zn$^{2+}$ triggers high [Zn$^{2+}$]$_i$ rises and preferential ROS generation in highly Ca-A/K channel-expressing, (Ca-A/K$^+$), neurons.

**Top panel:** Time course of cytosolic [Zn$^{2+}$]$_i$ rises, as indicated by changes in Newport Green fluorescence, in cortical neurons exposed for 5 min to both glutamate (Glu) and Zn$^{2+}$. Note that preferential [Zn$^{2+}$]$_i$ increases are observed in a subpopulation of neurons that were later identified as Ca-A/K$^+$ neurons by KA-stimulated Co$^{2+}$ staining.

**Middle panel:** The same cultures as above, loaded with the ROS-sensitive fluorescent probe hydroethidine (HEt) and exposed for 10 min to Glu+Zn$^{2+}$, show preferential ROS generation in Ca-A/K$^+$ neurons. (1) Baseline images obtained under visible light before exposure to Glu+Zn$^{2+}$. Pseudocolor HEt fluorescence images obtained (2) before and (3) 20 min after the end of the exposure. (4) Post-identification of Ca-A/K$^+$ neurons by KA-stimulated Co$^{2+}$ staining.

**Bottom panel:** Time course of Glu/Zn$^{2+}$-triggered ROS generation in HEt-loaded cortical neurons. Note the preferential increases in HEt fluorescence of Ca-A/K$^+$ neurons. (Modified from [150])

B. Preferential Zn$^{2+}$ influx through Ca-A/K channels of pyramidal neurons in an in vitro model of transient global ischemia.

Murine hippocampal slices were exposed for 5 min to oxygen and glucose deprivation either alone (OGD); in the presence of the NMDA antagonist MK-801 and the VSCC antagonist Gd$^{3+}$ (+MK, Gd$^{3+}$); in presence of the Ca-A/K channel blocker 1-naphthylacetyl spermine (+NAS); or in the presence of the extracellular Zn$^{2+}$ chelator Ca-EDTA (+CaEDTA). One control slice was exposed to oxygen- and glucose-containing media (+O$_2$). After 4 h slices show different degrees of injury as assessed by confocal imaging of propidium iodide uptake in the CA1 pyramidal cell layer. **Left column,** set of confocal images of propidium iodide uptake displayed on an 8-bit pseudocolor scale. **Right column,** matched slices from a single experiment were sectioned to 25 microns following imaging, and stained with toluidine blue. Note the alteration of neuronal morphology and cell loss (indicated by voids) in the CA1 pyramidal cell layer after OGD alone or with MK-801 and Gd$^{3+}$, and the relatively-preserved morphology in other conditions. Bars = 50 µm. (Modified from [156]).
But while the preceding studies support the concept of MTs as a “passive” cellular defense mechanism against toxic [Zn²⁺] elevations, emerging evidence suggests that, in other instances, MTs may in fact act mainly as a source of injurious Zn²⁺ release. In principle, the ability of MTs to release Zn²⁺ upon changes in the cellular redox state renders these proteins a reservoir of readily-available Zn²⁺ under the conditions of oxidative stress seen in ischemia. Indeed, the additional knockout of MT-3 in ZnT-3 KO mice results in substantial protection from the excitotoxic injury observed in CA1 [169]. Furthermore, recent preliminary data indicate that NO-triggered [Zn²⁺], rises and subsequent neuronal loss in the CA1 region are substantially reduced in MT-3 KO mice compared to wild type animals [170]. MTs may thus ultimately act not only as Zn²⁺ buffers, but also as sources for potentially deleterious cation release.

As mentioned, another site of intracellular Zn²⁺ release is offered by mitochondria [38, 89], and the implications of this mitochondrial Zn²⁺ release in ischemic injury will be discussed in further detail below (cf. section IVA.2). Overall, recent findings seem to indicate that the mechanisms underlying injurious Zn²⁺ accumulation may be more multifaceted than previously thought and encompass pre-synaptic (vesicular/non-vesicular) as well as post-synaptic sources, as summarized in Fig. (4).

3. Zn²⁺ Influx and Homeostatic Mechanisms in Ischemic Conditions

Thus far, we have presented two general models of post-synaptic Zn²⁺ accumulation. To date, the majority of studies of Zn²⁺ transmembrane movement, such as those identifying routes of extracellular Zn²⁺ entry or elucidating homeostatic mechanisms, have been conducted in vitro under “normal” physiological conditions. Given the significant and characteristic acidosis which develops in ischemic tissue (cf. [96]), however, it may be pertinent to consider how the systems involved in each model might respond in the specific environment of ischemia, and thus potentially contribute to the development of ischemic pathology.

Interestingly, the Zn²⁺ permeability of both VSCC and Ca-A/V channels appears to be increased at acidic pH while the Ca²⁺ permeability of these channels (as well as NMDAR permeability) is decreased under the same conditions [72, 152, 171, 172]. One must keep in mind that the relative permeability of Zn²⁺ is less than that of Ca²⁺ for each of these channels [36, 72, 149, 173]; however, given that Zn²⁺ shows far greater potency than Ca²⁺ in promoting ROS generation (cf. IVB.1c), these findings may support a model which privileges the role played by Zn²⁺ in the evolution of ischemic injury.

Acidosis may also modulate other transporter systems involved with Zn²⁺ fluxes. A recent report demonstrated an inhibition of ⁶⁵Zn²⁺ accumulation in cortical neurons by extracellular acidosis (pH 6.0), suggesting the presence of a putative plasma membrane Zn²⁺/proton antiporter. Through use of the Zn²⁺-sensitive fluorescent probe Zinquin, the same study also found that Zn²⁺ accumulated in cytosolic organelles under basal conditions. Interestingly, this accumulation was sensitive to changes in the acid-base equilibrium, and Zn²⁺ uptake by these cytoplasmic organelles could be blocked by extracellular acidification [174]. The organelles were later tentatively identified as mitochondria, as the pattern of punctate Zinquin staining co-localized almost completely with staining observed using the mitochondrial marker Mitotracker Green 4

An additional clue to the potential role played by acidosis in modulating [Zn²⁺], homeostasis comes from the observation that, on a strictly biochemical level, acidic shifts appear to rapidly destabilize the interaction between MTs and Zn²⁺, favoring an overall release of Zn²⁺ [47, 55]. This phenomenon has now been confirmed in intact neurons, where intracellular acidification has been found to dramatically increase [Zn²⁺], rises induced by DTDP oxidation of MTs. Interestingly, these acid-induced [Zn²⁺] rises have been also found to interfere with mitochondrial function by triggering partial depolarization of these organelles [38].

An acidic environment may thus influence Zn²⁺ homeostasis through modulation of Zn²⁺ influx, transport, and/or intracellular mobilization. Zn²⁺ dyshomeostasis may in turn disrupt neuronal acid-base equilibrium, thereby creating a potential feed-forward loop. A recent study reports that Zn²⁺ is able to both induce intracellular acidification and/or prolong recovery from intracellular acidosis [175]. The Zn²⁺-induced acidification is dependent on extracellular Ca²⁺ entry through the activity of the Na⁺/Ca²⁺ exchanger, whereas the delayed recovery from intracellular acidosis is attributed to Zn²⁺ inhibition of proton efflux by the Cl⁻/HCO₃⁻ exchanger, one of the major systems involved in maintaining neuronal acid-base equilibrium [175-177]. Moreover, persistent intracellular acidosis might promote perturbation of [Zn²⁺] and subsequent neuronal injury by interfering with the overall neuronal redox state, either by reducing the activity of cellular antioxidant enzymes or increasing hydroxyl radical formation [178].

Finally, another key factor in the development of ischemic neuronal injury is glutamate-induced intracellular Ca²⁺ overload, and a recent study has reported the intriguing finding that Zn²⁺ may have modulatory effects on Ca²⁺ homeostasis. The study demonstrates that extracellular Zn²⁺ can specifically induce Ca²⁺ mobilization from the endoplasmic reticulum of non-neuronal cells, via a G-protein/phospholipase C-mediated mechanism. In addition, these Zn²⁺-triggered [Ca²⁺], rises are able to
Zn$^{2+}$ is transported into pre-synaptic vesicles of certain glutamatergic neurons by the vesicular transporter, ZnT-3 (1), and is co-released with glutamate into the synaptic cleft upon sustained synaptic activation. Synaptic Zn$^{2+}$, extracellular H$^+$, and physiological Mg$^{2+}$ substantially block NMDAR (2), likely resulting in a net inhibition of Ca$^{2+}$ influx during ischemia. Some neurons (i.e., the highly TGI-vulnerable CA1 pyramidal neurons) express AMPAR that are highly permeable to Zn$^{2+}$ as well as to Ca$^{2+}$ (Ca-A/K channels; 3). Zn$^{2+}$ may also flux across the plasma membrane through largely extra-synaptic routes such as voltage-sensitive Ca$^{2+}$ channels (VSCC; 4) and the putative Na$^+$/Zn$^+$ exchanger (5). The Zn$^{2+}$ transporter, ZnT-1 (6) promotes Zn$^{2+}$ efflux from neurons and likely helps to maintain low [Zn$^{2+}$]$_i$ under physiological conditions. Metallothioneins are important regulators of [Zn$^{2+}$]$_i$ which may act either as a Zn$^{2+}$-buffering system or, under oxidizing conditions, as a source for Zn$^{2+}$ release into the cytosol (7). Rapid Zn$^{2+}$ entry through Ca-A/K-receptor channels can cause mitochondrial dysfunction and reactive oxygen species (ROS) generation (8), which in turn may further inhibit NMDAR activity (9), and/or induce release of Zn$^{2+}$ from MT (7). Zn$^{2+}$ might also interfere with transcription factors, leading to GluR2 subunit downregulation and therefore promoting an increase in the number of Ca-A/K channels (10). Note that acidosis blocks NMDAR activity (also blocked by Zn$^{2+}$), yet increases Zn$^{2+}$ (but not Ca$^{2+}$) permeability of both Ca-A/K channels and VSCC (cf. section IVA.3).

to increase the activity of the Na$^+$/H$^+$ exchanger, thus potentially interfering with the intracellular acid-base equilibrium as well [179].

B. Mechanisms of Zn$^{2+}$-Dependent Neurotoxicity

The mechanisms by which intracellular Zn$^{2+}$ promotes cell death are only beginning to be understood. Although Zn$^{2+}$-dependent “death” pathways are likely to be complex, overlapping and/or interrelated given the cation’s multidirectional modulation of cell physiology reviewed above, one well-established mechanism of cellular injury by intracellular Zn$^{2+}$ again underscores parallels between Zn$^{2+}$ and Ca$^{2+}$. Analogous to Ca$^{2+}$, metabolic inhibition and ROS generation may be crucial to both rapid and slow Zn$^{2+}$ neurotoxicity [149, 180]. Mitochondria are the major cellular source of ROS, which are routinely produced and rapidly used as recyclable co-factors in the electron transport chain, but are otherwise “contained” within the mitochondrial membranes under normal physiological conditions where they cannot adversely affect cellular function. Significantly, several studies have suggested that these organelles are important sites for the toxic effects of both elevated intracellular Ca$^{2+}$ and Zn$^{2+}$ [181, 38, 86, 87, 89, 149, 150, 182].

1. Mitochondrial Mechanisms of Neurotoxicity

a. Mitochondrial ROS Generation

Mitochondrial uptake likely plays an important role in Zn$^{2+}$ homeostasis (cf. section IIC), but as is the case with Ca$^{2+}$, the cation sequestration is not always benign. Profound disruption of mitochondrial function occurs following rapid and massive cytosolic Zn$^{2+}$ loads, most likely mediated by cation entry through the highly Zn$^{2+}$-permeable Ca-A/K channels.
Indeed, activation of these channels by selective agonists such as AMPA or kainate, but most importantly by the endogenous agonist glutamate, results in preferentially large cytosolic \([\text{Zn}^{2+}]_i\) rises and strong mitochondrial dysfunction in the Ca-A/K(+) neuron subpopulation (Figs. (3A),(4)). This dysfunction manifests as a pronounced loss of mitochondrial membrane potential (\(\Delta \Psi_m\)) and increased generation of ROS [149, 150, 87], which are then able to diffuse out of these organelles and likely disrupt plasma membrane lipids, ultimately promoting neuronal death.

\(\text{Zn}^{2+}\) can trigger ROS production through multiple pathways which are only partially understood at present, but the cation has long been known to inhibit cellular respiration in isolated mitochondria at low micromolar concentrations [137-140]. As far as localizing specific site(s) of action for \(\text{Zn}^{2+}\), previous studies have identified a potential site for electron transport chain (ETC) inhibition at cytochrome \(b_{c1}\) in complex III, while more recent evidence suggests an additional site of action farther upstream [141, 183, 184]. For instance, \(\text{Zn}^{2+}\) appears to interfere with mitochondrial activity at complex I by inhibiting \(\alpha\)-ketoglutarate dehydrogenase (KGDHG), as a greater sensitivity to \(\text{Zn}^{2+}\) inhibition was found for mitochondria respiring on complex II substrates [184].

Further studies have characterized additional loci where the cation can promote ROS production. A recent report indicates that \(\text{Zn}^{2+}\) inhibition of LADH, a component of the KDGHC complex, may provide a novel mechanism for \(\text{Zn}^{2+}\)-dependent ROS generation at complex I [142]. LADH catalyzes NADH oxidation by oxygen, generating ROS (largely in the form of hydrogen peroxide) as a by-product, and this reaction has been found to be accelerated 5-fold by \(\text{Zn}^{2+}\) [142]. \(\text{Zn}^{2+}\) may also contribute to cellular oxidative burden indirectly through inhibition of the "reverse" LADH reaction, thus decreasing the formation of dihydrolipoic acid, a potent ROS scavenger and antioxidant regenerator [185]. This \(\text{Zn}^{2+}\)-mediated increase in oxidative stress may in turn promote the redox-sensitive induction of the mitochondrial permeability transition pore (mPTP) [186, 187].

b) Mitochondrial Release of Pro-Apoptotic Factors

The rapid and dramatic increase of cytosolic \(\text{Zn}^{2+}\) mediated by cation entry through Ca-A/K channels most likely results in a precipitous decline in intracellular energy levels and mitochondrial function, thus promoting necrotic neuronal death. This injurious cascade likely leads to the selective neuronal injury observed in TGI. In contrast, milder cytosolic \(\text{Zn}^{2+}\) loads may reasonably be expected to be seen in neurons lacking Ca-A/K channels and in the penumbral areas of TGI [145] as a result of cation entry through less permeable but more ubiquitously-expressed routes (e.g., VSCC). These lower \([\text{Zn}^{2+}]_i\) rises may elicit less fulminant disruption of mitochondrial function, allowing neurons to activate their apoptotic machinery instead. Indeed, \(\text{Zn}^{2+}\) has been implicated in the induction of both necrotic and apoptotic processes [188].

Classical apoptosis and necrosis are in fact two coexisting phenomena which can occur in parallel in cells exposed to the same stimulus. Often, the intensity of the insult as well as the status of cellular energy levels decides which process is more prevalent, and mitochondria, by controlling ATP production, seem to act as a switch between a more prominent necrotic or apoptotic demise. For example, a subacute insult may leave affected neurons with injured but still partially-functioning mitochondria, therefore generating sufficient ATP levels to allow apoptosis to fully develop. Fulminant insult, by contrast, results in the rapid compromise of intracellular energy levels and mitochondrial function, leaving cells no other option than to abort the apoptotic program and switch to necrosis [189].

How might \(\text{Zn}^{2+}\) and its interactions with mitochondria fit in to this scheme of "switching" between apoptotic and necrotic pathways? Recent studies in isolated mitochondria suggest that low levels of \(\text{Zn}^{2+}\) cause increased mitochondrial respiration and decreased ROS generation, consistent with mPTP induction [38, 86, 186]. Furthermore, patch clamp recording in mitoplasts has demonstrated that submicromolar \(\text{Zn}^{2+}\) can induce a multi-conductance cation channel activity in the inner mitochondrial membrane, a phenomenon that is often blocked by the mPTP inhibitor cyclosporin A (CSA) [38]. A likely consequence of mPTP opening is the catastrophic release of pro-apoptotic mitochondrial proteins such as cytochrome C (Cyt-C), and this was indeed observed in isolated mitochondria exposed to similar submicromolar \(\text{Zn}^{2+}\) concentrations [86].

The idea that \(\text{Zn}^{2+}\) may promote apoptosis via its interaction with mitochondria is further strengthened by recent findings in intact neurons. VSCC-mediated \([\text{Zn}^{2+}]_i\) rises in cortical neurons (estimated to be in the submicromolar range by microfluorimetry) were demonstrated to be sufficient to trigger substantial mitochondrial swelling and release of pro-apoptotic mediators such as Cyt-C and apoptosis inducing factor (AIF). The additional observation that inhibition of mPTP opening attenuates both \(\text{Zn}^{2+}\)-triggered release of pro-apoptotic factors as well as subsequent neuronal loss further supports a critical role for \(\text{Zn}^{2+}\) in modulating apoptotic neuronal death [86].

c) Relative Potency of \(\text{Zn}^{2+}\) Effects on Mitochondria

Thus far, the mechanisms of \(\text{Zn}^{2+}\)-dependent neurotoxicity seem to share several common targets with Ca-A2+-dependent mechanisms of injury. For instance, both \(\text{Zn}^{2+}\) and Ca-A2+ can trigger different degrees of mitochondrial dysfunction and in doing so, promote either acute necrotic or slower apoptotic forms of neuronal injury. Where the two cations diverge, however, is in the potency of their disruptive
effects. In the case of acute necrotic processes triggered by large cytosolic rises of either cation, comparison between achieved intracellular cation concentration and subsequent neurotoxicity indicate, surprisingly, that intracellular Zn\textsuperscript{2+} acts far more robustly than Ca\textsuperscript{2+} in promoting neuronal dysfunction and degeneration [148, 149, 190]. For instance, in Ca-A/K(+) neurons, recovery from Ca-A/K channel-mediated [Zn\textsuperscript{2+}] rises much slower than recovery of similarly-induced [Ca\textsuperscript{2+}] increases, despite the lower absolute [cations] observed in the case of Zn\textsuperscript{2+} [87, 149]. In parallel with these different temporal profiles of cytosolic clearance, Zn\textsuperscript{2+}-triggered disruption of mitochondrial function (i.e., loss of $\Delta\psi_m$ and ROS generation) persists longer than that induced by Ca\textsuperscript{2+} [87]. In terms of apoptotic processes, Zn\textsuperscript{2+} has also been found to have a much higher potency than Ca\textsuperscript{2+} in inducing mPTP opening, mitochondrial swelling, and release of pro-apoptotic factors [86].

On a final note, Zn\textsuperscript{2+}-triggered perturbation of mitochondrial function should also be considered in light of the fact that the ischemic brain is, in most cases, also an aging brain. In older neurons, Zn\textsuperscript{2+}-induced disruption of mitochondrial function would almost certainly have a greater impact on cell viability than what foregoing models have proposed, as these models are largely based on experimental findings gathered from young, healthy neuronal preparations. Indeed, the capability of these organelles to cope with stressors and death signaling dramatically decreases with aging. Although individual mitochondria have a relatively rapid rate of turnover, neurons in general do not. Thus, mutations in mitochondrial DNA (mtDNA) induced by increased oxidative stress inside and outside of the organelles are perpetuated and accumulate in neurons over time. mtDNA mutations may result in the expression of defective mitochondrial proteins and therefore less-efficient metabolism. Altered mitochondrial metabolism may in turn disrupt the critical recycling of ROS, leading to increased mitochondrial oxidative stress and further effects on mtDNA, thus producing a vicious, feed-forward cycle [191]. In this scenario, such an injurious loop would be further enhanced by any ischemia-dependent derangement of mitochondrial Zn\textsuperscript{2+} content.

2. “Extra-Mitochondrial” Mechanisms of Neuronal Injury

a) Cytosolic ROS Generation

Mild cytosolic [Zn\textsuperscript{2+}] rises likely activate a number of “extra-mitochondrial” pathways of injury in addition to the apoptotic cascade described above (cf. section IVB.1b). Such pathways may involve Zn\textsuperscript{2+}-dependent oxidative stress independent of mitochondria, as the cation is known to modulate a number of cytosolic enzymes which generate ROS secondary to their main physiological activity. For example, Zn\textsuperscript{2+} appears to induce the activity of NADPH oxidase (a multi-subunit enzyme widely expressed in central neurons) via protein kinase C (PKC) activation [180, 192], which in turn generates ROS (Fig. (5)). Another likely effector of Zn\textsuperscript{2+}-mediated free radical production in the cytosol is neuronal nitric oxide synthase (nNOS), which together with superoxide can produce the injurious free radical peroxynitrite (ONOO\textsuperscript{−}). Despite the fact that high (micromolar) concentrations of Zn\textsuperscript{2+} have been reported to inhibit nNOS activity in a biochemical assay in vitro [193], cortical neurons exposed to Zn\textsuperscript{2+} show nNOS activation and increased levels of nitric oxide and nitrates [180, 194]. Interestingly, the number of neurons expressing nNOS may increase following focal cerebral ischemia [195; Fig. (5)].

In addition to effects such as lipid membrane degradation, oxidative stress can also result in DNA strand breakage and activation of poly(ADP ribose) polymerase (PARP), consumption of NAD\textsuperscript{+} during the formation of poly(ADP-ribose) polymers, and ultimately, death through ATP depletion. PARP is a self-regulated enzyme which rapidly triggers its own inhibition by transferring poly(ADP ribose) to itself, but can also be reactivated via cleavage of poly(ADP ribose) from PARP by poly(ADP ribose) glycohydrolase (PARG). Thus, the PARP/PARG cycle seems to be necessary for persistent PARP-dependent activity leading to NAD\textsuperscript{+} and ATP depletion and eventual cellular injury (Fig. (5)). In cortical neurons, brief exposure to Zn\textsuperscript{2+} triggers neurotoxicity which appears to involve robust activation of the PARP/PARG cycle, as pharmacological inhibition of both these enzymes results in significant neuroprotection [180, 194].

Furthermore, oxidative stress may also act as a critical trigger for additional intracellular Zn\textsuperscript{2+} release, thereby enabling a dangerous feed-forward cycle of cellular injury. For instance, NO and ONOO\textsuperscript{−} induce release of Zn\textsuperscript{2+} from MTs [58-60, 196], and it is conceivable that an injurious “loop” could develop in which NO oxidizes MT, promoting Zn\textsuperscript{2+} release, which then in turn activates nNOS, producing more NO [180, 194; Fig. (5)].

Finally, oxidation-induced [Zn\textsuperscript{2+}] rises may also activate a parallel pathway of neuronal death involving perturbation of intracellular K\textsuperscript{+} content, which has been shown to play a key role in neuronal apoptosis (cf. [197]). Experiments carried out in neuronal culture have demonstrated that oxidizing agents such as DTDP are able to mobilize Zn\textsuperscript{2+} from MTs, ultimately triggering neuronal apoptosis [57]. This DTDP-induced neuronal apoptosis was attenuated by high levels of extracellular K\textsuperscript{+} or by addition of the voltage-gated K\textsuperscript{+} channel blocker tetraethylammonium (TEA), suggesting that the injury cascade initiated by [Zn\textsuperscript{2+}] release involves Zn\textsuperscript{2+}-dependent intracellular K\textsuperscript{+} depletion (Fig. (5)). Zn\textsuperscript{2+}-triggered K\textsuperscript{+} depletion appears to be mediated by MAP kinase p38-dependent phosphorylation of K\textsuperscript{+} channels [198].
Zn\textsuperscript{2+} activates multiple intracellular pathways leading to both necrotic and apoptotic neuronal death. Zn\textsuperscript{2+} can trigger necrosis by promoting ATP depletion through direct inhibition of glycolytic pathways (i.e., GADPH), or indirectly by decreasing NAD\textsuperscript{+} via Zn\textsuperscript{2+}-induced mitochondrial dysfunction and/or another as yet unknown mechanism. Zn\textsuperscript{2+} may also induce oxidative stress by either triggering release of mitochondrial ROS or by favoring ROS generation through the activation of cytosolic enzymes such as NADPH oxidase and nNOS. Oxidative stress can then promote further Zn\textsuperscript{2+} release from MTs, as well as contribute to NAD\textsuperscript{+} depletion upon ROS-induced PARP/PARG activation. Apoptotic neuronal death may also be initiated by Zn\textsuperscript{2+}-triggered activation of apoptotic factors. Zn\textsuperscript{2+} can induce p38-dependent K\textsuperscript{+} efflux leading to depletion of intracellular K\textsuperscript{+} (a well-known trigger of neuronal apoptosis), and can also promote apoptotic signaling through induction of p75/NADE and Egr-1. Note the potential for interaction between pathways; for example, Zn\textsuperscript{2+}-triggered mitochondrial dysfunction can also favor the release of pro-apoptotic factors such as cytochrome-C (Cyt-C) and apoptosis inducing factor (AIF).

**b) Disruption of Cellular Metabolism**

Zn\textsuperscript{2+} might also contribute to neurotoxicity by directly modulating neuronal metabolism. Biochemical assays have demonstrated that Zn\textsuperscript{2+} can inhibit key enzymes in the glycolytic pathway, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [199], phosphofructokinase [200], and NAD\textsuperscript{+} glycohydrolase [201]. Recent data obtained in cortical neurons supports the idea of Zn\textsuperscript{2+}-dependent disruption of neuronal metabolism, and further characterizes a possible mechanism of action. The study shows that submicromolar cytosolic Zn\textsuperscript{2+} rises are sufficient to trigger a powerful inhibition of GAPDH, leading to ATP depletion and neuronal death (Fig. (5)). This Zn\textsuperscript{2+} inhibition of GAPDH involves decreased levels of cytosolic NAD\textsuperscript{+}, mediated by an as-yet unknown mechanism.

Restoring levels of cytosolic NAD\textsuperscript{+} by the addition of pyruvate resulted in robust neuroprotection against Zn\textsuperscript{2+}- (but not Ca\textsuperscript{2+}-) dependent toxicity, suggesting a critical role for this pathway in neuronal injury triggered by moderate intracellular Zn\textsuperscript{2+} loads [202]. Interestingly, pyruvate has also been found to dramatically decrease both ischemic \([\text{Zn}^{2+}]\text{i}\) rises and injury in an animal model of TGI [203].

**c) Zn\textsuperscript{2+} Set-Point" and Neuronal Injury by Zn\textsuperscript{2+} Depletion**

Thus far, our discussion has encompassed the toxic effects of too much Zn\textsuperscript{2+}, but it appears that too little Zn\textsuperscript{2+} is also able to promote injury [204], suggesting the intriguing possibility that cells possess a finely tuned "Zn\textsuperscript{2+} set-point." Indeed, Zn\textsuperscript{2+} depletion induced by cation-specific chelation has
been reported to induce apoptosis in several different cell lines as well as in neurons (cf. [204] for review). In neurons, Zn\(^{2+}\) has been shown to inhibit caspase-3, one of the major cell death proteases involved in apoptosis. Caspase-3 is activated by oxidation of specific sulfhydryl groups on its inactive form, and one of the postulated mechanisms of the anti-apoptotic activity of Zn\(^{2+}\) is its capability to bind to and mask these sulfhydryl groups, thus preventing ROS-driven activation of the protease [204, 205].

Zn\(^{2+}\) depletion has also been reported to induce apoptotic death by enhancing endonuclease activity [206, 207]. In fact, Zn\(^{2+}\)’s effect on endonucleases may ultimately have a bidirectional effect on overall neuronal health, whereby Zn\(^{2+}\) activation of an inducible, Mg\(^{2+}\)-dependent endonuclease promotes injury while Zn\(^{2+}\) inhibition of constitutive proton- and Ca\(^{2+}/Mg\(^{2+}\) dependent endonucleases supports cell survival [206, 207]. Moreover, in addition to this effect on endonucleases, Zn\(^{2+}\) has also been very recently found to exert an anti-apoptotic effect on neurons by inhibiting the activation of the two pro-apoptotic oncogenes, Bax and Bak [208].

Finally, a set of studies conducted in Hela cells indicates that several Zn\(^{2+}\)-binding metalloproteins, including a large number of transcription factors (such as the transcription factor Sp family), are specifically degraded during apoptosis induced by Zn\(^{2+}\) chelation [209]. Although the complex interaction between Zn\(^{2+}\) and transcription machinery is still largely unexplored, Zn\(^{2+}\) has been shown to effectively permeate the nuclear membrane, and the nuclear envelope possesses specific ion channels modulating the ionic exchange between the cytoplasm, the perinuclear space and the nucleoplasm [210].

It is worth noting that most of these potential mechanisms of Zn\(^{2+}\) depletion-induced apoptosis favor a model in which the cation serves as a tonic inhibitor of apoptosis in normal cell physiology. Thus, the possibility of neuronal injury by Zn\(^{2+}\) depletion in vivo may be a relevant consideration in the implementation of Zn\(^{2+}\)-chelating strategies in the aftermath of an ischemic insult.

d) Modulation of Intracellular Signaling Pathways

Zn\(^{2+}\) has been reported to specifically induce both the low-affinity neurotrophin receptor p75(NTR) and its associated death executor NADE in cortical neurons (Fig. (5)), while exposure to various other neurotoxic conditions (e.g., NMDAR activation, Ca\(^{2+}\) overload, and oxidative stress) fails to induce activity of either. Zn\(^{2+}\) exposure also increases the expression and secretion of neuronal NGF, the p75(NTR) agonist, which has been found to enhance Zn\(^{2+}\)-dependent neurotoxicity. This neuronal loss is blocked by caspase inhibitors, supporting the idea that Zn\(^{2+}\)-induced association between p75(NTR) and NADE triggers neuronal injury by apoptosis. In vivo, both p75(NTR) and NADE were induced in degenerating CA1 neurons in a model of TGI and extracellular Zn\(^{2+}\) chelation was found to completely block the induction of these two factors, further suggesting a positive correlation between Zn\(^{2+}\) accumulation and their induction [211]. These findings may be relevant to some forms of ischemic injury, as the p75(NTR)/NADE “death pathway” is activated in TGI [212, 213].

Zn\(^{2+}\) has also been reported to increase expression of early growth-response factor-1 (Egr-1) (Fig. (5)), an immediate-early gene transcription factor that is induced after cerebral ischemia [214]. In neurons, brief exposure to Zn\(^{2+}\) has been reported to trigger sustained extracellular signal-regulated kinase (Erk 1/2) activation, an event upstream of Egr-1 induction; pharmacological inhibition of Erk 1/2 activation blocked both Egr-1 activity and Zn\(^{2+}\)-dependent neurotoxicity. Most significantly, Zn\(^{2+}\) rather than Ca\(^{2+}\) influx appears to be the trigger for Erk 1/2 activation and Egr-1 induction, suggesting that Zn\(^{2+}\) and not Ca\(^{2+}\) may be the more critical factor in the induction of this ischemic injury pathway [215].

e) Zn\(^{2+}\) as a Modulator of Glutamate Receptor Trafficking?

A fascinating hypothesis regarding the effects of altered intracellular Zn\(^{2+}\) homeostasis is the potential role that the cation could exert in modulating the dynamic expression and trafficking of glutamatergic receptors, a process which has been implicated in both physiological and pathological conditions [216, 217]. Research into the mechanisms of both glutamate receptor subunit (GluR) trafficking and expression has flourished in recent years, but the ionic determinants of these phenomena are still largely unexplored. Given the critical role of the GluR2 subunit in determining the Ca\(^{2+}\) permeability of AMPAR, ways in which its expression might be specifically influenced by changes in intracellular ionic composition is a particularly interesting avenue of investigation.

The selective and delayed death of CA1 pyramidal neurons observed in TGI [144] displays a temporal profile which is compatible with the injury-driven transcriptional changes proposed by Zukin and Bennett in the “GluR2 hypothesis” [159, 160]. Recent findings from this group indicate a role for REST, a gene-silencing transcription factor that suppresses GluR2 promoter activity, in the selective downregulation of the subunit triggered by TGI. As REST contains a Ca\(^{2+}\)-dependent CREB/CARE regulatory element in its promoter region, Zukin and Bennett suggest that rapid ischemic changes in [Ca\(^{2+}\)]\(i\) could likely influence its activity. It is also worth noting that REST is a zinc-finger protein, and one might speculate that changes in [Zn\(^{2+}\)], or cellular redox state could also, in theory, influence the activity of this transcription factor. For instance, recent reports show that PKC activation is associated with Zn\(^{2+}\) release from its zinc-finger regulatory domain, supporting the notion that in some instances, Zn\(^{2+}\)’s presence at such regulatory
sites may fulfill more than a purely structural purpose [218, 219].

3. Interaction Between Pathways of Injury

On a final note, the distinction between mitochondrial and extra-mitochondrial sites of action is becoming increasingly blurred, and it is quite possible that the two pathways interact in a synergistic way. For instance, recent findings demonstrate how cytosolic PARP activation leads to a decreased NAD+ levels, an event that triggers the release of AIF from mitochondria. Cytosolic AIF eventually promotes the collapse of $\Delta \Psi_m$, the mitochondrial membrane potential, prompting the release of Cyt-C and initiating the apoptotic cascade [220, 221].

Moreover, the two pathways may conceivably be linked by the mobilization of intracellular Zn$^{2+}$ which occurs upon oxidative stress. Recent observations in intact neurons suggest that the level of DTDP- (and possibly NO-) induced Zn$^{2+}$ release from protein-bound stores is able to cause a partial loss of $\Delta \Psi_m$, and that, in isolated mitochondria, comparable [Zn$^{2+}$] rises are able to trigger a multi-conductance ion channel activity consistent with mPTP opening [38, 86]. Conversely, Zn$^{2+}$-induced mitochondrial ROS generation might promote yet more Zn$^{2+}$ release from the protein-bound pool. Thus, the two pathways, rather than acting separately, could together form a self-perpetuating and vicious cycle of neuronal injury (Fig. (5)).

In addition, one must keep in mind that PARP and other poly(ADP-ribosyl) transferases are localized within mitochondria as well as in the cytosol, and as very recently reported, activation of mitochondrial PARP is able to bring about loss of $\Delta \Psi_m$ and NAD+ depletion [221]. As discussed above, such reduced levels of NAD+ upon Zn$^{2+}$ exposure are instrumental in inhibiting the glycolytic pathway and triggering neuronal death [202], such that Zn$^{2+}$-dependent depletion of mitochondrial NAD+ may further enhance the injury cascade. But regardless of interdependencies, the cascades mediated by these Zn$^{2+}$-influenced cytosolic factors ultimately converge at the same endpoint: cell death (Fig. (5)).

V. THERAPEUTIC IMPLICATIONS

Although scientific interest in the field of Zn$^{2+}$ biology has steadily increased in recent years, our present understanding of the physiological and pathological significance of Zn$^{2+}$ signaling in the brain is still patchy, at best. A more thorough understanding of the Zn$^{2+}$-dependent mechanisms involved in ischemic injury might lead to more successful therapeutic strategies, as the diversity of presently-known intracellular targets of Zn$^{2+}$ neurotoxicity already suggests a very promising, yet complex, therapeutical road map. Different forms of ischemia may also require different therapeutic approaches to ameliorate Zn$^{2+}$-induced injury.

For instance, in TGI, inhibition of Zn$^{2+}$ translocation through Ca-A/K channels might prove to be an important target to promote neuronal survival. Compared to other ionotropic glutamate receptors such as NMDAR, these highly Zn$^{2+}$-permeable receptor channels exhibit a more selective distribution with relative abundance on the dendrites of hippocampal neurons most vulnerable to ischemic injury, making them attractive candidates for therapeutic/pharmacological intervention [155, 156]. Regardless of ongoing debate of levels of synchronously-released Zn$^{2+}$ (cf. section I), Ca-A/K channels are the most likely mediators of Zn$^{2+}$-dependent neuronal injury. Where expressed, these channels would act as the major route of Ca$^{2+}$ entry during ischemia, as NMDAR are not expected to allow any significant Ca$^{2+}$ influx under the ischemic acidic shift. Thus, in addition to their high Zn$^{2+}$ permeability, Ca-A/K channels could presumably also promote [Zn$^{2+}$]i accumulation through Ca$^{2+}$-dependent intracellular Zn$^{2+}$ release. In fact, the selective Ca-A/K channel antagonist 1-naphthylacetyl spermine (NAS) has been shown to afford promising neuroprotection in an in vitro model of TGI [156]. Moreover, in contrast to the minimal effect seen with NMDAR antagonists [222, 223], NAS effectively blocks apoptotic signaling and delayed injury in in vivo models of TGI [224, 225]. Targeting downstream mechanisms of Zn$^{2+}$ injury in TGI may also be worth further exploration. The dramatic neuroprotective effect exerted by pyruvate in a TGI animal model may in fact offer the most immediate translation to clinical strategies, given its nature as an inexpensive, safe, and readily-available compound [203].

Growing evidence suggests that Zn$^{2+}$ may be linked to neuronal injury in focal ischemia as well, as extracellular Zn$^{2+}$ chelation has proven beneficial in animal models [145]. Moreover, it is conceivable that in a focal insult, Zn$^{2+}$-induced cytosolic injury cascades triggered by lower, presumably VSCC-mediated [Zn$^{2+}$]i rises might contribute significantly to neuronal demise. The wide array of factors involved in these cascades, as discussed above and illustrated in Fig. (5), implies a wealth of heretofore untapped therapeutic potential, yet the complexity presented by possible interactions among these pathways makes further knowledge of Zn$^{2+}$ neurobiology essential. As one example, Zn$^{2+}$ release from MTs may be an important injurious factor in this scenario, yet the dual role of these proteins as intracellular Zn$^{2+}$ buffers as well as sources of Zn$^{2+}$ release presents a therapeutic challenge. The recent report demonstrating the specificity of NO as an endogenous modulator of Zn$^{2+}$ release from MT-3 may offer some food for thought regarding novel therapeutic strategies, but also underscores the relative adolescence of this avenue of investigation and the importance of ongoing research.

Overall, in both transient global and focal ischemia, Zn$^{2+}$ chelation may be the most productive
approach to neuroprotection at present. Zn\(^{2+}\) chelation strategies employed in the field of Alzheimer’s disease have shown promising results [226], and could potentially be applied to stroke intervention as well. For example, the retired United States Pharmacopoeia antibiotic clioquinol, an orally bioavailable Zn\(^{2+}\) (and Cu\(^{2+}\)) chelator that is able to cross the blood-brain barrier, might be considered. Given that neurons appear to possess a finely-tuned “Zn\(^{2+}\) set point” (cf. section IVB.2c), clioquinol may be better suited for ischemic therapy than other Zn\(^{2+}\) chelators, as it possesses a relatively weak affinity for Zn\(^{2+}\) and thus may be able to bind toxic levels of cytosolic Zn\(^{2+}\) without interfering with the much lower Zn\(^{2+}\) levels needed for physiological signaling.

VI. CONCLUDING REMARKS: NEW PARADIGMS IN EXCITOTOXICITY

Finally, on a more speculative note, a potentially intriguing area of investigation may exist in the interplay between Ca\(^{2+}\) and Zn\(^{2+}\) in mediating excitotoxic neuronal injury. The deleterious synergism between the two cations, though not yet fully explored, might offer fascinating new perspectives on the ionic determinants of ischemic injury. Given the emerging role of intracellular Zn\(^{2+}\) release in neuronal death, and the fact that the cation appears to induce injury with greater potency compared to Ca\(^{2+}\), Zn\(^{2+}\) may be an underappreciated mediator of excitotoxicity, which has for the most part been thought of as a purely Ca\(^{2+}\)-dependent phenomenon. Moreover, the deleterious effects of cytosolic [Ca\(^{2+}\)] increases in ischemia should perhaps be re-evaluated in light of emerging data regarding the Ca\(^{2+}\) dependence of intracellular Zn\(^{2+}\) release. The observation that such release of Zn\(^{2+}\) from mitochondria is particularly prominent in the case of large, glutamate-triggered [Ca\(^{2+}\)] \(_i\) rises, coupled with the likely probability that Ca\(^{2+}\)-induced mitochondrial ROS generation would also promote Zn\(^{2+}\) release from MTs, offers the possibility of a more complex and dramatically shifted paradigm. In such a model, glutamate-driven [Ca\(^{2+}\)] \(_i\) rises might actually play a more modest role than currently perceived, instead serving as an “accomplice” to spark the release of the true ionic mediator of ischemic neuronal injury: Zn\(^{2+}\).

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Zn(^{2+})</td>
<td>“Free” ionic zinc</td>
</tr>
<tr>
<td>[Zn(^{2+})](_i)</td>
<td>Intracellular Zn(^{2+}) concentration</td>
</tr>
<tr>
<td>[Zn(^{2+})](_o)</td>
<td>Extracellular Zn(^{2+}) concentration</td>
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<tr>
<td>TGI</td>
<td>Transient global ischemia</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>MT</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>CDF</td>
<td>Cation Diffusion Facilitator</td>
</tr>
<tr>
<td>ZIP</td>
<td>Zn(^{2+})-regulated metal transporter, Iron-regulated metal transporter-like Protein</td>
</tr>
<tr>
<td>ZnT</td>
<td>Zn(^{2+}) Transporter</td>
</tr>
<tr>
<td>DTP</td>
<td>2,2'-dithiodipyridine</td>
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<td>GSSG</td>
<td>Glutathione disulfide</td>
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<td>GSH</td>
<td>Glutathione</td>
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<tr>
<td>NO</td>
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<tr>
<td>NADP+/</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>VSCC</td>
<td>Voltage sensitive Ca(^{2+}) channel</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
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<td>AMPAR</td>
<td>Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor</td>
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<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
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<tr>
<td>EAAT</td>
<td>Excitatory amino acid transporters</td>
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<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
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<td>MF</td>
<td>Mossy fiber</td>
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<tr>
<td>fEPSP</td>
<td>Field excitatory post-synaptic potential</td>
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<td>HFS</td>
<td>High-frequency stimulation</td>
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<tr>
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<td>(\alpha)-ketoglutarate dehydrogenase</td>
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<td>Glycerol-3-phosphate dehydrogenase</td>
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<tr>
<td>TPEN</td>
<td>N,N,N',N'-tetakis(2-pyridylmethyl) ethylenediamine</td>
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<td>Ca-A/K channels</td>
<td>Ca(^{2+})-permeable AMPA/kainate receptor-mediated channels</td>
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<tr>
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<td>Strongly Ca-A/K channel-expressing neurons</td>
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<td>ZnT-3 knockout</td>
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<td>(\Delta\Psi)_m</td>
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<td>Mitochondrial permeability transition pore</td>
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Rethinking the Excitotoxic Ionic Milieu