INTERACTIONS BETWEEN ZINC AND MITOCHONDRIA IN NEURONAL INJURY

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Zinc is a ubiquitous heavy metal that binds to proteins involved in critical cellular processes. Apart from its necessary role, excessive release of intracellular free zinc (Zn^{2+}) is neurotoxic under stressed conditions characteristic of ischemic or epileptic neuronal injury.

Our earlier results indicated that Zn^{2+}-induced cell death is exacerbated in neurons compared to supporting neuroglia, suggesting that astrocytes have means to upregulate Zn^{2+} buffering mechanisms, i.e. the Zn^{2+}-binding protein metallothionein (MT). The first aim of this dissertation sought to address whether MT can effectively maintain Zn^{2+} levels at a non-toxic minimum. From these studies, we have identified a dichotomous role for MT – protective as a Zn^{2+} buffering agent and detrimental as an oxidant-labile source for toxic Zn^{2+}.

Previous studies demonstrated the role of Zn^{2+} as a mitochondrial toxin. Although it has been widely speculated that Zn^{2+} is taken up by the mitochondrial calcium uniporter, the evidence is not entirely convincing. In the second specific aim, we addressed the specific hypothesis that mitochondrial Zn^{2+} uptake occurs though the

uniporter. Using a novel model involving isolated mitochondria pre-incubated with a Zn^{2+}-selective fluorophore and attached to glass coverslips, we demonstrated for the first time direct visualization of mitochondrial Zn^{2+} transport.

The third specific aim addresses the importance of mitochondria as dynamic intracellular ATP factories, whose intracellular trafficking is critical for neuronal viability. We hypothesized that elevated Zn^{2+} would attenuate mitochondrial trafficking. Our results revealed that Zn^{2+} inhibited mitochondrial movement at pathophysiological levels. Intriguingly, acute activation of phosphotidyl inositol 3- kinase was implicated in both Zn^{2+}-mediated movement inhibition and toxicity, providing a novel role for this traditionally pro-survival signaling pathway.

In summary, this dissertation identifies intracellular targets for Zn^{2+}-mediated neurotoxicity. We specifically emphasize the relevance of mitochondria as a Zn^{2+} target under two circumstances which are critically dependent on the Zn^{2+} concentrations established – direct mitochondrial interactions that may involve Zn^{2+} transport, and indirect mitochondrial interactions that affect intracellular mitochondrial trafficking.

FORWARD

Dedicated to my parents

Ramu & Rathna Malaiyandi

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The work presented here would not have been possible without the contribution, both direct and indirect, of many individuals.

I express my deepest gratitude to my family for their unconditional love and encouragement, throughout not only my successes, but as well as my failures. I have been fortunate to come from a long line of scientists and critical thinkers and there has been no shortage of role models growing up.

I feel a great sense of filial pride when I speak of my advisor, Ian Reynolds. Ian approaches life with an optimistic and adventurous outlook and he applies this also to science: to challenge oneself, not to be afraid to take risks, and more importantly, to have fun in the process. He was recently described as “a mentor’s mentor”; this could not more perfectly describe his role of molding of young graduate students into research investigators and educators.

My most rewarding experiences in the lab stem from the everyday interactions with the colleagues who have become my extended family in Pittsburgh. In addition to sharing their workspace, ideas and suggestions with me, these individuals have shared important moments of their lives, and have served both as counselors and confidantes. I feel somewhat fraudulent by taking sole credit for my accomplishments. How I can simply explain it is that I have been truly fortunate to be in the right place, at the right time, amongst a wonderful group of people. Thanks for the memorable experience.

Latha M. Malaiyandi

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LIST OF ABBREVIATIONS

$\Delta\Psi_m$	mitochondrial membrane potential
Aβ	amyloid β
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
apoCA	apocarbonic anhydrase
APP	amyloid precursor protein
ARE	antioxidant electrophilic response element
ARF6	ADP-ribosylation factor 6
BDNF	brain-derived nerve growth factor
Ca^{2+}	free ionic calcium
$[Ca^{2+}]_i$	intracellular free calcium
Ca^{2+}-A/K	calcium-permeable AMPA/kainate receptors
CDF	cation diffusion facilitator
CQ	clioquionol
2,2'-DTDP	2,2'-dithiodipyridine
Drp1	dynamin-related protein 1
DTT	dithiothreitol
EDTA	ethylenediamine tetratacetic acid
EGTA	ethyleneglycotetraacetic acid
ERK	extracellular signal-regulated kinase
FCCP	carbonylcyanide-p-trifluoromethoxyphenylhydrazone
FRET	fluorescence resonance energy transfer
GABA	γ-amino butyric acid
GAPDH	glyceraldehydes 3-phosphate dehydrogenase
GSH	glutathione (reduced)
GSSG	glutathione (oxidized)
JNK	c-Jun N-terminal kinase
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
Mfn	mitofusin
MIT	2-methyl-4-isothiazolin-3-one
MRE	metal response element
MT	metallothionein
mt-eYFP	mitochondrially-targeted enhanced yellow fluorescent protein
MTF-1	metal response element-binding transcription factor-1
NAD^+	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NF	neurofilament
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
PARP	poly-[ADP-ribose] polymerase
PD	Parkinson's disease
PI 3-kinase	phosphatidyl inositol 3-kinase
PKC	protein kinase C
ppm	parts per million

Pyr	sodium pyrithione or 1-hydroxypyridine-2-thione, sodium salt
Rh123	rhodamine 123
ROS	reactive oxygen species
RuRed	ruthenium red
SOD1	copper/zinc superoxide dismutase
TEA	tetraethylammonium
TMRM	tetramethylrhodamine methyl ester
TOR	target of rapamycin
tPA	tissue plasminogen activator
TPEN	N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine
TSQ	6-methoxy-8-quinolyl-*p*-toluenesulfonamide
VSCC	voltage-sensitive calcium channels
Zn^{2+}	free ionic zinc
$[Zn^{2+}]_i$	intracellular free zinc
ZIP	Zrt-Irt like proteins
ZnT	zinc transporter

Chapter 1. Introduction

1.1 Preface

Zinc is a heavy metal common to all tissues of the human body and is relevant as a nutritional requirement. A large body of evidence attests to the critical role of zinc in the human diet, and zinc deficiency is associated with numerous pathologies, including cognitive dysfunction. More recently however zinc has been studied because of its neurotoxic potential. This perspective stands apart from traditional zinc biology, because it concerns the disruption of zinc homeostasis already present in the body, as opposed to zinc-related disorders that have as their root cause a nutritional excess or deficiency. Thus, the main focus of this dissertation concerns the neurotoxic properties of zinc.

The introduction begins with a description of zinc in a neurophysiologic context: where it is found, what are the suspected functions, and how it is detected (Section 1.2). We then discuss the distribution (Section 1.2.1) and methods of measuring (Section 1.2.2) the three distinct pools of zinc in the brain. Zinc is an essential component in zinc-requiring proteins and enzymes (Section 1.2.3) and has been described as a neuromodulator of synaptic transmission (Sections 1.2.4. and 1.2.5). Since intracellular zinc concentrations are so tightly and precisely regulated, the cell contains inherent homeostatic mechanisms to maintain the free zinc concentrations within cells, including the metallothionein family of zinc-binding proteins (Section 1.3.1) and the family of membrane-bound zinc transporters (Section 1.3.2). In considering the different mechanisms for mobilization of intracellular free zinc, we re-explore the "zinc translocation hypothesis" (Section 1.4.1), which was once considered the primary mechanism for zinc mobilization between neurons inherent for proper cell-cell

communication, but also which may exacerbate neuronal injury. With emerging evidence against the zinc translocation hypothesis, we then discuss recent theories for zinc mobilization within cells in strong oxidizing environments (Section 1.4.2), which may contribute more importantly to zinc-mediated neurotoxicity compared to excessive zinc influx. With respect to disease states, pathogenic roles for zinc have been proposed in the mechanisms of several pertinent neurodegenerative disorders (Sections 1.5.1-1.5.6). At the more cellular level, we discuss proposed mechanisms for zinc-mediated toxicity that may contribute to the overall neuropathogenesis (Sections 1.6.1-1.6.5).

The introduction concludes with a general overview of the dissertation (Section 1.7), describing each specific aim and the global conclusions reached in asking specific questions related to (1) determining the role of metallothionein as an intracellular zinc buffer under toxic conditions, (2) investigating the mechanisms of mitochondrial zinc transport during injury, and (3) exploring the mechanisms for zinc-mediated inhibition of mitochondrial trafficking. Given the extensive literature on zinc-induced injury, this introduction will mostly concentrate on the existing evidence for the pathological role for zinc in the brain, whereas the more controversial topic of zinc actions on mitochondria and the more novel topics of mitochondrial trafficking and zinc-mediated cellular signaling pathways will be reserved for the discussion of the dissertation.

1.2 Physiological zinc in the nervous system

In the brain, zinc is among the twenty most abundant elements and is second only to iron among the transition elements. Bound to several proteins and enzymes, neural zinc is necessary for regulation of critical cellular processes and functions. Nutritional zinc deficiency is documented to cause teratogenic and CNS disturbances in animals

(O'Dell, 1993; Sandstead, 1994), specifically by reducing synaptic activity (Wensink et al., 1987) and causing hippocampal dysfunction (Hesse, 1979) upon chronic zinc deficiency. Traditionally designated as a trace element, it was difficult to ascertain a significant and more precise concentration of zinc in the brain. This is not reflected by its description as a "trace" element, but because available detection techniques underestimated physiological concentrations of zinc in the brain. Newer generation methods for detecting neural zinc are now more sensitive and are capable of staining brain zinc pools that were previously inaccessible to dyes. Indeed, estimated concentrations of total brain zinc far exceed that of classic neurotransmitters (10-100 times higher) and neuropeptides ($10^3 – 10^4$ times higher) (Frederickson, 1989). This emphasizes the pertinent role of zinc in the brain, and suggests its involvement in many critical cell functions.

1.2.1 Neural zinc distribution

The brain contains heterogeneous zinc distributions. Evidence from the literature suggests that there are three disparate pools of zinc in the central nervous system: vesicular zinc, protein-bound zinc, and free ionic zinc. Each of these pools of zinc will be separately discussed below in decreasing order of magnitude.

Protein-bound zinc

The majority of intracellular zinc is bound tightly to proteins and zinc-containing enzymes. Zinc ions are thought to be incorporated into these proteins at the time of synthesis where they remain, with minimal transfer of zinc ions at a later point (Frederickson, 1989). Therefore, the pool of protein-bound zinc is relatively stable and may only be involved in the specific function of the zinc-containing enzyme. However,

since protein-bound zinc accounts for nearly 85-95% of zinc in the brain, the array of zinc binding proteins is from a quantitative standpoint the most important in terms of regulating zinc levels in neural cells.

Vesicular zinc

A special class of zinc-containing neurons, localized almost exclusively in the forebrain, sequesters zinc into presynaptic vesicles in some glutamatergic neurons. Within these presynaptic vesicles, ionic zinc is packaged along with glutamate at zinc concentrations estimated to be in excess of 1 mM (Frederickson et al., 1989). The vesicles are accessible to zinc-specific permeable dyes and staining methods, and therefore the zinc residing within them can be histochemically stained (Szerdahelyi and Kasa, 1984; Frederickson, 1989). Neurobiologists have been particularly interested in vesicular zinc because it is mobilized during synaptic activity. Upon synaptic stimulation, the zinc is released along with glutamate, and traverses the synaptic cleft where it may have some impact on neurotransmission at the postsynaptic membrane (Choi and Koh, 1998). Indeed much evidence suggests that this specific pool of zinc may affect the activities of several neurotransmitter receptors. While it may have relevant consequences on neuronal function, vesicular zinc comprises scarcely 15% of total brain zinc (Frederickson, 1989). In addition, it must be emphasized that the zinc-containing neuron is only a select subset of glutamatergic neurons and not all forebrain neurons contain histochemically-reactive zinc. Apart from neuromodulatory activity, a second explanation for the function of vesicular zinc may involve a role in stabilizing stored glutamate and retaining the neurotransmitter within vesicles (Frederickson, 1989).

However, the observation that not all glutamatergic neurons contain Zn^{2+} excludes this explanation.

Free ionic zinc

Given the large number of intracellular zinc binding sites, intracellular free zinc ($[Zn^{2+}]_i$) is very small. Limitations in detecting $[Zn^{2+}]_i$ have made it particularly difficult to study Zn^{2+} dynamics in cells, but it is estimated that under normal conditions $[Zn^{2+}]_i$ is roughly femtomolar to picomolar concentrations. In fact, O'Halloran contends that there is no free zinc in the cell (Outten and O'Halloran, 2001). It is conceivable that $[Zn^{2+}]_i$ rises and declines as zinc is mobilized between storage sites, metal chaperones, and zinc-requiring proteins. $[Zn^{2+}]_i$ may have functions similar to Ca^{2+} in that acute zinc pulses could also act as intra- and intercellular signals (Frederickson, 1989). While the amount and function of this pool of zinc is still speculative, it is clear that relatively small increases in the amount of $[Zn^{2+}]_i$ are lethal to neurons.

1.2.2 Detecting neural zinc

As mentioned above, the study of physiological zinc in the brain is limited by difficulties in measuring cellular zinc. This is perhaps not surprising, given that most zinc is tightly bound to and perhaps masked by proteins. Additionally, zinc biologists must face the obstacles that (1) the traditional detection techniques used to stain large and complex proteins cannot be applied for the zinc ion and (2) zinc is a common element that is found everywhere and therefore, contamination must be carefully avoided (Frederickson, 1989). The method for detecting zinc in the brain depends on the nature of the sample, i.e. different techniques are applied for whole tissue vs. cells. The differences in these procedures will be discussed below.

In whole brain tissue

Wet human brain tissue contains approximately 10 μg/g of zinc, and total intracellular zinc is approximated at 150 μM (Wallwork, 1987; Frederickson, 1989). However, zinc in interstitial fluid contributes to the quantities detected in wet tissue and makes it difficult to precisely determine intracellular total zinc content (Frederickson, 1989). Investigating zinc distribution in the human central nervous system per weight of dry tissue (denoted in ppm; parts per million), cortical gray matter (69-82 ppm) typically contains more zinc than cortical white matter (33 ppm) (Smeyers-Verbeke and Verbeelen, 1985). The highest concentrations of zinc were found in the hippocampus (108 ppm), with lower amounts (76 ppm) in the cerebellum (Frederickson, 1989). Zinc contribution to tissue fluid may explain the large difference between white and gray matter, given that white matter has a lower extracellular fluid volume. If so, zinc in the extracellular fluid would account for the ~2.5-fold difference (Frederickson, 1989). Factors that commonly contribute to analytical error may include loss of sample during its processing, zinc precipitation or fluid evaporation from samples. Also, zinc can quickly bind glass and plastic surfaces of containers holding the sample (Frederickson, 1989). Such technical problems must be considered when quantifying precise zinc concentrations from whole brain tissue poses.

In brain slices

In spite of quantitative limitations, qualitative data of zinc staining in brain sections gives some picture of regional zinc distribution in the brain. Apparently the pool of zinc that is histochemically reactive, or most readily stained, is located in specific presynaptic zinc-containing neurons of the CNS. This pool of zinc is not bound to

proteins and is also highly concentrated within relatively small vesicles, making it easier to detect this stored pool compared to the much smaller Zn^{2+} pool that may transiently exist in the cytoplasm.

Early evidence for stored Zn^{2+} was observed when the colorimetric zinc-binding reagent dithizone was injected intraperitoneally, producing bright red staining in the rat hippocampus (Frederickson et al., 1981). This suggested that histochemically-accessible Zn^{2+} is abundant in specific CNS neurons *in situ*. Limitations of dithizone include contamination of signal by trace amounts of copper, weak signals (unless zinc is very high) and susceptibility to degradation upon light exposure (Choi and Koh, 1998). Therefore, although dithizone is sufficient for gross staining in brain regions where Zn^{2+} is heavily concentrated, it is not useful for detailed detection of $[Zn^{2+}]_i$.

Other methods corroborated that stored Zn^{2+} was localized to presynaptic vesicles of a subset of CNS neurons. The silver amplification method of Timm demonstrated metal precipitation *in situ* by forming metallic silver shells around weakly-bound metal in postmortem histologic brain sections (Frederickson, 1989). Refined variants of the Timm method are used for selective detection of transition metals, such as copper, iron and zinc. The more recent utilized Timm-Danscher method specifically stains presynaptic vesicles of zinc-containing neurons in the mammalian CNS (Danscher, 1981; Danscher et al., 1985). The Zn^{2+} staining in presynaptic vesicles was confirmed by a second labeling method using 6-methoxy-8-quinolyl-*p*-toluenesulfonamide (TSQ), which brightly fluoresces when in complex with Zn^{2+}. This probe effectively traverses both the plasma and vesicular membranes where it can come into direct contact with Zn^{2+}. The observation that no fluorescence is produced when TSQ is in complex with other

transition metals suggests that this indicator is selective for vesicular Zn^{2+} (Frederickson et al., 1987). The combination of both silver amplification and fluorescent labeling further confirms the presence of this Zn^{2+} pool that may have important consequences for synaptic transmission. TSQ and its derivatives (Zinquin and TFL-Zn) penetrate readily into synaptic vesicles and bind Zn^{2+} very well (K_d ~ nM) but do not remain within vesicles (Snitsarev et al., 2001), and therefore, may not be entirely useful apart from a qualitative standpoint. The newer generation of fluorescent Zn^{2+} probes, Zinpyr-1 and its derivatives are more stable under physiological conditions, remain within vesicles and their fluorescence upon Zn^{2+} binding can be resolved in acute brain slices using standard confocal imaging (Burdette et al., 2001). However, since Zinpyr-1 has low nanomolar affinity for Zn^{2+} and vesicles are estimated to contain millimolar levels of Zn^{2+}, Zinpyr-1 may not be the ideal indicator for detecting vesicular Zn^{2+}.

In cells

$[Zn^{2+}]_i$ is detected with a variety of membrane-permeant fluorescent indicators. These indicators get trapped inside the cell where they can be used to monitor Zn^{2+} changes in living cells. These include single wavelength excitation probes, such as Newport Green or FluoZin-3, or dual wavelength dyes, such as mag-fura-2 or FuraZin-1, which facilitate Zn^{2+} detection through ratiometric imaging compared to the former two. It is difficult to accurately convert fluorescence signals to actual $[Zn^{2+}]_i$ values here because such calculations previously used in ratiometric imaging may be misleading (Dineley et al., 2002). However, these probes do provide a semi-quantitative method to detect timing and magnitude of Zn^{2+} fluxes within neurons. In addition, the membrane-

impermeant forms of these dyes are routinely used to detect extracellular Zn^{2+} that is released from presynaptic terminals (Li et al., 2001).

Tracking the migration of zinc within the cell can be difficult, given that cytosolic concentrations are putatively small and tightly regulated. Recently, Sastry and Lastoskie (2004) used computer-based cellular models to predict how biosensors can be used to detect metals such as copper and zinc by determining the optimal placement of biosensors within the complex and heterogeneous cellular environment. Biosensors are becoming more sophisticated tools for measuring both $[Zn^{2+}]_i$ and extracellular Zn^{2+} release. For the former, Pearce and colleagues (2000) developed a FRET-based construct to detect $[Zn^{2+}]_i$, whereby a conformational change in the construct indicates rapid and selective release of Zn^{2+} from metallothionein. More recently, Hara and Aizenman (2004) demonstrated that a metal response element (MRE)-driven luciferase reporter gene can be used for detecting increases in intracellular Zn^{2+} concentrations in cultured neurons. For detecting Zn^{2+} release from vesicles, Thompson and colleagues (2000) used the protein apocarbonic anhydrase (apoCA) tagged with a fluorescent indicator that increases in fluorescence when apoCA binds to Zn^{2+}. These probes, with the exception of the MRE-driven luciferase construct, allow real-time visualization of Zn^{2+} in living cells.

1.2.3 Zinc requirements for protein and enzyme function

Zinc interacts with proteins in three conformations: zinc-containing proteins (also including zinc-containing enzymes), zinc-regulated proteins, and zinc-protein complexes. In each of these categories, the zinc ion plays a distinct role. The zinc-containing proteins include metalloenzymes such as polymerases found in the nucleus, electron

transport enzymes in the mitochondria, and phospholipases associated with the cell membrane (Frederickson, 1989). These metalloenzymes bind zinc very stably and presumably would allow very little zinc exchange. Evidence indicates that because of their firm stability, zinc metalloenzymes are relatively unaffected by acute changes in $[Zn^{2+}]_i$. However, there is some evidence that high affinity metal chelators can affect zinc metalloenzyme stability by disrupting coordination of the zinc ions (Vallee and Galdes, 1984).

Zinc-containing proteins also include those in which zinc has a non-catalytic role, for example in stabilizing DNA or RNA structure (Vallee, 1988). In addition, 1% of the human genome is estimated to encode for zinc finger proteins. The observation that many of these zinc finger proteins are highly conserved implies that zinc plays a global role in control of gene expression in tissues where these proteins are expressed (Berg, 1988). Apart from playing structural roles, the zinc-containing protein can also play a scavenging role in the brain. For example, the tripeptide protein glutathione is an important zinc-binding molecule in certain brain regions, such as the hippocampus and cerebellum. Approximately 5-10% of total zinc in the brain is estimated to be associated with glutathione (Sato et al., 1984). The observation that levels of zinc-glutathione complexes are region-specific and variable implies that glutathione may regulate zinc levels in areas where required.

Unlike zinc-containing proteins, zinc-regulated proteins have been demonstrated to change shape and activity in the presence of cytoplasm zinc. For example, it has been reported *in vitro* that tubulin binds zinc with high affinity (Gaskin et al., 1978; Kress et al., 1981). The bound zinc ions affect tubulin phosphorylation, whereby low zinc-tubulin

association facilitates microtubule assembly, and high zinc-tubulin association deters microtubule assembly (Haskins et al., 1980; Hesketh, 1984a, b). In addition to tubulin, there are reports of over a dozen enzymes shown to be activated (Parisi and Vallee, 1969) or inhibited (Zaslavsky, 1979; White et al., 1986) by zinc *in vitro*.

Like zinc-regulated proteins, the zinc–protein complex includes any protein loosely aggregated with zinc ions, where the metal is neither tightly nor stoichiometrically coordinated with the protein (Frederickson, 1989). Examples of this include zinc-insulin (Gold and Grodsky, 1984) complexes, which may act as important storage complexes for CNS zinc.

1.2.4 Zinc modulation of synaptic transmission

With the observation that certain neurons can sequester zinc into synaptic vesicles, initial studies sought to determine the mechanism of zinc release. Zinc release was detected in brain slices stimulated by depolarization with high K^+ buffer, kainate (Assaf and Chung, 1984) or electrical stimulation (Howell et al., 1984). This zinc release was determined to be calcium-dependent, strengthening the physiological relevance of the phenomenon. Several lines of evidence suggest that once released zinc behaves as a neurotransmitter by acting on and modulating various ion channels and classic neurotransmitter receptors on the postsynaptic membrane. The effects of Zn^{2+} on synaptic neurotransmission has been investigated with several approaches, for example by adding exogenous Zn^{2+} to cultured neurons and brain slices, chelating endogenous Zn^{2+} to understand the consequences of $[Zn^{2+}]_i$ release, or using knockout animals to interfere with physiological Zn^{2+} signaling pathways (Smart et al., 2004). These approaches and their findings will be discussed in detail below.

Glutamate receptors

Since zinc is co-localized and released with glutamate in a subpopulation of glutamatergic neurons, it is plausible that zinc is also a modulator of glutamate receptors. Indeed, evidence suggests that Zn^{2+} elicits subunit-dependent regulation of NMDA receptors, specifically that Zn^{2+} concentrations as low as several nanomolar can inhibit NMDA receptors (Smart et al., 1994; Paoletti et al., 2000). This indicates that Zn^{2+} inhibits excitatory postsynaptic potentials (EPSPs) mediated by NMDA receptors. Early evidence for EPSP inhibition by zinc was demonstrated by applying micromolar Zn^{2+} concentrations to cortical brain slices (Smart and Constanti, 1983). Moreover, this inhibitory effect was prevented by Zn^{2+} chelation with TPEN (Ueno et al., 2002). More recently, a low-affinity, modulatory Zn^{2+} binding site on the NMDA receptor subunit NR2B has been identified and raises the possibility that Zn^{2+} may mediate a concentration- and subunit-dependent inhibition of excitatory synaptic activity (Rachline et al., 2005). On the other hand, low millimolar concentrations of Zn^{2+} significantly increased AMPA binding to its receptor and also potentiated whole cell current responses to AMPA in a concentration-dependent manner (Bresink et al., 1996), implying that Zn^{2+} may also modulate AMPA receptor properties.

GABA receptors

Endogenous Zn^{2+} has also been shown to regulate many aspects of GABAergic synaptic activity. Specifically, Xie and Smart (1991) demonstrated in adult hippocampal slices the ability of Zn^{2+} through interaction with $GABA_A$ receptors to increase GABA neurotransmitter release from presynaptic vesicles, thereby causing large depolarizing potentials. In later studies, Zhou and Hablitz (1993) demonstrated that low micromolar

applications of Zn^{2+} induced spontaneous large-amplitude inhibitory presynaptic potentials (IPSPs) mediated via GABA receptor activity. Together, this suggested that Zn^{2+} released from presynaptic nerve terminals may facilitate GABA release and subsequent induction of GABA receptor-mediated potentiation of IPSPs.

Glycine receptors

Zn^{2+} is thought to modulate glycine receptor activity in a concentration-dependent fashion. Very low Zn^{2+} concentrations enhance glycine receptor activity by competing and displacing glycine from its receptor (Laube et al., 2000; Suwa et al., 2001). Higher concentrations of Zn^{2+} inhibit glycine receptor function (Bloomenthal et al., 1994; Laube et al., 1995), implying a concentration-dependent modulation of receptor activity. It was recently demonstrated that Zn^{2+} mediates subtype selective inhibition of glycine transport by glycine transporter subtype 1b (Ju et al., 2004).

In summary, Zn^{2+} has been shown to modulate synaptic neurotransmission by both excitatory and inhibitory receptors at specific receptors in the nervous system. Given that histochemically-reactive Zn^{2+} is present in a minority of synaptic terminals in the brain and spinal cord, what remains questionable is the precise role of this Zn^{2+} pool in modulating synaptic activity under physiological conditions and its consequences on postsynaptic function. The latter will be discussed in subsequent sections with respect to pathophysiological levels of $[Zn^{2+}]_i$.

1.2.5 Zinc modulation of synaptic transmission

As mentioned above, zinc may be an important modulator of the NMDA subtype of glutamate receptors. A recent study demonstrated that Zn^{2+} release upon synaptic excitation can modulate NMDA receptor-mediated changes in neighboring synapses,

suggesting a heterosynaptic action of zinc on NMDA receptors (Ueno et al., 2002). Since it is well recognized that NMDA receptors are involved in physiological processes such as learning and memory, it is possible that this Zn^{2+} mobilization could regulate cognitive processes as well. The induction of long-term potentiation (LTP) is a form of synaptic plasticity critical in the cellular mechanism of learning. Indeed, Li and colleagues (2001) claimed that $[Zn^{2+}]_i$ accumulation in CA3 pyramidal neurons was mandatory for LTP induction. Furthermore, they showed that the depletion of histochemically reactive Zn^{2+} impaired LTP. However, there is increasing evidence against synaptic translocation of Zn^{2+} as knockout mice devoid of histochemically-reactive Zn^{2+} are not cognitively impaired (Cole et al., 2000; Lopantsev et al., 2003). Therefore, this argues against the physiological role of vesicular Zn^{2+} or suggests an alternative Zn^{2+} source for LTP induction.

1.3 Maintaining cellular zinc homeostasis

$[Zn^{2+}]_i$ is maintained at a minimum ($< 10^{-12}$ M) by specific metal sensors, transporters and binding proteins (Outten and O'Halloran, 2001). This extremely small free cytosolic concentration is a reflection of the abundance of metal-binding components in the intracellular environment. In addition, the ability of these regulatory systems to act as switches that turn on or off under conditions of zinc excess or deficiency suggests that cells do not tolerate large fluctuations in cytoplasmic $[Zn^{2+}]$.

1.3.1 Metallothioneins

The most well characterized Zn^{2+}-binding protein is metallothionein (MT), a low molecular weight protein (~7 kDa) that sequesters and distributes metal ions, regulates the biosynthesis and activity of zinc metalloproteins, and provides cytoprotection from

free radical damage (Aschner et al., 1997). First isolated by Vallee and colleagues in 1966, the expression of MT in a plethora of organisms, including bacteria, plants, eukaryotic species, and all mammalian cells, suggests an important role in maintaining cellular metal homeostasis. MT has high affinity for not only essential metals (zinc and copper), but also non-essential metals (cadmium and mercury).

Since copper and zinc are physiologically relevant metals, it is plausible that MT may function as a cellular metal chaperone for zinc-requiring proteins and enzymes (Costello et al., 2004), while in addition to acting as a zinc reservoir to prevent its cellular accumulation. Studies have demonstrated that both MT knockout cell and animal models exhibit increased susceptibility to oxidative damage (Palmiter, 1995; Kelly, 1996), whereas MT over expression in these systems confers resistance to heavy metal toxicity (Beach and Palmiter, 1981; Liu et al., 1995). However, regardless of the extensive research that has been done on this family of proteins, a true physiological role for MT in zinc homeostasis still remains uncertain. For example, genetic experiments reveal that mammalian cells that are MT-deficient grow as well as cells expressing MT (Hamer, 1986; Palmiter, 1987). In addition, specific MT isoform knockout mice develop, grow and reproduce normally (Palmiter, 1998), arguing against a critical role for MT as a metal chaperone delivering zinc to metalloproteins. Together, these results imply that MT is protective during zinc toxicity, but may be unimportant during basal conditions. Perhaps cells deficient in MT have alternative methods to remove toxic accumulations of $[Zn^{2+}]_i$, for example, with metal transporters that facilitate the efflux of zinc. Or alternatively, perhaps cells do not experience elevated $[Zn^{2+}]_i$ under normal conditions, thereby not requiring buffering capacity of MT.

Biochemistry of metallothionein

Zinc is redox inactive, but it can coordinate with proteins in different structural geometries. With respect to MT, high resolution X-ray and NMR analyses have revealed the structural detail of the protein and its zinc binding sites. Thionein, the apo-form of MT, is a cysteine-rich molecule that can bind up to seven zinc ions forming the metal-bound form of the protein ($K_d \sim 4 \times 10^{-13}$ M), indicating that MT may be a key intracellular player in maintaining $[Zn^{2+}]_i$ at a minimum (Maret, 2003). Thionein consists of two metal clusters that selectively coordinate the metal, such that each metal atom is held tetrahedrally by four cysteine ligands. The thiolate clusters of MT are of two types: the ß-domain contains cysteine ligands that coordinate 3 zinc ions in one site, whereas the α-domain consists of cysteine ligands that coordinate 4 zinc ions in two identical zinc sites. The protein folds in such a manner to envelope the two clusters in a dumbbell shape (Maret and Vallee, 1998), thereby protecting the bound zinc ions from solvent exposure. Despite this, the thiolate cluster coordination of zinc ions still allows the facile exchange of the metal with other metalloproteins and metalloenzymes.

The two domains are significantly different with regard to zinc binding and release (Jiang et al., 2000). For instance, the higher number of disulfide bridges that hold the zinc ions together in the α-domain correlate to the higher stability of this cluster and also the higher affinity for zinc ions. There is some evidence that metals in the ß-domain exchange more readily and rapidly compared to those in the α-domain. The α-domain is more thermodynamically stable, thereby making the ß-domain a better zinc donor. Although both the α- and ß-domains are unstable by themselves, mutual interactions between the two domains are responsible for stabilizing the entire protein and this

determines the reactivity of MT (Maret and Vallee, 1998). In this respect, MT may be a unique zinc-containing protein in that it controls the biological availability of zinc, which is different in other cases, where zinc controls the biological function of many metalloproteins.

Although zinc binding to MT occurs with high affinity, this reaction is reversible in cell-free systems, as the multiple cysteine residues of MT can be oxidized, releasing bound metal in the process (Jacob et al., 1998; Jiang et al., 1998). Although Zn^{2+} can be transferred to apo-proteins in both directions (from MT to apo-enzymes and enzymes to thionein), not all 7 zinc ions are transferred at once. In addition, transfer is dependent on the modification of the thiol groups, such that some zinc ions are more susceptible to be transferred than others. A study by Maret and Vallee (1998) demonstrated that exposure of MT protein to disulfide reagents, such as 2,2'-dithiodipyridine (2,2'-DTDP) *in vitro* enhanced the transfer of zinc from MT to apoenzymes. The cycle of zinc binding and release is carefully orchestrated via redox reactions associated with glutathione, the principle free radical scavenger in the cytosol (Jiang et al., 1998). Reduced glutathione (GSH) converts to oxidized glutathione (GSSG) as catalyzed by glutathione peroxidase. In other words, in a reduced environment MT remains metallated, whereas Zn^{2+} ions are released under more oxidizing conditions. This strongly suggests that MT is not simply a metal detoxification mechanism, but part of a more complex system that mobilizes Zn^{2+} in response to the redox status of the cell.

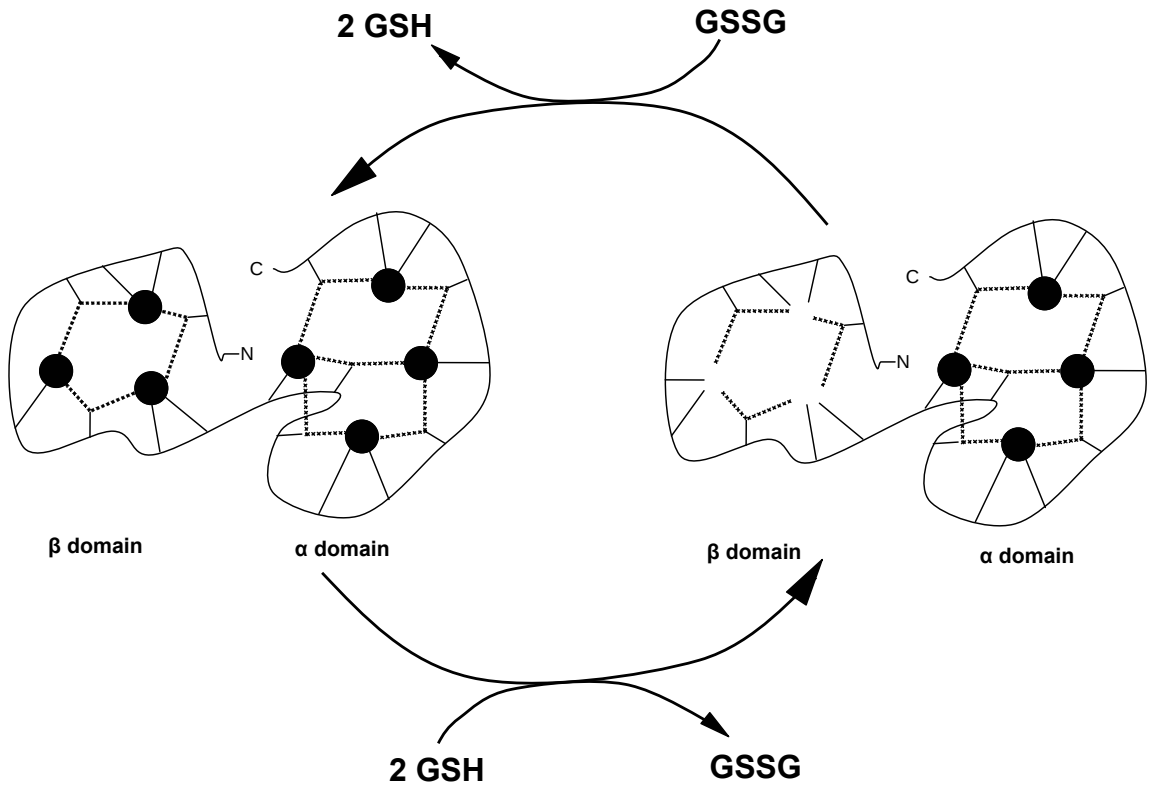


Figure 1.1 Zinc binding and release by metallothionein is orchestrated by glutathione.

Metallothionein isoforms

The mouse genome possesses 4 MT genes located on chromosome 8, whereas in humans at least 16 MT genes are clustered on chromosome 16 (West et al., 1990; Quaife et al., 1994). The idea of multiple MT genes suggests either redundancy in function or divergence of function dependent on cell-specific expression. Four isoforms of mammalian MT protein have been identified. MT-I and MT-II are expressed throughout development in all tissues; their expression is regulated in the presence of metals, glucocorticoids and inflammation (Palmiter, 1987). The other two isoforms display more restricted expression, which might imply more specialized functions. MT-III expression

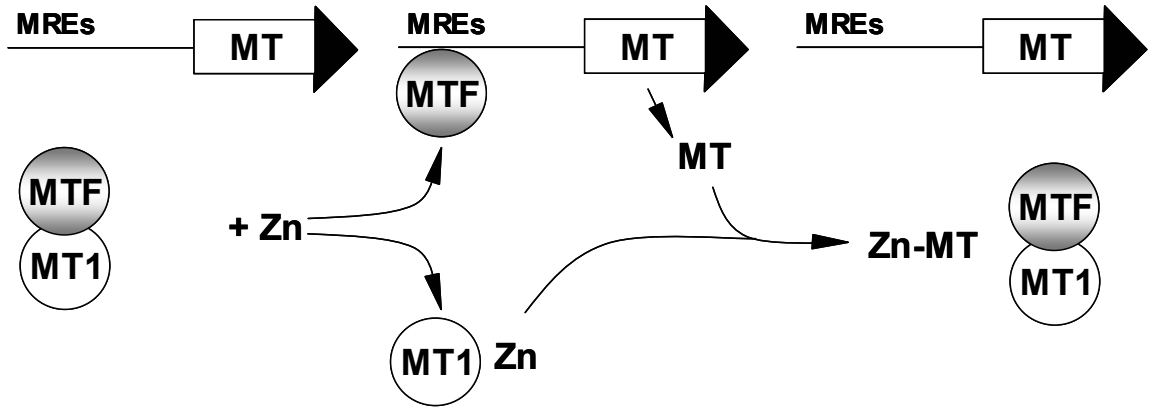
is localized to neurons and was found to inhibit growth in cultured cells (Uchida et al., 2002). Specifically, Palmiter (1995) demonstrated that under conditions of zinc deficiency, the persistent expression of MT-III (but not MT-I) inhibits growth of baby hamster kidney (BHK) cells, implying that this MT isoform competes with zinc binding proteins when the cells undergo zinc starvation. These results are intriguing, because *in vitro* evidence shows that MT-III does not differ appreciably from MT-I in its Zn^{2+} binding dynamics. In any case, the superior Zn^{2+} scavenging activity of MT-III presumably facilitates the degradation of other newly synthesized apo-proteins which require Zn^{2+} for proper structure and function. This hypothesis is consistent with the results that MT-I protein levels diminish when zinc is depleted and only become induced when zinc is abundant, while MT-III expression is unaltered by metals or inflammatory stress.

Initially identified as growth inhibitory factor (GIF) isolated from human brain extracts, MT-III was found to deficient in individuals with Alzheimer's disease (Uchida et al., 1991). However in a later study, Erickson *et al.* (1994) observed that although MT-III may inhibit neuronal growth, neither MT-III mRNA nor protein levels were significantly reduced in AD brain extracts. A direct correlation between MT-III expression and the pathology of AD remains controversial. Suggesting a physiological role for MT-III in the storage and transport of vesicular Zn^{2+}, high levels of MT-III protein are found in the hippocampal neurons that sequester Zn^{2+} in vesicles (Masters et al., 1994). However, mice deficient in MT-III do not display any behavioral or neurological abnormalities, and grow, develop and reproduce normally. Yet they are more susceptible to increased neuronal death resulting from kainate-induced seizures

(Erickson et al., 1997). Together, this suggests that MT-III may regulate $[Zn^{2+}]_i$ in pathological situations, but may not be as relevant under physiological conditions.

The most recently identified isoform, MT-IV, is unique to epithelial cells, where its expression is restricted to stratified epithelium of the skin, tongue and upper alimentary canal (Quaife et al., 1994). Although MT-IV has the conserved metal thiolate clusters that bind Zn^{2+}, this is the least well characterized of the MT proteins. Like the other MT isoforms, the functional significance of this protein remains unknown.

The expression of certain MT isoforms are metal-induced: when $[Zn^{2+}]_i$ accumulates, a metal response element-binding transcription factor (MTF-1) binds several metal response elements (MREs) located upstream of the promoter on the MT gene. This binding, in turn, upregulates MT expression. Richard Palmiter (1994) has demonstrated that MTF-1 is a metalloregulatory protein whose DNA-binding activity is reversibly activated in response to changes in $[Zn^{2+}]_i$.



Adapted from RD Palmiter, 1994

Figure 1.2 Model of metallothionein gene regulation by metals

Although all MT isoforms may be somewhat protective, each is differentially regulated based on its cell-specific expression. For example, it appears that both MT-I and MT-II are directly inducible in astrocytes, while the predominantly neuronal MT-III is relatively unresponsive to induction (Palmiter, 1998). This cell-specific level of MT expression predisposes cells to certain fates or pathways. Indeed, we have previously shown that astrocytes are more resistant than neurons to toxic levels of $[Zn^{2+}]_i$, perhaps due to glial-specific buffering mechanisms (Dineley et al., 2000). In addition, the observation that astrocytes have ten times more basal MT-I than neurons and are able to up regulate expression considerably (Radtke et al., 1993) may explain why astrocytes are more resistant to zinc-mediated cell death than neurons (Dineley et al., 2000). Whether MT-I and MT-II serve as glial-specific buffering mechanisms remains to be determined. Moreover, inducing MT expression in a zinc toxicity model may indicate a protective role in maintaining zinc homeostasis.

With respect to MT protection *in vivo*, a study by van Lookeren Campagne and colleagues (1999) demonstrated that transgenic mice overexpressing MT-I displayed reduced infarct size and sensorimotor deficits after focal cerebral ischemia, supporting the contribution of free radicals and heavy metal toxicity to the pathogenesis of stroke. The same group (van Lookeren Campagne et al., 2000) showed that induction of MT-I expression immediately after cerebral ischemic insult was in response to an antioxidant electrophilic response element (ARE) binding to the MT-I promoter, suggesting that MT-I is an early gene activated specifically in brain microvascular endothelial cells and astrocytes as a protective mechanism after stroke. Also, Tredelenburg and colleagues (2002) used serial analysis of gene expression (SAGE) to show that MT-II mRNA is the

most highly upregulated transcript after focal cerebral ischemia in mice, indicating that it is a neuroprotective gene that ameliorates stroke-induced neuronal damage.

Zinc release from metallothionein

Extensive evidence also suggests that MT can protect against oxidative damage in a range of different cells and tissue. Yeast which cannot synthesize MT and also which lack functional superoxide dismutase are more sensitive to oxidative stress (Tamai et al., 1993). It has been demonstrated in cells and *in vivo* that agents that induce oxidative stress, such as cytokines (De et al., 1990), paraquat and H_2O_2 (Min et al., 1991; Bauman et al., 1992; Dalton et al., 1994), induce MT-I and MT-II expression, and that this reflects a defense mechanism to protect the organism against oxidative damage.

We previously stated that astrocytes are more resistant to zinc-mediated cell death than neurons (Dineley et al., 2000), perhaps due to the upregulated zinc-buffering capabilities of the glial-specific MT isoform. However, it must also be recognized that there is the potential for $[Zn^{2+}]_i$ release from MT stores in response to an oxidative shift in the cellular environment (Aizenman et al., 2000), thereby making cells more prone to oxidant-mediated cell injury and death. Perhaps the existence of more oxidative-sensitive Zn^{2+} pools in neurons compared to atsrocytes may explain their heightened vulnerability to elevated $[Zn^{2+}]_i$. In addition, if oxidant-induced zinc release exacerbates cell injury in this model, MT may be viewed more as a cellular liability rather than as an advantage.

1.3.2 Zinc transporters

As mentioned above, organisms have evolved sophisticated homeostatic mechanisms to maintain $[Zn^{2+}]_i$ at its required minimum. In addition to intracellular binding proteins such as metallothionein, many prokaryotic and eukaryotic cells express

two families of zinc transport proteins to allow Zn^{2+} influx with deficiency and to remove cytosolic Zn^{2+} with toxic accumulations. (1) ZIP or Zrt-Irt like proteins, first identified in *Saccharomyces cerevisiae* (Zrt) and *Arabidopsis thalina* (Irt), have eight transmembrane-spanning domains, and are proposed to be involved in cellular Zn^{2+} uptake, although precise mechanisms are not fully understood. Currently 15 ZIP proteins have been identified (Liuzzi and Cousins, 2004). (2) The CDF or cation diffusion facilitator family, containing proteins that confer resistance to heavy metal toxicity in many eukaryotic cell types, consists of six transmembrane domains (Gaither and Eide, 2001).

The most familiar members of the CDF family are the zinc transporters (ZnT), of which at least nine proteins have been identified (Liuzzi and Cousins, 2004). Expressed in the brain as well as many other tissues, these transporters are localized to both plasma and intracellular membranes of cells that express them. Zinc transporters are involved in three basic maintenance functions: (*i*) cellular uptake to replenish $[Zn^{2+}]_i$, (*ii*) removal of excess $[Zn^{2+}]_i$ and (*iii*) sequestration of $[Zn^{2+}]_i$ into intracellular compartments when appropriate. In summary, zinc transporters mediate the influx/efflux of Zn^{2+} and they also control the intracellular availability of Zn^{2+} for important proteins that modulate cell activity. Although few are understood, mechanisms for how these transporters interact with other zinc-binding proteins and reasons for why they sequester Zn^{2+} are being identified.

Cellular zinc uptake

The precise role of the ZIP family of Zn^{2+} transporters has not been well characterized *in vivo*. There is limited evidence that cell lines treated acutely with elevated $[Zn^{2+}]_i$ have reduced ZIP mRNA levels (Costello et al., 1999). Others have

demonstrated that Zn^{2+} deficiency in brain epithelial cells resulted in increased Zn^{2+} uptake (Lehmann et al., 2002), presumably through upregulation of ZIP proteins. Extensive studies in yeast more conclusively showed that ZIP transporters are constitutively expressed and are the primary means of Zn^{2+} uptake in the presence of physiological $[Zn^{2+}]_i$. ZIP expression is induced as a result of zinc starvation, (Zhao and Eide, 1996; Zhao and Eide, 1997). However, ZIP is altered by elevated $[Zn^{2+}]_i$, suggesting that under these conditions, the ZnT family more likely maintains $[Zn^{2+}]_i$.

Removal of cytoplasmic zinc

One hypothesis for zinc efflux is under normal resting conditions, metallothioneins and other zinc-containing proteins are presumably saturated with Zn^{2+} (Colvin et al., 2003). Under these conditions, the plasma membrane Zn^{2+} transporter, ZnT-1 plays a pertinent role in the removal of excess $[Zn^{2+}]_i$. Studies have demonstrated that ZnT-1 expression in cultured cells counteracts increases in $[Zn^{2+}]_i$. Reduced $[Zn^{2+}]_i$ levels and subsequent resistance to Zn^{2+}-mediated toxicity was observed in PC-12 cells overexpressing ZnT-1 (Kim AH et al., 2000). More recent evidence indicates that ZnT-1 expression in astrocytes protects against Zn^{2+}-mediated toxicity by reducing toxic $[Zn^{2+}]_i$ accumulation (Nolte et al., 2004). Palmiter (2004) recently reported that baby hamster kidney (BHK) cells that have inactive ZnT-1 and MT genes, have high levels of $[Zn^{2+}]_i$. Restoration of MT expression moderately improved cell survival, but only slightly reduced $[Zn^{2+}]_i$. Expression of both ZnT-1 and MT drastically improves cell survival and significantly lowers $[Zn^{2+}]_i$, suggesting the codependence between ZnT-1 and MT in regulating cellular Zn^{2+} homeostasis. In any case, ZnT-1 must have some critical role, as mice lacking ZnT-1 die *in utero* (Andrews et al., 2001).

Intracellular zinc compartmentalization

Several ZnTs are localized to intracellular membranes and mainly function in Zn^{2+} sequestration. ZnT-2 was discovered on the membranes of acidic cytoplasmic vesicles that pump Zn^{2+} into these vesicles when extracellular Zn^{2+} is elevated. Zn^{2+} stored in a subset of glutamatergic neurons are thought to be pumped into vesicles by ZnT-3, which lies on the vesicular membrane (Palmiter et al., 1996).

Zn^{2+} concentrations within these synaptic vesicles are estimated to be up to millimolar levels (Frederickson et al., 1983) and could account for almost 15% of total brain Zn^{2+} (Cole et al., 1999). The ability of this transiently-released Zn^{2+} (which may reach local concentrations of hundreds of micromolar in the synapse) to regulate ion channels and receptors on the postsynaptic neuronal membrane (Li et al., 2001; Thompson et al., 2002; Ueno et al., 2002) suggests a neuromodulatory role for Zn^{2+} in brain regions containing histochemically-visible Zn^{2+} pools. However, the mechanism of how vesicles are replenished remains unknown; it is hypothesized that Zn^{2+} reservoirs like MT could release stores that are pumped into vesicles by ZnT-3 (Colvin et al., 2003). The importance of this transporter is questioned by data from knockout mice: while they presented with selective and complete depletion of vesicular Zn^{2+} in the brain (Cole et al., 1999), the animals displayed no developmental or behavioral deficits. Moreover, electrophysiological properties of the CA3 pyramidal neurons were unaltered (Cole, 2000; Lopantsev et al., 2003). ZnT-3 knockouts do experience more severe seizures and hippocampal cell death in response to kainate, suggesting that (1) vesicular Zn^{2+} pools may not be necessary to mediate toxicity, and (2) an alternative excitotoxic Zn^{2+} source may contribute more to neuronal death.

Also expressed in the brain, ZnT-4 is mainly expressed in luminal cells of milk ducts (Michalczyk et al., 2002) and is involved in the transport of Zn^{2+} into milk during lactation (Kelleher and Lonnerdal, 2002). Mice deficient in ZnT-4 have reduced quantities of Zn^{2+} in milk (Huang et al., 2002). ZnT-5 is predominantly expressed in pancreatic ß cells and is specifically important for vesicular sequestration of Zn^{2+} in the pancreas (Kambe et al., 2002). ZnT-6 and -7 are localized to the Golgi body and cytoplasmic vesicles, respectively, and are expressed in a range of tissues, including brain, lung and small intestine (Huang et al., 2002; Kirschke and Huang, 2003). ZnT-8 has been recently identified in pancreas and liver, ZnT-9 has been identified in both cytoplasmic and nuclear fractions of various tissues (Liuzzi and Cousins, 2004). In fact, it is important to note that for the majority of these "transporters", there is not yet hard evidence that they actually transport zinc.

Table 1.1 CDF family of zinc transporters

	Membrane localization	**Tissue specificity**	**Function**
ZnT-1	Plasma membrane	All cells	Extrude cellular Zn^{2+}
ZnT-2	Acidic cytoplasmic vesicles	All cells	Store Zn^{2+}
ZnT-3	Synaptic vesicles	Brain and testis	Store Zn^{2+}
ZnT-4	Luminal cell vesicles	Mammary gland	Induce lactation
ZnT-5	Vesicles	Pancreatic ß cells	Sequestration of Zn^{2+}
ZnT-6	Golgi body	Brain, lung and small intestine	Sequestration of Zn^{2+}
ZnT-7	Cytoplasmic vesicles	Brain, lung and small intestine	Sequestration of Zn^{2+}
ZnT-8	Vesicles	Pancreas and liver	Not yet known
ZnT-9	Cytoplasmic and nuclear vesicles	Various tissues	Not yet known

Adapted from Liuzzi and Cousins, 2004

1.4 Mechanisms of zinc elevation

As mentioned above, cells contain sophisticated mechanisms to maintain $[Zn^{2+}]_i$ at a minimum. However, under pathophysiological conditions, $[Zn^{2+}]_i$ can become elevated where these regulatory mechanisms are overcome, leading to Zn^{2+}-mediated cellular disruptions and subsequent neuronal death. The questions that arise are when most neural zinc is tightly coordinated to proteins and/or sequestered in intracellular compartments, (*i*) what is the source for elevations of $[Zn^{2+}]_i$ and (*ii*) what factors or stimuli trigger its intracellular elevation ?

1.4.1 Zinc translocation hypothesis

It has been known for some time that toxic levels of Zn^{2+} induce neuronal death (Choi et al., 1988), presumably through translocation of elevated Zn^{2+} from presynaptic to postsynaptic neurons. Based on observations that zinc is released from specific glutamatergic neurons and modulates the activities of postsynaptic receptors, it was hypothesized that large amounts of synaptically-released zinc could mediate neuronal pathogenesis. This was demonstrated from evidence showing histochemically-reactive pools of zinc are readily depleted upon excessive neuronal stimulation and appear in dying CA3 and CA1 pyramidal neurons. Furthermore, selective chelation of Zn^{2+} in the synapse with a membrane-impermeant chelator reduced postsynaptic Zn^{2+} accumulation and subsequent cell death (Koh et al., 1996).

Zn^{2+} released into the synapse can enter postsynaptic cells through a number of Ca^{2+}-permeable pathways. Zn^{2+} permeates the NMDA receptor (Koh and Choi, 1994), which is ubiquitously present on most neuronal membranes and gate Ca^{2+}-permable channels. In addition, it is thought that Zn^{2+} can enter via Ca^{2+}-permeable AMPA/kainate

receptors (Ca^{2+}-A/K), which are found on subpopulations of neurons (Weiss et al., 1993) and also through voltage-sensitive calcium channels (VSCC). Studies by Sensi and Weiss (1997) in cultured neurons claim that Zn^{2+} in order of preference enters cells through Ca^{2+}-A/K channels followed by VSCCs, and finally by NMDA receptor channels. In addition to these three main routes mentioned above, it is also speculated that Zn^{2+} can permeate a unique Na^{+}-Zn^{2+} exchanger, although it is entirely possible that it simply substitutes for Ca^{2+} in the known plasma membrane Na^{+}-Ca^{2+} exchanger (Sensi et al., 1997; Cheng and Reynolds, 1998).

The hypothesis that Ca^{2+}-A/K channels are the predominant route for Zn^{2+} entry is supported by antagonists of these receptors providing protection against oxygen-glucose deprivation-induced Zn^{2+} accumulation and neuronal loss (Yin et al., 2002). In addition, hippocampal pyramidal neurons that are susceptible to Zn^{2+}-induced neurodegeneration preferentially express Ca^{2+}-A/K receptors, suggesting that this specific type of channel may be upregulated in response to stress conditions involving toxic Zn^{2+} translocation (Weiss and Sensi, 2000). Direct evidence for this has been provided by Liu *et al*. (2004), who recently demonstrated that selective blockade using genetic manipulations of Ca^{2+}-A/K receptors in adult rats rescues susceptible CA1 pyramidal neurons from forebrain ischemic injury. With respect to the localization of Ca^{2+}-A/K receptors, it must be noted with caution that not all electrophysiological studies have confirmed their presence in hippocampal regions (Weiss and Sensi, 2000). In addition, not all neurons expressing high densities of Ca^{2+}-A/K receptors are equally vulnerable or susceptible to excitoxicity, implying that additional factors contribute to the mechanism of neuronal injury.

Because Zn^{2+} and Ca^{2+} are both prevalent intracellular divalent cations with neurotoxic capacities, it is perhaps useful to compare their physiological characteristics. However in practicality, the two ions are maintained by different gradients. Zinc is mostly tethered to proteins, allowing $[Zn^{2+}]_i$ to remain relatively small, whereas $[Ca^{2+}]_i$ is bound to fewer proteins until it is released transiently between signaling as a second messenger. Another important difference between the two cations is that whereas extracellular Ca^{2+} is mostly free and therefore Ca^{2+}-regulated stimuli simply open membrane channels for its entry, hardly any extracellular Zn^{2+} is free, thereby requiring its release before Zn^{2+}-induced signals can open membrane channels which the ion permeates. The last disparity between calcium and zinc are the numbers of scavenging, sequestering and releasing proteins available in the intracellular environment: there are over a dozen identified Ca^{2+}-binding proteins, such as calbindin and calmodulin, however, metallothionein is the only known Zn^{2+}-binding protein that regulates its intracellular availability (Frederickson and Bush, 2001).

Table 1.2 Comparison of zinc and calcium signal properties

Attribute	Zinc (Zn^{2+})	Calcium (Ca^{2+})
~ Free ion extracellularly	Not established, likely 1 pM	mM
~ Free ion in cytosol	Not established, ~ pM range	~ nM
~ Ion in storage (releasable pool)	~ mM in presynaptic vesicles	Low μM in SER
Specific membrane-bound storage organelles ?	YES; presynaptic vesicles and secretory granules	YES; SER and neuronal synaptic vesicles
Specific releasable pools	YES; presynaptic vesicles and MT	YES; SER
Ion-sequestering proteins ?	YES (few described, i.e. MT)	YES (dozens described, ie cabining and calmodulin)
Permeation thru ion channels ?	YES (NMDA, AMPA/KA, VSCC)	YES (NMDA, AMPA/KA, VSCC)
Ion modulated enzymes	YES	YES
Cytotoxicity?	YES, nM fatal	YES, μM fatal
Number of ion-containing proteins	Hundreds of zinc finger and other zinc-containing proteins	Many calcium storage and calcium-modulated proteins

Adapted from CJ Frederickson and AI Bush, 2001

1.4.2 Oxidative stress-induced zinc release

Since animals lacking vesicular zinc still exhibit neuronal injury due to toxic Zn^{2+} accumulation, this suggests that toxic $[Zn^{2+}]_i$ can emanate from other sources. The large reservoir of zinc bound to intracellular proteins within the cytosol represents a potential source of toxic zinc. Indeed, it has been shown *in vitro* that modification of thiolate clusters of MT by disulfide agents is a mechanism to mobilize Zn^{2+} from an otherwise stable zinc-protein complex in a non-oxidizing environment (Maret and Vallee, 1998). This suggests that MT acts not only as a zinc buffer but also as a zinc donor, and that this cycle of zinc binding and release is carefully orchestrated by the intracellular redox status. A shift in the redox balance towards a more oxidizing state may contribute to increased zinc release that leads to pathogenesis in many neurodegenerative diseases.

To mimic oxidative stress, the sulfhydryl oxidizing agent, 2,2'-dithiodipyridine (2,2'-DTDP) has been used to show Zn^{2+} release from MT protein in cell-free systems. In addition, application of 2,2'-DTDP increased $[Zn^{2+}]_i$ in cultured cortical neurons, presumably by mobilizing metal bound to proteins. Neuronal injury by 2,2'-DTDP was confirmed to be Zn^{2+}-induced, since membrane-permeant Zn^{2+} removal significantly protected against toxicity (Aizenman et al., 2000). Using another form of oxidative stress characteristic in stroke and ischemia, Zhang and colleagues (2004) recently showed that peroxynitrite-induced neuronal apoptosis is mediated by zinc release from intracellular stores, which in turn activated 12-lipoxygenase. Peroxynitrite-mediated cell death was prevented by inhibitors of lipoxygenase and other downstream players in the apoptotic cascade.

In addition to oxidative stress, nitrosylative stresses have also been shown to liberate zinc from metalloproteins. After administration of nitric oxide (NO) donors to rats, brain tissue showed histochemically-reactive zinc in hippocampal neurons using Timm's and TSQ fluorescence methods (Cuajungco and Lees, 1998). A following study by Frederickson and colleagues (Frederickson et al., 2002), confirmed that NO caused zinc accumulation in hippocampal neurons, specifically from zinc depletion in presynaptic terminals. However, the NO-sensitive Zn^{2+} pool could result from a general mobilization of intracellular stores, since Zn^{2+} accumulation was also observed in cerebellar neurons, where there is little to no vesicular zinc.

Specific evidence for NO-mediated zinc released from MT was first demonstrated in cell-free systems, where the mechanism involves electrophilic attack of NO on thiolate clusters (Aravindakumar et al., 1999), and the production of these free radicals causes

zinc release from MT. Another study confirmed this mechanism, and moreover showed that NO released Zn^{2+} from the β-domain of the protein, while leaving the α-domain relatively untouched (Zangger et al., 2001). Investigating the role of MT in NO signaling was facilitated in cells using a FRET-based green fluorescent fusion protein to study the direct interaction between MT and NO (Pearce et al., 2000). NO caused conformation change of and subsequently released metal from the protein. Furthermore, enhanced expression of this FRET-MT construct in mouse lung fibroblasts prevented NO-induced conformational changes in MT and subsequent Zn^{2+} release (St. Croix et al., 2002), suggesting that MT content can regulate cellular Zn^{2+} homeostasis in response to nitrosylative stress. With respect to *in vivo* data, mice deficient in MT-III exhibited reduced cytoplasmic zinc accumulation and neuronal death in the hippocampal CA1 region after kainate-induced seizures compared to wild type mice (Lee et al., 2003). This suggests that zinc release from MT may contribute to neuronal death following acute brain injury.

Although MT is accepted as the predominant zinc-binding protein in the cytoplasm, there are other sources for oxidant-labile Zn^{2+}. For example, Knapp and Klann (2000) observed that superoxide exposure activates PKC in enzyme preparations by interaction with and thiol oxidation of the cysteine-rich, zinc finger motif of the enzyme. In summary, their data showed that (1) Zn^{2+} chelation stimulated PKC activity, (2) superoxide induced PKC activity by promoting Zn^{2+} release from the enzyme, and (3) oxidized and active PKC contained less Zn^{2+} than reduced PKC. A subsequent study by Korichneva *et al*. (2002) provided *in vitro* evidence that oxidation of PKC by reactive oxygen species triggers the release of zinc ions in NIH 3T3 cells.

1.5 Zinc and neurodegeneration

Evidence from a number of whole animal studies implicated zinc as a potent neurotoxin under acute neurological conditions, such as ischemia and epilepsy. In addition, there is emerging evidence that Zn^{2+} may contribute to chronic and selective neurodegeneration associated with as Alzheimer's and Parkinson's. The role(s) of Zn^{2+} in each of these disorders will be discussed in detail below.

1.5.1 Cerebral ischemia

A toxic role for Zn^{2+} in the pathology of cerebral ischemia or stroke was first identified by Tonder *et al.* (1990) and later confirmed by Koh and colleagues (1996), where toxic $[Zn^{2+}]_i$ selectively accumulated in dying postsynaptic neurons in the CA1 pyramidal region following transient global ischemia in rats. The Zn^{2+} accumulation and subsequent neuronal death was prevented with a membrane-impermeable Zn^{2+} chelator, suggesting that Zn^{2+} translocated from presynaptic sites and subsequently accumulated specifically in postsynaptic neurons. Similarly, Lee *et al.* (2003) claimed that transient focal ischemia in rats also causes Zn^{2+} translocation from pre- to postsynaptic neurons and subsequent cell death may accelerate cerebral infarct growth following the ischemic insult.

1.5.2 Epileptic seizures

Although extensive evidence portrayed Zn^{2+} as a seizure stimulant in animal models of epilepsy (Pei, 1983; Pei and Koyama, 1986), opposing studies claim that endogenous Zn^{2+} can have anticonvulsant properties as well. For example, Mitchell and Barnes (1993) demonstrated that chelation of endogenous Zn^{2+} reduced the threshold for convulsions and augmented the severity of kainate-induced seizures, thereby implying

that zinc release may be protective against epileptic seizures. Other studies showed that Zn^{2+} chelation did not protect hippocampal neurons against accumulated Zn^{2+}-mediated neuronal injury in seizure-induced mice (Cuajungco and Lees, 1997). Therefore, it remains controversial whether the protection is mediated directly by zinc chelation or a side effect of the zinc-chelating agents. Additionally, ZnT-3 knockout mice experience more intense seizures in response to kainate. Whether or not zinc is involved in precipitating seizure events, there is extensive evidence that zinc accumulates in dying neurons after seizure. The Zn^{2+} damage to CA3 neurons after seizures is exacerbated in MT-III knockout mice (Lee et al., 2003). In addition, observations were made by Assaf and Chung (1984) where elevated K^{+}-induced depolarization in rat hippocampal slices induced massive Zn^{2+} release producing extracellular levels up to 300 μM, which may be sufficient to cause neuronal cell death. Other studies have shown that this Zn^{2+} accumulation generates neurotoxicity in cultured cells (Pollard et al., 1994b; Pollard et al., 1994a; Charriaut-Marlangue et al., 1996), it remains to be elucidated whether toxic doses of Zn^{2+} that are pro-convulsant are released from neurons to the same extent *in vivo*.

1.5.3 Alzheimer's disease

One of the neuropathological hallmarks of Alzheimer's disease (AD) is cortical tissue atrophy and the loss of functional neurons, especially in hippocampal regions (Cuajungco and Lees, 1997). The tissue characteristically includes significant accumulation of plaques, mainly composed of amyloid-ß protein (Aß), which contributes to the neurodegenerative mechanisms associated with AD (Selkoe et al., 1990). Aß originates from the cleavage of amyloid precursor protein (APP; Checler, 1995), and

overexpression of APP is thought to contribute to the etiology of AD. APP expression is tightly regulated by zinc-containing transcription factors, and itself contains a zinc-binding site (Quitschke, 1994; Grilli et al., 2000). The suspected role of Zn^{2+} in regulation of Aß accumulation stems from the interactions between Zn^{2+} and APP. Binding of Zn^{2+} to APP not only stimulates its synthesis, but also prevents its degradation to form potential toxic products, such as Aß (Bush et al., 1993; Bush et al., 1994a; Bush et al., 1994b; Bush et al., 1994c). Thus, the binding of Zn^{2+} may be important for maintaining the stability of APP and preventing the subsequent accumulation of Aß.

The accumulation of Aß has been shown to be cytotoxic to both cultured cells (Gschwind and Huber, 1995) and animal models (LaFerla et al., 1995), presumably through oxidative injury that can lead to nuclear and mitochondrial DNA damage and malfunctioning of mitochondrial respiration (Mecocci et al., 1994; Mutisya et al., 1994; Hutchin and Cortopassi, 1995), all of which may contribute to AD pathogenesis. It is also well documented that oxidative stress can cause Zn^{2+} release from intracellular stores (Aizenman et al., 2000), and it is speculated that $[Zn^{2+}]_i$ at toxic levels can in turn promote Aß aggregation (Bush et al., 1994a; Bush et al., 1994b; Bush et al., 1994c). However, whether Zn^{2+} prevents or enhances senile plaques remains to be further elucidated.

1.5.4 Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) primarily causes degeneration of motor neurons in the spinal cord, brain stem and cerebral cortex affecting motor function. Genetic mutations in the superoxide dismutase type 1 (SOD1) gene is thought to account for the 5-10% of familial ALS cases, where etiology is a gain of function in the mutant

enzyme, as opposed to a loss of function (Cuajungco and Lees, 1997). SOD1 encodes for the cytosolic antioxidant enzyme, copper/zinc-SOD, which catalyses the dismutation of superoxide free radicals to hydrogen peroxide and oxygen (Olanow, 1993). The SOD protein contains binding sites for two zinc ions and two copper ions, all four of which are critical for proper SOD activity. Although copper binding is essential for the catalytic activity, zinc is thought to be more important for maintaining structural integrity. SOD binds Zn^{2+} with high affinity and the Zn^{2+} ions within the binding sites of the enzyme are not susceptible to removal with chelators (Cuajungco and Lees, 1997). However, it has been demonstrated that mutations in SOD that affect Zn^{2+}-binding sites could result in enzyme instability of (Lyons et al., 1996), which could substantially alter SOD ability to scavenge free radicals in highly oxidative environments.

Another factor which could reduce Zn^{2+} binding to SOD is the formation of neurofilament (NF) aggregates in ALS pathology. NF has high affinity for zinc and has been shown to compete for Zn^{2+} with both wild type and mutant SOD. A study in 1997 (Crow et al., 1997) demonstrated that decreased SOD-Zn^{2+} affinity led to enhanced SOD activity for damaging cellular proteins by peroxynitrite-mediated tyrosine nitration. A subsequent study by Estevez and colleagues (1999) observed that overexpression of zinc-deficient SOD encouraged NO-mediated apoptosis in motor neurons. This neuronal injury was prevented when the enzyme was replete with Zn^{2+}, suggesting that unstable structure led to improper enzyme function and subsequent accumulation of toxic free radicals. Beyond this, further investigation needs to address how altering metal binding affects ALS disease progression *in vivo*.

1.5.5 Parkinson's disease

Along with ALS, the etiologic mechanism leading to Parkinson's disease (PD) also involves disruption of cellular redox balance (Jenner et al., 1992). Postmortem studies identified lower levels of polyunsaturated fatty acids and elevated levels of malondialdehyde (which is indicative of increased lipid peroxidation) in substantia nigra tissue extracted from PD patients compared to controls (Dexter et al., 1989b). Zinc levels were observed to be markedly increased in dopaminergic neurons of PD brains (Dexter et al., 1989a; Dexter et al., 1991; Jenner et al., 1992). It has been demonstrated that overwhelming the redox status of the cell may cause Zn^{2+} release from metalloproteins (Aizenman et al., 2000), which may in turn generate more free radicals, increasing oxidation-induced neuronal injury. A more definitive pathogenic role for Zn^{2+} in Parkinson's disease is yet unidentified.

1.5.6 Hyperzincemia

A recently described metabolic syndrome associated with toxic Zn^{2+} overload has been defined as hyperzincemia, which is a condition that occurs in relation with copper deficiency. Studies reveal that increased zinc absorption appears to be the primary defect, and decreased plasma copper presents as the secondary defect (Prodan et al., 2002). The high intake of zinc was speculated to be from either high intestinal absorption or reduced intestinal elimination of zinc. This occurs presumably from induction of MT in the intestines, thereby blocking copper absorption and producing the secondary copper deficiency. Previously, hypocupremia has been shown to cause neurological disorders in both humans and animals, but no known neurodegeneration has been associated with increased zinc ingestion. The symptoms described in patients with hyperzincemia-

induced copper deficiency include severe CNS demyelination, axonal degeneration and widespread neuronal loss (Hedera et al., 2003). Plasma copper levels can be restored with copper supplements, but this does not prevent the worsening of neurological conditions.

1.6 Mechanisms of zinc-mediated toxicity

Based on the evidence described above, $[Zn^{2+}]_i$ accumulation contributes to neurodegeneration during excitotoxic conditions. *In vivo* studies have shown that intense $ZnCl_2$ treatments of rat hippocampus were detrimental to both neurons and glia and that this cell death was overcome by co-treatment with a zinc chelator (Lees et al., 1990; Cuajungco and Lees, 1996). Moreover, there are extensive *in vitro* reports identifying $[Zn^{2+}]_i$ as a potent neurotoxin to cells maintained in culture, suggesting that high extracellular Zn^{2+} exposure (300-600 μM) may disrupt neuronal homeostasis (Choi et al., 1988; Koh and Choi, 1994). Although an unambiguous mechanism for Zn^{2+}-mediated neurotoxicity has not been delineated, several potential pathways have been identified. These mechanisms are only starting to be elucidated and many are analogous to Ca^{2+}-induced injury mechanisms. It is highly improbable that one single mechanism mediates Zn^{2+}-induced neurotoxicity. It is more probable that Zn^{2+} overload involves multiple cellular disturbances that converge or act in parallel to cause cell death. The specific circumstances under which these mechanisms are involved in Zn^{2+}-induced cell death will be discussed below.

1.6.1 Oxidative stress

Regardless of the Zn^{2+}-induced pathway to neuronal death, the convergence on oxidative stress is prominent in Zn^{2+}-mediated neurodegeneration. Likewise, oxidative

stress can result in toxic zinc burden, and it is documented that oxidant exposure can trigger the release of Zn^{2+} from intracellular stores (Aizenman et al., 2000). In turn, it is plausible that elevated $[Zn^{2+}]_i$ may produce further free radical accumulation. Indeed, Kim *et al.* (1999) demonstrated that toxic Zn^{2+} entry in mouse cortical cells resulted in toxic free radical generation and produced neuronal degeneration. The resulting cell death from Zn^{2+}-induced free radical generation may be due to the downregulation of antioxidant mechanisms, such as glutathione. A recent study by Chen and Liao (2003) demonstrated that Zn^{2+}-induced neuronal injury depleted glutathione pools in neurons, while replenishment of glutathione significantly attenuated Zn^{2+} neurotoxicity. More specific evidence for Zn^{2+}-induced free radical generation was shown by Noh and colleagues (1999) where toxic exposure of mouse cortical cultures to Zn^{2+} activates membrane PKC, with subsequent free radical generation and neuronal death. This neuronal injury was inhibited by application of PKC inhibitors or antioxidants, suggesting PKC-generated oxidative stress as one important mechanism in Zn^{2+}-induced neuronal death. Noh and Koh (2000) later confirmed that PKC activates the free radical-generating enzyme NADPH oxidase, as one possible effector for oxidative stress in zinc-overloaded cortical neurons.

1.6.2 Zinc-induced apoptosis vs. necrosis

Studies have elucidated a dose-dependent activation of cell death, where at high concentrations zinc-induced cell death reflects necrosis, characterized by rupturing of the plasma membrane and cell swelling (Yokoyama et al., 1986; Choi et al., 1988). Interactions of Zn^{2+} with plasma membrane-bound neurotransmitter receptors and ion channels present one mechanism for necrotic death. As mentioned earlier, toxic Zn^{2+}

translocation inhibits NMDA and GABA receptors, but potentiates both Ca^{2+}-A/K and VSCC. In addition to acting on the receptors themselves, Zn^{2+} is also thought to inhibit enzymes critical for synthesizing neurotransmitters (Frederickson, 1989). Moreover, Zn^{2+} interaction with structural proteins may be characteristic of necrotic cell death. One of the first direct evidence portraying neuronal functional disruptions came from earlier studies in the late 1970s investigating how Zn^{2+} influences cytoskeletal assembly (Gaskin and Kress, 1977). Physiological Zn^{2+} levels in the brain coordinate assembly and disassembly of tubulin (Eagle et al., 1983) and other microtubule-associated proteins *in vitro* (Gaskin et al., 1978). Toxic levels of Zn^{2+}, on the other hand, interfere with microtubule structure and alter the cytoskeleton (Kress et al., 1981), thereby disrupting global neuronal function.

On the other hand, lower zinc concentrations reflect a more apoptotic cell injury, characterized by cell body shrinkage, chromatin condensation and DNA fragmentation (Sunderman, 1995). Neuronal apoptosis is associated with both acute neurodegeneration, described in traumatic brain injury or cerebral ischemia, but also is evident during the progression of chronic diseases, such as Alzheimer's and Parkinson's. The mechanisms of Zn^{2+}-mediated apoptosis is only recently being explored, but is considered relevant as many zinc-dependent transcription factors are required for normal cell functioning (O'Halloran, 1993), and therefore may be impaired during apoptotic injury. Early evidence showing the specific Zn^{2+} translocation into dying postsynaptic neurons following cerebral ischemia or seizures, claim that the neurons die by apoptotic mechanisms (Koh et al., 1996). Zn^{2+} activation of immediate early genes, such as *c-jun* and *c-fos* are known to play a role in the cascade of events leading to apoptosis in non-

neuronal cells (Xu et al., 1996). In addition, toxic Zn^{2+} was shown to induce DNA laddering, a consequence of DNA fragmentation and a hallmark of apoptosis, which activates poly-[ADP-ribose] polymerase (PARP), an enzyme which recognizes and repairs damaged DNA strands (Shimizu et al., 1990). Sheline and colleagues (2003) recently demonstrated that only acute, and not chronic, exposure of Zn^{2+} to cortical neurons activated PARP and furthermore, that Zn^{2+} neurotoxicity was diminished in mutant mice lacking the PARP gene. In addition, Zn^{2+} mediation of apoptosis through caspase-dependent mechanisms appears to be favored. For example, Aizenman and colleagues (2000) demonstrated that 2,2'-DTDP-induced neurotoxicity was prevented by pretreatment with a broad spectrum caspase inhibitor. Further evidence for caspase-dependent apoptosis in Zn^{2+} neurotoxicity comes from studies by Park and colleagues (2000) demonstrating that exposure of cortical neurons to elevated Zn^{2+} activated the $p75^{NTR}$ and $p75^{NTR}$-associated death executor, which promote caspase-dependent apoptosis. Zn^{2+} has been shown to activate the sphingomyelinase (Spence et al., 1989; Schissel et al., 1996), which leads to production of the known apoptogen, ceramide (Hannun, 1994; Brugg et al., 1996).

1.6.3 Zinc activation of MAP kinases

As mentioned above, generation of oxidative stress is induced by intracellular Zn^{2+} overloads. Oxidative stress is one mechanism implicated in the cell death that occurs during neurodegenerative disorders and several studies have observed that oxidant radicals trigger cell death via activation of several signaling pathways, such as the mitogen-activated protein kinase (MAPK) pathways (Alessandrini et al., 1999; Bhat and Zhang, 1999; Stanciu et al., 2000; Wang et al., 2002). The ubiquitous MAPK family

consists of c-Jun N-terminal kinase (JNK), p38 MAPK, and the extracellular signal-regulated kinases (ERK), all of which are protein kinases that activate their substrates through phosphorylation of serine or threonine residues, and are involved in maintenance of basic cell functions as well as induction of cell death. JNK and p38 mediate cellular mechanisms in response to stress, whereas ERK is mainly activated by growth factors and mediates signaling relevant for cell proliferation and survival.

However, growing evidence shows that ERK is also activated during, and may contribute to neuronal death caused by zinc overload. Indeed, Seo and colleagues (2001) showed that $[Zn^{2+}]_i$ elevations mediated cell death in differentiated PC12 cells involved JNK and ERK, but not p38 activation. A separate study (Daniels et al., 2004) use a neuroblastoma cell line to show that high levels of extracellular Zn^{2+} rapidly activated p38, but downregulated ERK. These opposing conclusions could reflect differences between the experimental model system and the concentrations and duration of Zn^{2+} exposure. As opposed to models of direct Zn^{2+} exposure, Aizenman and colleagues have demonstrated through a series of studies that $[Zn^{2+}]_i$ release upon oxidation in primary neurons activates MAPK members during the course of neuronal apoptosis. In the first study (McLaughlin et al., 2001), they observed that Zn^{2+} release by sulfhydryl oxidation in neurons caused cell death by oxidative stress and p38 activation. Only p38 inhibition blocked the 2,2'-DTDP-induced toxicity, implying that p38 is an early event, whereas energetic dysfunction, caspase activation or DNA damage are late events in oxidant-induced apoptosis. In a following study (Du et al., 2002), they applied the industrial biocide 2-methyl-4-isothiazolin-3-one (MIT) to enhance $[Zn^{2+}]_i$ in cortical cultures through oxidative stress via the 12-lipoxygenase pathway. MIT application induced early

ERK activation, which led to NADPH oxidase activation, DNA damage, and PARP activation, all of which was independent of caspase activation. Taken together, these results suggest a role for MAPK in $[Zn^{2+}]_i$-induced neuronal death..

1.6.4 Involvement of potassium channels

Reports show that blocking K^+ efflux by increasing extracellular K^+ or inhibiting K^+ channels could inhibit apoptotic processes and protect neurons from a wide variety of apoptotic stimuli (Hughes and Cidlowski, 1999). Specifically, studies of oxidant-induced Zn^{2+} release in neurons suggest that 2,2'-DTDP-induced cell death can be prevented by high extracellular K^+ or by inhibiting K^+ channels using tetraethylammonium (TEA) (Aizenman et al., 2000). With respect to specificity of the K^+ channels that are activated during Zn^{2+}-induced injury, recent work by Pal *et al.* (2003) identified Kv2.1 as the main K^+ channel activated after toxic doses of 2,2'-DTDP or staurosporine in neurons. This was shown by targeted disruption of the Kv2.1-encoded K^+ channel, which prevented the enhanced K^+ currents and also protected neurons against apoptogen-stimulated cell death.

1.6.5 Inhibition of energy production

The observation that mitochondria are the major cellular generators of free radicals suggests that these organelles are intracellular targets of toxic $[Zn^{2+}]_i$. We and others have demonstrated that toxic Zn^{2+} exposure causes loss of mitochondrial membrane potential ($\Delta\Psi_m$), enhances reactive oxygen species (ROS) production, and inhibits mitochondrial respiration by blocking specific sites of electron transport. Each of these direct effects of Zn^{2+} on mitochondrial respiration will be discussed more thoroughly in the discussion.

1.7 The Dissertation

As an extension of previous work completed in the lab, this dissertation initially sought to further understand the neurotoxic properties of zinc, primarily by means of manipulating intracellular free zinc levels under toxic situations. To accomplish this, the first aim investigated the zinc buffering properties of metallothionein (MT) in primary astrocytes. Astrocytes were a convenient model to overexpress the zinc-binding protein, but the model also reiterated findings that only astrocytes, and not neurons, primarily have the ability to induce MT expression under elevated $[Zn^{2+}]_i$. From MT over expression in astrocytes, we observed that cells were more capable of buffering $[Zn^{2+}]_i$ accumulation, implying a protective role of MT under toxic injury. On the other hand, exposure to strong oxidizing agents enhanced zinc accumulation in MT-overexpressing cells, presumably as zinc is released from MT upon oxidation, thereby rendering intracellular MT populations as toxic sources of zinc. This first study proposed a dichotomous nature of MT: as potentially protective and toxic under different toxic exposures.

We and others previously demonstrated that toxic zinc exposure to isolated brain mitochondria produces disturbances in mitochondrial function. Methods used to show zinc uptake into mitochondria primarily through the calcium uniporter remain inconclusive. In addition, we have demonstrated that removal of extramitochondrial zinc protects against zinc-mediated mitochondrial defects. Therefore a fundamental question remains whether zinc is physically transported into mitochondria or does zinc act at an external site without requiring mitochondrial entry to generate its toxic consequences. To address this very specific question, we used a novel method to fluorescently track zinc

changes in glass coverslip-adherent isolated brain mitochondria using fluorescence microscopy. We observed that mitochondria do indeed accumulate zinc when exposed to pathophysiological levels. The ability of mitochondria to take up zinc was reduced, but not completely abolished, when the mitochondria were less polarized and when the calcium uniporter was blocked, suggesting a number of pathways for zinc action on mitochondria that are concentration-dependent.

The last aim of the thesis slightly deviates from the more traditional studies in zinc-induced neurotoxicity and is more closely associated with the mainstream interests of our lab. Although still within the realm of zinc and mitochondria, it introduces the more novel concept that mitochondria are not simply static cellular energy-generating factories, but are quite dynamic entities that are constantly moving and changing shape. It is hypothesized that this so called "trafficking" of mitochondria is essential for proper energy distribution and functioning of neurons. In recent years, our lab has become increasingly interested in the consequences of neuronal injury on the trafficking of mitochondria to appropriate region of neurons where needed. We have been able to successfully visualize and track mitochondria within living neurons through methods of fluorescent labeling of mitochondrial proteins. Our earlier studies have published results suggesting that glutamate excitotoxicity not only impairs mitochondrial movement in neurons, but also dramatically alters their morphology. Together, these two events may contribute to both mitochondrial dysfunction and their ability to deliver ATP and maintain calcium levels within various regions of the neuron and may overall contribute to cellular injury.

We extended this study to investigate the effects of zinc, as an effective neurotoxin and mitochondrial toxin, on mitochondrial trafficking. Toxic loads of zinc effectively impair mitochondrial movement, but unlike glutamate, do not cause any gross morphological changes. In exploring the mechanism responsible for this zinc-mediated effect, we identified the transient activation of PI 3-kinase as necessary for movement inhibition. Interestingly, blocking activation of this protein kinase prevented movement inhibition and neurotoxicity induced with toxic zinc elevations, suggesting a common pathway between changes in mitochondrial trafficking and zinc-induced injury. Also notable is the rare evidence for PI 3-kinase activity involvement in cell death, whereas the major body of literature describes it as a mediator of cell survival.

Chapter 2. Divergent consequences arise from metallothionein overexpression in astrocytes: zinc buffering and oxidant-induced zinc release

2.1 Abstract

Excessive accumulation of the heavy metal zinc is cytotoxic. As a consequence, cellular vulnerability to zinc-induced injury may be regulated by the abundance of proteins that maintain intracellular free zinc concentrations ($[Zn^{2+}]_i$). In this study, we over expressed the zinc-binding protein metallothionein-II (MT) in astrocytes to assess its impact as (*i*) an acute zinc buffering mechanism and (*ii*) an oxidant-releasable zinc pool. Over expression of MT in primary astrocyte cultures was accomplished using an adenoviral vector. Using the zinc-sensitive fluorescent indicator mag-fura-2, we monitored $[Zn^{2+}]_i$ after stimulating zinc influx or oxidant treatment. With MT over expression, we observed an acute buffering effect manifested as a dampening of stimulus-induced increases in $[Zn^{2+}]_i$. Contrastingly, we also saw enhanced zinc release with application of the sulfhydryl oxidizing agent 2,2'-dithiodipyridine. These results indicate that over expression of a zinc-binding protein can quickly diminish $[Zn^{2+}]_i$ following zinc influx, but elevate $[Zn^{2+}]_i$ under conditions of oxidative stress, providing protective yet potentially endangering effects.

2.2 Introduction

Intracellular free zinc ($[Zn^{2+}]_i$) is important for the activities of many cellular enzymes and proteins. However, recent studies have identified it as a toxin to cells maintained in culture (Choi et al., 1988; Koh and Choi, 1988; Weiss et al., 1993; Koh and Choi, 1994; Manev et al., 1997). In addition to *in vitro* paradigms, studies using animal models suggest that elevated $[Zn^{2+}]_i$ may contribute to neurodegeneration in conditions such as ischemia (Koh et al., 1996), epilepsy (Frederickson et al., 1988) and head trauma (Suh et al., 2000). It has been demonstrated that zinc differentially affects neural cell types. The finding that astrocytes require much higher concentrations for zinc-mediated injury compared to neurons (Dineley et al., 2000) suggests unique characteristics of this cell type, such as glial-specific zinc buffering mechanisms (Choi et al., 1988).

It is likely that cells have intrinsic mechanisms for controlling and preventing accumulation of $[Zn^{2+}]_i$. For example, the total intracellular zinc concentration is approximately 150 μM, whereas the free zinc concentration is much lower, in the pM range (Outten and O'Halloran, 2001). This presumably reflects the abundance of intracellular zinc binding components in the cytoplasmic milieu. Several candidate homeostatic mechanisms for maintaining $[Zn^{2+}]_i$ have been identified. These include zinc transporters as well as the zinc-binding protein metallothionein (MT). Certain members of these two families are thought to be regulated by the metal response element-binding transcription factor (MTF-1), so that the expression levels of these proteins are increased in response to increases in $[Zn^{2+}]_i$ (Palmiter, 1994; Langmade et al., 2000).

The most well characterized example of a zinc-binding protein is metallothionein (MT), a low molecular weight (6-7 kDa) protein that binds and sequesters metal ions,

such as copper, zinc and cadmium (Shaw et al., 1991). MT is a cysteine-rich molecule, which can bind up to seven zinc ions with high affinity, $K_D \sim 1.4 \times 10^{-13}$ M, (Maret and Vallee, 1998) forming metal thiolate clusters (Arseniev et al., 1988). Four isoforms of mammalian MT have been identified, designated I-IV: MT-I and MT-II are expressed ubiquitously (Palmiter, 1987), MT-III is found primarily in neurons (Masters et al., 1994), and MT-IV is expressed in differentiated squamous epithelial cells (Quaife et al., 1994). Endogenous MT-I and MT-II mRNA are directly inducible in cells by various stimuli (i.e. metals, glucocorticoids and oxidative stress) while the primarily neuronal MT-III mRNA is relatively unresponsive to induction (Kramer et al., 1996). In addition to behaving as a heavy metal scavenger, other functions of MT include regulating the biosynthesis and activities of zinc metalloproteins and metalloenzymes (Cherian, 1977) and providing cytoprotection from free radical damage (Schwarz et al., 1994).

The over expression of MT both in cell culture and animal models results in resistance to heavy metal toxicity (Hidalgo et al., 2001). This suggests that MT may be an important player in protecting cells from elevated levels of heavy metals. Interestingly, a recent study demonstrated induction of the MT-II transcript in mice following transient focal cerebral ischemia (Trendelenburg et al., 2002), supporting the idea of a more neuroprotective role for MT-II. However, despite available evidence on this family of proteins, an explicit physiological role for MT in zinc homeostasis still remains unknown.

Aside from its intrinsic ability to bind zinc, MT also functions dynamically by releasing zinc ions under certain conditions. Specifically, the multiple cysteine residues of the protein are susceptible to oxidation, thereby releasing bound metal in the process.

Indeed, the oxidant-induced release of zinc ions from MT has been demonstrated in cell-free systems (Maret and Vallee, 1998). In addition, previous studies have suggested the release of zinc ions from metalloproteins in neuronal cultures under oxidative conditions (Aizenman et al., 2000). Furthermore, it has been shown that nitric oxide can liberate zinc ions presumably from metalloproteins in the brain *in vivo* (Cuajungco and Lees, 1998). These lines of evidence oppose a protective role, but rather suggest a detrimental function for MT under an oxidative burden.

In this study, we investigated the role of MT in zinc homeostasis in primary astrocyte cultures to identify an explicit buffering effect of MT on $[Zn^{2+}]_i$. We demonstrate the novel effect of MT-over expressing cells to maintain lower $[Zn^{2+}]_i$ compared to wild-type cells following an equivalent zinc load. On the other hand, we also demonstrate that MT-over expressing cells exhibit increased oxidant-induced $[Zn^{2+}]_i$ release compared to control cells, implicating MT as a deleterious source of $[Zn^{2+}]_i$. This study thus identifies a dual role for MT in regulating $[Zn^{2+}]_i$.

2.3 Materials & Methods

Cell Culture

Procedures involving animals were in agreement with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Primary cultures of Type I astrocytes were prepared as previously described (Kress et al., 2002) from cortices removed from one day-old Sprague-Dawley rats and plated on 75 mm^2 flasks in Basal Medium Eagle's (BME) supplemented with 15% fetal bovine serum (FBS), 0.1% glutamine, 0.6% glucose, 100 U/mL penicillin and 100 μg/mL streptomycin. Medium was changed every other day for ten consecutive days after plating. The flasks were then shaken for 15-18 hours at 37˚C to remove other glial types. The remaining astroglial cells were removed by trypsin and plated on poly-D-lysine-coated 31 mm glass coverslips placed in 6-well plates in supplemented BME. All experiments were done on cells 1-7 days after being plated on coverslips.

Infections

Astroglial cultures were infected with an MT replication deficient adenovirus, constructed from an E1a-deleted replication-deficient adenoviral vector expressing the human metallothionein-II_A using a kit from Microbix Biosystems, Inc. and a bacterial plasmid containing the MT insert (Sciavolino et al., 1992). Media were aspirated from the wells of the culture plates and 250-400 μL of infected media (diluted appropriately from a stock concentration of 1.8×10^{11} PFUs/mL) were added per well and incubated for

one hour at 37˚C, during which the plates were shaken every ten minutes. After one hour, the virus was diluted with 1.5 mL fresh media per well and plates were incubated for an additional 16-24 hours before performing experiments.

Immunocytochemistry

MT-infected astrocyte cultures were fixed in 4% paraformaldehyde for 30 minutes at room temperature prior to blocking in 10% BSA (in PBS) for an additional 30 minutes. Cells were then incubated in a mouse monoclonal MT antibody (Dako Corp, Carpinteria, CA) overnight at 4˚C at a 1:500 dilution (in 3% BSA/0.4% Triton-X in PBS). Following a series of 1X PBS washes, cells were then placed in a goat anti-rat secondary antibody conjugated with Cy3 (1:1000 in 3% BSA/0.4% Triton-X in PBS) for 2 hours at room temperature. Plates were rinsed in 1X PBS again before viewing under the microscope.

Reagents and solutions

During microfluorimetric $[Zn^{2+}]_i$ recordings, cells cultured on coverslips were perfused at a constant rate with HEPES-buffered salt solution (HBSS), containing 140 mM NaCl, 5 mM KCl, 0.9 mM $MgSO_4$, 1.4 mM $CaCl_2$, 10 mM $NaHCO_3$, 0.6 mM Na_2HPO_4, 0.4 mM KH_3PO_4, 20 mM HEPES and 5.5 mM glucose, adjusted to pH 7.4 with NaOH. Amounts of zinc were diluted from 1000X stocks of $ZnCl_2$ made in 25 mM HCl. The zinc-specific ionophore sodium pyrithione (Sigma, St. Louis, MO) was added at a concentration of 20 μM from a stock of 20 mM in DMSO. The membrane-permeant heavy metal chelator N,N,N',N'-tetrakis(2-pyridalmethyl)ethylenediamine (TPEN)

(Molecular Probes, Eugene, OR) was added at a concentration of 25 μM from a 25 mM stock in DMSO. The sulfhydryl oxidizing agent 2,2'-dithiodipyridine was added at a concentration of 100 μM from a stock of 100 mM, prepared fresh each time in DMSO.

Fluorescence Microscopy

$[Zn^{2+}]_i$ measurements were recorded from control, LacZ-infected and MT-infected cells using the zinc-sensitive fluorophore mag-fura-2 (Molecular Probes, OR). Coverslips were loaded at room temperature for 20 minutes with mag-fura-2 diluted in 5 mg/mL BSA/HBSS loading buffer for a final concentration of 5 μM before being mounted in a recording chamber with constant perfusion at a rate of 20 mL/min. The fluorophore was alternatively illuminated at wavelengths 335 and 375 nm. Emitted light passed through a 400 nm dichroic and a 510/80-wideband emission filter. The PC-based imaging system consisted of a monochromator-driven xenon light source (ASI, OR), Nikon Diaphot 300 microscope with a 40X oil immersion objective, a CCD camera (Hamamatsu Photonics, Japan) and a PC-based acquisition program (SimplePCI, Compix, PA). All microfluorimetric measurements were performed at room temperature.

Data Analysis

Data were represented as a comparison of the mag-fura-2 ratio changes that occurred prior and subsequent to an added stimulus. This ratio change was generated by subtracting the baseline ratio value from the ratio value achieved at the end of the stimulus. Each experiment averaged 5-10 cells in a single field of a coverslip, and experiments were repeated in cultures from 3-5 independent infections. Values were

analyzed with the appropriate statistical tests using the software package GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA).

2.4 Results

MT over expression in astrocytes

Previous studies have investigated zinc homeostasis by over expressing human metallothionein-II_A in epithelial cells using a replication-deficient adenoviral vector (St Croix et al., 2002). Here, we employed this viral construct to over express MT in primary astrocyte cultures. To determine optimal infection efficiency, cells were infected with an adenovirus containing the β-galactosidase gene using a range of viral concentrations from 10^6-10^9 plaque forming units/mL (PFUs/mL). The extent of infection was visualized through β-galactosidase staining. From this preliminary experiment, an optimal viral titer of 10^7-10^8 PFUs/mL, which typically infected approximately 60% of cells on any given coverslip, was established for all subsequent infections involving the MT adenovirus. To confirm MT over expression in infected cultures, immunocytochemistry was performed using a mouse monoclonal anti-MT antibody along with a Cy3-conjugated goat anti-rat secondary antibody. Protein expression was detected using fluorescence microscopy (Figure 2.1). Comparing non-infected (A) to infected (B) cells, we showed that marked MT immunofluorescence was detected only in infected cultures, consistent with virus-induced MT over-expression. It is not reasonable to conclude an absence of endogenous MT in our astrocyte cultures. Indeed, similar cultures stained with the same antibody did reveal MT in wild-type cells

using Western techniques (Rising et al., 1995). However, under these immunocytochemical conditions endogenous MT was not detected in non-infected cells. The inability to detect MT-II in non-infected cells precludes estimating the extent of over expression through the immunocytochemical procedures we used.

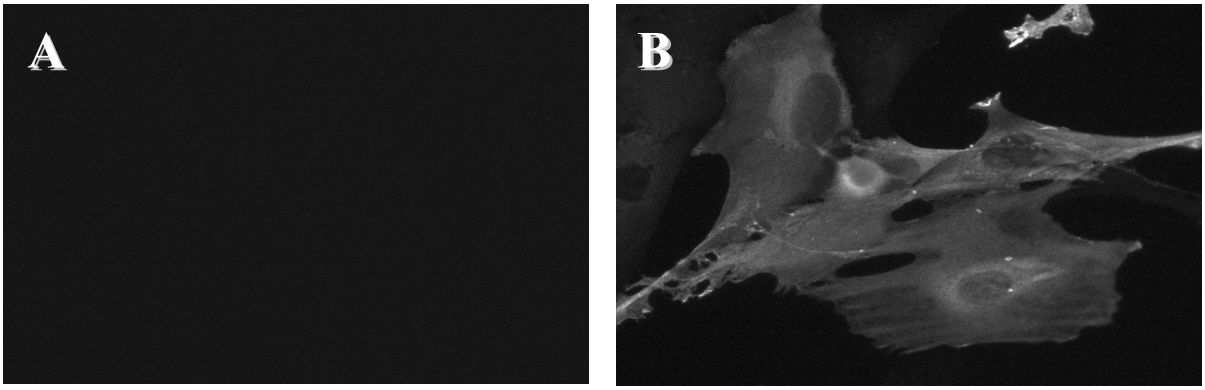


Figure 2.1 Adenoviral infection induces MT protein expression in astrocytes. Primary astrocyte cultures were infected with the MT-II adenovirus at a viral titer of 10^7-10^8 plaque forming units/mL (PFUs/mL) and stained with a monoclonal mouse anti-MT antibody. Fluorescent images from (A) control and (B) MT-infected cells confirm that expression is detected only in infected cultures. Results are representative of 3 individual experiments. Magnification 40X.

Ionophore-induced $[Zn^{2+}]_i$ changes

Having established over expression of MT in primary astrocytes, we then measured $[Zn^{2+}]_i$. For monitoring $[Zn^{2+}]_i$, we employed a member of the fura-2 dye family, mag-fura-2 (Cheng and Reynolds, 1998), which is commonly used to measure changes in $[Ca^{2+}]_i$ and $[Mg^{2+}]_i$, but has a much higher affinity for Zn^{2+}, $K_D \sim 20$ nM (Molecular Probes, Inc., Eugene, OR). To examine the buffering ability of MT in

response to acute changes in $[Zn^{2+}]_i$ (Figure 2.2), a brief stimulus of $ZnCl_2$ (1μM) in the presence of the zinc ionophore sodium pyrithione (20 μM) was applied to cells (Dineley et al., 2000). As seen in the representative traces shown in Figure 2.2A, ionophore-induced $[Zn^{2+}]_i$ increases are smaller in MT-infected cells compared to controls. The signal remains elevated during the washing out of zinc and pyrithione, indicating that $[Zn^{2+}]_i$ levels also prevail until the addition of the membrane-permeant heavy metal chelator TPEN (25 μM). The reversal of mag-fura-2 fluorescence back to baseline levels suggests that the signal reflects $[Zn^{2+}]_i$ and not increases in $[Ca^{2+}]_i$ or $[Mg^{2+}]_i$. Fluorescence signals were not converted to actual $[Zn^{2+}]_i$ values here because we have recently demonstrated that such calculations previously used in ratiometric imaging may be misleading (Dineley et al., 2002). Cells infected with the LacZ virus were included in this paradigm as a negative control to show that the effects seen were not due to the induction of endogenous zinc buffering components as a stress response to the virus itself. These results, summarized in Figure 2.2B, demonstrate the significantly reduced responses to ionophore-induced $[Zn^{2+}]_i$ changes in MT-infected cells compared to both control and LacZ-infected cells. Since we generate a sub-maximal efficiency for each infection, these results actually underestimate the true magnitude of the effect. Regardless, these observations suggest that MT can act as a rapid buffering mechanism in the presence of $[Zn^{2+}]_i$ accumulation.

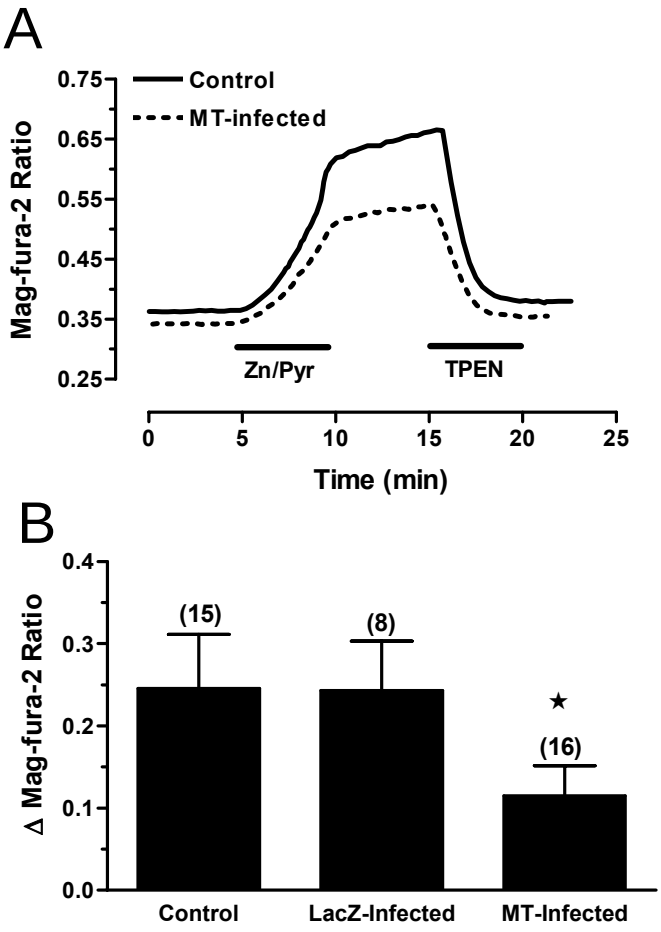


Figure 2.2 MT overexpression reduces ionophore-induced $[Zn^{2+}]_i$ elevations.
(A) Representative traces are shown from $[Zn^{2+}]_i$ recordings using mag-fura-2, where cells were stimulated for 5 minutes with 1 μM $ZnCl_2$ in the presence of 20 μM sodium pyrithione. Following a brief HBSS rinse, fluorescence levels were returned to baseline values with a 5-minute exposure to 25 μM TPEN. Control cells (as indicated by the solid line) are distinguished from those cells infected with the MT adenovirus (indicated by the dotted line). Each trace represents the mean of approximately 5-10 astrocytes from a single coverslip. (B) The graph represents a compilation of all experiments from control, LacZ-infected and MT-infected cells. The values above each column indicate the number of coverslips included in each condition. Bars represent means ± S.E. * $P<0.05$, one-way ANOVA, Tukey's multiple comparison test, $F = 1.981$.

Oxidant-induced $[Zn^{2+}]_i$ release

Previous studies in cell free systems have shown that zinc ions are released from MT upon oxidative stimuli (Maret and Vallee, 1998). In addition, Aizenman *et al.* have demonstrated that zinc-induced neurotoxicity can occur upon exposure to a sulfhydryl oxidizing agent, presumably from metalloproteins releasing zinc (Aizenman et al., 2000). This led us to investigate whether MT over expression in astrocytes affects the pools of oxidant-releasable $[Zn^{2+}]_i$ (Figure 2.3). In this experimental paradigm, cells were initially exposed to 25 μM TPEN to chelate any unbound or loosely-bound $[Zn^{2+}]_i$, followed by a 10-minute perfusion with the sulfhydryl oxidizing agent 2,2'-dithiodipyridine (2,2'-DTDP, 100 μM). As seen in the sample traces in Figure 2.3A, the response to 2,2'-DTDP is larger in MT-infected cells compared to non-infected controls. These results are compiled in Figure 2.3B, where the change in mag-fura-2 ratio upon 2,2'-DTDP treatment is significantly greater in MT-over expressing cells compared to control cells. These data confirm the ability of MT to release $[Zn^{2+}]_i$, as was suggested by previous studies in cell-free systems. In addition, these effects suggest that MT can be a significant intracellular source of oxidant-releasable zinc.

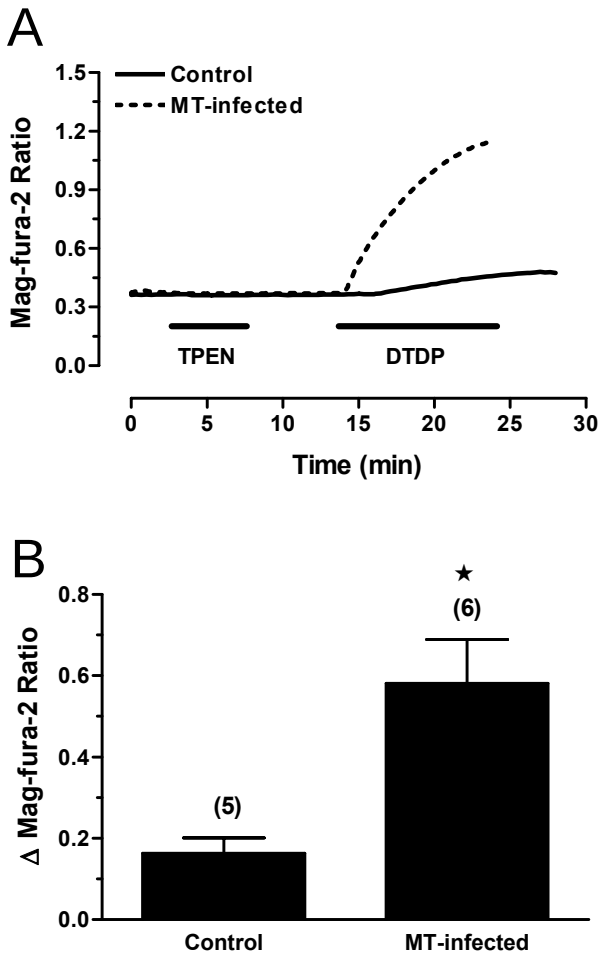


Figure 2.3 Overexpression of MT enhances oxidant-induced $[Zn^{2+}]_i$ release.

(A) Representative traces of $[Zn^{2+}]_i$ recordings from both non-infected (solid line) and MT-infected (dotted line) astrocytes. Following a 5-minute perfusion with 25 μM TPEN to chelate unbound or loosely-bound $[Zn^{2+}]_i$, 100 μM 2,2'-DTDP was administered for 10 minutes. (B) The graph represents a summary of the 2,2'-2,2'-DTDP-induced $[Zn^{2+}]_i$ changes in control and MT-infected cell populations as indicated by changes in mag-fura-2 ratio values. Values above each column represent the number of experiments done under each condition. Bars represent means ± S.E. * $P<0.05$, $t = 3.375$.

Acute zinc pre-loading of cellular thionein

The ability of MT-infected cells to buffer $[Zn^{2+}]_i$ implies that the MT adenovirus generates a significant pool of the apo-form of MT, or thionein (St Croix et al., 2002). We next investigated whether altering the cellular MT: thionein ratio in infected astrocytes would have any consequences on the potential amounts of oxidant-releasable $[Zn^{2+}]_i$ (Figure 2.4). Larger quantities of zinc loaded MT would presumably (*i*) decrease the buffering capability of MT and (*ii*) increase the amounts of oxidant-releasable zinc from MT-over expressing cells. To acutely increase the proportion of zinc-bound MT, control and infected cells were pre-loaded with an initial zinc stimulus as described above (Figure 2.2), after which the oxidant 2,2'-DTDP was introduced (Figure 2.4A). As a control for pre-loading, cells were perfused with sodium pyrithione alone for the same period of time (Figure 2.4B). With pyrithione treatment alone, we occasionally observed increases in signal, which reflect ambient zinc in the buffer, and such responses did not vary between control and infected cells. As anticipated, MT over expression increased the response to 2,2'-DTDP. However, the pre-loading treatment with zinc and sodium pyrithione did not significantly enhance the subsequent response to 2,2'-DTDP in either of the conditions tested (Figure 2.4C). These series of experiments conclude that acute zinc pre-loading of MT does not enhance oxidant-releasable zinc.

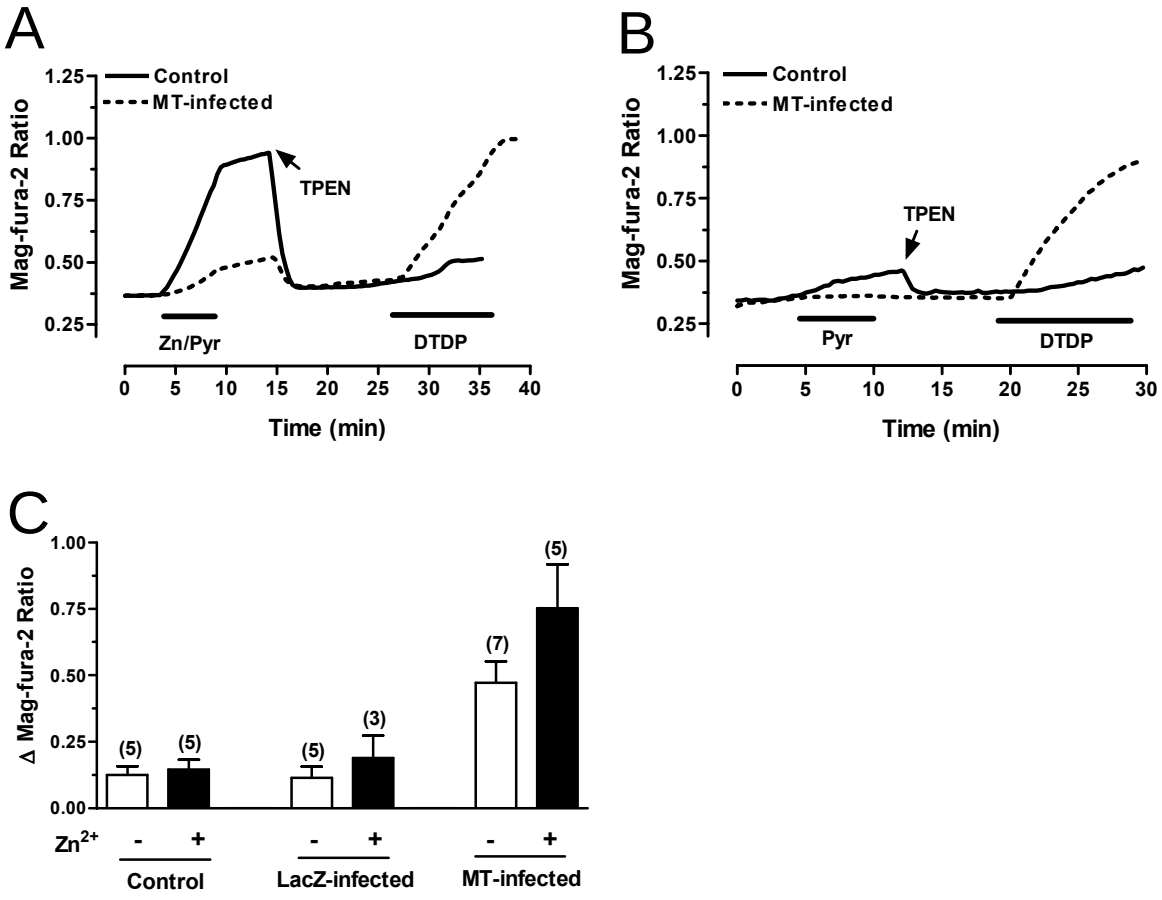


Figure 2.4 $[Zn^{2+}]_i$ release is not enhanced in zinc pre-loaded MT-infected cells.
Oxidant-induced $[Zn^{2+}]_i$ was recorded from control (solid line) and MT-infected (dotted line) cells in the (A) presence or (B) absence of an initial 1 μM $ZnCl_2$ stimulus facilitated by 20 μM sodium pyrithione. Following this 5-minute stimulus and a brief HBSS wash, 25 μM TPEN was administered to chelate any free $[Zn^{2+}]_i$. After a subsequent HBSS rinse, 100 μM 2,2'-DTDP was applied for approximately 10 minutes as described in Figure 3. (C) The graph represents a summary of all experiments done using this paradigm under control, LacZ-infected and MT-infected conditions, where open bars represent those coverslips pre-loaded with sodium pyrithione alone and solid bars represent those coverslips pre-loaded with sodium pyrithione in the presence of $ZnCl_2$. Numbers above columns represent the number of times experiments were repeated. Bars represent ± S.E. * $P<0.05$, $t = 1.666$.

2.5 Discussion

The findings from this study demonstrate that MT over expression in cultured cells results in enhanced $[Zn^{2+}]_i$ buffering following an acute zinc load. On the contrary, MT over expression also enhanced $[Zn^{2+}]_i$ release in response to an oxidative stimulus, thus revealing potentially endangering effects of increasing the levels of this protein in cells. Earlier studies found associations between MT expression and neuroprotection in both cells and animals that over express MT (Durnam and Palmiter, 1987; van Lookeren Campagne et al., 1999), presumably through buffering of $[Zn^{2+}]_i$. However, we are not aware of any studies that have explicitly demonstrated that up regulating MT expression can actually alter $[Zn^{2+}]_i$. Thus, the findings presented here provide a direct demonstration that over expression of MT can decrease $[Zn^{2+}]_i$.

Comparison to cytosolic calcium-binding proteins

Although these findings are novel with respect to $[Zn^{2+}]_i$ buffering, our findings are analogous to previous studies that had over expressed cytosolic calcium binding proteins. For example, Lledo and colleagues showed that overexpressing calbindin-D28k in GH3 cells decreased the magnitude of intracellular calcium transients (Lledo et al., 1992). Similar results have been reported in PC12 cells (McMahon et al., 1998) and HEK293 cells (Rintoul et al., 2001). The overexpression of calcium binding proteins therefore provides a model that is quite analogous to the results reported here.

Cellular redox environment modulates zinc binding and release

Several lines of evidence suggest that the cellular redox environment mediates zinc binding and release. When MT is oxidized in the presence of glutathione, it releases free zinc ions, while under reducing conditions the protein remains metallated (Maret and Vallee, 1998). Therefore, changes in cellular redox status can modulate zinc release. It is likely that the modification of the thiol ligands in the MT clusters that house the zinc ions ultimately determines the extent of zinc mobilization (Jacob et al., 1998). It was previously shown that under conditions of oxidative stress in cultured neurons, the zinc released from intracellular stores can contribute to neuronal injury via an apoptotic cascade (Aizenman et al., 2000). This was accomplished with the sulfhydryl oxidizing agent, 2,2'-dithiodipyridine (2,2'-DTDP), which we also used in our experiments. Although 2,2'-DTDP produced significantly and consistently larger increases in fluorescence signal in MT-overexpressing cells compared to those containing basal levels of expression, 2,2'-DTDP is far removed from a true physiological stimulus. Nitric oxide (NO), on the contrary, has been demonstrated to mobilize $[Zn^{2+}]_i$ both *in vivo* (Cuajungco and Lees, 1998) and *in vitro* (St Croix et al., 2002), presumably through its release from metalloproteins. It is also conceivable that a protein other than MT contributes to the oxidant-induced release of zinc in cells since there are many other zinc-bound cellular proteins and enzymes. For example, it has been suggested that protein kinase C (PKC), an abundant intracellular protein, can release zinc in the presence of oxidants (Knapp and Klann, 2000). Thus, even though we were unable to identify neither the endogenous oxidant nor the metalloprotein species responsible for zinc release in wild-type cells, this

study provides an interesting observation that enhanced oxidant-sensitive release of zinc is a possible outcome if MT protein expression is altered.

Regulating zinc loading of thionein

A previous study by St. Croix *et al.* has specifically demonstrated NO-induced zinc release from MT in epithelial cells (St Croix et al., 2002). They concluded that the overall cellular ratio of MT to non-metal bound thionein is the critical factor that determines the buffering power of the system. The authors observed an NO-induced zinc release from MT with adenoviral infection alone, which was then enhanced when cells were grown overnight in zinc-supplemented media. This implies that infected cells contain mainly the apo-form of MT, or thionein. In our experiments we applied a strategy for potentially converting thionein to MT by acutely exposing cells to a zinc pre-loading stimulus prior to assessing the effects of oxidants. However, we did not observe enhanced oxidant-induced zinc release when astrocytes were pre-loaded with a brief zinc stimulus regardless of MT over expression. This suggests that our astrocyte cultures, when infected with virus, contain mostly metallated protein, as opposed to thionein. However, the profound buffering effect that we observed in our MT-over expressing cells argues against this hypothesis. In addition, the limited time frame of the pre-loading experiments suggests that saturating MT may not occur as rapidly or efficiently. Perhaps a more chronic or prolonged zinc exposure of MT-infected cells would more efficiently load zinc onto MT. It is also possible that there are essentially two pools of sulfhydryl groups that can bind and buffer zinc, but that only one of the two pools is oxidant sensitive. If this oxidant-sensitive pool is fully metallated, while the second pool is not

saturated and thus available to bind zinc, this would generate conditions where the zinc buffering was enhanced while the oxidant-releasable zinc pool was not increased by zinc loading. This could explain the findings we report here, although further experiments would be required to fully evaluate this hypothesis. One could also speculate that MT acts as a metal chaperone to deliver zinc to an undetermined oxidant-sensitive zinc pool. It is conceivable that because the timed nature of these experiments, an acute zinc pre-loading followed by application of the oxidant is too rapid for MT to deliver metal to this unknown zinc-binding protein. However, we cannot conclude this based on the results presented here since little is known about the dynamics of the mechanisms that regulate zinc loading of thionein in astrocytes.

Are astrocytes more resistant to zinc-mediated injury compared to neurons ?

We have previously demonstrated that astrocytes are less vulnerable to both zinc- and iron-induced injury when compared to neurons (Dineley et al., 2000; Kress et al., 2002). Astrocytes are also relatively resistant to the toxic effects of DTDP (Aizenman et al., 2000). Therefore, the primary astrocyte cultures used here was a sub-optimal system for studying zinc-induced cytotoxicity. However, unlike neurons, which are synaptically excitable cells, astrocytes lack the various neurotransmitter receptors that act as potential entry routes for zinc. This benefits our aim of primarily studying the cytosolic binding and release of zinc without the added burden of considering its cytotoxic influences. Unlike primary neuronal cultures, astrocytes are also more easily manipulated for biochemical purposes. We have demonstrated previously that astrocytes are more resistant to the toxic consequences of elevated $[Zn^{2+}]_i$ compared to neurons (Dineley et

al., 2000). It is possible that either the elevated endogenous levels of MT-I or MT-II or else the induction of MT-I and MT-II in astrocytes may explain this zinc resistance. However, it is also possible that the targets of zinc-mediated injury are relatively resistant in astrocytes compared to neurons. Unfortunately, we cannot make this distinction from the present study. Regardless, we present an interesting dichotomous nature of MT during ischemic conditions: in one aspect behaving as an important $[Zn^{2+}]_i$ buffering agent, but on the other hand existing as an intracellular source for toxic amounts of zinc during oxidative stress.

Chapter 3. Direct visualization of mitochondrial zinc accumulation reveals uniporter dependent and –independent transport mechanisms

3.1 Abstract

Current evidence suggests that zinc may kill neurons by disrupting energy production, specifically by inhibiting mitochondrial function. However it is unclear if the inhibitory effect requires zinc accumulation, and if so, precisely how zinc enters mitochondria. Here, using fluorescence microscopy to visualize individual rat brain mitochondria, we detected matrix zinc uptake using the fluorophore FluoZin-3. Fluorescence increased rapidly in mitochondria treated with micromolar free zinc, and was quickly returned to baseline by membrane permeant chelation. Zinc uptake occurred through the calcium uniporter, because depolarization or uniporter blockade reduced fluorescence changes. However, increased fluorescence under these conditions suggests that zinc can enter through a uniporter-independent pathway. Fluorescence steadily declined over time and was unaffected by acidification or phosphate depletion, suggesting that zinc precipitation is not a mechanism for reducing matrix zinc. Uniporter blockade with ruthenium red also did not change the rate of zinc loss. Instead, zinc appears to exit the matrix through a novel efflux pathway not yet identified. Interestingly, dye-loaded mitochondria showed no fluorescence increase after treatment with strong oxidants, arguing against oxidant-labile intramitochondrial zinc pools. This study is the first to directly demonstrate zinc accumulation in individual mitochondria and provides insight about mechanisms mediating mitochondrial zinc uptake and efflux.

3.2 Introduction

Mitochondria are semi-autonomous organelles that play a critical role in maintaining energy gradients required for neuronal survival through control of ATP production, calcium (Ca^{2+}) mobilization, and reactive oxygen species (ROS) accumulation. During glutamate excitotoxicity, which is most commonly used to model ischemic injury, intracellular Ca^{2+} reaches pathophysiological levels. This in turn leads to mitochondrial perturbations such as Ca^{2+} overload, dissipation of mitochondrial membrane potential ($\Delta\Psi_m$), and decreased ATP production. Consequently, the impaired mitochondria may initiate a cell death cascade involving the release of pro-apoptotic molecules and further ROS accumulation (Nicholls and Budd, 2000).

In vivo evidence suggests that intracellular free zinc ($[Zn^{2+}]_i$) contributes to neurodegeneration in transient global (Koh et al., 1996) and focal (Lee et al., 2002) cerebral ischemia. Moreover, there are extensive *in vitro* reports identifying exogenous Zn^{2+} as a potent neurotoxin to cells in culture (Choi et al., 1988; Koh and Choi, 1994). Other evidence suggests that neurotoxic $[Zn^{2+}]_i$ can be mobilized from intracellular stores in response to oxidative stress (Aizenman et al., 2000). Precisely how Zn^{2+} kills neurons remains unclear, but a growing body of evidence suggests that Zn^{2+} may impair neuronal energy production, specifically by inhibiting mitochondria (Dineley et al., 2003). Indeed, as early as 1967, Skulachev suggested that Zn^{2+} blocks complexes of the electron transport chain, findings that were generally supported by subsequent studies (Skulachev et al., 1967; Nicholls and Malviya, 1968; Kleiner, 1972, 1974; Lorusso et al., 1991; Link and von Jagow, 1995; Mills et al., 2002). Other possible sites of Zn^{2+} action include the TCA cycle (Brown et al., 2000; Gazaryan et al., 2002). The consequences of Zn^{2+}

inhibition are potentially numerous, including swelling and cytochrome *c* release (Jiang et al., 2001), loss of $\Delta\psi_m$, and increased ROS production (Sensi et al., 1999; Sensi et al., 2000; Dineley et al., 2005).

While it is obvious that Zn^{2+} can disrupt mitochondrial function, it unclear whether Zn^{2+} acts externally or if mitochondria import Zn^{2+} into the matrix. Several studies show that the Ca^{2+} uniporter blocker ruthenium red partially protects against Zn^{2+} effects on mitochondria (Brierley and Knight, 1967; Saris and Niva, 1994; Jiang, 2001), implying that Zn^{2+} enters via a pathway that normally accommodates Ca^{2+}. However these studies provide no direct evidence that Zn^{2+} is actually imported into the matrix.

In this report, we have used a novel mitochondrial preparation technique (Vergun et al., 2003b) to study Zn^{2+} transport in individual mitochondria. This approach is superior to previous experiments using mitochondrial suspensions because it employs fluorescent indicators to detect matrix Zn^{2+}, allows substrates and solutions to be perfused onto mitochondria when desired, and removes the ambiguity of signals arising from dye leakage. Using the high affinity Zn^{2+} indicator FluoZin-3, we provide evidence for two distinct mechanisms for Zn^{2+} import. The first was consistent with a uniporter-like mechanism because it required $\Delta\psi_m$, was inhibited by ruthenium red, and was relatively high capacity. A second mode of Zn^{2+} import occurred in the absence of $\Delta\psi_m$, was insensitive to uniporter blockade, and was relatively low capacity. Additionally, the results and methods presented here provide a new practical and conceptual framework for investigating mitochondrial Zn^{2+} homeostasis.

3.3 Experimental Procedures

Materials

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Isolation of brain mitochondria

All procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Rat brain mitochondria were isolated from the cortex of adult Sprague Dawley rats as previously described by Vergun *et al*. (2003b). Briefly, mitochondria were isolated using a Percoll gradient method described by Sims (1991) with minor modifications. Isolation buffer contained (in mM) 225 mannitol, 75 sucrose, 0.5 EDTA, 5 HEPES, and 1 mg/ml fatty acid free BSA, pH adjusted to 7.4 with KOH. All isolation procedures were carried out at 0-2^{o}C. Mitochondria in isolation medium were stored on ice until use.

Fluorescence Microscopy

All imaging experiments were performed at room temperature in KCl-based buffer containing (in mM) 125 KCl, 2 K_2HPO_4, 5 HEPES, 5 $MgCl_2$, 0.02 EDTA, 5 malate, 5 glutamate and 0.05 ATP, pH adjusted to 7.0 with KOH. Mitochondria were added to this buffer at a final concentration of 0.75-1 mg protein/ml immediately before each experiment. For phosphate-free experiments, K_2HPO_4 was replaced with 2 mM $KC_2H_3O_2$. Thirty-one mm glass coverslips were washed with 70% ethanol, then with

H_2O and dried prior to use. A 20 µl drop of mitochondrial suspension was placed in the middle of the coverslip for 5-7 min. Coverslips were then placed into a 700-µl perfusion chamber and mounted onto a microscope fitted for fluorescence imaging as described below. The coverslip was superfused with KCl buffer at 5 ml/min; after 1 min of perfusion unattached mitochondria were effectively washed out of the recording chamber. Typically, mitochondria remained attached at a density of 400,000 – 500,000 per square millimeter. Thus, approximately 2,000 mitochondria were present in a field observed with a 100x objective.

For $\Delta\Psi m$ measurements we used the potentiometric probes rhodamine 123 (Rh123) and tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR) at nonquenching concentrations. Rh123 (200 nM) or TMRM (40 nM) was present in the perfusion medium during the experiment (no preloading was necessary). To monitor Zn^{2+} uptake, the mitochondria were loaded with 50 µM FluoZin-3 AM (Molecular Probes Eugene, OR) in substrate-free KCl buffer containing 200 µM EGTA for 20 min at 37°C, and then placed onto the coverslip as described above. Polarized FluoZin-3 loaded mitochondria were pre-selected by transiently adding TMRM to the perfusate. After preselection, TMRM was no longer included in the buffer. Under such conditions TMRM is completely gone from the matrix in 1 to 2 minutes, allowing us to measure "pure" FluoZin-3 changes. Treatments of $ZnCl_2$ and other reagents were diluted in KCl buffer from stock concentrations and perfused onto mitochondria at 5 ml/min.

For the fluorescence recording we used a BX50WI Olympus Optical (Tokyo, Japan) microscope fitted with an Olympus Optical LUM PlanFI 100x water immersion quartz objective. The fluorescence was monitored using excitation light provided by a

75W xenon lamp-based monochromator (T.I.L.L. Photonics GmbH, Martinsried, Germany), and emitted light was detected using a CCD camera (Orca; Hamamatsu, Shizouka, Japan). Rh123 or FluoZin-3 was illuminated at 490 nm, and light passed through a 500 nm long pass dichromatic mirror and a 535/25 nm band pass filter (Omega Optical, Brattleboro, VT). TMRM was excited at 550 nm, light passed through a 570 nm long-pass dichromatic mirror and a 605/55 nm band pass filter. Fluorescence data was acquired and analyzed using Simple PCI software (Compix Inc, Cranberry PA). Fluorescence was measured in 50-100 individual mitochondria for each coverslip. Objects smaller than 0.3 μm in diameter were not analyzed. Background fluorescence was collected from 3-4 mitochondrion-free regions and subtracted from all the signals. All experiments were repeated 4-6 times using mitochondria from at least three different animals.

Statistics

Statistical analysis was performed using Prism 4.01 (Graph Pad Software, San Diego, CA). All the data are presented as mean ± S.E. Comparisons were made using Student's t test and one-way ANOVA with Dunnett posttest to compare all conditions to control, with p values of less than 0.05 regarded as significant.

3.4 Results

Visualization of Zn^{2+} uptake into single mitochondria

We visualized mitochondrial Zn^{2+} uptake using the high affinity Zn^{2+} indicator FluoZin-3 AM (K_D ~ 15 nM), which was loaded into isolated rat brain mitochondria prior to attachment onto glass coverslips (Vergun et al., 2003b). FluoZin-3 signal was dim in unstimulated mitochondria (Figure 3.1A), but increased greatly upon addition of 30 μM $ZnCl_2$ to the perfusate (Figure 3.1B). We used the potentiometric probe TMRM in these same mitochondria to show that most were polarized at the outset as indicated by a bright TMRM signal (Figure 3.1C), but depolarized in response to Zn^{2+} (Figure 3.1D). A representative FluoZin-3 trace (Figure 3.1E) shows that an acute Zn^{2+} stimulus produced a rapid and robust increase in FluoZin-3 signal in mitochondria that was quickly reversed to baseline by the membrane-permeant heavy metal chelator TPEN (25 μM). As noted in materials and methods, TMRM was used transiently at the beginning of the experiment to allow pre-selection of healthy, polarized, FluoZin-3 loaded mitochondria. In a typical experiment, >90% of attached mitochondria were polarized; mitochondria depolarized at the outset were not selected for analysis.

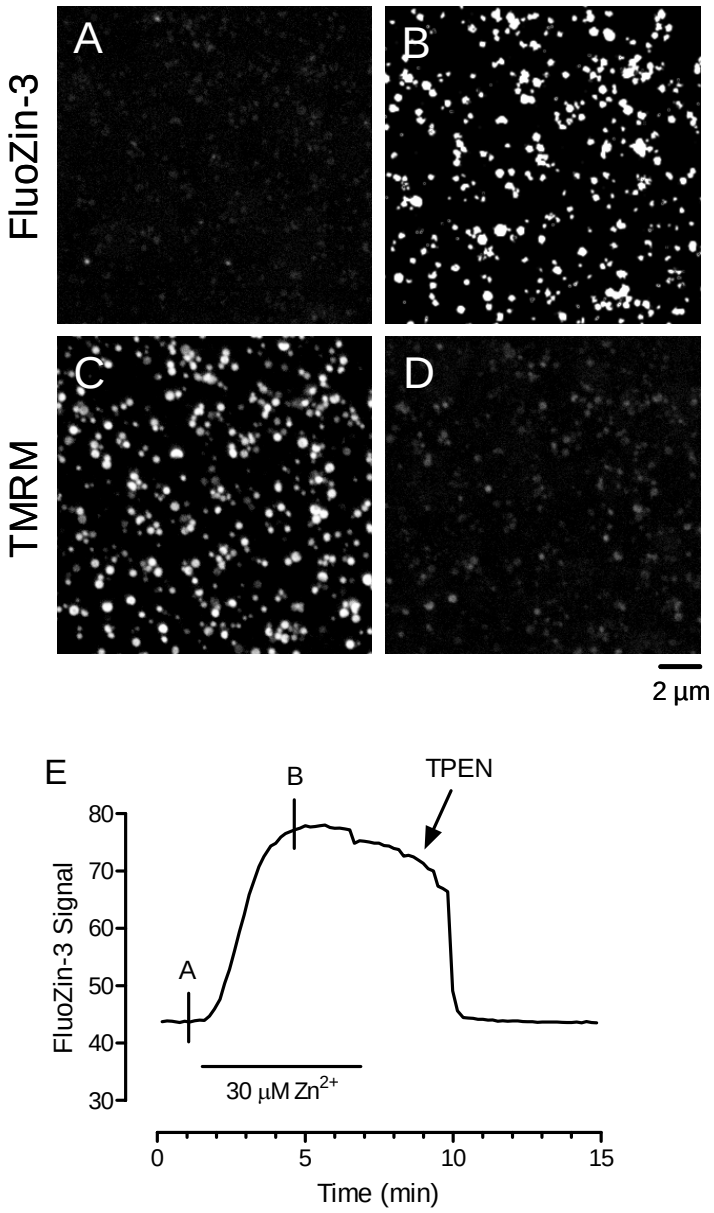


Figure 3.1 FluoZin-3 detects Zn^{2+} uptake in single mitochondria. Isolated mitochondria loaded with FluoZin-3 AM were perfused with 30 μM added $ZnCl_2$ to stimulate mitochondrial Zn^{2+} accumulation. FluoZin-3 images were captured before (A) and after (B) Zn^{2+} exposure. Corresponding TMRM images were taken before the start of the experiment (C) to select polarized mitochondria, and after Zn^{2+} exposure (D) to demonstrate Zn^{2+}-induced mitochondrial depolarization. A representative trace (E) shows that Zn^{2+} produces a rapid and robust increase in FluoZin-3 signal which is reversed by 25 μM TPEN. Data from (E) represent the mean ± S.E. from approximately 50-100 mitochondria within a given field.

FluoZin-3 detects matrix Zn^{2+} in a concentration-dependent manner

To determine the lowest concentration of Zn^{2+} that produces an increase in FluoZin-3 fluorescence, we perfused mitochondria with a range of added $ZnCl_2$ concentrations (0, 21, 25, 30 and 100 μM). Using the web-based program MAXCHELATOR (http://www.stanford.edu/~cpatton/maxc.html), we estimated approximate free Zn^{2+} concentrations of 0, 1, 5, 10, and 80 μM given the chelator present in the KCl buffer (20 μM EDTA) and the binding affinity of EDTA for Zn^{2+} ($K_D \sim 7.6 \times 10^{-14}$ M). This is likely an overestimation of free $[Zn^{2+}]$ as it only takes into consideration the added $[Zn^{2+}]$ and [EDTA], but not other buffering components in the medium. From this approximation, concentrations of Zn^{2+} from here on will be described as free Zn^{2+} values. As the representative traces in Figure 3.2A show, this range of free Zn^{2+} produced concentration-dependent FluoZin-3 responses, which reached maximum levels at 10 μM free Zn^{2+}. Perfusion with TPEN alone (25 μM) did not significantly decrease the basal signal, indicating that the endogenous mitochondrial free Zn^{2+} pool is small and contributes minimally to background fluorescence (Figure 3.2B).

We observed fluorescence increases in response to Zn^{2+} stimuli within distinct, single mitochondria. A recent study used membrane-impermeant Ca^{2+} indicators to detect free Ca^{2+} in the intermembrane space (Chalmers and Nicholls, 2003). To be convinced that FluoZin-3 was detecting matrix Zn^{2+} and not Zn^{2+} trapped in the intermembrane space, we incubated mitochondria with the membrane impermeant salt form of FluoZin-3 (Figure 3.2B) that would be expected to enter the intermembrane space. Exposure to 10 μM free Zn^{2+} did not increase fluorescence. This data suggests that FluoZin-3 AM is indeed detecting matrix free Zn^{2+}. To be certain that FluoZin-3 was selective for Zn^{2+} in

this context, we perfused dye-loaded mitochondria with 200 μM $CaCl_2$ (Figure 3.2B). Consistent with cell-free dye measurements, high Ca^{2+} caused no FluoZin-3 increase (Devinney et al., 2005). This additionally demonstrated that large Ca^{2+} loads did not elevate matrix free Zn^{2+}.

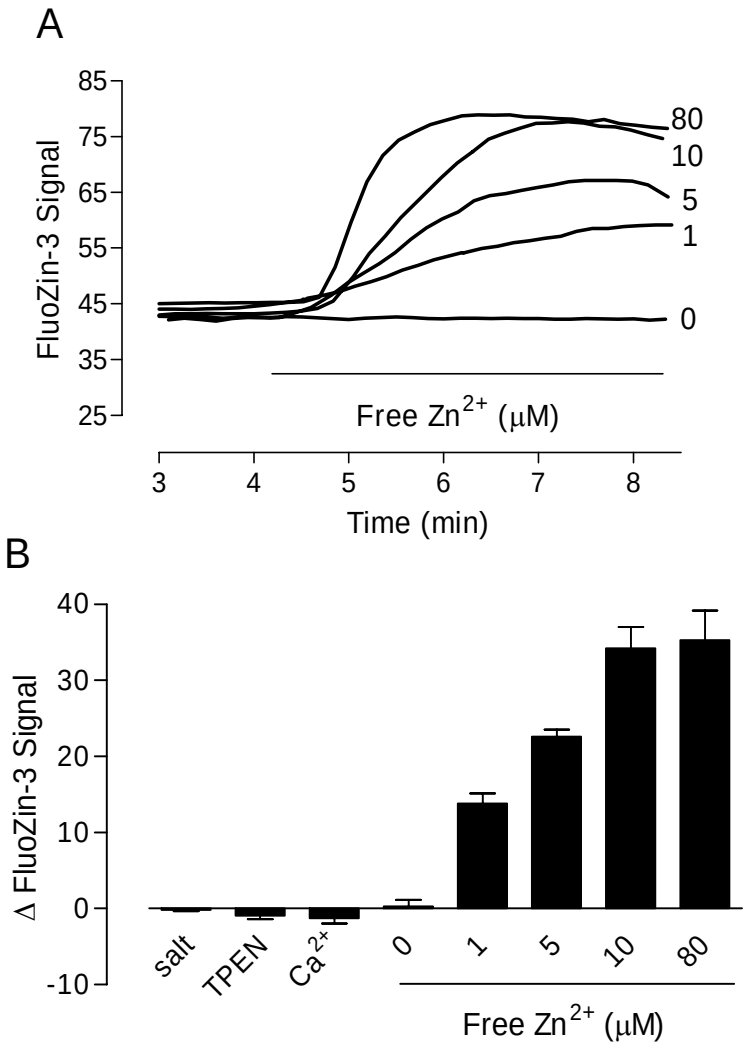


Figure 3.2 Mitochondria accumulate matrix Zn^{2+} in a concentration-dependent manner. (A) Representative traces from mitochondria exposed to increasing concentrations of added $ZnCl_2$ (0, 21, 25, 30 and 100 μM) display increasing FluoZin-3 responses. In considering the concentration of chelator present during experiments (20 μM EDTA), we have estimated free Zn^{2+} concentrations of 0, 1, 5, 10 and 80 μM, respectively. (B) Summary of FluoZin-3 responses indicate that the signal reaches maximal levels at approximately10 μM free Zn^{2+}. Controls: (1) mitochondria incubated with the membrane impermeant salt form of FluoZin-3 and perfused with 10 μM free $ZnCl_2$ ("salt"), (2) perfused with 25 μM TPEN alone ("TPEN"), and (3) perfused with 200 μM $CaCl_2$ alone ("Ca^{2+}"). Data from (A) represent the means ± S.E. from approximately 50-100 mitochondria within a given field. Data from (B) represent the mean ± S.E. of 4-6 experiments from at least three different mitochondrial preparations.

Loss of $\Delta\Psi_m$, uniporter blockade, or co-application of Ca^{2+} reduces mitochondrial Zn^{2+} uptake

Next, we examined if $\Delta\Psi_m$ drives Zn^{2+} uptake into mitochondria (Figure 3.3). Through TMRM fluorescence we have established that approximately 90% of attached mitochondria are polarized at the start of the experiment prior to addition of Zn^{2+} (Figure 3.1C), and only these mitochondria were selected for analysis. Mitochondria were depolarized prior to Zn^{2+} with a 5-pre-treatment with (*i*) the uncoupler FCCP (250 nM) or (*ii*) high Ca^{2+} (200 μM) (Figure 3.3A). Both of these maneuvers reduced Zn^{2+} uptake by as much as much as 80% suggesting that $\Delta\Psi_m$ drives matrix Zn^{2+} import.

Several studies suggested that mitochondrial Zn^{2+} uptake occurs through the Ca^{2+} uniporter (Brierley and Knight, 1967; Saris and Niva, 1994; Jiang et al., 2001). To determine if the Ca^{2+} uniporter may be a route for Zn^{2+} uptake, we applied the uniporter blocker ruthenium red (2 μM) prior to and during Zn^{2+} exposure in one set of experiments, and in another we co-applied high Ca^{2+} with Zn^{2+} (Figure 3.3B). Both maneuvers significantly reduced Zn^{2+} uptake, which is compatible with a uniporter mechanism that is responsible for large capacity Zn^{2+} uptake. However, it is important to note that in all these conditions Zn^{2+} still increased FluoZin-3 fluorescence. This suggests that some Zn^{2+} uptake occurs through a relatively low capacity, high affinity mechanism(s) independent of $\Delta\Psi_m$ and the uniporter.

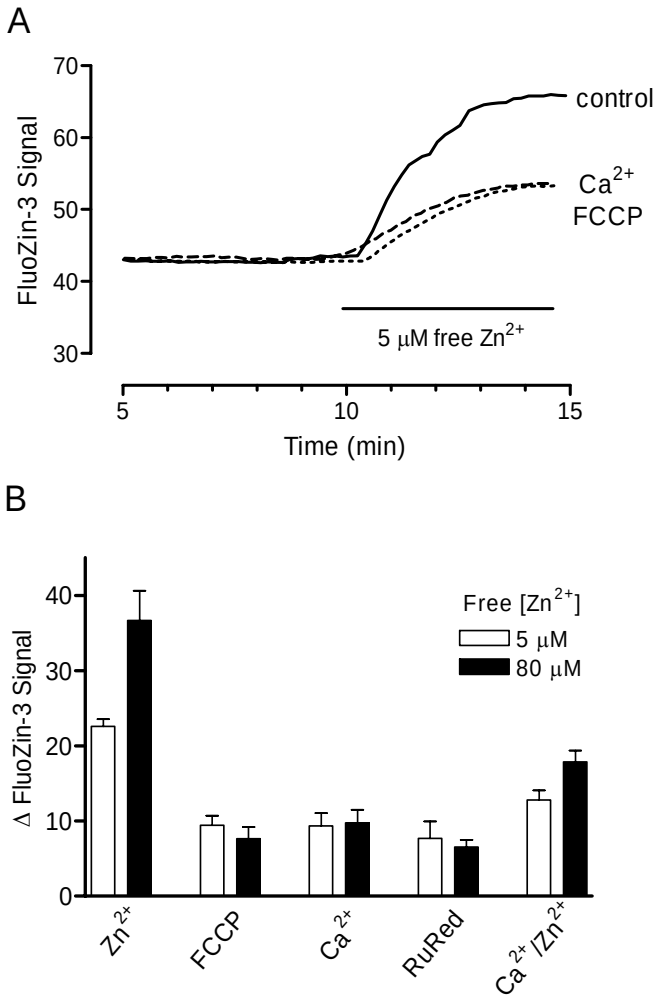


Figure 3.3 Mitochondrial Zn^{2+} uptake is partially mediated by the Ca^{2+} uniporter and depends on $\Delta\Psi_m$. (A) Representative traces of FluoZin-3 responses in mitochondria exposed to 5 μM free Zn^{2+} in the presence or absence of two depolarizing conditions: (i) 250 nM FCCP was perfused for an additional 5 minutes prior to co-perfusing 5 μM free Zn^{2+} or (ii) 200 μM $CaCl_2$ was perfused for 5 minutes prior to 5 μM free Zn^{2+}. (B) Summary graph of FluoZin-3 responses produced by 5 μM (open bars) or 80 μM (solid bars) free Zn^{2+}. These control (Zn^{2+} alone) values were compared to Zn^{2+} uptake after depolarization (with FCCP or high Ca^{2+}), treatment with the Ca^{2+} uniporter blocker ruthenium red (RuRed, 2 μM), or in the presence of competing Ca^{2+} (200 μM). Data from (A) represent the mean ± S.E. from approximately 50-100 mitochondria within a given field. Data from (B) represent the mean ± S.E. of 4-6 experiments from at least three different mitochondrial preparations. All treatments were significantly different when compared to both control (Zn^{2+} alone) treatments. $p < 0.0001$ for one-way ANOVA.

Uniporter blockade partially protects against Zn^{2+}-induced depolarization

Our previous observations suggest that Zn^{2+} depolarizes isolated mitochondria in suspension, which is partially protected by blocking the Ca^{2+} uniporter with ruthenium red (Dineley et al., 2005). However, this partial protection is overcome with higher Zn^{2+} concentrations, suggesting that Zn^{2+}-induced depolarization does not require Zn^{2+} entry. Here, we were able to distinguish between intra- and extramitochondrial Zn^{2+}-induced depolarizations using the potentiometric dye Rhodamine 123 (Rh123) (Figure 3.4).

As demonstrated in Figure 3.4A, 10 μM free Zn^{2+} partially depolarized mitochondria, as indicated by decreased Rh123 fluorescence. It is important to note that these mean traces represent incomplete depolarizations throughout the entire mitochondria population, as opposed to an effect that produces two distinct subsets, i.e. one completely depolarized and another fully polarized. Depolarization by 10 μM free Zn^{2+} (~50% decrease in fluorescence) was partially blocked by ruthenium red. In Figure 3.4B, however, mitochondria exposed to higher free Zn^{2+} (80 μM) depolarized to a greater extent (~80% decrease in fluorescence) and were not protected with ruthenium red. These findings are consistent with the suggestion that the predominant effect of Zn^{2+} on $\Delta\Psi_m$ is mediated by a site outside of the mitochondrial matrix.

In panel 3.4D, we show Ca^{2+}-induced depolarization, which was completely blocked with ruthenium red. While the data serves as a positive control supporting the feasibility of this particular approach, it also reveals interesting differences between the effects of Zn^{2+} and Ca^{2+}: depolarization by high Zn^{2+} was slower, partially reversible, and insensitive to ruthenium red, whereas depolarization by high Ca^{2+} during the brief

time frame was immediate, not rapidly reversible and completely sensitive to ruthenium red.

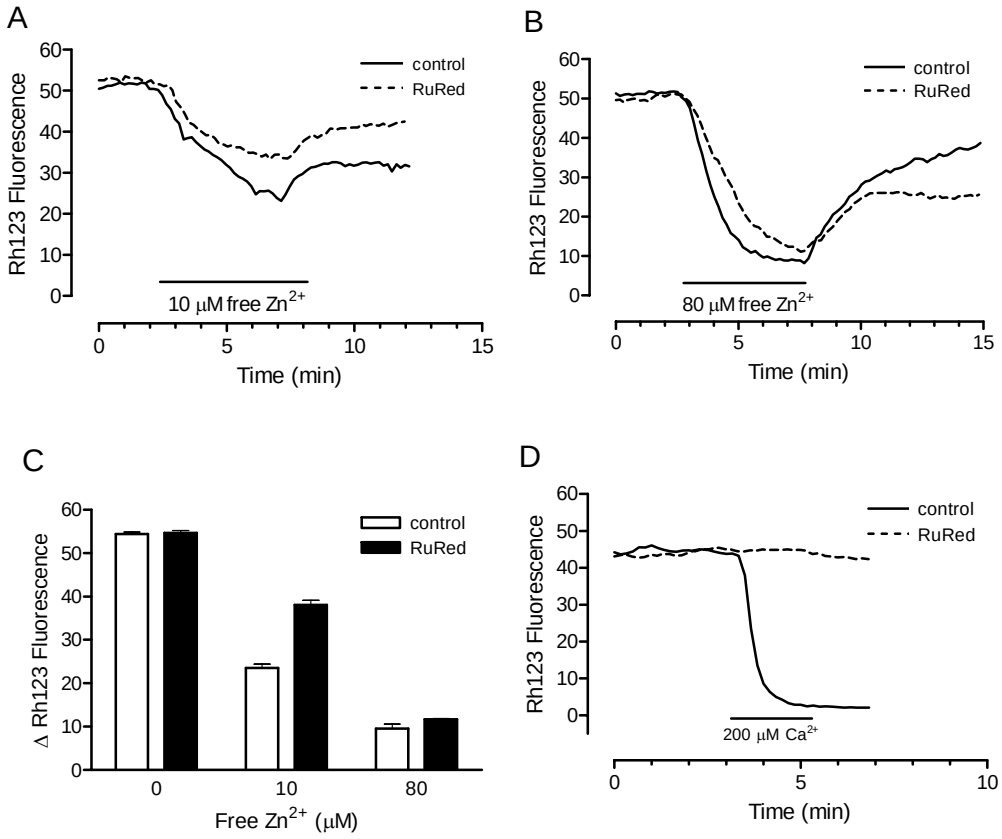


Figure 3.4 Ruthenium red partially protects against Zn^{2+}-induced mitochondrial depolarization. Using the $\Delta\Psi_m$-sensitive dye Rhodamine 123 (Rh123), the extent of depolarization (determined by decreased fluorescence) was measured in mitochondria perfused with two different Zn^{2+} concentrations. (A) Representative trace of mitochondria exposed to 10 μM free Zn^{2+} depolarized (solid line), and was partially blocked by ruthenium red (RuRed, 2 μM). (B) Representative traces showing mitochondria exposed to 80 μM free Zn^{2+} fully depolarized (solid line) and are not protected by ruthenium red (dotted line). (C) Graph summarizing mitochondrial depolarization with 0, 10 or 80 μM free Zn^{2+} without (open bars) or with (closed bars) ruthenium red. (D) Positive control to demonstrate that mitochondria rapidly depolarized in the presence of 200 μM Ca^{2+} (solid line), which is completely inhibited in the presence of ruthenium red (dotted line). Data from (A), (B), and (D) represent the mean ± S.E.

from approximately 50-100 mitochondria within a given field. Data from (C) represent the mean ± S.E. of 4-6 experiments from at least three different mitochondrial preparations.

Zn^{2+}-induced FluoZin-3 fluorescence slowly decreases over time

Following Zn^{2+} treatment, the FluoZin-3 signal did not remain elevated and stable, but instead declined slowly over several minutes (Figure 3.5A). To rule out dye leakage or photobleaching, we did control experiments where mitochondria were given two pulses of Zn^{2+} separated by ~ 5 minutes (Figure 3.5B). The second pulse of 80 μM free Zn^{2+} did not re-elevate fluorescence levels, which is unsurprising given that mitochondria were depolarized by the first pulse (as in Figure 3.4B). However, when the ionophore sodium pyrithione was used to deliver the second pulse, maximum fluorescence was roughly equal to that achieved by the first pulse (Figure 3.5C). These data suggest that there is no substantial FluoZin-3 leakage or photobleaching in this paradigm.

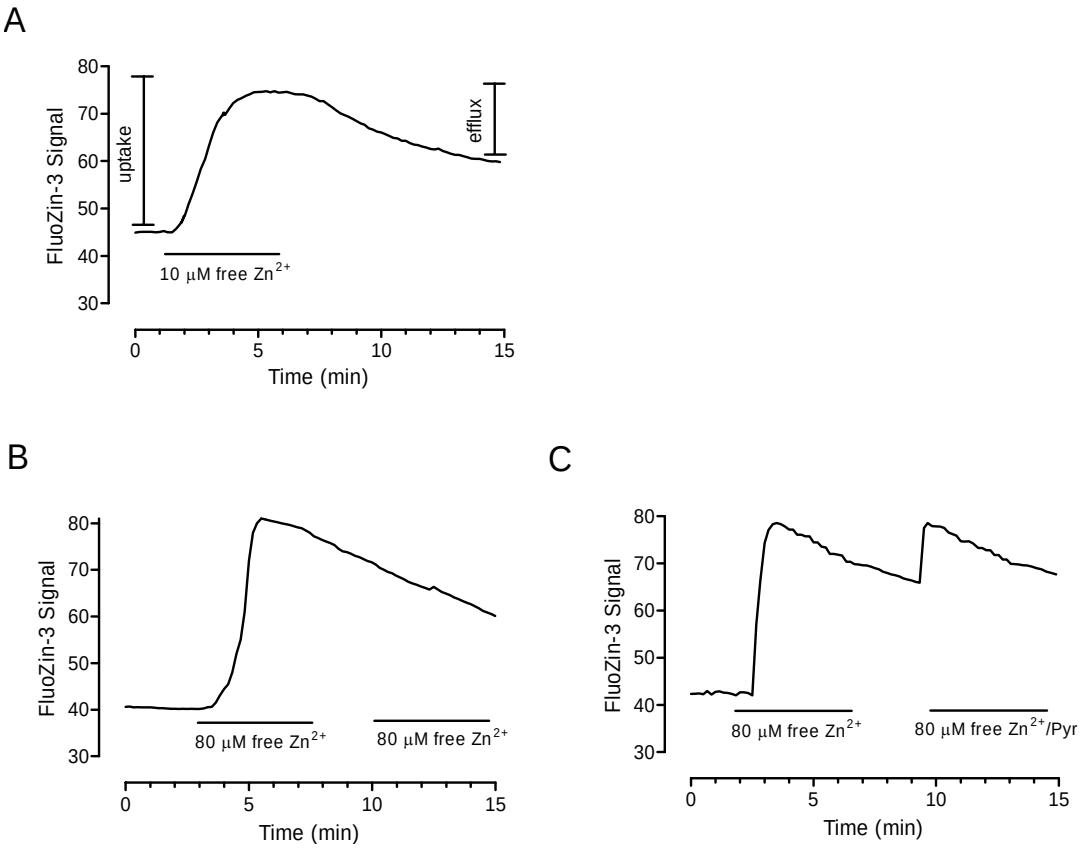


Figure 3.5 FluoZin-3 fluorescence slowly decreases over time, but is not a result of dye leakage or photobleaching. (A) Representative trace of mitochondria perfused with 10 μM free Zn^{2+} followed by 10-washout period with KCl buffer demonstrates slow decline of FluoZin-3 fluorescence over minutes. For summary data, uptake was calculated as the difference from baseline to maximum fluorescence, while efflux was calculated as the difference between maximal fluorescence to fluorescence values at the end of the washout period. (B) To determine if decreasing signal resulted from dye leakage or photobleaching, mitochondria were challenged with consecutive free Zn^{2+} (80 μM) stimuli separated by 2-3 minutes. (C) When the second pulse was delivered in the presence of sodium pyrithione, FluoZin-3 change was equivalent to the first response, arguing that dye leakage from mitochondria is not contributing to the decreasing signal. Data represent mean traces from approximately 50-100 mitochondria within a given field and are representative of 3-4 additional experiments.

Slow decline of matrix Zn^{2+} is unaffected by phosphate removal, decreased matrix pH, or uniporter blockade

A second possibility for the declining signal may be Zn^{2+}-phosphate precipitatation within the matrix, which would decrease the amount of Zn^{2+} available to bind FluoZin-3. In previous studies of mitochondrial Ca^{2+} homeostasis, acetate was substituted for buffer phosphate as a means of decreasing Ca^{2+}-phosphate precipitation in the matrix (Zoccarato and Nicholls, 1982; Chalmers and Nicholls, 2003). We used the same maneuver here to determine if phosphate depletion would affect the FluoZin-3 recovery. However, neither the apparent uptake nor removal of Zn^{2+} was altered when phosphate was replaced with acetate (Figure 3.6A). Furthermore, matrix acidification with FCCP, which should promote the dissolution of metal-phosphate complexes, also did not alter FluoZin-3 recovery (Figure 3.6B). Taken together, these data suggest that phosphate does not significantly participate in controlling matrix Zn^{2+}.

Lastly, we tested for uniporter-mediated Zn^{2+} efflux by following the Zn^{2+} stimulus with 2 μM ruthenium red (Figure 3.6C). Ruthenium red did not affect the rate of signal decline, arguing against uniporter-mediated Zn^{2+} efflux. The only effective method for significantly altering the Zn^{2+} efflux was with TPEN (25 μM). This reversal in signal depends on membrane permeation, as the impermeant chelator EDTA did not accelerate Zn^{2+} removal compared to control (Figures 3.6B and 3.6C).

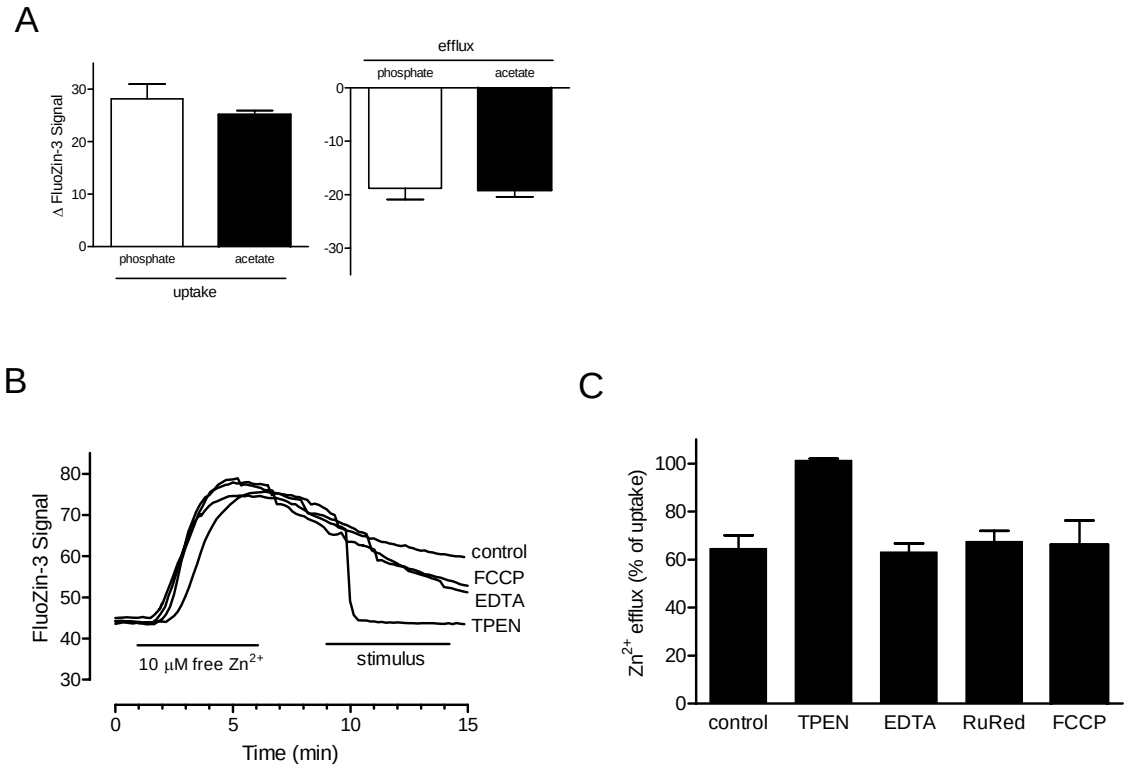


Figure 3.6 Matrix Zn^{2+} declines slowly and is unaffected by phosphate removal, matrix acidification or uniporter blockade. (A) Summary graph of experiments as shown in Figure 5A compare the uptake and efflux changes in mitochondria stimulated with Zn^{2+} in either phosphate buffer or phosphate-free (acetate) buffer. (B) Representative traces of mitochondria exposed to 10 μM free Zn^{2+} followed by washout (control), 250 nM FCCP, 100 μM EDTA or 25 μM TPEN. (C) Summary graph of mitochondria exposed to 10 μM free Zn^{2+} followed by washout, TPEN, or FCCP as shown in (B). Similar experiments were done with Zn^{2+} exposure followed by 100 μM EDTA or 2 μM ruthenium red. Only TPEN was significantly different from washout with control buffer. $p < 0.0015$ by one-way ANOVA. Data from (B) represent the mean ± S.E. from approximately 50-100 mitochondria within a given field. Data from (A) and (C) represent the mean ± S.E. of 4-6 experiments from at least three different mitochondrial preparations.

Strong oxidant exposure does not increase matrix Zn^{2+}

Previous reports suggested that mitochondria possess oxidant-sensitive sources of Zn^{2+} (Sensi et al., 2003b), including metallothionein (Ye et al., 2001), and Zn^{2+}-sensitive fluorophores have been used to detect oxidant-mobilized free Zn^{2+} in cells (Aizenman et al., 2000). We used FluoZin-3 here to determine if mitochondria possess oxidant labile Zn^{2+}. However, mitochondria treated with high concentrations of either 2,2'-DTDP (300 μM) or H_2O_2 (1 mM) showed no fluorescence increase (Figure 3.7). Additionally, 2,2'-DTDP did not further elevate FluoZin-3 fluorescence in Zn^{2+}-loaded mitochondria (data not shown). Together, these results suggest that the pool of oxidant-sensitive Zn^{2+} binding proteins in the mitochondrial matrix is minimal.

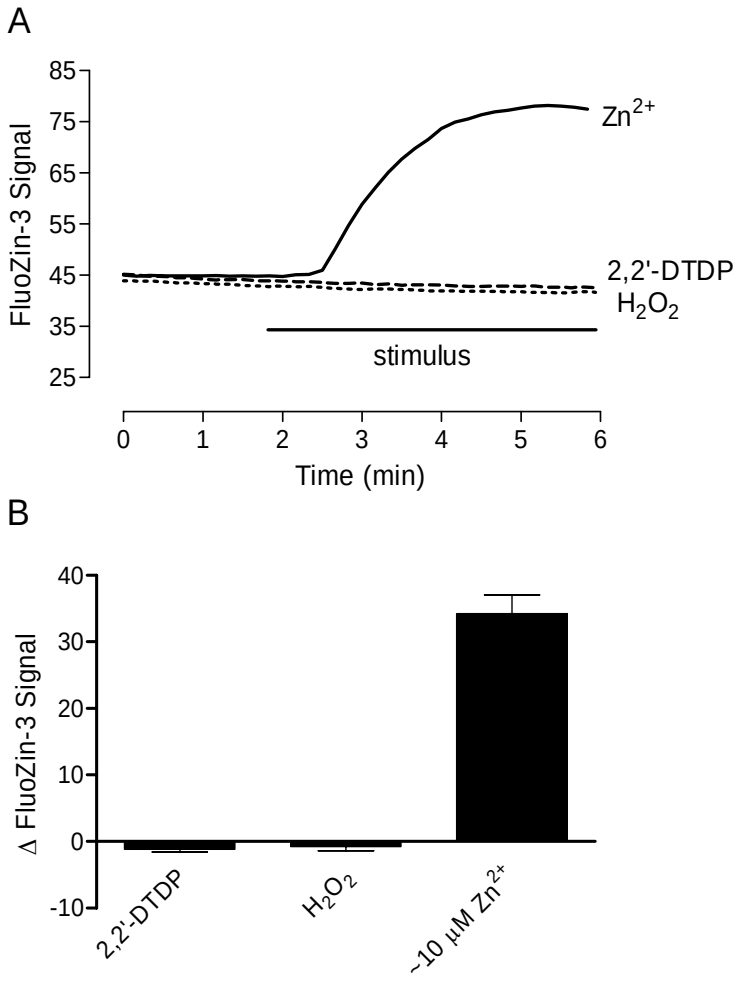


Figure 3.7 Strong oxidation does not increase matrix free Zn^{2+}. (A) Representative mean traces of mitochondria exposed to 10 μM free Zn^{2+}, 300 μM 2,2'-DTDP, or 1 mM H_2O_2. (B) Summary data show that the oxidative stimuli did not increase FluoZin-3 fluorescence. Data from (A) represent the mean ± S.E. from approximately 50-100 mitochondria within a given field. Data from (B) represent the mean ± S.E. of 4-6 experiments from at least three different mitochondrial preparations.

3.5 Discussion

Advantages of experimental system for studying Zn^{2+} transport

In this study we provide direct evidence for mitochondrial Zn^{2+} uptake. FluoZin-3-loaded mitochondria allowed the detection of robust and reproducible increases in free Zn^{2+} within the mitochondrial matrix. The ability to perfuse solutions directly onto adherent mitochondria is an advantage over previous reports using isolated mitochondrial suspensions, because solution exchange is feasible. In addition, the use of superfused mitochondria eliminates contributions of extra-matrix dye to the overall dye signal, which would otherwise be hard to avoid. Our prior study shows that the preparation is bioenergetically functional and fully capable of maintaining a membrane potential over the duration of these experiments (Vergun et al., 2003b). Thus, isolated attached mitochondria provide a useful model system in which to characterize Zn^{2+} transport. Our mitochondrial preparation probably generates a mixture of both neuronal and glial mitochondria. Unfortunately it is currently not feasible to separate or distinguish between neuronal and non-neuronal mitochondria from a whole-brain preparation. However, our observations show that mitochondria respond uniformly to the stimuli used here. The absence of any subpopulations suggests that neural mitochondria, regardless of cellular origin, are more similar than different with respect to zinc handling mechanisms.

Uniporter-dependent and –independent mechanisms for mitochondrial Zn^{2+} uptake

There appear to be two mechanisms of Zn^{2+} accumulation, one of which appears to be the calcium uniporter while the other is a novel pathway. Mitochondrial Zn^{2+}

uptake occurs partially via a $\Delta\Psi_m$- and Ca^{2+} uniporter-sensitive pathway because the accumulation of Zn^{2+} was partially inhibited by ruthenium red and by mitochondrial depolarization with FCCP. This is consistent with previous reports that Zn^{2+} uptake is mediated by the uniporter in isolated bovine heart and rat liver mitochondria. Brierley and Knight (1967) reported that Zn^{2+} uptake could be detected in bovine heart mitochondria using atomic absorption at supraphysiological levels and only in the absence of other permeant ions. Saris and Niva (1994) demonstrated that Zn^{2+}-induced mitochondrial swelling in rat liver was inhibited by ruthenium red, which implies a uniporter-mediated Zn^{2+} accumulation. However, these studies used considerably higher concentrations of Zn^{2+}, which are likely to greatly exceed free Zn^{2+} concentrations found within cells under any reasonable circumstance. On the contrary, between 20 and 40% of the accumulation of Zn^{2+} was insensitive to uniporter inhibition, uncoupling or the addition of Ca^{2+} (Figure 3.3). This accumulation presumably occurs through a separate and as yet unidentified pathway. In the presence of ruthenium red or in the absence of a membrane potential, the amount of Zn^{2+} accumulated through this $\Delta\Psi_m$- and uniporter-independent pathway is the same in the presence of 5 or 80 μM free Zn^{2+}. This suggests that this mechanism is already saturated by Zn^{2+} at the lower concentration. However, the driving force for this Zn^{2+} accumulation remains unclear.

Identification of a novel mitochondrial Zn^{2+} efflux pathway

Along with Zn^{2+} import, this method is useful for monitoring Zn^{2+} efflux. These studies reveal that Zn^{2+} efflux occurs in a uniporter-independent fashion. It is notable, too, that neither the accumulation nor efflux of Zn^{2+} is altered when phosphate was

replaced by acetate in the superfusion solution. Additionally, FCCP did not affect efflux. When calcium is transported by mitochondria it accumulates in an insoluble phosphate precipitate (Zoccarato and Nicholls, 1982). Addition of uncouplers alters the pH of the matrix allowing solubilization of the precipitate and subsequent efflux of calcium (Nicholls and Chalmers, 2004). The absence of effect of phosphate substitution or FCCP on efflux rate argues that Zn^{2+} does not form insoluble phosphate salts after accumulation. Since Zn^{2+} is a critical ion necessary for many cellular processes, cells have evolved specific membrane-bound Zn^{2+} transport proteins to supply free Zn^{2+} to the various subcellular compartments. Evidence accumulated to date has identified at least 14 specific Zn^{2+} transporters in mammalian cells that are localized to the plasma membrane and intracellular vesicles (Liuzzi and Cousins, 2004). Thus far there is no evidence that any member of this family is associated with mitochondria, and a recent report on the human heart mitochondrial proteome (Taylor et al., 2003) did not identify any of the known Zn^{2+} transporters. Thus, the mechanism of Zn^{2+} efflux remains unclear.

Are there oxidant-labile intramitochondrial Zn^{2+} pools ?

Approximately 80% of intracellular Zn^{2+} is thought to be protein bound, and it plausible that Zn^{2+}-protein complexes may regulate cellular and subcellular Zn^{2+} homeostasis (Frederickson, 1989). Ye *et al.* demonstrated that isolated liver mitochondria import exogenously added metallothionein, raising the possibility that metalloproteins can regulate respiration by controlling Zn^{2+} availability (Ye et al., 2001). Unfortunately this intriguing hypothesis is not yet supported by studies in intact cells. It is known that mice lacking MT-I and -II show no bioenergetic abnormalities (Palmiter,

1998), and no MT isoforms were identified in the heart mitochondrion proteome (Taylor et al., 2003). In any case, we were unable to detect oxidant-labile zinc in the preparation used in this study. Ostensibly then our data argues that mitochondria contribute little to the oxidant-induced $[Zn^{2+}]_i$ loads observed in several different cell types (Cuajungco and Lees, 1998; Aizenman et al., 2000; St. Croix et al., 2002; Bossy-Wetzel et al., 2004).

The ability to monitor subcellular zinc transport in intact cells would present obvious advantages, and in fact this work was preceded by a number of reports claiming evidence for mitochondrial zinc transport in cultured neurons (Sensi et al., 2000; Sensi et al., 2003b; Sensi et al., 2003a; Bossy-Wetzel et al., 2004). These studies relied solely on live cell fluorescent indicator that purportedly localize to mitochondria. The approach however is fraught with a number of confounds including dye labeling of subcellular structures inconsistent with mitochondrial morphology, labeling that does not or only partially co-localizes with other established mitochondrial markers, and dye responses to zinc that occur not just within mitochondria, but throughout the entire cell body. Given these complications, it is premature to conclude that mitochondria have a substantial role in accumulating or releasing zinc in the intact cell, and definitive proof of such requires the development of a zinc sensor that is unambiguously specific for mitochondria.

Zn^{2+} concentrations allow the study of different effects in cells vs. in mitochondria

Studies of Zn^{2+} transport by brain mitochondria are relevant to the role of Zn^{2+} in neuronal injury. As a neurotoxin, Zn^{2+} appears to have an important impact on neuronal energy metabolism (Choi and Koh, 1998; Koh, 2001; Dineley et al., 2003; Sensi and Jeng, 2004), and this appears to be due at least partly to an interaction with mitochondria.

However, it is much less clear whether the impact of Zn^{2+} on mitochondrial function depends on Zn^{2+} accumulation into the matrix, or whether actions of Zn^{2+} on the exterior of the mitochondrion could suffice. The present studies clearly reveal evidence for the ability of Zn^{2+} to impair mitochondrial function. Thus, Zn^{2+} diminishes $\Delta\Psi_m$ quite effectively (Figure 3.4), an effect that is partly sensitive to ruthenium red at low ion concentrations. This impairment of $\Delta\Psi_m$ can then account for the failure of mitochondria to accumulate Zn^{2+} from a second exposure (Figure 3.5), even though the dye is clearly capable of reporting more Zn^{2+} under these circumstances. The limited sensitivity of the mitochondrial depolarization to ruthenium red suggests that $\Delta\Psi_m$ loss is due to an external effect of Zn^{2+}. This conclusion is consistent with other findings showing that Zn^{2+} mediated mitochondrial impairment can be reversed by membrane-impermeant chelators (Dineley et al., 2005). However, this argues against the notion that mitochondrial transport of Zn^{2+} is necessary for neurotoxicity (Jiang et al., 2001; Sensi et al., 2003b). The relevance of this transport phenomenon may also depend on the concentrations of cytoplasmic Zn^{2+} that are achieved during injury to neurons, a parameter that is difficult to determine quantitatively (Dineley et al., 2002). Although mitochondrial depolarization can clearly occur as the result of Zn^{2+} exposure (Sensi et al., 2003b), it is clearly also possible to kill neurons without causing overt mitochondrial dysfunction (Dineley et al., 2000). It seems likely that there are multiple mechanisms of Zn^{2+} -mediated injury that can be expressed according to the magnitude of the intracellular Zn^{2+} load. Whether mitochondrial depolarization and bioenergetic failure represent the primary effects of Zn^{2+} at the lowest concentrations remain to be determined.

Chapter 4. Zinc inhibits mitochondrial movement in neurons by PI 3-kinase activation

4.1 Abstract

Mitochondria have been identified as targets of the neurotoxic actions of zinc, possibly through decreased mitochondrial energy production and increased reactive oxygen species accumulation. It has been hypothesized that impairment of mitochondrial trafficking may be a mechanism of neuronal injury. Here, we report that elevated intraneuronal zinc impairs mitochondrial trafficking. At concentrations just sufficient to cause injury, zinc rapidly inhibited mitochondrial movement without altering morphology. Zinc chelation initially restored movement, but the actions of zinc became insensitive to chelator in less than 10 minutes. A search for downstream signaling events revealed that inhibitors of PI 3-kinase prevented this zinc effect on movement. Moreover, transient inhibition of PI 3-kinase afforded neuroprotection against zinc-mediated toxicity. These data illustrate a novel mechanism that regulates mitochondrial trafficking in neurons, and also suggests that mitochondrial trafficking may be closely coupled to neuronal viability.

4.2 Introduction

Neural zinc is a highly regulated ion, much of which is tightly coordinated to proteins, leaving a small pool of zinc that remains free and mobile (Frederickson, 1989). Elevated intracellular free zinc ($[Zn^{2+}]_i$) results from entry through several Ca^{2+}-permeable pathways (Weiss et al., 1993; Koh and Choi, 1994; Yin and Weiss, 1995; Sensi et al., 1997; Cheng and Reynolds, 1998) and also from oxidant-mediated release from intracellular stores (Aizenman et al., 2000). Elevated $[Zn^{2+}]_i$ results in neuronal injury *in vitro* (Choi et al., 1988; Koh and Choi, 1994), and it has been suggested that its accumulation may contribute to neurodegeneration associated with ischemia (Koh et al., 1996; Lee et al., 2002), epileptic seizures (Frederickson et al., 1988), and head trauma (Suh et al., 2000).

Although an unambiguous mechanism for Zn^{2+}-mediated neurotoxicity has not been identified, several lines of evidence suggest mitochondria and energy metabolism as subcellular targets for the toxic actions of $[Zn^{2+}]_i$ (Dineley et al., 2003; Sensi and Jeng, 2004). Zinc can inhibit glycolysis (Sheline et al., 2000), the tricarboxylic acid cycle (Brown et al., 2000) and complexes in the electron transport chain (Skulachev et al., 1967; Kleiner, 1972; Kleiner, 1974; Link and von Jagow, 1995; Mills et al., 2002). It has also been shown that Zn^{2+} dissipates mitochondrial membrane potential ($\Delta\Psi_m$), decreases oxygen consumption, and enhances reactive oxygen species (ROS) accumulation (Dineley et al., 2005). However, it remains unclear which, if any, of these processes represent the critical target for the neurotoxic actions of zinc.

It has long been appreciated that mitochondria constantly move, divide and fuse throughout the cell (Bereiter-Hahn and Voth, 1994). It is reasonable to suggest that

mitochondrial trafficking in neurons serves to position mitochondria to deliver ATP to regions of high energy demand (van Blerkom, 1991) and to aid in the regulation of local Ca^{2+} homeostasis (Spira et al., 2001; Yi et al., 2004), although direct evidence that mitochondrial trafficking is driven by energy demand remains sparse. Nevertheless, recent studies suggest that interruption of trafficking is one consequence of excessive activation of N-methyl-D-aspartate (NMDA) receptors and the subsequent calcium entry (Rintoul et al., 2003). Other potential neurotoxins, such as nitric oxide (Rintoul *et al.* 2004, *SFN Abstract*) and ATP depletion (Rintoul et al., 2003) also inhibit mitochondrial movement, suggesting that impaired delivery of mitochondria could be an important contributor to neuronal injury.

In this report, we have investigated the effects of $[Zn^{2+}]_i$ on mitochondrial trafficking. Using a mitochondrially-targeted enhanced yellow fluorescent protein (mt-eYFP), we observed that movement is substantially diminished with ionophore-induced elevations of $[Zn^{2+}]_i$, that are just sufficient to cause injury. We further show that these actions of zinc are mediated by a signaling cascade that involves activation of PI 3-kinase, and that transient inhibition of PI 3-kinase prevents both the impairment of trafficking and zinc-induced neuronal injury.

4.3 Materials & Methods

Materials

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Cell Culture

All procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Primary cultures of rat forebrain neurons were prepared from embryonic Sprague Dawley rat pups (17 days in gestation) and grown in a 37˚C incubator containing 5% CO_2. Cortices were removed, trypsinized at 37˚C for 30 minutes, and plated on poly-D-lysine-coated 31 mm glass cover slips. Cells were plated in medium containing Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cultures contain less than 5% glia in total cell population. Five hours after initial plating, plating medium was completely removed and replaced with N2-NB media (Neurobasal media with 0.5% penicillin/streptomycin and 1% N2 supplement). Cells were re-fed with N2-NB media 4 days after plating, whereby approximately half of the volume was removed and replaced with an equal volume of fresh N2-NB media. Cells were fed 8 and 11 days after plating with B27-NB media (Neurobasal media with 0.5% penicillin/streptomycin and 2% B27 Supplement Minus AO) using the same feeding protocol described above. For preparation of the insulin-depleted cells, cultures were fed

only with B27-NB media beginning at 5 hours after initial plating. All experiments were performed on neurons after 13-15 days in culture.

Transfection using DNA constructs

The mitochondrial-targeted enhanced yellow fluorescent protein (mt-eYFP) construct (Llopis et al., 1998) consists of eYFP fused to the mitochondrial targeting sequence from subunit IV of human cytochrome *c* oxidase. The luciferase reporter plasmid consists of the luciferase gene placed in a mammalian expression vector under the control of a CMV promoter (Gossen and Bujard, 1992). After 10-12 days *in vitro*, neurons were transiently transfected using a modified calcium phosphate transfection technique (Xia et al., 1996). This procedure typically generates 1-2% efficiency; with maximal protein expression 24-72 hours post transfection.

Fluorescence microscopy

All experiments were performed using a Hepes-buffered salt solution (HBSS) of the following composition: 137 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 0.6 mM KH_2PO_4, 0.6 mM Na_2HPO_4, 0.9 mM $MgSO_4$, 1.4 mM $CaCl_2$, 20 mM Hepes and 5.5 mM glucose (pH adjusted to 7.4 with NaOH). Neurons were perfused for 5 ml/min during the course of each experiment and the chamber temperature was maintained at 37˚C with heated buffer. The PC-based system for data acquisition is as previously described (Dineley et al., 2002) using SimplePCI imaging software (Compix Inc, Cranberry, PA). Using a 40X water immersion objective, mitochondrial movement in a field containing a single cell were observed by illuminating at 490 nm and acquiring images every 6 seconds.

Following a brief 3 to 5-minute rinse in HBSS buffer, cells were perfused with the appropriate stimulus (diluted in buffer from stock concentrations).

For $[Zn^{2+}]_i$ measurements, we used the Zn^{2+}-sensitive fluorophore mag-fura-2-AM (Molecular Probes, Eugene, OR). Coverslips were incubated with 5 μM mag-fura-2 for 20 minutes at 37˚C. With constant perfusion at 10 ml/min, fluorescence was measure by illuminating the indicator alternatively at 335 and 375 nm. For $\Delta\Psi_m$ experiments, neurons were incubated with 5 μM Rhodamine 123 (Molecular Probes, Eugene, OR) for 10 minutes at 37 ˚C (Vergun et al., 2003a). Cells were excited at 490 nm and fluorescence was measured in 10-20 individual neurons for each coverslip. For nucleic acid staining, coverslips were incubated in HBSS containing 1.5 μM Hoechst-33342 for 15 minutes at room temperature (Dineley et al., 2000). Cells were illuminated at 340 nm and fluorescent images were obtained randomly from 3-6 cell-containing regions per coverslip.

Quantification of mitochondrial movement

Mitochondrial movement was analyzed using a macro-based analysis program as previously described (Rintoul et al., 2003). Briefly, a 255 x 255 pixel subfield containing mitochondria was selected where pixel images in successive images are subtracted. A movement event for each pixel was registered if the change in fluorescence between successive fields exceeds 20 fluorescence units and a quantitative measurement of movement was obtained by combining pixel events in a field over 2 minutes. Movement was represented as average event counts/ pixel, which are normalized to pre-stimulus movement values.

Toxicity

For the simplicity of assaying only cells that express mt-eYFP, we transfected neurons with the luciferase construct to measure neuronal injury. For toxicity experiments, coverslips of luciferase-transfected neurons were washed three times with HBSS. Reagents were diluted in HBSS at the desired concentration, and 1 ml of solution was applied to the coverslips for the desired time at room temperature. Stimuli were terminated by washing at least 3 times with excess HBSS. The wash buffer was then replaced with conditioned media and returned to the incubator. Twenty four hours later, cells were rinsed and lysed in PBS. Lysates were used to measure luciferase enzyme activity using the BriteLite Ultra-High Sensitivity Luminescence Reporter Gene Assay System (Perkin Elmer Life Sciences, Boston, MA) according to manufacturer's instructions. Arbitrary luminescence units were normalized to control, non-treated cells.

Western blotting

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 % Triton X-100, 5 mM EGTA, 20 μM Leupeptin, 1 mM AEBSF, 1 mM $NaVO_3$, 10 mM NaF, and 1 tablet of protease inhibitor). Protein concentration was determined by a micro-protein assay using the BCA protein assay kit according to manufacture's instruction (Pierce Biotechnology, Rockford, IL). Approximately 30 μg of lysates were mixed with equal volumes of 2 x SDS sample loading buffer (60 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, and 2% SDS) and size-fractionated by electrophoresis on 4-15% Tris-HCl Ready Gels (Bio-Rad Labs, Hercules, CA) at 30 mA for 1 hour followed by electrotransfer onto a nitrocellulose

membrane at 80 volts for 2 hours. The membrane was pre-blotted with 5% dry milk in PBS-Tween (1X PBS, 0.1% Tween 20) at room temperature for 1 hour. The blots were probed with rabbit antiserum raised against Akt, and phosphosphorylated-Akt (Cell Signaling Technology, Beverly, MA) antibodies. Goat anti-rabbit IgG coupled to horseradish peroxidase (1:1000, Pierce Biotechnology, Rockford, IL), was used as the secondary antibodies. Bands were visualized by the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL).

Statistical Analyses

Statistical analysis was performed using Prism 4.01 (Graph Pad Software, San Diego CA). All the data are presented as mean ± S.E. consisting of 4-6 experiments for each condition from at least three different cell culture preparations. Comparisons were made using Student's t test and one-way ANOVA with Dunnett post test to compare all conditions to control, with p values of less than 0.05 regarded as significant.

4.4 Results

Concentrations of Zn^{2+} that inhibit mitochondrial movement parallel Zn^{2+} concentrations that are neurotoxic.

We fluorescently labeled neuronal mitochondria using a mitochondrially-targeted enhanced yellow fluorescent protein (mt-eYFP). The ionophore sodium pyrithione was used to increase $[Zn^{2+}]_i$ (Dineley et al., 2000). As shown in Figure 4.1a, buffer, sodium pyrithione alone (20 μM), or 0.3 μM Zn^{2+}/Pyr did not affect mitochondrial movement.

However, higher concentrations of Zn^{2+} significantly inhibited mitochondrial movement, with concentrations as low as 1 μM resulting in a significant decrease in movement. Notably, this stimulus consistently stopped movement of all mitochondria in a given field and did not preferentially alter the movement of retrogradely vs. anterogradely moving mitochondria. This Zn^{2+}-mediated effect was irreversible up to 20 minutes after the stimulus was washed out. Interestingly, this concentration-dependent movement inhibition corresponded to the concentration-dependence of Zn^{2+} toxicity (inset).

To establish that this was not a Ca^{2+}-induced phenomenon, we perfused cells with Zn^{2+}/Pyr under Ca^{2+} free conditions to confirm that the stimulus was still effectively inhibiting movement (Figure 4.1b). There is evidence that under depolarizing conditions, cellular Zn^{2+} entry occurs through plasma membrane Ca^{2+}-permeable pathways (Sensi et al., 1999). As a more physiological approach to elevate $[Zn^{2+}]_i$, we observed that mitochondrial movement was significantly inhibited with elevated Zn^{2+} (300 μM) in the presence of high K^+ buffer (Figure 4.1b). Evidence that exposure to strong oxidizing agents can generate $[Zn^{2+}]_i$ fluxes in neurons is indicative of Zn^{2+} release from cytosolic reservoirs, including metallothionein (Aizenman et al., 2000). Application of the sulfhydryl oxidizing agent, 2,2'-DTDP (10 μM) significantly inhibited mitochondrial movement, suggesting that $[Zn^{2+}]_i$ release in addition to Zn^{2+} entry is sufficient to induce the effect (Figure 4.1b). Our previous study showed that Ca^{2+}-induced cessation of movement was also associated with a substantial change in mitochondrial morphology (Rintoul et al., 2003). However, the representative fluorescent images taken before (Figure 4.1c) and after (Figure 4.1d) Zn^{2+} exposure demonstrated that there are no gross morphological changes that occur with Zn^{2+} exposure.

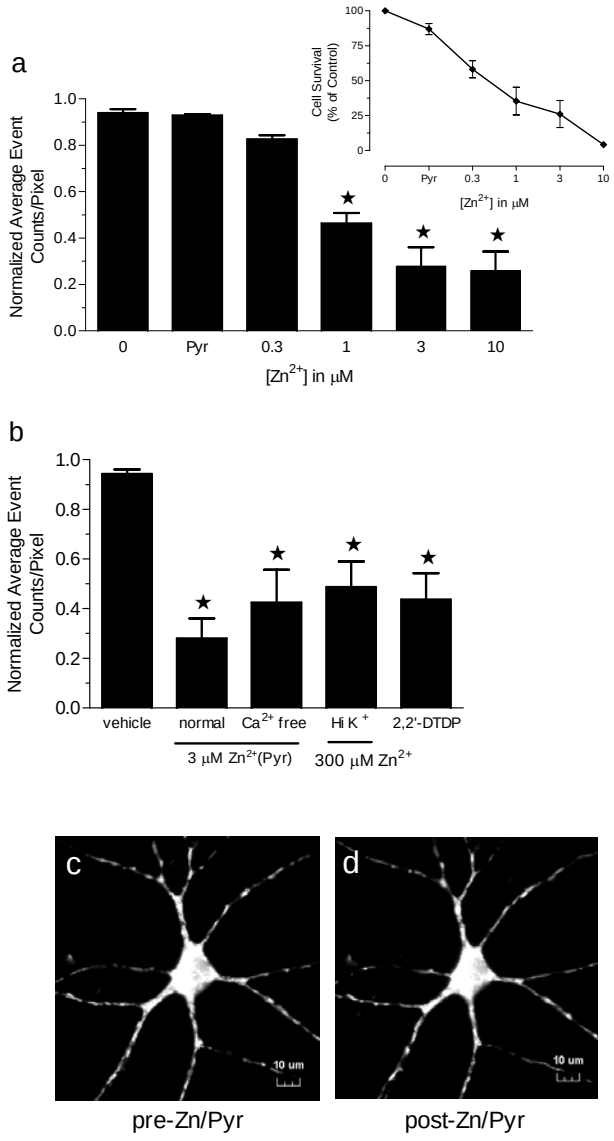


Figure 4.1 $[Zn^{2+}]_i$ inhibits mitochondrial movement at cytotoxic concentrations. (a) Neurons expressing mito-eYFP were perfused with buffer, pyrithione (20 μM) alone or in the presence of a range of [$ZnCl_2$] (0.3-10 μM) for 10 minutes. Bars represent mitochondrial movement measured at the end of the stimulus. Inset demonstrates that neurons undergo Zn^{2+}-mediated toxicity at parallel concentrations that affect mitochondrial movement. (b) Mitochondrial movement is inhibited when neurons are perfused with (*i*) 3 μM Zn^{2+}(Pyr) in Ca^{2+}-free buffer, (*ii*) 300 μM Zn^{2+} in an elevated K^+ buffer, or (*iii*) 10 μM 2,2'-DTDP. Fluorescent images were taken prior to (c) and after (d) 3 μM Zn^{2+} (Pyr) to show that there are no gross morphological changes with acute Zn^{2+} treatment. Treatments were significantly different when compared to control (buffer alone). $p < 0.01$ for one-way ANOVA with Dunnett's post test.

Zn^{2+} inhibition of mitochondrial movement is partially sensitive to chelation.

To determine whether chelation can restore Zn^{2+}-mediated movement inhibition, we applied membrane-permeant heavy metal chelator TPEN (50 μM) immediately following Zn^{2+}/Pyr exposure (Figure 4.2a). Notably, TPEN partially restored movement when applied after 1 μM Zn^{2+}, but was ineffective at restoring movement when applied ten minutes after 3 μM Zn^{2+}. Additionally, Figure 4.2b shows that TPEN restored movement when applied after shorter exposures to 3 μM Zn^{2+}, but became ineffective after treatments longer than 6 minutes. We also observed that TPEN rescued neurons from injury when applied 5 minutes after Zn^{2+}/Pyr exposure, but did not protect against toxicity after a 10-Zn^{2+} treatment (Figure 4.2c and 4.2d). Together, these results indicate that the TPEN effects are concentration- and time sensitive-dependent. The insensitivity to TPEN after longer or larger Zn^{2+} exposures suggested the initiation of a signaling cascade, which is irreversible upon simple Zn^{2+} chelation and therefore becomes independent of Zn^{2+}.

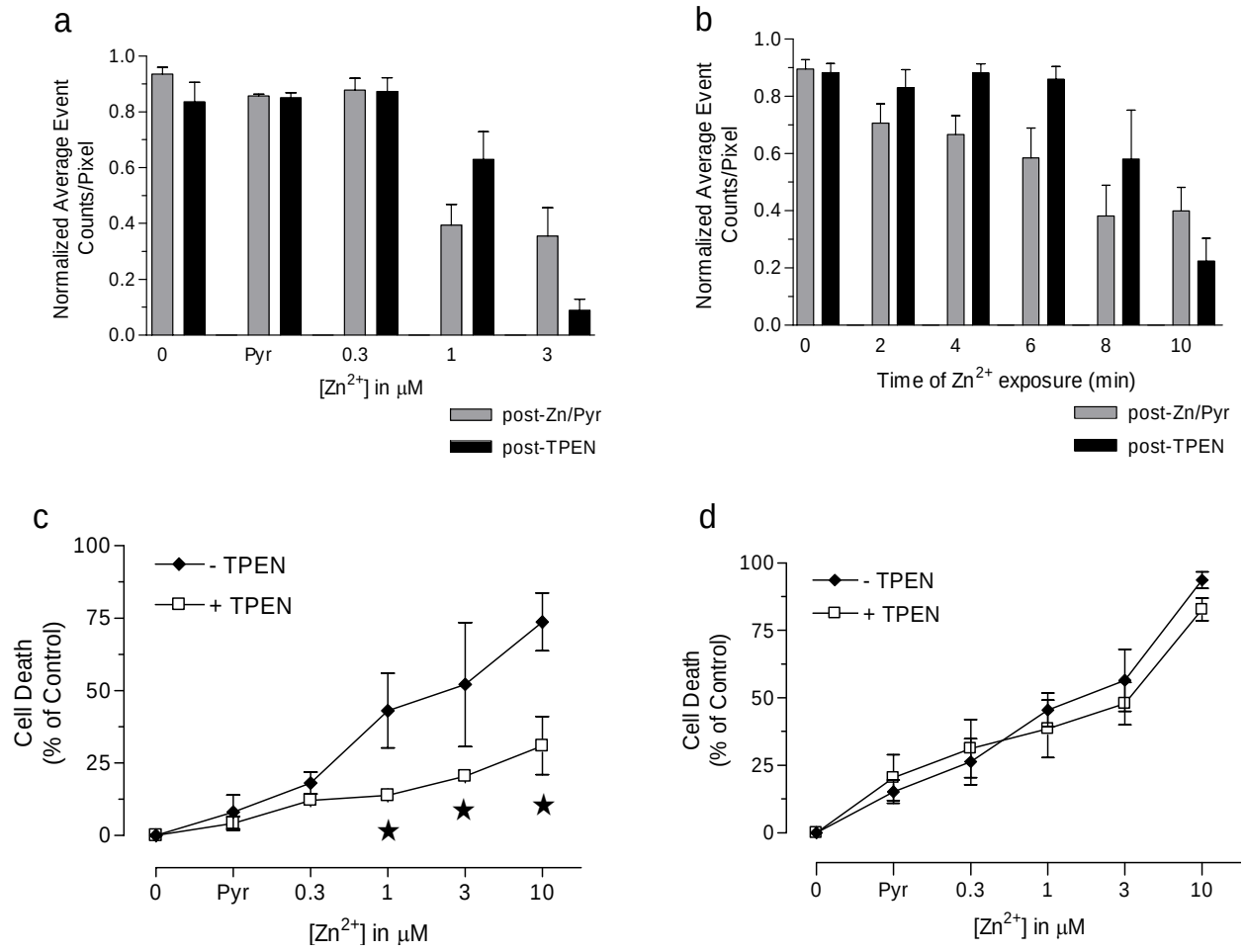


Figure 4.2 Zn^{2+} removal restores movement inhibition in a concentration- and time-sensitive manner. (a) Neurons were perfused with Zn^{2+}/Pyr (0-10 μM) for 10 minutes followed by 50 μM TPEN for 10 minutes. Mitochondrial movement was measured both after the Zn^{2+} (gray bars) and TPEN stimuli (black bars). (b) Neurons were perfused with 3 μM Zn^{2+}(Pyr) for varying times (0-10 minutes) followed immediately by TPEN. Zn^{2+}-induced toxicity was assayed in neurons exposed to 0-10 μM Zn^{2+} (Pyr) for 5 minutes (c) or 10 minutes (d) immediately followed by TPEN treatment. $p < 0.05$ by paired Student's t test.

Inhibitors of PI 3-kinase prevent Zn^{2+}-induced movement inhibition.

To identify the Zn^{2+}-activated signaling cascade involved in the movement effect, we investigated several agents that selectively inhibit protein kinases. Of all the compounds which we tested in our movement paradigm, only the PI 3-kinase inhibitors, wortmannin (1 μM) and LY294002 (30 μM), were effective in that they (*i*) did not affect mitochondrial movement by themselves, and (*ii*) blocked the Zn^{2+}-mediated effect at the concentration (3 μM Zn^{2+}/Pyr) and time (10 minutes) which was TPEN insensitive (Figure 4.3a). One concern was that perhaps wortmannin was preventing ionophore-induced $[Zn^{2+}]_i$ accumulation by blocking its cellular entry or chelating the $[Zn^{2+}]_i$ before it can mediate any effects on movement. We excluded this possibility by monitoring $[Zn^{2+}]_i$ in mag-fura-2-loaded neurons which were perfused with 3 μM Zn^{2+}/Pyr, either with or without wortmannin (1 μM) pretreatment (Figure 4.3b). Both conditions produced equal mag-fura-2 responses that were TPEN-reversible, confirming that wortmannin was not preventing entry or binding $[Zn^{2+}]_i$.

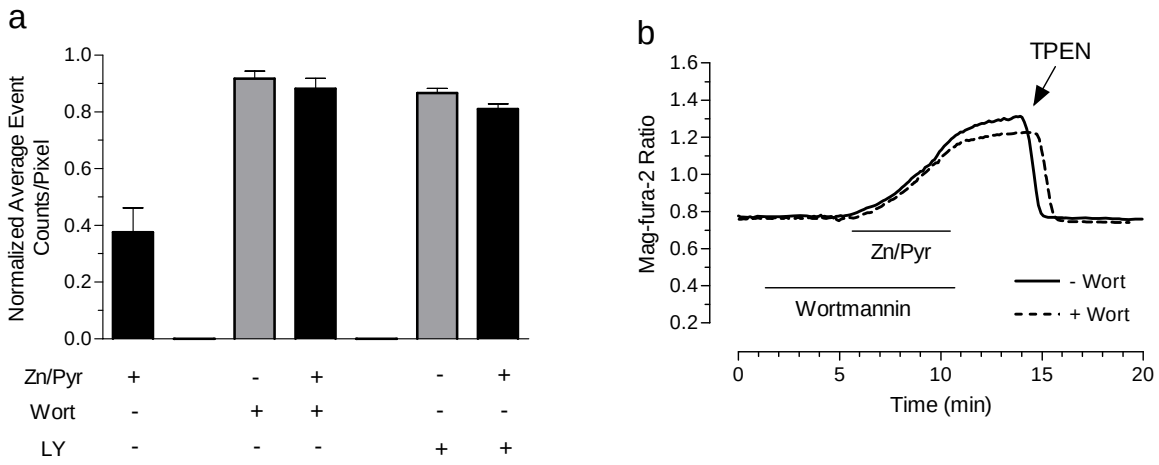


Figure 4.3 PI 3-kinase inhibitors prevent Zn^{2+}-induced movement inhibition. (a) Neurons were perfused with either wortmannin (1 μM) or LY294002 (30 μM) for 10 minutes prior to and during 3 μM Zn^{2+}(Pyr) treatment and mitochondrial movement was analyzed after application of inhibitors (gray bars) and Zn^{2+} in the presence of inhibitors (black bars). (b) Neurons loaded with mag-fura-2 were exposed to 3 μM Zn^{2+}(Pyr) for 5 minutes either without (solid line) or pre-treated with 1 μM wortmannin (dotted line). In both conditions, TPEN (50 μM) was applied after the Zn^{2+} stimulus.

Preventing Akt phosphorylation does not prevent Zn^{2+}-induced movement inhibition.

As a possible downstream effector of PI 3-kinase, we investigated the role of Akt phosphorylation in Zn^{2+}-induced movement inhibition. We monitored phosphorylated Akt levels in neurons treated with 3 μM Zn^{2+}/Pyr for 10 minutes, either in the presence or absence of wortmannin (1 μM) or a putative Akt inhibitor (1L-6-Hydroxymethyl-*chiro*-inositol-2-(R)-2-O-methyl-3-O-octadecykcarbonate) (10 μM; Calbiochem, San Diego, CA) (Figure 4.4a). This phosphatidylinositol ether analog has been reported to selectively inhibit Akt phosphorylation and prevent growth of HT-29, MCF, HeLa and PC-3 cancer cell lines. This novel class of Akt inhibitors selectively blocks Akt activation and

downstream substrates without affecting upstream kinases or other kinase pathways. We used insulin (100 nM) as a positive control in these experiments. As demonstrated in Figure 4.4a, both insulin and Zn^{2+} rapidly induced Akt phosphorylation within 10 minutes in a wortmannin-sensitive manner. Interestingly, treatment with Akt inhibitor alone increased phosphorylation from control levels, and this compound did not significantly reduce phosphorylation in neurons compared to either insulin or Zn^{2+} by themselves.

We also monitored the effects of these treatments on mitochondrial movement. As shown in Figure 4.4b, perfusion with 100 nM insulin for 10 minutes did not significantly decrease mitochondrial movement compared untreated cells, even though a ten-stimulus of 100 nM insulin is sufficient for Akt phosphorylation in primary neurons. Treatment with the Akt inhibitor itself slightly decreased mitochondrial movement and also did not prevent the Zn^{2+}-induced movement effect. Although previous studies have used this inhibitor to prevent Akt phosphorylation in cell lines (Kozikowski et al., 2003), it appears to be less effective in neurons.

As an alternative to the Akt inhibitor, we transiently co-transfected an HA-tagged dominant negative Akt (K179M) plasmid along with the mitochondrially-targeted eYFP plasmid. Over expression of DN-Akt did not prevent the Zn^{2+}-mediated movement inhibition (data not shown), arguing against Akt activation by PI 3-kinase as a downstream event. We additionally tested other targets of PI 3-kinase that are of Akt-independent pathways. Blocking ARF6 phosphorylation using a dominant negative construct (Langille et al., 1999) or inhibiting TOR phosphorylation using rapamycin (Lynch et al., 2001) did not prevent the Zn^{2+}-mediated inhibition (data not shown), indicating that these protein kinases are not directly involved in the movement paradigm.

Thus, although PI 3-kinase activation appears to be an essential component of the effect of zinc, it is apparently not sufficient to account for the actions of zinc on mitochondrial movement.

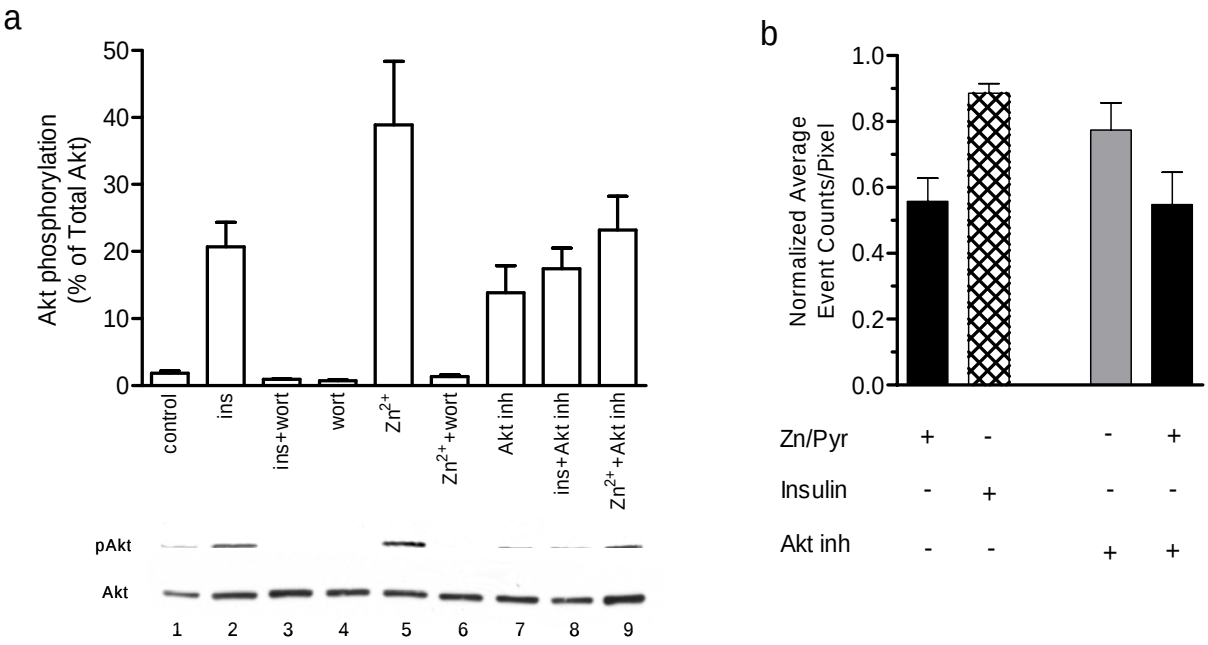


Figure 4.4 Akt inhibitor does not prevent Zn^{2+}-induced mitochondrial movement inhibition. (a) Representative western blot for phosphorylated Akt from neurons treated for 10 minutes with 3 μM Zn^{2+}(Pyr) or 15 minutes with 100 nM insulin, either in the presence or absence of wortmannin (1 μM) or Akt inhibitor (10 μM) pretreatment for 10 minutes. The lane numbers correspond to the bar conditions depicted in the graph directly below. Relative protein levels were normalized to total Akt protein levels as shown in the graph, which is a compilation of results from at least 3 different culture preparations. (b) Neurons were exposed to 3 μM Zn^{2+}(Pyr), 100 nM insulin or pretreated for 10 minutes with Akt inhibitor (10 μM) prior to Zn^{2+}. Movement was measured after insulin (hatched bar), inhibitor (gray bar) and Zn^{2+} (black bar) treatments.

Zn^{2+} effects on mitochondrial movement do not result from compromised mitochondrial function.

We have previously shown that acute application of the mitochondrial uncoupler FCCP, which rapidly dissipates $\Delta\Psi_m$, decreased mitochondrial movement in primary neurons (Rintoul et al., 2003). Zinc at some concentrations can also impair mitochondrial function (Sensi et al., 1999; Dineley et al., 2000). However, an examination of the effects of these zinc treatments on mitochondrial membrane potential showed that zinc only produced a significant depolarization at relatively high concentrations (Figures 4.5a and 4.5b). In addition, pretreating neurons with wortmannin (1 μM) did not prevent the inhibition of mitochondrial movement produced by FCCP (750 nM) exposure (Figure 4.5c). Together, these results argue against mitochondrial dysfunction as the key mechanism in Zn^{2+}-mediated movement inhibition.

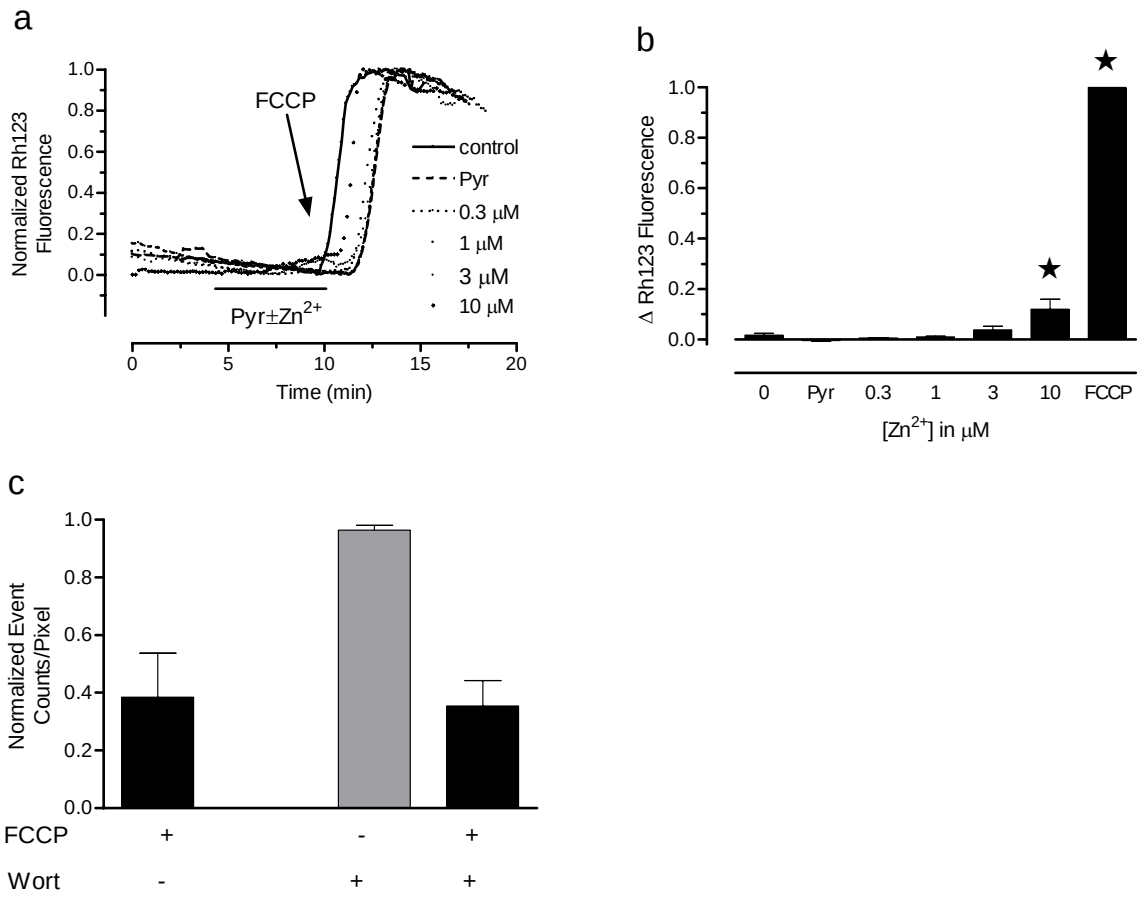


Figure 4.5 Zn^{2+}-induced movement inhibition does not grossly affect mitochondrial function. (a) Representative traces of $\Delta\Psi_m$ changes in neurons using Rhodamine 123 exposed to pyrithione (20 μM) alone or in the presence of Zn^{2+} (0.3-10 μM) for 5 minutes. FCCP (750 nM) was applied at the end of each experiment. (b) Summary graph of Rhodamine 123 changes in neurons exposed to Zn^{2+}(Pyr). (c) Mitochondrial movement in neurons was measured after FCCP (750 nM) exposure for 5 minutes, either without or with 10-minute wortmannin (1 μM) pretreatment. $p < 0.01$ by ANOVA with Dunnett's post test.

Wortmannin protects against Zn^{2+}-induced neurotoxicity.

As shown in figure 3a, pre-perfusion with wortmannin prevented Zn^{2+}-mediated inhibition of mitochondrial movement. However, the effectiveness of wortmannin was decreased when Zn^{2+} was increased from 3 to 10 μM (Figure 4.6a). Interestingly, wortmannin protected against Zn^{2+}-induced neurotoxicity with concentrations at or less than 3 μM Zn^{2+}/Pyr, but not with 10 μM (Figures 4.6b and 4.6c), suggesting a correlation between PI 3-kinase activation in Zn^{2+}-mediated movement inhibition and kinase activity during neurotoxicity.

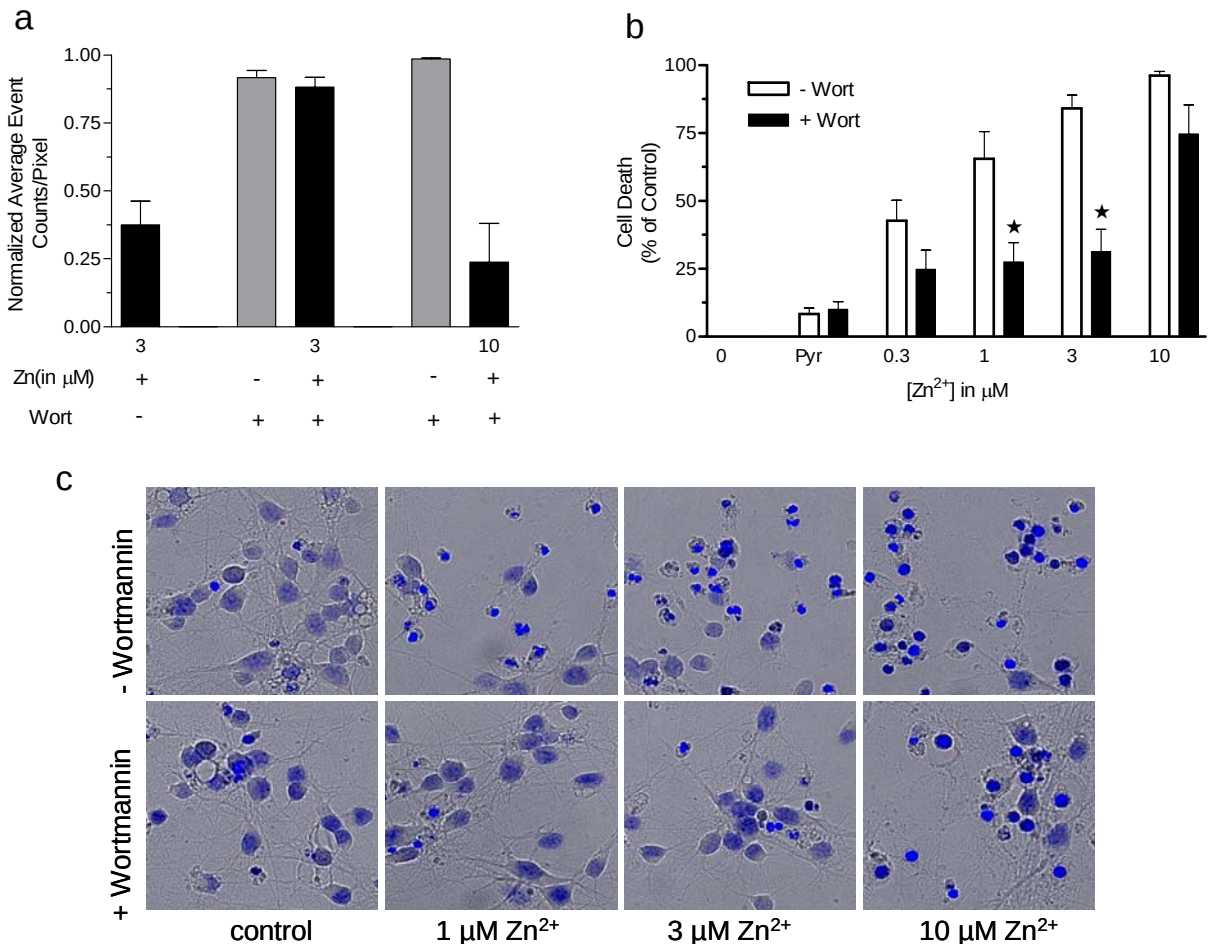


Figure 4.6 Wortmannin prevents both Zn^{2+}-mediated movement inhibition and neurotoxicity in a concentration-sensitive manner. (a) Movement was analyzed in neurons following 3 or 10 μM Zn^{2+}(Pyr) stimuli pretreated with 1 μM wortmannin. (b) Extent of cell death was measured in neurons pretreated with wortmannin (1 μM) prior to Zn^{2+} (0-10 μM) exposure. (c) Representative Hoechst staining in neurons exposed to varying Zn^{2+} (Pyr), either with or without wortmannin, where cell death is represented by extensive nuclear condensation (bright blue staining) compared to more healthier-looking cells (diffuse blue staining). $p < 0.005$ by Student's t test.

Transient, as opposed to sustained, PI 3-kinase activation prevents Zn^{2+}-mediated neurotoxicity.

Although PI 3-kinase is generally considered to be a pro-survival signaling mechanism, a recent study demonstrated that transient PI 3-kinase inhibition protects primary neurons from oxidative stress (Levinthal and DeFranco, 2004). As described above, our data suggests that wortmannin protected against Zn^{2+}-induced neurotoxicity at concentrations that inhibited mitochondrial movement. Although wortmannin is a fast-acting inhibitor (half-life of 90 minutes), it is also relatively unstable in aqueous solution. The other selective PI 3-kinase inhibitor LY294002 has a longer half-life (3.5 hours) and produces a more sustained kinase inhibition (Jones et al., 1999). Since we were unable to prevent Zn^{2+}-induced neurotoxicity with LY294002 (Figure 4.7a), we speculated that the neuroprotection that we observed with wortmannin could be explained by a transient inhibition of the kinase. To explore this possibility, neurons were repeatedly exposed to wortmannin during the course of Zn^{2+} toxicity. Sustained kinase inhibition with wortmannin no longer prevented neurons against elevated $[Zn^{2+}]_i$ (Figure 4.7b), in agreement with the hypothesis that only transient inhibition of PI 3-kinase affords protection against Zn^{2+} toxicity.

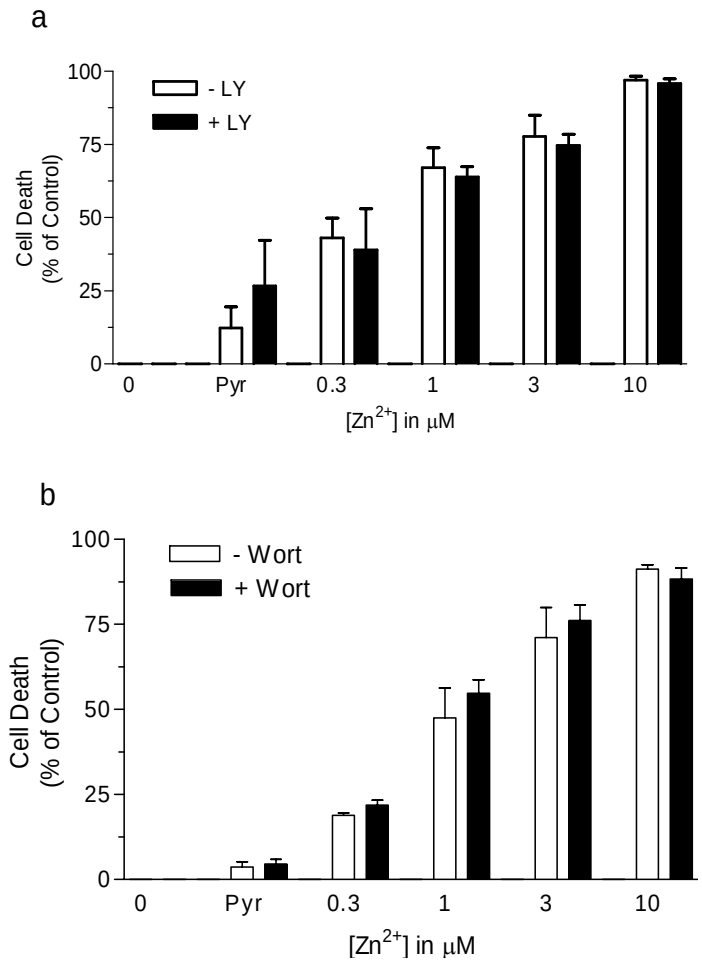


Figure 4.7 Transient inhibition of PI 3-kinase affords protection against Zn^{2+}-mediated toxicity. (a) Extent of cell death was measured in neurons pretreated with LY294002 (30 μM) prior to Zn^{2+} (0-10 μM) exposure. (b) Toxicity assayed in neurons pretreated with wortmannin (1 μM) followed by 0-10 μM Zn^{2+} (Pyr) were given additional wortmannin pulses 3 and 6 hours after Zn^{2+} application.

4.5 Discussion

The main finding from this study is that elevated $[Zn^{2+}]_i$ modulates mitochondrial movement through a signaling mechanism that is most commonly portrayed as a pro-survival pathway. In our experiments, acute application of pathophysiological $[Zn^{2+}]_i$ consistently stopped mitochondrial movement in cortical neurons. This effect was reversible only with early Zn^{2+} chelation, indicating that a Zn^{2+}-induced signaling cascade had been initiated which subsequently mediated movement inhibition. Interestingly, both Zn^{2+}-induced movement changes and neurotoxicity were prevented with inhibitors of the PI 3-kinase pathway. Thus, we have demonstrated a signaling mechanism that regulates both mitochondrial trafficking and neuronal viability in parallel.

Comparison to NGF modulation of mitochondrial movement in neurons

Recent studies have identified several modulators of mitochondrial trafficking in central neurons. In particular, elevated intracellular Ca^{2+} is an effective inhibitor of movement, an effect that is likely to be mediated by inhibition of ATP synthesis as well as potentially disruption of the cytoskeleton (Rintoul et al., 2003). Nitric oxide also inhibits movement (Rintoul *et al.* 2004, *SFN Abstract*), probably as a result of inhibition of the electron transport chain. However, the mechanism by which Zn^{2+} inhibits movement is distinct in that it appears to occur without clear disruption of mitochondrial function. Our findings of a role for PI 3-kinase are consistent with a recent report by Chada and Hollenbeck, which reported that focal stimulation of NGF receptors in sympathetic neurons causes mitochondria to "dock" or stop in the region of NGF

application via a mechanism sensitive to inhibition by wortmannin (Chada and Hollenbeck, 2003), and likely by undergoing docking interactions with the actin cytoskeleton (Chada and Hollenbeck, 2004). Interestingly, this phenomenon did not occur with global application of NGF, which would more closely parallel the conditions used here. Nevertheless, the basic concept of a PI 3-kinase-mediated control of mitochondrial trafficking is clearly similar between the two model systems. From our experiments, it is appears that PI 3-kinase is involved in the actions of zinc described here, but the downstream target of the signaling cascade is less obvious. Although the conditions used here result in Akt phosphorylation, agents like insulin that mimic this action of zinc do not recapitulate the effects on trafficking. Also, neither Akt inhibitors nor dominant negative Akt prevent the effects of zinc. Likewise, neither TOR nor ARF6 inhibition interrupted the actions of zinc. Thus, the step between PI 3-kinase activation and the mitochondrial target remains unidentified. Moreover, recent studies have highlighted the role of other signaling cascades in the regulation of mitochondrial trafficking, including interactions with phosphatidylinositol (4,5) bisphosphate species (De Vos et al., 2003), activation of protein kinase A (Okada et al., 1995) and tumor necrosis factor-induced p38 activation (De Vos et al., 2000). In addition to mitochondria, others have implicated the regulation of synaptic vesicular transport by kinesin motor proteins (Zhao et al., 2001; Okada et al., 1995). With regard to the current study, we have also demonstrated that mitochondrial trafficking is regulated by a signaling mechanism critical for cell survival.

What effects are mediated at the lowest, most physiologically relevant Zn^{2+} levels ?

A number of different mechanisms for zinc-mediated toxicity have been suggested. The data shown here illustrate the point that there are multiple effects of Zn^{2+} which occur over a range of concentrations. We observed that low concentrations of Zn^{2+} that inhibit movement and produce injury have no effect on $\Delta\Psi_m$. Higher concentrations of zinc produce a form of injury that is not prevented by wortmannin, and is also associated with mitochondrial depolarization. Zinc mediated mitochondrial depolarization has been reported in a number of studies, both in intact cells (Sensi et al., 1999) as well as in isolated mitochondria (Dineley et al., 2005). However, these results suggest that while mitochondria are clearly a target for zinc action, the most sensitive mechanisms associated with cytotoxicity do not involve gross disruption of mitochondrial function.

Secondary effects of oxidants in inhibiting mitochondrial movement

Evidently, this movement phenomenon is not strictly dependent on the source of zinc. To mimic oxidant-labile $[Zn^{2+}]_i$, we applied 2,2'-DTDP to neurons and observed that mitochondrial movement significantly decreased with concentrations as low as 10 μM. This was surprisingly low, given that 100 μM 2,2'-DTDP was applied to cells in order to see mag-fura-2 responses in neurons (Aizenman et al., 2000). In addition, we also observed movement inhibition with 100 μM H_2O_2 (unpublished observations), which was used to mimic ROS exposure. Interestingly, TPEN did not restore mitochondrial movement when applied after either 2,2'-DTDP or H_2O_2. Together, these results raise

the possibility that apart from a direct Zn^{2+} effect, a secondary ROS-induced mechanism may also modulate mitochondrial movement.

PI 3-kinase activation by zinc in different cell types

Indirect PI 3-kinase activation by Zn^{2+} has been reported in other studies. Eom *et al.* (2001) found that Zn^{2+}-induced stimulation of JNK through the PI 3-kinase pathway in primary mouse cortical neurons in culture, and Kim *et al.* (2000b) demonstrated that extracellular Zn^{2+} activates p70 S6 kinase through PI 3-kinase signaling in Swiss 3T3 cells. These findings differ from our own in that PI 3-kinase activation is dependent on the activation of other pathways, which may be attributable to the exposure of 10 times higher concentrations of Zn^{2+} (although in the absence of ionophore), longer Zn^{2+} exposures necessary to achieve Akt phosphorylation, or varying sensitivities among cell types. We did not observe that the activation of other protein kinases plays a direct role in the Zn^{2+}-mediated movement effect, as inhibitors of these pathways did not protect against movement inhibition (data not shown). We cannot entirely exclude the possibility that these kinases are either activated downstream of or in parallel with PI 3-kinase, however, their activation did not affect Zn^{2+}-mediated changes in mitochondrial trafficking or neuronal injury. Zn^{2+} activates a number of other protein kinases involved both in pro- and anti- cell survival mechanisms. For example, studies have demonstrated that $[Zn^{2+}]_i$ can activate members of the MAP kinase family. Zn^{2+} activation of the stress-activated p38 (McLaughlin et al., 2001), as well as its activation of the traditionally pro-survival ERK (Du et al., 2002), is hypothesized to contribute to neuronal injury and subsequent cell death. In addition, activation of PKC in neurons plays a role in Zn^{2+}-

induced oxidative neuronal injury (Noh et al., 1999). Since signal transduction pathways are rarely activated in a linear fashion, it is more than likely that there is crosstalk between these pathways with respect to Zn^{2+}-mediated neuronal injury.

What is the role of PI 3-kinase activity during neuronal injury ?

PI 3-kinase activity has traditionally been associated with many key cellular processes, including cell growth and survival, membrane trafficking, neurite outgrowth and cytoskeleton reorganization (Foster et al., 2003). Indeed, extensive literature demonstrates that growth factor-mediated activation of the PI 3-kinase/Akt pathway, by insulin (Patel et al., 1993) or IGF-1 (Dudek et al., 1997; Schubert et al., 2004), NGF (Chada and Hollenbeck, 2003), and BDNF (Zheng and Quirion, 2004), is neuroprotective against a range of cellular stresses. In addition, Luo *et al.* (2003) have demonstrated that constitutive activation of Akt is neuroprotective in, whereas Akt deactivation promotes multiple models of neuronal cell death, including NMDA excitotoxicity, or NO- and H_2O_2-elicited injury. Including the present study, however, only one other report by Levinthal and DeFranco (2004) recently provided an example of PI 3-kinase activity associated with cell death. The authors demonstrated that glutamate-induced oxidative neuronal injury is mediated by activation of PI 3-kinase. Moreover, a window of transient PI 3-kinase inhibition afforded protection against oxidative glutamate toxicity, which is consistent with our results involving Zn^{2+} toxicity. We also observed that transient PI 3-kinase inhibition was neuroprotective, but this protection was overcome with sustained kinase inhibition. This provides insight about the bipolar nature of PI 3-

kinase: short term activation may contribute to cell death, whereas long term activation is essential for cell survival.

Chapter 5. Discussion

5.1 General outline

This discussion focuses on the impact of zinc on neuronal mitochondria and is divided into two parts. The first involves mitochondrial zinc transport and the direct impact of zinc on isolated neural mitochondria. Here we take some effort to extract a long and confused history of mitochondrial-Zn^{2+} investigation into an accessible summary, to which our own findings are related. The second part describes our novel findings that elevated intracellular zinc interferes with mitochondrial movement in primary cultures of neurons. This part describes the relatively young but growing body of literature regarding the relevance of mitochondrial trafficking in normal cell function and during injury. Special emphasis is given to the role of Zn^{2+} in cytoskeletal organization and Zn^{2+} activation of cellular signaling pathways. The discussion concludes with potential therapeutic interventions into zinc-induced neuronal injury and a look into possible future directions for the zinc neurobiologist.

5.2 Mitochondrial dysfunction is an early event in neurodegeneration

Mitochondria are critical for neuronal function as they maintain energy gradients required for cell survival. The mitochondrial membrane potential ($\Delta\Psi_m$) is necessary for normal ATP production, Ca^{2+} storage and release, and control of reactive oxygen species (ROS) production. Perhaps our best understanding of the role of mitochondria in neuronal death comes from studies of glutamate excitotoxicity that occurs in ischemia (Nicholls et al., 2000). In this scenario, high levels of extracellular glutamate cause massive accumulation of intracellular calcium. As mitochondria become overloaded with calcium from the cytosol, membrane potential is compromised, along with the capacity to

synthesize ATP production via oxidative phosphorylation. The disruption of mitochondrial function eventually leads to a cell death cascade involving the release of pro-apoptotic molecules and accumulation of reactive oxygen species (Nicholls et al., 2000).

Zn^{2+} is also thought to cause neuronal injury by targeting mitochondria, and it is useful to compare Ca^{2+} toxicity to Zn^{2+} toxicity. However, there are significant differences. For instance, it is clear that in normal healthy cells, mitochondria labor continuously to take up and release Ca^{2+}, while similar regulation of zinc is only suspected. Additionally, while mitochondria have calcium transport pathways that can accommodate zinc, it is unclear whether zinc has access to these pathways particularly when calcium reaches micromolar levels that occur during excitotoxic stimulation.

5.2.1 Mitochondria can import zinc by two pathways

From circumstantial evidence, previous studies suggested that Zn^{2+} could enter mitochondria through the mitochondrial calcium uniporter. Studies conducted as early as 1967 in isolated bovine heart mitochondria observed Zn^{2+} accumulation in mitochondria by atomic absorption, which was dependent upon respiration and inhibited by uncouplers (Brierley and Knight, 1967). However, the conditions used a sucrose buffer without other cations, and added micromolar levels of Zn^{2+}. Saris and Niva (1994) proposed a role for uniporter-mediated transport, based on ruthenium red inhibition of Zn^{2+}-induced swelling in rat liver mitochondria. However, swelling was modest even at large concentrations of free zinc, and interpretation of the data was further complicated by the use of a variety of assay conditions. Using isolated rat brain mitochondria, Jiang and colleagues (2001) observed similar swelling that was inhibited by ruthenium red, and also

cytochrome c release, which they claimed occurred at free zinc levels as low as 10 nM. Curiously, their data suggested that mitochondria take up more zinc when exposed to zinc and calcium combined. In any case, none of these studies presented any conclusive evidence for zinc import.

Thus our work using FluoZin-3 in isolated, attached brain mitochondria is the first to directly show zinc accumulation in the matrix (Chapter 3). Moreover, we found evidence for two distinct modes of zinc import. The first is a high capacity transport mechanism that is consistent with a uniporter mode because it is inhibited by ruthenium red, and depends on $\Delta\Psi_m$. The second is a low capacity, presumably energy-independent mechanism that occurs in the presence of ruthenium red and in the absence of $\Delta\Psi_m$. We are unable to further characterize this second pathway. Speculation might include a mitochondrial zinc transporter, but this seems improbable given that the heart mitochondria proteome revealed no ZIP or CDF-like proteins (Taylor et al., 2003). Because our recording conditions contained no added Na^+, it is also unlikely that Zn^{2+} could be substituting for Ca^{2+} in the mitochondrial Na^+-Ca^{2+} exchanger. It might be argued that our isolation procedure or treatments disrupted mitochondrial membranes, allowing free diffusion of zinc, but if so we would also expect significant leakage of dye, which was not the case. A possible mechanism is suggested by Zazueta *et al*. (2004), who found evidence for the distinct subunit localization for the inhibition and transport properties of the uniporter. By reconstituting solubilized mitochondrial proteins into cytochrome oxidase vesicles, a bimodal calcium uptake activity was measured, where one pathway was sensitive to the ruthenium red and Ru_{360}, and the other was insensitive. One could propose a similar explanation for Zn^{2+} actions, in that ruthenium red may bind

to the inhibition subunit that would prevent most Zn^{2+} uptake, but still allows some Zn^{2+} to bind the transport subunit. Further characterization of the calcium uniporter is required to elucidate this mechanism. Our data also provide evidence for zinc efflux (Chapter 3). However, similar to the low capacity influx, we are unable to further characterize this mechanism. We know only that it is apparently unaffected by mitochondrial phosphate pools and proceeds relatively slowly compared to Zn^{2+} influx, although the latter could result from buffering of Zn^{2+} by FluoZin-3.

5.2.2 Where do zinc actions on mitochondria occur ?

Numerous groups including ours have shown that zinc depolarizes mitochondrial membrane potential (Sensi et al., 1999; Sensi et al., 2000; Dineley et al., 2005). However, it has been a matter of disagreement as to whether zinc exerts this effect from the inside or outside of mitochondria. Using isolated brain mitochondria in cuvette-based experiments, we showed that free zinc at about 100 nM effectively depolarizes mitochondria, and that membrane potential could be partially or even wholly rescued by an impermeant chelator (Dineley et al., 2005). This suggests that zinc is acting externally. Our recent data (Chapter 3) are in agreement. Using adherent mitochondria loaded with Rh123, we showed that as low as 10 μM free Zn^{2+} depolarized mitochondria, and repolarization was observed with subsequent application of an impermeant chelator (EDTA). Moreover, ruthenium red, which completely blocks calcium-induced depolarization, only protected against Zn^{2+}-induced depolarization at low free Zn^{2+} concentrations (10 μM) but had no effect at higher free zinc concentrations (80 μM). We can not rule out a depolarizing effect of small amounts of zinc that enter mitochondria in

the presence of ruthenium red, but it seems unlikely given the robust repolarization achieved with EDTA.

With respect to Zn^{2+} effects on respiration, several studies in isolated mitochondria have delineated specific inhibitory sites on the electron transport chain (Kleiner and von Jagow, 1972; Kleiner, 1974; Link et al., 1995; Mills et al., 2002). These results support the specific actions of Zn^{2+} on the electron transport chain, but do not explicitly confirm whether Zn^{2+} inhibits respiration from within the matrix or by binding to an external site. In some of these situations, Zn^{2+} inhibition was shown to be reversible upon membrane-impermeant Zn^{2+} chelation (Kleiner, 1974; Link et al., 1995; Mills et al., 2002), suggesting an external association between the electron transport chain and Zn^{2+}. In a recent study (Dineley et al., 2005), we specifically demonstrated that Zn^{2+} inhibits oxygen consumption in isolated brain mitochondria when mitochondria are supplied with substrates that feed into specific complexes. Again, extramitochondrial Zn^{2+} chelation with EGTA restored respiration in the presence of complex I and II substrates, suggesting that Zn^{2+} inhibition of these complexes occurs in the intermembrane space side of the electron transport chain. Our interpretation from Zn^{2+} effects on $\Delta\Psi_m$ (Chapter 3) or respiration (Dineley et al., 2005) is in agreement with crystallography studies that revealed zinc binding sites near the externally accessible bc1 complex (Berry et al., 2000).

5.2.3 Evidence for zinc effects on mitochondria in cells

Previous groups have demonstrated that lethal doses of $[Zn^{2+}]_i$ *in vitro* result in general mitochondrial injury. Specifically, Manev and colleagues (1997) observed that brief exposure of high micromolar Zn^{2+} concentrations to cultured cerebellar neurons

resulted in early global mitochondrial dysfunction prior to cell injury. A more specific study demonstrated that toxic levels of Zn^{2+} inhibit glycolysis at glyceraldehyde phosphate dehydrogenase (GAPDH; Sheline et al., 2000), resulting in progressive reduction of ATP and NAD^{+}. In addition, elevated nanomolar Zn^{2+} was also shown to cause mitochondrial swelling in neurons loaded with MitoTracker Green to visualize mitochondria (Jiang et al., 2001). However, there were problematic issues associated with the results: (1) the swelling was only assessed qualitatively, employing an ill-defined arbitrary swelling index and (2) since no zinc buffering components were used to establish more physiological intracellular conditions, they could not define any concentration dependency to their measured parameters.

Others have previously demonstrated that excitoxicity-induced $[Zn^{2+}]_i$ accumulation dissipates $\Delta\Psi_m$ in cultured primary neurons (Sensi et al., 1999; Sensi et al., 2000). In contrast, we never observed Zn^{2+}-mediated depolarizations in primary neurons. These were reasonable ionophore-mediated rapid Zn^{2+} elevations that were sufficient to cause neuronal injury (Dineley et al., 2000, Chapter 4). Perhaps these different findings can be explained by the role of Zn^{2+} during excitotoxicity. We know that Ca^{2+} by itself is an effective and direct mitochondrial toxin. During glutamate stimulus, both $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$ become elevated. The question is: does Zn^{2+} become elevated enough in the presence of Ca^{2+} to mediate direct effects on mitochondrial function ? In the studies by Sensi and colleagues, they applied high extracellular Zn^{2+} in the presence of depolarizing stimuli or kainate treatment, which would allow Zn^{2+} influx presumably through Ca^{2+}-permeable pathways. The estimated nanomolar Zn^{2+} levels cannot compete as an excitotoxin with Ca^{2+}. In this case, the mitochondrial depolarization observed by others

would be predominantly Ca^{2+}-induced. In our paradigm, where we attempt to elevate Zn^{2+} levels comparable to Ca^{2+} by introducing Zn^{2+} in cells with the ionophore, we do not see loss of $\Delta\Psi_m$. Here, it is reasonable to speculate that Zn^{2+}-induced ROS production or Ca^{2+} mobilization is the more direct mitochondrial effect, as opposed to Zn^{2+} directly acting on mitochondria. Together, this speculates the ancillary role of Zn^{2+} during excitotoxicity. This proposed role is not meant to demote the importance of Zn^{2+} as a neurotoxin. Indeed, we recently demonstrated that glutamate exposure in neurons stimulated large FluoZin-3 increases, which were reduced with TPEN (Dineley *et al.* 2004, SFN Abstract). We know that cellular Zn^{2+} elevations occur during glutamate excitotoxicity, however, once free inside the cell, perhaps Zn^{2+} is more indirectly involved in mitochondrial dysfunction compared to Ca^{2+}.

5.3 Metallothioneins and mitochondria

5.3.1 Metallothionein modulation of mitochondrial function

Ye and colleagues (2001) observed that MT localized to the intermembrane space of isolated rat liver mitochondria, where micromolar protein levels inhibited respiration. Added free Zn^{2+} inhibited respiration to the same extent, suggesting that Zn^{2+} release from MT mediates this effect. The apo-form of MT, thionein, was quite effective at reversing Zn^{2+} inhibition of respiration, suggesting that MT delivers Zn^{2+} to the mitochondrial inner membrane without necessary matrix entry of MT. The primary issue which failed to be addressed in the study by Ye and colleagues are that micromolar concentrations of exogenous MT protein may artificially force MT into isolated mitochondria, thereby being displaced from a more physiological investigation of its function. Secondly, there is no current evidence that mitochondria contain any MT-like

proteins (Taylor et al., 2003). In isolated mitochondria, we did not observe any oxidant-induced Zn^{2+} signals, suggesting that MT does not serve in mitochondrial Zn^{2+} sequestration (Chapter 3). Indeed, very few mitochondrial proteins have been identified as Zn^{2+}-binding proteins (Taylor et al., 2003).

The only study which remotely provides a physiological link between MT and mitochondria comes from Sakurai and colleagues (1993), where they measured levels of MT protein in rat liver by radioimmunoassay. In subcellular fractions, MT was highest in cytosol, followed by nucleus, mitochondria and microsomal fractions. Although the authors claimed that mitochondria were the organelles with the highest MT concentrations, they did not analyze MT content in other organelles or cytosolic compartments. Another problematic issue with this study was that strong MT staining was only evident in livers of rats that developed hepatitis compared to much weaker staining in control rats, suggesting that the disease condition may have induced protein expression and may not be a physiological phenomenon. Together, there is no conclusive data for the endogenous presence of MT in mitochondria.

5.3.2 Is metallothionein a mitochondrial zinc chaperone ?

As mentioned above, it was hypothesized that mitochondrial Zn^{2+} transport occurs via the Ca^{2+} uniporter. Although we have shown that uniporter blockade partially inhibits direct mitochondrial Zn^{2+} uptake (Chapter 3) and Zn^{2+}-induced mitochondrial perturbations (Dineley et al., 2005), we cannot exclude the possibility of a novel, uncharacterized transport mechanism. Two recent studies by Leslie Costello and colleagues have demonstrated the existence of a novel mitochondrial Zn^{2+} uptake process, whereby Zn^{2+} ions are thought to be donated from loosely-bound zinc-ligand

complexes to a putative zinc transporter on the inner membrane. In the first study (Guan et al., 2003), mitochondria isolated from rat prostate were subjected to radioisotope labeling with ^{65}Zn to measure uptake rates with different Zn-ligand complexes. Prostate cells contain 3 to10-fold more Zn^{2+} compared to other cells and citrate is a prominent Zn^{2+}-ligand in the prostate. Kinetic transport assays exhibited that uptake rates from Zn^{2+}-ligands, such as cysteine, citrate or aspartate were essentially the same as for free Zn^{2+}. Notably, no uptake was determined in the presence of EDTA or EGTA, indicating that undissociated Zn^{2+} is not available for transport across the inner membrane. In addition, none of the Zn^{2+}-ligands themselves were permeable across the membrane.

The more recent study by Costello *et al.* (2004) proposed MT as a chaperone for mitochondrial zinc uptake in rat prostate and liver. Exogenous MT enhanced liver mitochondrial Zn^{2+} uptake as effectively as Zn^{2+}-citrate described in the previous study. On the other hand, prostate mitochondria do not accumulate Zn^{2+} from MT sources, but depend on the presence of other Zn^{2+}-ligand complexes, suggesting a novel transport uptake mechanism in prostate vs. liver tissues. Although Zn^{2+} transport may be regulated differently in brain vs. prostate and liver, it is possible that this putative transporter is the uniporter-independent mechanism that we hypothesize in our studies (Chapter 3).

Several differences arise when comparing approaches of our studies vs. Costello *et al.*: (1) Since our preparations are devoid of cytosol and we did not introduce MT, we are not concerned with MT acting as a donor to the transport mechanism. (2) We used fluorescent indicators to directly visualize free Zn^{2+} accumulation inside individual isolated mitochondria, whereas the other studies used radiolabeling, which does not allow distinction between free and bound Zn^{2+} in isolated mitochondria in suspension.

(3) When we exposed our isolated mitochondria to strong sulfhydryl oxidants we observed no oxidant-labile Zn^{2+} pool, such as MT. In summary, perhaps mitochondria import Zn^{2+} by transporter-dependent mechanisms under basal conditions, whereas import is more energy-independent with elevated $[Zn^{2+}]_i$.

As one potential candidate for Zn^{2+} chaperone, the mitochondrial copper chaperone, Cox17, was recently identified and shown to deliver copper into mitochondria for incorporation into and activation of cytochrome *c* oxidase. Interestingly, the molecular weight of this copper chaperone is quite similar to MT, their sequences align at lysine and cysteine residues critical for metal binding, and they both lack a defined mitochondrial localization sequence but still manage to localize to the intermembrane space. A recent study by Palumaa and colleagues (2004) demonstrated through electrospray ionization-mass spectroscopy that purified Cox17 can bind and transfer up to 4 copper ions and two zinc ions regardless of the redox status of the protein. Given these similarities between the two proteins, it would be interesting to examine whether Cox17 can transport Zn^{2+} into mitochondria.

5.4 Measuring mitochondrial zinc pools

5.4.1 Oxidant-labile Zn^{2+} exchange between the cytosol and mitochondria ?

One hypothesis for Zn^{2+}-induced neurotoxicity was that perhaps Zn^{2+} is also taken up by mitochondria like Ca^{2+} for it to depolarize mitochondria and generate ROS as necessary stages leading to global cellular dysfunction (Sensi et al., 2000). To investigate the hypothesis that mitochondria take up Zn^{2+} from oxidant-sensitive cytosolic pools and release Zn^{2+} for cytosolic Zn^{2+} proteins, Sensi and colleagues (2003b) sought to visualize Zn^{2+} mobilization between cytoplasm and mitochondria by treatments with 2,2'-DTDP

and FCCP, respectively. Neurons exposed to FCCP followed by 2,2'-DTDP exhibited increased FluoZin-3 responses, suggesting the movement of Zn^{2+} released from mitochondria to protein-bound cytosolic sites. However, it should be noted that FCCP also acidifies the intracellular environment, potentially destabilizing Zn^{2+}-protein complexes in the cytosol. One would therefore expect that FCCP would alone increase cytosolic Zn^{2+}. The reverse however, 2,2'-DTDP followed by FCCP, did not exhibit similar increases in $[Zn^{2+}]_i$. The authors claimed that this reduced response is due to the rapid sequestration of mitochondrially-released Zn^{2+} into protein-bound pools. Given the highly oxidized cytosolic environment resulting from 2,2'-DTDP exposure, it is difficult to imagine that the redox state of MT under these conditions would allow it to quickly bind Zn^{2+}. Indeed, in previous studies of 2,2'-DTDP treatment of non-stimulated neurons, the Zn^{2+} response remains elevated and relatively stable until Zn^{2+} chelation (Aizenman et al., 2000). This response must be relatively large since it was detected with the low-affinity cytosolic indicator Newport Green. Approaches to visualize and distinguish mitochondrial vs. cytosolic pools of Zn^{2+} may provide more convincing evidence of intracellular Zn^{2+} mobilization. Techniques that attempted to demonstrate this will be discussed below.

5.4.2 Use of mitochondrial-specific zinc fluorescent indicators

The specific visualization of mitochondrial Zn^{2+} was confounded by the lack of Zn^{2+}-selective mitochondrial probes. Initial studies by Sensi and colleagues (2000) sought to adapt the divalent cation indicator, rhod-2, since it was used to observe glutamate-stimulated intramitochondrial Ca^{2+} accumulation in neurons (Peng et al., 1998). Using this dye in primary neurons, Sensi *et al*. (2000) observed Zn^{2+}-dependent

speckled fluorescent changes in the perinuclear region, which they concluded as mitochondrial Zn^{2+} sequestration. However, the failure to demonstrate co-localization with a $\Delta\Psi_m$-sensitive dye did not confirm that the speckled pattern actually corresponds to mitochondria.

More recently, a novel Zn^{2+} indicator with nanomolar affinity, thought to localize to mitochondria has been used to monitor changes in intramitochondrial free Zn^{2+}. In a methods study (Sensi et al., 2002) to characterize the properties of RhodZin-3, high co-localization was observed in neurons labeled with RhodZin-3 and MitoTracker Green. The staining of both dyes was predominantly in the perinuclear region and spread throughout the processes. However, basal RhodZin-3 fluorescence in a resting neuron was observed as speckles throughout the cell body, which may or may not correspond to mitochondria. In our experiments visualizing mitochondria using GFP-tagged mitochondrial protein (Chapter 3), the cell bodies are completely and consistently bright due to the high concentration of mitochondria localized within a focused region, whereas individual mitochondria can be distinguished in the processes as elongated structures. Therefore, we rarely have observed speckled or punctuate mitochondria within the cell body or processes of healthy neurons. In contrast, Sensi and colleagues observed that in stimulated cells, only certain speckles increased modestly in fluorescence, as opposed to entire mitochondrial structures or the entire cell body.

In summary, studying $[Zn^{2+}]_i$ has been difficult because of limitations in reagents. However, even with recently emerging Zn^{2+} fluorescent indicators, detecting mitochondrial Zn^{2+} still remains a challenge, due to the indeterminate localizations of these probes and their uninterpretable signals. In order to convincingly detect

mitochondrial Zn^{2+} in living cells, perhaps designing a mitochondrially-targeted Zn^{2+} sensor (i.e. mitochondrially-targeted MT) may provide more accurate localization of mitochondrial Zn^{2+} in the absence of cytosolic noise.

5.4.3 Detected mitochondrial zinc signals are dependent on experimental model

As mentioned above, currently available fluorescent indicators do not allow the precise separation between Zn^{2+} signals arising from the cytosol and mitochondria. To more directly observe mitochondrial Zn^{2+} accumulation without confounding signals from the cytosol, we investigated mitochondrial Zn^{2+} transport in isolated mitochondria. Our initial studies effectively demonstrated Zn^{2+}-induced mitochondrial dysfunction using $\Delta\Psi_m$- and ROS-sensitive indicators (Dineley et al., 2005). However, we were unsuccessful at demonstrating direct Zn^{2+} uptake in isolated mitochondria using fluorescent Zn^{2+} indicators in a spectrofluorimetric assay. In loading isolated mitochondria with Newport Green, we observed some dye leakage. Also, because of the nature of the cuvette-based assays, it was difficult to determine precise free zinc concentrations in the presence of chelator. Since experiments were performed on isolated mitochondria in suspension, it was difficult to determine the location of Zn^{2+} actions on mitochondria; distinguishing between dye binding Zn^{2+} within the matrix vs. dye binding Zn^{2+} on external sites. These problems prevented us from accurately interpreting the signals we detected in mitochondria upon Zn^{2+} exposure.

We overcame some of these issues in using a novel experimental system, whereby isolated brain mitochondria were attached to glass coverslips and were capable of taking up FluoZin-3 to investigate mitochondrial Zn^{2+} transport. The advantages this model has over the previous one are as follows: (*i*) the mitochondria retained dye fairly well and we

were able to detect matrix Zn^{2+} changes, (*ii*) chelator concentrations were maintained relatively stable as it was present in the isolation procedure and perfused throughout the experiment, (*iii*) as opposed to cuvette-based experiments, individual attached mitochondria can be imaged, where solutions and treatments can be perfused over the sample when desired and (*iv*) adherent mitochondria allow direct visualization of mitochondrial Zn^{2+} uptake, since it is not possible to observe direct transport in mitochondrial suspensions. Although this model is devoid of cytosolic components and therefore does not account for any Zn^{2+} mobilization between different intracellular pools, it presents many advantages to directly visualize mitochondrial Zn^{2+} uptake.

5.5 Mitochondrial trafficking in neurons

The concept of mitochondria as dynamic entities was described almost 90 years ago (Lewis and Lewis, 1915). However, based on the availability of imaging methods, it has been difficult to study their movements in cells. The recent advent of techniques to visualize mitochondria in living cells has revolutionized our investigations of these semi-autonomous organelles that constantly divide, fuse, and move throughout the cell (Bereiter-Hahn and Voth, 1994). Although we lack a complete understanding of the forces that drive mitochondrial dynamism, there are emerging suggestions for the necessity of their cellular distribution. Mitochondrial motility and distribution become important parameters, especially when considering cellular polarity, or the presence of distinct subcellular domains and the demands and needs with them. For example, neurons have a highly polarized morphology, consisting of processes that originate from a central cell body, forming the basis for directionalized signaling. By delivering ATP to

areas of high metabolic demand, mitochondria could play an important role in organizing neuronal polarity (Mattson, 1999).

The need for mitochondrial movement in neurons has been hypothesized in several ways: (*i*) Diffusion of ATP within neurons seems inadequate to support energetic homeostasis. Therefore, the positioning of mitochondria to regions where required ATP consumption is high appears favorable for cell survival (van Blerkom, 1991). (*ii*) Calcium is essential for the activation of critical cellular enzymes and acts as a second messenger in signal transduction processes, in addition to regulating neurite growth and axonal development (Spira et al., 2001). By regulating local calcium homeostasis, mitochondria may deliver calcium to and sequester calcium at required regions of the cell. Specifically, the efflux of Ca^{2+} by mitochondria has been shown to enhance neurotransmitter release at nerve terminals, indicating that mitochondria also play a role in modulating local synaptic transmission (Tang and Zucker, 1997). This emphasizes the need to deliver mitochondria to pre-synaptic terminals. How mitochondrial functions affect mitochondrial movement in neurons has only recently been addressed. Indeed, evidence has suggested that differences in metabolic properties can affect frequency and distance of organelle motility in axons and dendrites of primary neuronal cultures (Overly, 1996).

5.5.1 Regulation of mitochondrial trafficking

Role of the cellular cytoskeleton

Aside from bioenergetic-associated changes in mitochondrial movement, there is evidence that the cytoskeleton regulates organelle distribution. Several studies have addressed aspects of mitochondrial movement with respect to their interaction with the

cytoskeleton. Specifically, it is known that mitochondrial movement is microtubule-based. For example, Ligon and Steward (2000b) demonstrated that using cytoskeleton-disrupting drugs that depolymerize microtubules, but not actin filaments, attenuated mitochondrial movement in both axons and dendrites. An earlier study by Krendel and colleagues (1998) demonstrated that disassembly of actin filaments resulted in increased rate and frequency of mitochondrial movement along microtubules, confirming that most of the movement occurs along microtubules, although actin filaments may modulate movement by tethering mitochondria to specific regions and controlling rate of microtubule-based movement. In addition, physical associations between microtubules and mitochondria have been identified (Price *et al.*, 1991), further supporting the concept of mitochondrial movement along these cytoskeletal "tracks". More recently, mitochondria-neurofilament interactions were noted *in vitro* as another cytoskeletal component that modulates mitochondrial motility (Wagner et al., 2003). The physical association was dependent on $\Delta\Psi_m$ and also the phosphorylation status of the neurofilament subunits, suggesting that neurofilament activity may also control mitochondrial distribution in neurons.

Contributions of motor proteins to mitochondrial movement

In addition to microtubules, molecular motor proteins of the dynein and kinesin families also mediate mitochondrial movement. These proteins provide the bidirectional movement of mitochondria (Hirokawa, 1996), specifically anterograde and retrograde transport in neurons, through their phosphorylation/dephosphorylation. Anterograde axonal transport of mitochondria is supported by kinesin motors, driving movement towards the plus ends of microtubules. In contrast, retrograde movement is supported by

the dynein motors, driving movement towards the minus ends of microtubules (Schroer and Sheetz, 1991).

Although the exact properties and functions of these motor proteins are not completely known, evidence suggests that these motor proteins distinguish mitochondrial axonal movement from the axonal movement of other organelles and vesicles. Firstly, the observation that mitochondria globally move slower than other organelles suggests that in addition to simply having a larger mass, they contain specific motors that control or regulate the velocity of their movement (Morris and Hollenbeck, 1993). For example, both mitochondrial and vesicular movement in axons was demonstrated to be regulated by two distinct isoforms of kinesin (Elluru et al., 1995), suggesting that the specific isoform associated with the organelle may explain why vesicles move faster than mitochondria. Secondly, mitochondrial movement is described as bidirectional and saltatory (Morris and Hollenbeck, 1993), whereas vesicular movement is generally unidirectional and smooth (Allen et al., 1982). The need to constantly change direction in a less smooth fashion could reflect the larger responsibility of the motor proteins in directional transport and the overall slower mitochondrial movement in axons. And thirdly, the physical association of mitochondria with motor proteins in discrete patches that are widely-spaced along the mitochondrial surface (Martz et al., 1984) provides the advantage of more precise control of cytoskeleton-based movement.

5.5.2 Mitochondrial trafficking during cell injury

It is thought that mitochondrial biogenesis, or the generation of new mitochondria, occurs in the perinuclear region (Davis and Clayton, 1996) and the mitochondria then move anterogradely to provide energy for growth of new processes

(Ligon and Steward, 2000a). Furthermore, it may be hypothesized that under injury conditions, mitochondria may be transported retrogradely or are accumulated and degraded in the periphery. Although there is this notion that mitochondrial distribution can be regulated in part via communication with the surrounding cytoskeleton, the mechanisms by which this occurs have not been defined. However, *in vitro* studies have demonstrated that targeted disruption of kinesin results in an abnormal perinuclear clustering of mitochondria (Tanaka *et al.*, 1998), suggesting that these motor proteins may be regulators of mitochondrial distribution.

Indeed as evidence for mitochondrial degradation near the cell body, Miller and Sheetz (2004) demonstrated that ~ 90% of mitochondria maintaining high potential were transported anterogradely towards the growth cone, whereas ~80% of mitochondria with reduced potentials were transported towards the cell body. Previous studies have shown that depolarized or damaged mitochondria are targeted for elimination and removal by the process of autophagy (Xue et al., 2001). Autophagy involves an intracellular vacuole composed of ER membranes and contains a lysosome used to engulf and degrade old, damaged mitochondria. If damaged or non-functioning mitochondria predominantly return back towards the cell body, perhaps high concentrations of these autophagosomes reside in the perinuclear region and serve as a mitochondrial "burial ground". These notions are purely speculative and no conclusive evidence so far demonstrates that mitochondria are destroyed near the cell body, although it would be convenient to recycle old mitochondrial components in regions proximal to where new mitochondria are generated.

Neurodegeneration models and altered mitochondrial axonal transport

Emerging data suggest that neuronal mitochondrial distribution can change in response to injury, although we know relatively little about how organelle transport processes change under physiological conditions. Using glutamate excitoxicity as a model for stroke, we demonstrated impaired mitochondrial movement and morphology in neurons (Rintoul et al., 2003). A failure to transport mitochondria to peripheral cell compartments were observed in response to overexpression of tau protein (Ebneth *et al.*, 1998) or mutations in presenilin protein (Pigino et al., 2003) as a model for Alzheimer's disease, and mitochondrial perinuclear clustering and fragmentation in response to apoptotic stimuli (De Vos *et al.*, 1998), presumably from defective kinesin-mediated organelle movement. Brownlees and colleagues (2002) demonstrated that abnormal neurofilament assembly perturbs mitochondrial movement and localization in a neuronal model of Charcot-Marie-Tooth disease, an inherited disorder of the peripheral nervous system.

Hereditary spastic paraplegia (HSP) is a genetic heterogeneous condition characterized by selective axonal degeneration in corticospinal tracts, resulting in progressive weakness, spasticity, and loss of sensation in the lower limbs. Ferreirinha and colleagues (2004) developed a mouse model for HSP by mutating the mitochondrial ATPase paraplegin, which resulted in local mitochondrial morphological and functional abnormalities, followed by impairment of axonal transport and consequent axonal degeneration. As a classic example of a polyglutamine-related disease, Huntington's disease is also associated with altered mitochondrial trafficking in motor neurons (Piccioni et al., 2002; Trushina et al., 2004). These studies demonstrated that aggregation

of polyglutamine tract-containing proteins confounds axonal trafficking of mitochondria, presumably due to disruptions of cytoskeletal interactions. We have recently demonstrated that overexpression of mutant huntingtin protein creates a physical "roadblock" in axonal processes, thereby preventing mitochondria from getting around these barriers to neuronal regions where they are needed (Chang *et al.* 2003, SFN Abstract). Such pathological alterations in mitochondrial distribution may contribute to the neurodegenerative process.

Glutamate excitotoxicity

Glutamate excitoxicity has been extensively studied as a model for ischemia-induced neurodegeneration. Our earlier studies demonstrated that upon intense glutamate stimulation of primary neurons, mitochondria accumulate Ca^{2+} (White and Reynolds, 1997). Mitochondrial Ca^{2+} overload prevents the organelle from properly buffering cytosolic Ca^{2+} levels or generating necessary ATP. Additionally, Ca^{2+} overload causes the release of apoptotic factors and subsequent cell death. In a later study (Stout et al., 1998) our lab demonstrated that $\Delta\Psi_m$-driven mitochondrial Ca^{2+} uptake was necessary for glutamate-induced neuronal death.

The inability of mitochondria to regulate $[Ca^{2+}]_i$ and fulfill cellular ATP demands will inevitably affect neuronal viability. One hypothesis is that mitochondria cannot deliver energy and nutrients to neuronal regions with high metabolic demand. In turn, the failure to place mitochondria in regions of where ATP is needed may compromise neuronal viability. To examine the effects of glutamate on mitochondrial trafficking in primary neurons, we utilized a mitochondrially-targeted fluorescent protein to visualize and measure mitochondrial movement (Rintoul et al., 2003). We observed that toxic and

acute glutamate exposure drastically impaired mitochondrial movement and altered morphology from an elongated to punctuate structure. In elucidating a mechanism for these effects, we compared the actions of glutamate to FCCP to depolarize mitochondria and oligomycin to inhibit ATP synthesis. Both FCCP and oligomycin impaired mitochondrial movement, but did not alter morphology. We speculate that movement inhibition results from loss of ATP, whereas the morphological change results specifically from Ca^{2+} uptake generated by glutamate. This is consistent with our observations that acute Zn^{2+} elevations impair movement without affecting morphology (Chapter 4). In contrast, the effects of Zn^{2+} and Ca^{2+} on mitochondrial function differ, such that with glutamate treatment, mitochondrial undergo Ca^{2+}-mediated $\Delta\Psi_m$ depolarizations (White and Reynolds, 1996), whereas elevated $[Zn^{2+}]_i$ does not cause loss of $\Delta\Psi_m$ (Dineley et al., 2000, Chapter 4). These results suggest that $\Delta\Psi_m$ depolarization does not play a role in Zn^{2+}-induced inhibition of mitochondrial trafficking.

Perhaps one component of the glutamate effect is associated with changes in the cellular cytoskeleton. Indeed, Ackerley and colleagues (2000) demonstrated that toxic exposure of glutamate in primary cortical neurons generated increased phosphorylation and subsequent aggregation of neurofilament proteins, thereby slowing axonal transport of neurofilament. Although the study did not implicate mitochondrial associations, one could hypothesize that glutamate stops mitochondrial movement through disrupting mitochondrial-neurofilament interactions. The mechanisms behind glutamate-mediated modulation of mitochondrial trafficking are still under investigation.

Although glutamate and zinc are neurotoxins, it is interesting that only glutamate induced a Ca^{2+}-mediated change in morphology. A growing body of literature indicates

that mitochondria can modulate their shape by expression of specific cytoplasmic proteins, examples being dynamin-related protein 1 (Drp1) and mitofusin (Mfn). Although their physiological roles are not clear, upregulation of Drp1 and Mfn protein expression in neurons causes mitochondrial fission and fusion, respectively (Filiano *et al.*, 2002, SFN Abstract). With respect to cell injury, studies in non-neuronal cells demonstrated that apoptogen treatment induced mitochondrial fragmentation (Frank et al., 2001), suggesting that fission events may be upregulated during cell death. Although we have not yet investigated fission mechanisms in our glutamate paradigm, we recognize that morphological changes may be important with respect to cell death mechanisms. However, changes in morphology during cell injury will not be discussed further with respect to this dissertation because we did not observe it with Zn^{2+}.

Nerve growth factor (NGF)-induced modulation of axonal transport

The docking or stopping of mitochondria in certain regions of the neuronal process is hypothesized to be controlled by sensor molecules, which could include ions (such as Ca^{2+}), metabolites (such as ATP, ADP or NADH), second messengers (such as a small G-protein), or a signal transduction pathway (Hollenbeck, 1996). The sensor could either be freely dispersed in the cytosol or closely associated with mitochondria or the cytoskeleton (Miller and Sheetz, 2004). One such example comes from a recent study by Chada and Hollenbeck (2003) demonstrating that nerve growth factor (NGF) signaling attenuates mitochondrial motility in dorsal root ganglion (DRG) neurons. Here, the authors show that local application of NGF along the axons causes not only more mitochondria to enter these NGF-concentrated foci, but also decreases the number of mitochondria that leave these foci as well. Most intriguingly, inhibitors of

phosphatidylinositol 3-kinase (PI 3-kinase) prevented the mitochondrial congregation at these locales, suggesting that a sensor protein-induced signaling pathway that is mimicked by NGF mediates this docking phenomenon.

These observations strongly support our $[Zn^{2+}]_i$-mediated mitochondrial movement inhibition that is regulated by PI 3-kinase activity (Chapter 4). However, it is interesting that unlike Zn^{2+} or glutamate, NGF is not normally considered a neurotoxin. This suggests that NGF-induced mitochondrial docking may be a necessary event to control mitochondrial transportation within cells and may have a more global importance for neuronal survival (Reynolds and Rintoul, 2004). Indeed, Kuruvilla and colleagues (2000) observed that NGF-mediated PI 3-kinase activation in sympathetic neurons is critical for neuronal viability. It must be emphasized that the concept of mitochondrial docking is not well established. Therefore, questions that remain to be addressed are: (1) what are the identities of these docking proteins that cause mitochondria to stop at specific sites and (2) what signals guide mitochondria to docking stations ?

5.6 Associations between zinc and trafficking in neurons

We use the term "mitochondrial trafficking" here to describe not only mitochondrial distribution, but also the anterograde and retrograde translocation of mitochondria along neuronal processes. These properties of mitochondrial trafficking have only been recently addressed, and even less is known about the factors that regulate them during injury. Based on the current literature, however, there is the suggestion that neurotoxins may affect mitochondrial trafficking in neurodegenerative conditions. Given that $[Zn^{2+}]_i$ is both a neuronal and mitochondrial toxin, we proposed that $[Zn^{2+}]_i$ mediates neurotoxicity through changes in mitochondrial movement. Through investigation of

mitochondrial movement in neurons, we have identified possible mechanisms and consequences of toxic $[Zn^{2+}]_i$ accumulation on mitochondrial trafficking in neurons (Chapter 4). We observed that Zn^{2+} does not affect $\Delta\Psi_m$ at the exposure concentrations and times which inhibit mitochondrial movement. This is similar to Miller and Sheetz (2004) where treatment of mannitol stopped mitochondrial movement without affecting $\Delta\Psi_m$. This suggests that mitochondrial inhibition, which is mediated by local sensor molecules, and directional transport, which is determined by $\Delta\Psi_m$, are events that are independently regulated. Topics that speculate mechanisms in zinc regulation of mitochondrial trafficking will be discussed below.

5.6.1 Zinc and the cellular cytoskeleton

The earliest evidence for Zn^{2+} effects on the cytoskeleton in the nervous system was provided by Kress and colleagues (1981) where zinc wires were implanted into adult rat brains for several weeks. The exposure of zinc reduced the numbers of microtubules significantly in brain tissue, which was presumed to result from destabilizing tubulin assembly as opposed to directly interacting with microtubules. The slow release of zinc ions from the wire had no affect on actin filaments or neurofilaments. Interestingly, the mitochondria within the processes exposed to zinc were enlarged, swollen and started to disintegrate, since only remains of cristae were visible at the end of the wire implantation period. This suggested that microtubule disorganization and mitochondrial disruption may be potential mechanisms involved in zinc-induced neuronal injury.

We have previously demonstrated that acute exposure of cytoskeletal disrupting agents dramatically stops mitochondrial movement (Filiano *et al.* 2004, SFN Abstract). After drug treatment, our primary neurons display punctate and fragmented staining of

tubulin (for microtubule organization) and phalloidin (for actin filament organization). In our Zn^{2+} experiments, however, we were not able to sufficiently conclude that Zn^{2+} inhibition of mitochondrial movement was specifically associated with cytoskeletal components (Chapter 4, data not shown). In contrast to the positive control using cytoskeletal inhibitors, the staining for cytoskeletal proteins after acute and toxic Zn^{2+} exposure produced ambiguous results, implying weaknesses in the technique that failed to display cytoskeletal disorganization unless the cell was grossly disrupted.

5.6.2 Zinc activation of cellular signaling pathways

Zn^{2+} has been shown to activate a number of cellular signaling pathways, which highly depend on the conditions and concentrations of Zn^{2+} exposure. Having tested a number of protein kinase inhibitors for protection against our Zn^{2+}-mediated movement phenomenon (Chapter 4), we identified inhibitors of the PI 3-kinase pathway as positive "drug hits" in our assay since (1) they did not affect mitochondrial movement by themselves and (2) they prevented the Zn^{2+}-mediated movement effect in our primary neurons. Therefore, we will exclude the discussion of other signaling pathways here and will only focus on evidence for PI 3-kinase activation by Zn^{2+}.

Zinc activation of phosphatidylinositol 3-kinase

Extensive evidence exists for indirect activation of PI 3-kinase by Zn^{2+} in various cell types: (*i*) hundreds of micromolar Zn^{2+} activated PI 3-kinase in epithelial cells within 15 minutes (Tang and Shay, 2001), (*ii*) low micromolar concentrations of added Zn^{2+} activated p70 S6 kinase in cell lines, which was sensitive to PI 3-kinase inhibitors (Kim et al., 2000b), (*iii*) micromolar concentrations of Zn^{2+} activated p70 S6 kinase, regulated by both PI 3-kinase and MAPK pathways in neuroblastoma cells (An et al., 2005), (*iv*)

micromolar Zn^{2+} concentrations at chronic exposures induces c-Jun-N-Terminal kinase (JNK) through PI 3-kinase in primary cortical cells and cell lines (Eom et al., 2001) and (*v*) micromolar quantities of Zn^{2+} for only a few minutes robustly activated PI 3-kinase in primary neurons and cell lines by activating upstream tropomycin-related kinase (Trk) receptors (Hwang et al., 2005). The important distinction between these studies and our own (Chapter 4) is that opposed to relying on inherent mechanisms for cellular Zn^{2+} uptake, we rapidly and reliably elevated $[Zn^{2+}]_i$ using an ionophore (Dineley et al., 2000). Using this method for Zn^{2+} delivery, we consistently observed that pathological Zn^{2+} elevations increased phosphorylated Akt levels in primary neurons within 5 minutes of exposure, at the same time as mitochondrial movement was inhibited. PI 3-kinase inhibitor prevented both Akt phosphorylation and the Zn^{2+}-induced movement effect, confirming that PI 3-kinase activity modulates mitochondrial movement.

In our mitochondrial movement paradigm, we sought the role of Akt phosphorylation as the most obvious downstream factor in the PI 3-kinase pathway (Chapter 4). Bijur and Jope (2003) demonstrated rapid accumulation of Akt in mitochondria after PI 3-kinase activation, suggesting that Akt is the downstream mitochondrial target in the PI 3-kinase pathway in response to cellular stresses. However, we could not conclude that Akt phosphorylation was a downstream event in Zn^{2+}-induced PI 3-kinase pathway, though we speculate that other downstream targets of PI 3-kinase (ARF6, TOR) may identify a Zn^{2+} target on mitochondria. Apart from Akt, the several other downstream targets of PI 3-kinase that we investigated were also not involved in the Zn^{2+} inhibition of mitochondrial movement. The difficulties in identifying the correct protein kinase target stem from the multitude of kinase substrates

in the cell, as well as limitations in the available methods for detecting targets. These issues will be discussed below.

Specificity of protein kinase inhibitors

The eight isoforms of PI 3-kinase proteins make up a kinase superfamily involved in key cellular functions such as cell growth and proliferation, motility, differentiation, survival and intracellular trafficking (Foster et al., 2003). Therefore, nearly all important aspects of cellular function are regulated by the cycle of phosphorylation and dephosphorylation of these critical kinases. We can specifically study these critical functions by blocking them in cells. The two most commonly used, yet chemically unrelated, inhibitors are wortmannin and LY294002. Unfortunately, the limited selectivity of these inhibitors stems from the fact that they inhibit all known PI 3-kinase isoforms, therefore it is difficult to dissect the involvement of distinct PI 3-kinase proteins. The fungal metabolite wortmannin (isolated from *Penicillium wortmanni*) irreversibly inhibits PI 3-kinase in cell-based assays at > 100 nM (Ward et al., 2003). It is also capable of inhibiting the skeletal muscle isoform (SnMLCK) and other protein kinases, although at several micromolar concentrations. Derived from the bioflavanoid quercetin family of chromenones, LY294002 is a selective, but less potent, inhibitor of the PI 3-kinase family. LY294002 completely inhibits PI 3-kinase activity at 100 μM. Although less potent than wortmannin, LY294002 has the advantage of being more stable in solution. As mentioned above though, neither of these inhibitors is selective toward any PI 3-kinase isoform and may even inhibit other protein kinases outside of the PI 3-kinase family.

Developments of more selective inhibitors have utilized the structures and binding properties of wortmannin and LY294002. More recently, a second generation of PI 3-kinase inhibitors have been developed with specific selectivity towards the α, β, and δ isoforms, although detailed information about their structures and their commercial availability are unknown.

Identifying targets of protein kinases

The other issue involving the study of kinases is the enormous complexities of signaling pathways. Approximately one-third of mammalian cell proteins can be phosphorylated and there are roughly 1000 protein kinases encoded by the human genome, each of which phosphorylates an estimated 30 proteins (Davies et al., 2000). This number is probably an underestimate since proteins can be phosphorylation targets of multiple kinases. The identification of kinase substrates has been slow and difficult because the active domains of many protein kinases share a high degree of structural similarity. Also as mentioned above, many of the pharmacological inhibitors lack specificity and often exhibit overlapping sensitivities to many different protein kinases.

We established in primary neurons that Zn^{2+} attenuated mitochondrial movement through rapid PI 3-kinase activation (Chapter 4). Speculating that the most direct downstream target is also a protein kinase, we selectively blocked the activity of that particular kinase to determine its role in our movement paradigm. Of the number of PI 3-kinase targets that we tested, none of the potential candidates provided us with positive evidence. In finding this target, we contended with the following difficulties: (*i*) signaling pathways are never linear and distinct, and therefore are dependent on and regulate other pathways simultaneously, (*ii*) we are limited with a finite number of

reagents which are not entirely selective and may even block multiple kinases, depending on concentrations applied, and (*iii*) many protein kinases have critical functions in several cell processes, therefore blocking its activity to study one aspect may compromise other cellular functions. However, the PI 3-kinase inhibitors provided a direct and specific effect on mitochondrial movement compared to the other signaling pathway inhibitors tested. Despite the inability to completely dissect this pathway from all the others occurring in the cell, we demonstrated that inhibition of a specific kinase prevented an indirect interaction between Zn^{2+} and mitochondria.

5.7 Therapeutic implications of chelators in zinc-induced toxicity

Evidence for Zn^{2+} contribution to neuronal death suggests potential targets for neuroprotection, the most logical approaches involving Zn^{2+} chelation or blocking cellular Zn^{2+} entry. More specific methods of reducing Zn^{2+} neurotoxicity, specifically by chelating $[Zn^{2+}]_i$ will be discussed below.

5.7.1 Methods of zinc chelation

We have demonstrated that membrane-permeant (TPEN) and membrane-impermeant (EDTA) chelators can reverse Zn^{2+}-mediated effects both in cells (Dineley et al., 2000) and isolated mitochondria (Dineley et al., 2005), respectively. With respect to use of these chelating agents *in vivo*, Cuajungco and Lees (1996) demonstrated that the presence of TPEN during intrahippocamal administration of $ZnCl_2$ completely prevented Zn^{2+}-induced neuronal loss. This is consistent with our results in primary neurons, where maximal cell survival was observed when elevated Zn^{2+} was co-administered, or applied within 5 minutes of TPEN (Chapter 4). However, if TPEN was applied following a 10-minute Zn^{2+} exposure, Zn^{2+} chelation no longer afforded protection, suggesting that

TPEN has a critical window for therapeutic potential. In other studies, administration of EDTA significantly protected against Zn^{2+}-induced neurotoxicity (Frederickson et al., 2002) in animal models of ischemia (Koh et al., 1996) and traumatic brain injury (Hellmich et al., 2004). Although these lines of evidence suggest that these chelators may provide therapeutic value to neurodegenerative models, whether they interfere with physiological Zn^{2+}-mediated processes remains unknown. In addition, it is apparent that direct Zn^{2+} removal reduces its neurotoxic consequences. However, the window of time for chelation is critical in order to prevent the cascade leading to Zn^{2+}-mediated neurotoxicity (Chapter 4).

5.7.2 Zinc and tissue plasminogen activator

Tissue plasminogen activator (tPA) normally converts plasminogen into the active form plasmin and is observed to mediate neurodegeneration *in vivo* and in cell culture (Tsirka et al., 1995; Tsirka et al., 1996). In addition, cell death is limited in $tPA^{-/-}$ mice, indicating that tPA is a potent neurotoxin (Tsirka et al., 1997). Interestingly, in the context of zinc-mediated neurotoxicity, tPA can afford protection (Kim et al., 1999a). Siddiq and Tsirka (2004) have investigated the mechanism for tPA-mediated protection against Zn^{2+}. They show that zinc binds and inhibits tPA activity in a dose-dependent fashion. In addition, tPA modulates extracellular Zn^{2+} by binding to it and also facilitating its cellular entry through voltage-sensitive calcium channels and Ca^{2+}-permeable AMPA/KA channels. The presence of tPA has physiological significance for Zn^{2+} sequestration and neuroprotection, since primary neurons deficient for tPA exhibit increased sensitivity to Zn^{2+}. Based on these studies, it has been speculated that zinc and

tPA may act to inhibit the neurotoxic properties of the other, although how their interactions are regulated is not entirely clear.

5.7.3 Zinc and clioquinol

One example of a potential clinical candidate for Zn^{2+} chelation is the oral antibiotic clioquinol (CQ), which selectively binds Zn^{2+} and Cu^{2+}, possesses hydrophobic properties, and freely crosses the blood-brain barrier. Given that one of the proposed mechanisms for Alzheimer's disease development is the Zn^{2+}-induced promotion of Aβ plaques, CQ presented an ideal drug candidate to solubilize the plaques. Cherny and colleagues (2001) presented a study, whereby chronic CQ treatment in a transgenic mouse model of Alzheimer's disease markedly improved Aβ plaque deposition compared to non-treated controls. These structural brain changes were accompanied by significant improvement in general behavior. These primary results suggest that CQ may be useful in clinical trials involving AD patients. Indeed, a recent study by Ritchie and colleagues (2003) described a pilot phase 2 clinical trial in AD patients with CQ treatment to specifically detect cognitive changes. According to the study, CQ provided a beneficial effect on AD progression seen only in the most severely affected subjects by reduction in plasma Aβ, enhanced zinc levels and cognitive improvements. However, after extensive use for over 20 years as an antibiotic, CQ was withdrawn from oral use in 1970, primarily due to the safety concerns associated with it, such as risk for developing subacute myelo-optic neuropathy, therefore long-term effects with CQ treatment remain unknown.

5.8 Overall conclusions and future perspectives

5.8.1 Source and concentrations of zinc with relevance to neuronal injury

One of the most concerning factors with respect to investigating the toxic effects of Zn^{2+} in cell culture is determining what effects are generated under the most physiologically relevant conditions. Because it presents a simplified and basic system, isolated mitochondria allow one to dissect the precise location of Zn^{2+} actions. We have previously demonstrated that 5-20 μM additions of Zn^{2+} cause substantial $\Delta\Psi_m$ loss and also completely inhibit O_2 consumption (Dineley et al., 2005). However, the actual concentrations sensed by mitochondria are much lower, given that 10 μM EGTA is present in the suspension solution. In contrast, in a study by Sensi and colleagues (2003b), picomolar concentrations of Zn^{2+} caused substantial $\Delta\Psi_m$ depolarizations in isolated mitochondria. The high sensitivity of mitochondrial function to such small Zn^{2+} concentrations compared to what we observed may be accounted for by the differences in techniques used to measure mitochondrial depolarization – TPP^+ electrode vs. Safranin O fluorescence, respectively. Returning to the study by Sensi *et al*., the authors demonstrated that the same picomolar concentrations of Zn^{2+} increased mitochondrial respiration, whereas slightly higher concentrations in the nanomolar range significantly inhibited mitochondrial respiration. However, these values only represent stock Zn^{2+} concentrations added to a mg of mitochondrial protein and do not reflect final concentrations in the suspended solution.

In contrast to isolated mitochondria, it is much more difficult to determine precise $[Zn^{2+}]_i$ in cells, given the presence of Zn^{2+}-binding proteins and other buffering components in the cytoplasm. Using the potentiometric probe JC-1, we observed only a

modest mitochondrial depolarization in primary neurons exposed to 300 μM Zn^{2+} in the presence of the ionophore sodium pyrithione (Dineley et al., 2000). Similarly, we observed no significant depolarization of neurons exposed up to 30 μM Zn^{2+} in the presence of pyrithione using either Rh123 or TMRM (Chapter 4). On the contrary, Sensi and colleagues demonstrated in subsequent studies in 1999 and 2000, that neurons loaded with TMRE underwent substantial loss of $\Delta\Psi_m$ upon acute exposure to kainate and 100-300 μM Zn^{2+}. The contrasting effects on mitochondrial function may be explained by variable experimental parameters: differences in $[Zn^{2+}]_i$ accumulation achieved with ionophore vs. stimulus or variability in dye concentrations used.

Although several studies have established a range of Zn^{2+} concentrations that affect neuronal function in a variety of ways, the critical question is how much $[Zn^{2+}]_i$ is minimally sufficient to mediate substantial cellular and mitochondrial defects ? From the studies completed in this dissertation, we have demonstrated: (1) oxidant-induced $[Zn^{2+}]_i$ release in cells sufficient to induce toxicity, (2) mitochondrial transport mechanisms that are either uniporter-dependent or uniporter-independent based on the concentrations and capacities for Zn^{2+} uptake, and (3) a concentration-dependent effect on mitochondrial movement that occurs at pathophysiological levels. Interestingly, these effects are sensitive to various ranges of Zn^{2+} concentrations, given that low micromolar Zn^{2+} concentrations do not depolarize mitochondria in cells, yet they effectively stop mitochondrial movement (Chapter 4). Based on our results, it is clear that there are several toxic effects mediated by Zn^{2+} and we have demonstrated that they occur at the lowest, pathophysiologically relevant levels in our paradigms.

5.8.2 (Patho)physiological role of synaptic vesicular zinc

Defined in the early 1990s, the zinc translocation hypothesis claimed that synaptically-released Zn^{2+} from presynaptic vesicles that accumulates into postsynaptic neurons may contribute to the excitotoxic brain injury characteristic of stroke, traumatic brain injury, or epileptic seizures. This hypothesis was strengthened by the use of extracellular metal chelators (Koh et al., 1996) and specific blockade of Zn^{2+}-permeable channels on the postsynaptic membrane (Yin et al., 2002) that substantially reduced the Zn^{2+} accumulation in and subsequent cell death of postsynaptic neurons. What immediately brought the Zn^{2+} translocation hypothesis into question was the observation that in transgenic mice that were deficient in ZnT-3, postsynaptic neurons still accumulated Zn^{2+} upon stimulation and the mice still suffered from kainate-induced seizures (Lee et al., 2000). Interestingly, this Zn^{2+} accumulation was demonstrated to be blocked by EDTA chelation (Frederickson et al., 2002), suggesting either that the membrane-impermeant chelator may be able to leech Zn^{2+} out of the cell or there may be an unknown extracellular source for Zn^{2+} release. Accordingly, Alan Kay (2003) recently demonstrated the visualization of releasable Zn^{2+} pools from brain slices using the Zn^{2+} fluorophore, FluoZin-3. From these studies, very little chelatable Zn^{2+} (~ 2 nM) was detected during synaptic transmission, representing the pool released from synaptic vesicles into the extracellular space. In contrast, more Zn^{2+} (~ 6 nM) was extruded in the presence of high K^+, but was not seen as released from slices, suggesting that this pool is not synaptic, and may be part of a new extracellular pool of Zn^{2+} that is loosely-associated with proteins.

Although the extensive evidence described above argues against the zinc translocation hypothesis, recent studies have determined that synaptic zinc still has an important pathophysiological role during the course of neurodegeneration. For example, a study by Lee and colleagues (2003) demonstrated that amyloid plaque formation was reduced in Alzheimer's disease (AD) transgenic mice, which were also ZnT-3-deficient, suggesting that the synaptic Zn^{2+} is a major contributor to the progression of AD. In a subsequent study by the same group (Lee et al., 2004), they hypothesized that female sex hormones reduced synaptic Zn^{2+}, and diminished plaque formation and AD progression. The estrogen levels were also inversely related to amounts of ZnT-3 protein in the brain, suggesting that estrogen modulation of synaptic Zn^{2+} may be neuroprotective in AD. In a third and most recent study, Friedlich and colleagues (2004) observed a transgenic mouse model for AD, whereby an exchangeable pool of Zn^{2+} between ZnT-3 and the cerebrovascular wall promotes cerebral amyloid angiopathy. The authors claimed that the source of Zn^{2+} had to be synaptic since the angiopathy was reduced in ZnT-$3^{-/-}$ animals. Furthermore, the condition was only present in the brain and not in peripheral organs, and ZnT-3 protein was only found in brain tissue and not in vessel walls or other blood components.

The contradicting data in these most recent studies is comparison to previous ones that state an absence of physiological function of ZnT-3 stem from a number of issues. First, although ZnT-3 knockout animals exhibit ~ 15% reduction in total brain zinc, they are physiologically normal in learning and memory tasks, and are found to grow, reproduce and age normally as well. Second, the absence of histochemically-stainable Zn^{2+} does not preclude the presence of other synaptic pools that cannot be readily

detected. And finally, that under excitotoxic condition, mice lacking synaptic Zn^{2+} still exhibit neuronal damage from seizures, strongly suggesting that the Zn^{2+} accumulation must be derived from other sources and thereby demoting the role of ZnT-3 as a physiological modulator of synaptic Zn^{2+}.

Given the evidence against the zinc translocation hypothesis, a new model has been proposed whereby Zn^{2+} contribution to neuronal death may be derived from multiple sources: (*i*) from presynaptic vesicles, (*ii*) from Zn^{2+}-binding proteins, or (*iii*) from mitochondrial stores (Sensi et al., 2003b; Bossy-Wetzel et al., 2004) although we have found no evidence of this last pool (Chapter 3). One of the most likely examples is metallothionein, an example of the protein-derived Zn^{2+} pool which has been demonstrated to be a stress-induced "Zn^{2+} fountain" after nitrosylative (Cuajungco and Lees, 1998; Chen et al., 2002) and oxidative (Maret and Vallee, 1998; Aizenman et al., 2000) stresses. The observation that MT-III knockout mice exhibit reduced neuronal Zn^{2+} staining after excitotoxic insult (Lee et al., 2002; Lee et al., 2003) suggests that MT-III may be a major source of toxic Zn^{2+}. This is not surprising given that with the numerous Zn^{2+}-binding proteins and enzymes in the intracellular milieu, the presence of oxidant-sensitive Zn^{2+} stores is a likely and threatening contributing factor to Zn^{2+}-induced toxicity under stressed conditions.

5.8.3 Zinc and reactive oxygen species: cause or effect ?

Oxidative stress is known to disrupt metal homeostasis, but it has also been hypothesized that metal release causes neuronal injury through ROS production. This proverbial "chicken and egg" situation begs to question whether it is metal-induced ROS production or ROS-mediated metal release that causes neuronal injury. Indeed, studies

have demonstrated that elevated Zn^{2+} exposure *in vitro* mediates neuronal death by free radicals, since cells are protected by treatment with antioxidants, such as trolox (Kim et al., 1999b) or vitamin D (Lin et al., 2003). In our isolated mitochondria experiments, we observed ROS production upon direct and acute Zn^{2+} exposure (Dineley et al., 2005). Interestingly, as opposed to reversal of Zn^{2+}-induced depolarization or compromised respiration upon EGTA addition, we observed no reversal in ROS production with chelation, suggesting that ROS production occurs by a more permanent and damaging mechanism compared to other mitochondrial disruptions. In our experiments involving Zn^{2+} modulation of mitochondrial trafficking, we observed that both elevated Zn^{2+}, strong oxidizing agents (2,2'-DTDP) or mimicking ROS exposure (H_2O_2) inhibited mitochondrial movement at concentrations toxic to neuronal cultures (Chapter 4). To determine if the effect of Zn^{2+} on movement was mediated primarily by ROS production, we followed the treatments with the reducing agent dithiothreitol (DTT). DTT provided no restoration of movement, indicating that ROS exposure may contribute to the movement effect in an irreversible and permanent manner.

In the reverse manner, it has been demonstrated in cultured cells that both strong oxidative (Aizenman et al., 2000) and nitrosylative (Bossy-Wetzel et al., 2004) stresses induce high $[Zn^{2+}]_i$ in cultured neurons. It is hypothesized that the subsequent ROS and RNS act on thiol bonds of MT to free disulfide linkage-bound Zn^{2+}. Indeed, we demonstrated that 2,2'-DTDP treatment in MT-expressing astrocytes rapidly generated $[Zn^{2+}]_i$ elevations significantly higher than controls. These $[Zn^{2+}]_i$ rises were diminished quickly upon Zn^{2+} chelation, indicating that Zn^{2+} was directly released from MT stores upon oxidation (Chapter 2). MT provides a direct linkage between oxidative stress and

metal release given that (1) MT is a metal-binding and antioxidant protein, (2) MT mRNA is upregulated in response to both elevated Zn^{2+} and levels of oxygen, and (3) MT-deficient mice and cells exhibit increased sensitivity to oxidative stress (Lazo et al., 1995) and enhanced apoptosis (Kondo et al., 1997), respectively. Since mitochondria are the primary generators of free radicals within cells, they are another critical linkage between Zn^{2+} and ROS. The elevated free Zn^{2+} released from MT then acts on mitochondria at specific sites of electron transport to enhance further ROS accumulation (Figure 5.1). This "feed-forward loop" of metal release and oxidative stress suggests that regardless of which one begins the cycle, excitotoxic mitochondrial, and subsequent neuronal damage, stems from simultaneous potentiation of increased Zn^{2+} accumulation and oxidative stress. What appears to be a future direction for the field is perhaps investigation of how drastic alterations in cellular oxygen in the brain during neurological conditions of stroke or cerebral ischemia, contribute to disruption of metal homeostasis and subsequent mitochondrial and cellular dysfunction.

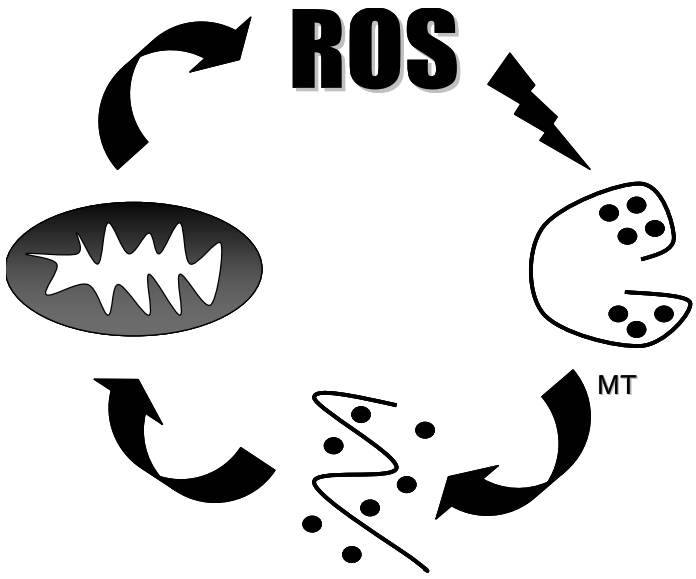


Figure 5.1 "Feed-forward loop" of zinc and reactive oxygen species

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