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Zn^{2+} transporters and Zn^{2+} homeostasis in neurons

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Abstract

Although the presence of Zn^{2+} in the brain has been known for nearly half a century, only recently has its precise location and potential roles as a neuromodulator and signaling molecule as well as neurotoxic agent come to the forefront. Unfortunately, our understanding of Zn^{2+} homeostatic mechanisms lags far behind. The recent identification of presumed Zn^{2+} transporters has opened new approaches to studying Zn^{2+} homeostatic mechanisms in neurons. Zn^{2+} transporters are involved in separate Zn^{2+} influx and efflux pathways in neurons. However, we are only beginning to understand the mechanism of Zn^{2+} transport and much more research needs to be done. We are only beginning to understand the transcriptional control and cellular location of Zn^{2+} transporters, as well. Finally, this review presents a working model of neuronal Zn^{2+} homeostasis and discusses the experimental evidence for the proposed roles that Zn^{2+} transporters might play.

Keywords: Metallothionein; Zn^{2+} trafficking; Transition element; Zinquin; Mitochondrion; Cultured cell; Fluorescence microscopy; (Rat)

1. The Zn^{2+}-containing cell: unifying concepts in Zn^{2+} transport and homeostasis

This review will combine old and new research findings addressing Zn^{2+} biology, building a conceptual framework describing our understanding of Zn^{2+} homeostasis in the neuron and the role played by Zn^{2+} transporters. We will certainly focus on new and exciting findings, areas of controversy, and will try to highlight gaps in our knowledge. Our first task will describe briefly the role Zn^{2+} plays in the nervous system and why Zn^{2+} homeostasis is important. Zn^{2+} is an essential trace element (Prasad et al., 1963) and the neurological consequences of dietary Zn^{2+} deficiency are well studied and characterized (Prasad, 1997). The developing organism is particularly sensitive to Zn^{2+} deficiency, Zn^{2+} being required for the proper formation of the nervous system (Prasad, 1997). Zn^{2+} deficiency in adult animals results in several behavioral symptoms including memory defects, lethargy, and greater susceptibility to stress (Sandstead, 1984). Similar symptoms are seen in humans suffering from various causes of Zn^{2+} deficiency (Prasad, 1997). Because Zn^{2+} has a myriad of biochemical actions (Vallee and Falchuk, 1993), and we have only a rudimentary knowledge of Zn^{2+} homeostasis and function in neurons, the basis for Zn^{2+}-deficient effects in the nervous system is not well established. For example, Zn^{2+} confers structural stability to the Zn^{2+} finger domains of DNA-binding proteins. This requirement for Zn^{2+} probably explains, in large part, the striking deleterious effects of Zn^{2+} deficiency on neonatal development. In addition, Zn^{2+} binds with high affinity to several enzymes (e.g., Cu^{2+}, Zn^{2+} superoxide dismutase) where it functions as a cofactor required for catalytic activity (Frederickson, 1989). Finally, more recent research has suggested that both extracellular and intracellular free Zn^{2+} can have important signaling functions, such as the inhibition of gamma-aminobutyric acid (GABA)ergic neurotransmission (Hosie et al., 2003) or modulation of protein kinase C signaling pathways (Korichneva et al., 2002). Although a dietary requirement for Zn^{2+} was established many years ago (Prasad et al., 1963), an appreciation that moderate increases in intracellular free Zn^{2+} are deleterious and can eventually lead to cell death has only recently emerged. Fig. 1 illustrates this concept in graphical terms. Depicted is a measure of cell viability and function as related to increasing intracellular free Zn^{2+}. Optimal cell health, growth, and function are achieved only within a narrow window of intracellular free Zn^{2+} concentrations. The resting intracellular free Zn^{2+} concentration in healthy neurons is currently a matter of great debate (see Section

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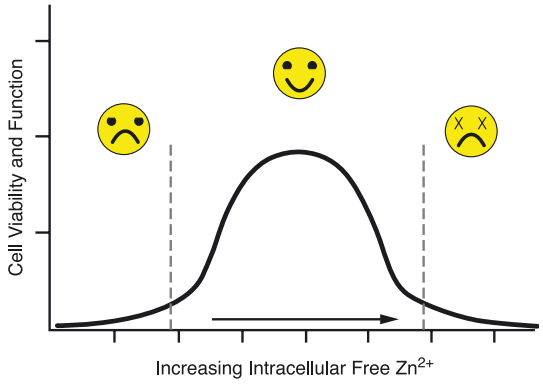

Fig. 1. This figure graphically illustrates the relationship between intracellular free Zn^{2+} concentrations and cell viability and function. To the left is shown the zinc-deficient state brought on normally by low dietary intake. Intracellular free Zn^{2+} concentrations are low, thus Zn^{2+} is not available to fulfill various cellular functions; cell signaling and cell division are impaired as well. To the right is shown zinc toxicity brought on by increases in intracellular free Zn^{2+}. Moderate increases in intracellular Zn^{2+} reaching concentrations of 400–600 nM are toxic to neurons. Increases in intracellular Zn^{2+} may be caused by increased Zn^{2+} entry or release of protein bound or sequestered Zn^{2+} within the cell.

2.1). Zinc-deficient status leads to derangements in cell function because Zn^{2+} is no longer available to fulfill its many required roles in association with Zn^{2+}-binding proteins and presumably as a signaling molecule. On the other hand, if intracellular free Zn^{2+} levels rise above critical levels, even transiently, important cellular functions are inhibited (see Section 2.5). Additionally, Zn^{2+} may initiate processes (particularly in association with other destructive factors such as reactive oxygen species) that can lead to cell death. Thus, proper Zn^{2+} homeostasis is a requirement for cell survival and all living organisms (prokaryotic and eukaryotic alike) have evolved complex and redundant mechanisms to regulate total cellular Zn^{2+} and intracellular free Zn^{2+}.

Although cellular mechanisms of Zn^{2+} homeostasis can be complex, when one looks across different cell types and phylogenetic levels, similarities and parallel pathways clearly emerge. A simplified model of how these mechanisms might work in concert in a generalized eukaryotic cell is illustrated in Fig. 2. The goal of these complex homeostatic mechanisms is to supply needed Zn^{2+} to the cell while maintaining low intracellular free Zn^{2+} concentrations. Even more important, when challenged with a Zn^{2+} load that could threaten to raise intracellular free Zn^{2+} to toxic levels, cytosolic Zn^{2+} must be chelated or sequestered in cytoplasmic organelles. If these processes fail to lower intracellular Zn^{2+}, cell function will certainly be compromised and death will likely ensue. The excess cellular Zn^{2+} must eventually be removed from the cell to ensure its survival. Illustrated in Fig. 2 are the several pathways by which Zn^{2+} enters and exits cells. Many cells, and in particular neurons, possess two pathways for Zn^{2+} entry: carrier-mediated transport and flux through receptor-operated and voltage-gated channels. The focus of this review article is transporters, so for the most part we will limit our discussion of channel-mediated Zn^{2+} fluxes (the reader is directed to recent excellent articles: Marin et al., 2000; Kerchner et al., 2000; Sensi et al., 2000; Jia et al., 2002; Sheline et al., 2002; Yin et al., 2002). Neurons, and probably most cells, express several different Zn^{2+} transporter proteins. These transporters are localized to the plasma membrane and intracellular membranes. Except in certain specialized tissues (e.g., intestinal uptake) plasma membrane transporters are involved in the cellular uptake and replenishment of Zn^{2+}, and in the removal of excess intracellular Zn^{2+}. Zn^{2+} transporters localized to intracellular membranes are involved in the sequestration of cytosolic Zn^{2+} (lowering Zn^{2+} concentrations when needed) and the intracellular trafficking and supply of Zn^{2+} to Zn^{2+}-binding proteins during synthesis. The mitochondria illustrated in Fig. 2 shows a major site within neurons for Zn^{2+} sequestration when cytosolic levels rise. Low intracellular free Zn^{2+} concentrations are maintained under normal homeostatic conditions by the actions of cytosolic metal-binding proteins, the most abundant of which is the metallothionein/thionein pair (Palmiter, 1998; Burdette and Lippard, 2003; Maret, 2003). Zn^{2+} binds with high affinity to the cysteines contained in this protein lowering intracellular free Zn^{2+} to the pico- to nanomolar range. The binding is redox-sensitive such that oxidation releases Zn^{2+} (Maret et al., 1999). Outten and O'Halloran (2001) and Finney and O'Halloran (2003) make the convincing argument that, at least in *Escherichia coli*, metal-binding proteins are so effective as to lower free Zn^{2+} and other metals to femtomolar levels. Metal-binding proteins probably perform two functions: they provide high capacity Zn^{2+} sequestration, but also

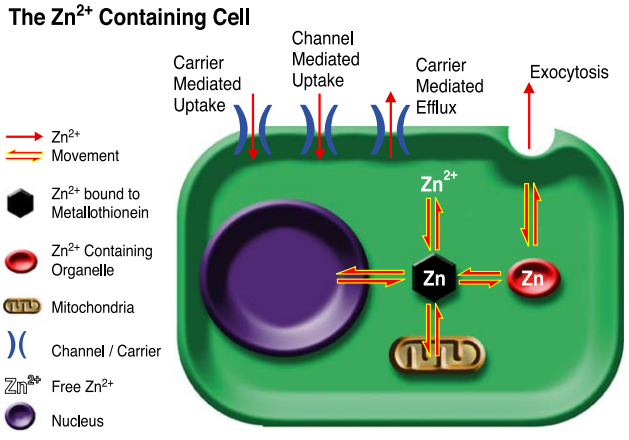

Fig. 2. This figure illustrates mechanisms of Zn^{2+} homeostasis in a generalized cell. Although this image is useful for illustrative purposes, it should be appreciated that individual cell types vary greatly in the mechanisms utilized. Zn^{2+} uptake occurs at the plasma membrane through carrier- and channel-mediated pathways. Distinct pathways of carrier-mediated Zn^{2+} efflux are likely to exist also. Intracellular free Zn^{2+} is maintained at very low levels by the actions of cytosolic Zn^{2+}-binding proteins (metallothioneins) or sequestration into organelles (mitochondria and secretory vesicles). Many cells, in particular glutamatergic neurons, can release large quantities of Zn^{2+} via secretory (synaptic) vesicles.

act as Zn^{2+} depots providing a source of Zn^{2+} for both intra- and extracellular Zn^{2+} signals and delivering Zn^{2+} to intracellular organelles. Very little is known about how Zn^{2+} transporters interact with and deliver Zn^{2+} to cytosolic metallothionein or how Zn^{2+} transporters contribute to Zn^{2+} compartmentalization and trafficking. Certain neurons and several other secretory cell types (e.g., insulin secreting β cells of the pancreas) store large amounts of Zn^{2+} in secretory vesicles and are sometimes termed "zincergic cells". The synaptic vesicles of glutamatergic neurons in the cerebral cortex and limbic system contain Zn^{2+} and significant amounts of Zn^{2+} can be released upon excitation (see circle, Fig. 2). In the nucleus, Zn^{2+} plays a critical role in the stabilization of zinc finger domains of DNA-binding proteins. We will now turn our focus to the Zn^{2+}-containing neuron and the unique problems faced by glutamatergic neurons in Zn^{2+} homeostasis and trafficking.

2. Cellular Zn^{2+} homeostasis: the neuron

2.1. What are the resting intracellular and extracellular concentrations of free Zn^{2+} in the brain?

The amount of Zn^{2+} in the mammalian brain averages about 10 μg/g (wet weight) and is fairly uniform over different regions or when comparing the grey and white matter (Frederickson, 1989). Although interesting developmental changes in brain Zn^{2+} occur, little change in total brain Zn^{2+} is observed with aging (Frederickson, 1989). Presumably then, the total amount of Zn^{2+} in the extracellular fluid and cytosol is fairly constant throughout the brain. Zn^{2+} concentration in the serum and extracellular fluid are estimated to be 0.15 μM (Takeda, 2000). Metal-binding proteins probably lower extracellular free Zn^{2+} to nanomolar or less concentrations (Takeda, 2000). Neurons, like most cells, accumulate Zn^{2+} (Colvin, 2002). Thus, total Zn^{2+} in the cytosol of a neuron may be as high as 150 μM (Takeda, 2000). Most of this Zn^{2+} is bound with high affinity to metallothionein. The intracellular free Zn^{2+} in neurons is estimated to be $\sim$ 1 nM using fluorescent probes (Canzoniero et al., 1997; Thompson et al., 2002). Dineley et al. (2002) explain how the ion sensitivity of fluorescent probes is dependent on their intracellular concentration. They show that micromolar affinity dyes have little difficulty detecting nanomolar changes in ion concentration when their intracellular concentration is high. They argue that estimates of intracellular free Zn^{2+} using fluorescent probes are unreliable. Thus, intracellular free Zn^{2+} may be much lower than 1 nM as suggested by Finney and O'Halloran (2003). The extremely low concentrations of extracellular and intracellular free Zn^{2+} presents a significant conundrum in the study of Zn^{2+} transport proteins that remains unsolved. Experimentation on the kinetic characteristics of Zn^{2+} transporters consistently reports an affinity for Zn^{2+} in the micromolar range. How can transporters with micromolar affinity efficiently transport ions with nanomolar steady state concentrations? It has been suggested that Zn^{2+} transporters are high capacity/low affinity systems (Gaither and Eide, 2000) or that they mediate the translocation of protein-bound Zn^{2+} (Frederickson, 1989). More experimentation on the mechanism of Zn^{2+} transport is needed to address this question.

2.2. Glutamatergic neurons that sequester Zn^{2+} in synaptic vesicles face unique problems of Zn^{2+} homeostasis

Fig. 3 illustrates a presynaptic glutamatergic neuron that sequesters Zn^{2+} in synaptic vesicles and its postsynaptic partner. Take note of the three pools of Zn^{2+} inside this neuron: free Zn^{2+}, protein-bound Zn^{2+}, and compartmentalized Zn^{2+}. Like any other cell, the vast majority of intracellular Zn^{2+} is tightly bound to metal-binding proteins and free Zn^{2+} is exceeding low (see Section 2.1). The unique feature of this cell, as it concerns Zn^{2+} homeostasis, is the existence of a significant pool of compartmentalized Zn^{2+} inside synaptic vesicles, which is apparently freely exchangeable. Sometimes, compartmentalized and free Zn^{2+} are termed "labile" Zn^{2+} because they are sensitive to metal chelators. The synaptic vesicle Zn^{2+} is also termed histochemically reactive (Frederickson, 1989) because it can be selectively stained by several histological procedures (see Franco-Pons et al., 2000; Jo et al., 2000; Wang et al., 2002c for examples of recent articles). Because of a neuron's architecture, the presynaptic glutamatergic neuron is challenged with the difficult problem of moving Zn^{2+} from likely sites of carrier-mediated uptake in the cell body to distant sites of Zn^{2+} sequestration in synaptic vesicles of axon terminals. The probable Zn^{2+} transport proteins expressed in neurons (ZnT and ZIP) and their subcellular location and function are discussed in detail below. The mechanisms and structures involved in the cellular trafficking of Zn^{2+} are still enigmatic. Zn^{2+} must be sequestered during this journey to protect the cell from the deleterious effects of free Zn^{2+}. This is confirmed by histological studies that show that during this journey, Zn^{2+} is in a state that is not histochemically reactive (Frederickson, 1989). Although metallothionein would be a likely candidate for Zn^{2+} sequestration during the journey, disruption of the metallothionein-III gene (the brain specific member of the metallothionein family) in mice does not affect levels of histochemically reactive Zn^{2+} (Erickson et al., 1997). Fig. 3 illustrates a purely speculative model where Zn^{2+} traverses the distance between cell body and axon terminal sequestered in mitochondria or other organelles including maturing synaptic vesicles. The model illustrates that both anterograde and retrograde movements of Zn^{2+} have been observed (Takeda, 2000). The suggestion that mitochondria contain endogenous releasable Zn^{2+} is problematic since mitochondria do not stain for histochemically reactive Zn^{2+} (Frederickson, 1989). This topic is discussed in greater depth in Section 2.6.

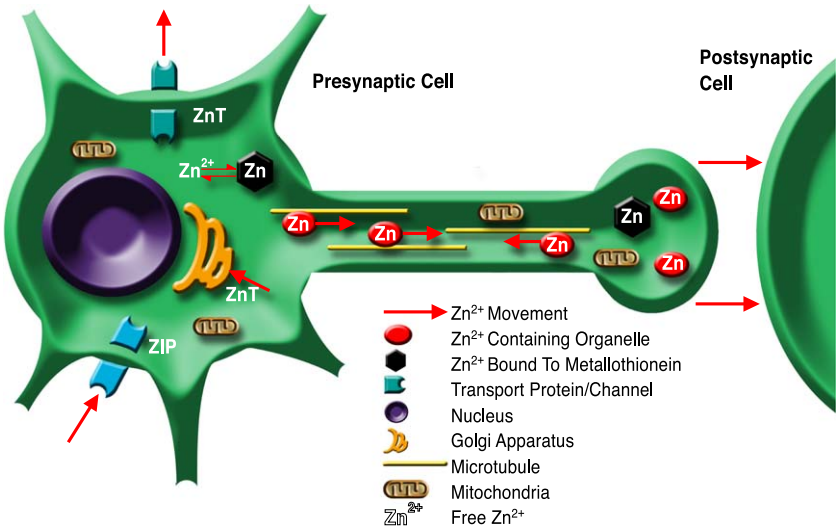

Fig. 3. This figure shows a schema illustrating the potential movements of Zn^{2+} within a presynaptic glutamatergic neuron. Carrier-mediated influx and efflux of Zn^{2+} takes place in the cell body. From here, intracellular free Zn^{2+} is bound to metallothionein or sequestered in cytoplasmic organelles. Carrier-mediated transport is most likely responsible for the organellar sequestration of free Zn^{2+}. These organelles could be mitochondria or maturing synaptic vesicles. At the axon terminal, Zn^{2+} is released into the synapse along with glutamate upon excitation. Adapted from Frederickson (1989).

2.3. Synaptic Zn^{2+}

Once Zn^{2+} is delivered to the axon terminal, it is concentrated in synaptic vesicles and becomes histochemically reactive. Within the presynaptic region, significant amounts of Zn^{2+} (as much as 20% of total brain Zn^{2+} (Cole et al., 1999)) are sequestered inside synaptic vesicles. The concentration of Zn^{2+} inside synaptic vesicles is micro- to millimolar (Frederickson et al., 1983). A putative Zn^{2+} transporter (ZnT-3) is known to be required for sequestration (Palmiter et al., 1996b) in the vesicles. Mitochondria and, of course, metallothionein are prevalent in the presynaptic space, but it is not known if Zn^{2+} can be exchanged between metallothionein, mitochondria and synaptic vesicles. Upon excitation, Zn^{2+} is released along with the neurotransmitter glutamate (Howell et al., 1984; Charton et al., 1985; Aniksztein et al., 1987; Varea et al., 2001; Thompson et al., 2002). Thus, neighboring cells are exposed to transient, but significant increases in extracellular free Zn^{2+} (see Fig. 4). Evidence suggests that synaptic levels transiently reach micromolar levels (Li et al., 2001b; Thompson et al., 2002; Ueno et al., 2002). The fate and physiological actions of synaptically released Zn^{2+} is only beginning to be understood. It is known that physiologically relevant concentrations of Zn^{2+} alter the activity of GABA and glutamate (NMDA receptor subtype) gated channels and other synaptic proteins. Thus, synaptically released Zn^{2+} is a potential modulator of both homo- and heterosynaptic inputs (Vogt et al., 2000; Frederickson and Bush, 2001; Ueno et al., 2002). Synaptically released Zn^{2+} may be required for the induction of long-term potentiation at the mossy fiber input to the CA3 pyramidal neurons of the hippocampus (Li et al., 2001a), this being a region of particularly abundant synaptic vesicle Zn^{2+} (Frederickson, 1989). Yet other studies provide evidence of a Zn^{2+} sensing receptor linking changes in extracellular Zn^{2+} to changes in intracellular Ca^{2+} (Hershfinkel et al., 2001). If Zn^{2+} is a

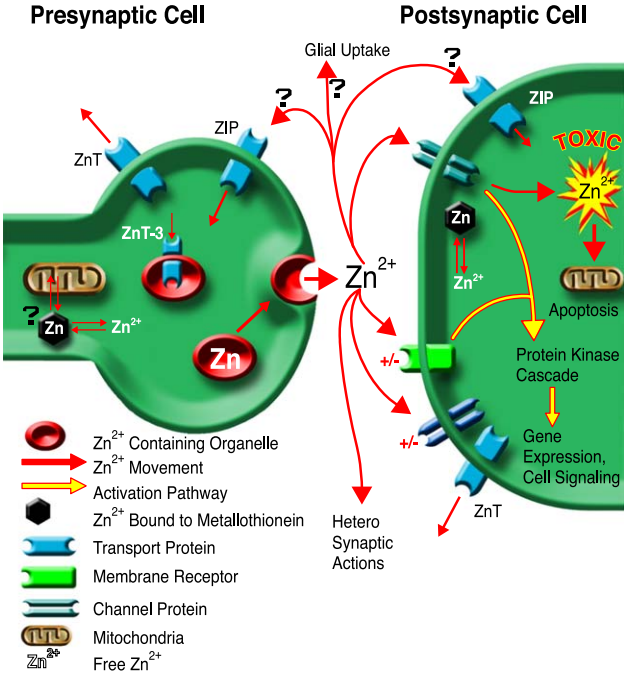

Fig. 4. This figure shows a schema illustrating the potential movements of synaptically released Zn^{2+}. Released Zn^{2+} may be recycled by Zn^{2+} transporters restoring synaptic vesicle Zn^{2+} levels. Alternatively, released Zn^{2+} may be taken up by glia. Additionally, released Zn^{2+} is translocated to the postsynaptic neuron, entering the cell via Ca^{2+}-permeable channels. Once inside the postsynaptic cell, Zn^{2+} potentially modulates several signaling pathways. Excitotoxic stimulation releases large quantities of Zn^{2+} into the synaptic space. The same conditions may release Zn^{2+} from intracellular-binding proteins. If intracellular free Zn^{2+} concentrations rise sufficiently, toxicity results.

neuromodulator, then a mechanism to clear the synapse of released Zn^{2+} and to refill synaptic vesicles must exist. Zn^{2+} transporters would be likely candidates to fill this need and ZIP like proteins on the presynaptic membrane could rapidly clear the synapse of Zn^{2+} and deliver Zn^{2+} to cytosolic metallothionein to be recycled to synaptic vesicles. In hippocampal slices, electrical stimulation enhances the uptake of exogenous Zn^{2+} (Howell et al., 1984). Alternatively, Zn^{2+} could escape the synapse by diffusion, and be taken up by glia. In the latter model, axonal transport of Zn^{2+} would be necessary to replenish synaptic vesicle stores (Budde et al., 1997). Additional research is needed to decide if either of these mechanisms is operative. As briefly mentioned above, Ca^{2+}-permeable channels on the postsynaptic membrane are permeable to Zn^{2+} such that Zn^{2+} enters the postsynaptic cell and cytosolic Zn^{2+} concentrations increase (Li et al., 2001b; Jia et al., 2002; Sheline et al., 2002). An interesting and emerging field of Zn^{2+} biology is the study of Zn^{2+} as an intracellular signaling molecule. Ever more signal transduction pathways are being recognized as Zn^{2+}-modulated. Unfortunately, this area of Zn^{2+} biology is beyond the scope of this review article (for a review, see Frederickson and Bush, 2001).

The selective and complete depletion of synaptic vesicle Zn^{2+} in mice with ZnT-3 gene disruption (Cole et al., 1999) has provided a useful experimental tool to test directly the physiological role(s) of synaptic Zn^{2+}. The results have been surprising. Brains of knockout mice are anatomically normal (Cole et al., 1999) and adult animals show no deficits in learning and memory in a panel of tests known to be dependent on hippocampal function (Cole et al., 2001). Furthermore, direct investigation of postsynaptic responses of CA3 pyramidal cells in hippocampal slices showed no effect of ZnT-3 knockout on excitability (Lopantsev et al., 2003). This suggests that either the neuromodulatory role of synaptically released Zn^{2+} is unimportant, or neurons can compensate for the loss of synaptic vesicle Zn^{2+} easily.

2.4. How will neurons cope with decreases or increases in cellular Zn^{2+}?

The schema presented in Figs. 3 and 4 show the many cellular mechanisms thought to be involved in neuronal Zn^{2+} homeostasis. Experimental evidence is still woefully inadequate, but we can begin to propose ways that these mechanisms work together to counteract increases or decreases in cellular Zn^{2+}. The primary mechanism by which neurons cope with decreasing availability of Zn^{2+} is likely to involve altering the ratio of "labile" Zn^{2+} to metallothionein bound Zn^{2+}. Metallothionein provides the neuron with a large capacity Zn^{2+} buffer, which can either supply Zn^{2+} for immediate needs or act as a reservoir. For longer-term adjustments, neurons probably alter the expression of metallothionein, which is under strict transcriptional control by Zn^{2+} (see Section 4.1). To illustrate how this model works, let us use the example of dietary Zn^{2+} deficiency. In this model, reduced dietary Zn^{2+} causes decreases in intracellular free Zn^{2+}. Over the short term, metallothionein releases Zn^{2+}, supplying the neuron's needs for "labile" Zn^{2+}. If the deficiency is prolonged, then decreased expression of the metallothionein gene reestablishes normal levels of "labile" Zn^{2+}. Thus, although dietary Zn^{2+} deficiency shows little alteration of total brain Zn^{2+} (see Section 4.2), a significant shift from metallothionein bound to "labile" Zn^{2+} could occur. Arguing against this central role of metallothionein in neuronal Zn^{2+} homeostasis are results of metallothionein-III gene knockout in mice (Erickson et al., 1997). These animals develop normally and show only show small changes in total brain Zn^{2+}, no change in histochemically reactive Zn^{2+}, normal behavior, and are not overly sensitive to Zn^{2+} exposure. The animals do show greater susceptibility to kainate induced seizures. One can only surmise that the necessary roles played by metallothionein-III can be filled by many proteins. It makes evolutionary sense that if metal binding is critical to survival, redundancy would be built into this function.

As brain Zn^{2+} levels are relatively constant (see Section 2.1), neurons would not be expected to be exposed to increases in extracellular Zn^{+}. However, this is not the case for the postsynaptic partners of Zn^{2+}-containing glutamatergic neurons, which are exposed to synaptically released Zn^{2+} and must deal with periodic increases in intracellular free Zn^{2+}. Similar (but in reverse) to what occurs when Zn^{2+} transiently decreases, metallothionein could be called upon to sequester excess intracellular Zn^{2+}. However, it is thought that metallothionein is nearly saturated with Zn^{2+} under normal conditions (Richard Palmiter, personal communication). Thus, Zn^{2+} transporters, particularly ZnT-1, may play an important role in reducing intracellular free Zn^{2+} under these conditions. Experiments show that the presence of ZnT-1 can counteract increases in intracellular Zn^{2+}. PC-12 cells stably expressing ZnT-1 demonstrate increased Zn^{2+} efflux and reduced sensitivity to Zn^{2+}-induced cell death (Kim et al., 2000). Furthermore, as synaptic activity results in net Zn^{2+} influx, these neurons must efflux Zn^{2+} to maintain cellular Zn^{2+} balance. Prolonged increases in cellular Zn^{2+} are dealt with by increasing the expression of metallothionein and ZnT-1 (see Section 4.1).

2.5. Zn^{2+} toxicity

With excitotoxic stimulation, which occurs under conditions of ischemia or sustained status epilepticus, large amounts of glutamate and Zn^{2+} are released and it is thought that massive influx of Zn^{2+} takes place in the postsynaptic cell, overwhelming Zn^{2+} homeostatic mechanisms resulting in large sustained increases in intracellular free Zn^{2+} (Frederickson et al., 1988; Frederickson et al., 1989; Suh et al., 2001; Li et al., 2001b) (see Fig. 4). Intracellular free Zn^{2+} concentrations reaching 400–600 nM trigger widespread death in neuronal cultures (Canzo-

niero et al., 1999; Sensi et al., 1999). There is convincing evidence that when neuronal cytosolic concentrations of Zn^{2+} rise under pathological conditions, Zn^{2+} is taken up into mitochondria with derangements of mitochondrial structure and function (Sensi et al., 1999, 2000, 2002, 2003; Wudarczyk et al., 1999; Jiang et al., 2001; Dineley et al., 2003) that have the potential to contribute to cell death involving apoptotic pathways (Budd et al., 2000). The exact mechanism(s) of Zn^{2+}-induced death are likely to include several pathways and the reader is directed to recent articles (Takeda, 2000; Sheline et al., 2000; Frederickson and Bush, 2001; Jiang et al., 2001; Kim and Koh, 2002; Dineley et al., 2003). Again, studies with ZnT-3 knockout mice seem to belie the importance of synaptically released Zn^{2+}. Knockout mice showed characteristic hippocampal cell death and accumulation of Zn^{2+} as a result of kainate induced seizures (Lee et al., 2000; Cole et al., 2000). More recent studies provide evidence that cytosolic protein-bound Zn^{2+} stores can be released by oxidation, causing increases in intracellular free Zn^{2+} and increased Zn^{2+} release (Cuajungco and Lees, 1998; Aizenman et al., 2000; Frederickson et al., 2002a). Internally released Zn^{2+} can be subsequently compartmentalized (Haase and Beyersmann, 2002). Intracellularly released Zn^{2+} may be responsible, at least in part, for Zn^{2+} toxicity in ZnT-3 knockout animals.

2.6. The evidence for extrasynaptic compartmentalization of Zn^{2+} in neurons

Zinquin fluorescence identifies numerous and widespread sites of Zn^{2+} compartmentalization in rat cortical neurons in primary culture (Colvin, 2002). Fig. 5A shows a typical image of punctate zinquin fluorescence in an untreated neuron. Zinquin fluorescence is metal-dependent and is sensitive to the addition of tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) (Colvin, 2002). When the neurons are briefly (5 min) exposed to 30 μM Zn^{2+} with pyrithione (a Zn^{2+} ionophore), zinquin fluorescence is still punctate, but is dramatically increased (see Fig. 9B). As it is well established (see Section 2.5) that mitochondria take up Zn^{2+} when cytosolic concentrations rise, the data in untreated neurons suggests the existence of endogenous Zn^{2+} in mitochondria. Additional support comes from Sensi et al., 2003. They show that RhodZin-3 (a Zn^{2+}-selective fluorescent probe that accumulates in mitochondria) yields TPEN-sensitive punctate staining similar to that seen with zinquin. In addition, they show that RhodZin-3 fluorescence colocalizes with Mitotracker Green confirming the mitochondrial identity of the Zn^{2+} compartments. How much Zn^{2+} is in mitochondria? Zinquin has a low affinity for Zn^{2+} (micromolar). Do mitochondria contain micromolar concentrations of Zn^{2+}? This cannot be the case, as much lower concentrations of Zn^{2+} are known to affect mitochondrial function (see Section 2.5). In addition, mitochondrial Zn^{2+} staining has never been seen with various highly sensitive silver staining techniques used in vivo (see Section 2.2). On the other hand, RhodZin-3 has a much higher affinity for Zn^{2+} (~ 65 nM) suggesting endogenous Zn^{2+} is in the nanomolar range (Sensi et al., 2003). Is it possible that zinquin could recognize such low concentrations of Zn^{2+}? Zinquin partitions into biological membranes (Snitsarev et al., 2001), such that very high concentrations may be obtained in mitochondria. Dineley et al. (2002) demonstrate that ion sensitivity is dependent on the intracellular dye concentration (see Section 2.1). Thus, zinquin could detect nanomolar concentrations of endogenous Zn^{2+} inside mitochondria.

If mitochondria contain endogenous Zn^{2+}, what role does it play? Sensi et al. (2003) could detect release of endogenous mitochondrial Zn^{2+} to the cytosolic compartment using FluoZin-3, a fluorescent probe with high sensitivity for Zn^{2+}. In addition, they show that released Zn^{2+} can be transferred to the cytosolic protein-bound pool

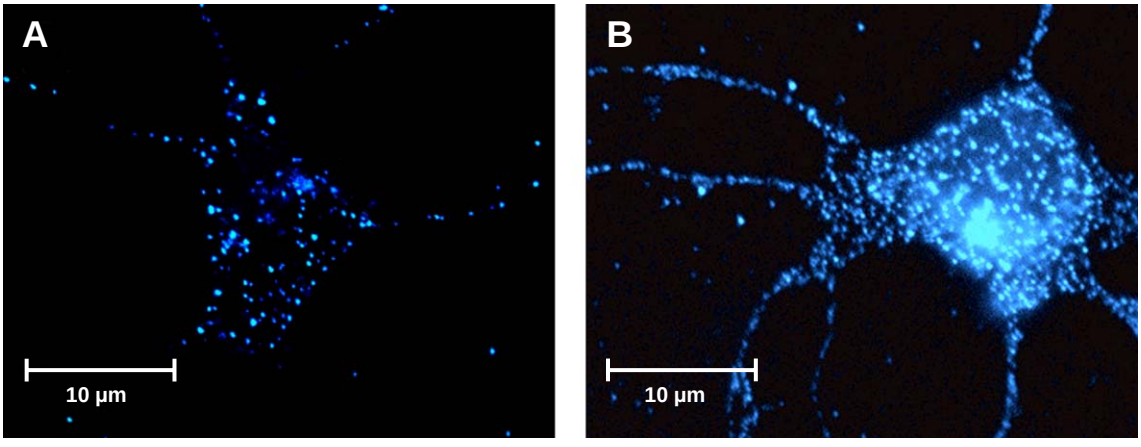

Fig. 5. Zinquin fluorescence in rat cortical neurons in primary culture. Cells grown on glass coverslips were maintained in Neurobasal media with B-27 supplement (Gibco) and all experiments were performed in the same media. Cells in B were treated with 30 μM Zn^{2+} and 25 μM pyrithione for 5 min at 37 °C, washed, and then both A and B were incubated with 25 μM zinquin for 30 min at 37 °C. Finally, the cells were washed and mounted with Antifade reagent (Molecular Probes).

(probably metallothionein) that is redox-sensitive (see Section 1). Thus, mitochondria could serve as Zn^{2+} depots, involved in the trafficking of Zn^{2+} throughout the cell. They also show that submicromolar Zn^{2+} modulates mitochondrial function.

2.7. The mechanism of Zn^{2+} transport by brain mitochondria

The studies described above suggest neuronal mitochondria contain endogenous Zn^{2+} and are an important intracellular compartment for Zn^{2+} buffering, exchange, and trafficking. If mitochondria were to function in this way, then a physiologically relevant mechanism for mitochondrial Zn^{2+} uptake and release must exist. Unfortunately, very little known about pathways of Zn^{2+} movement across mitochondrial membranes. In contrast, the role of mitochondria in cytosolic Ca^{2+} buffering is well accepted and uptake and release of Ca^{2+} by mitochondria have been extensively studied (for reviews, see Gunter and Pfeiffer, 1990; Gunter et al., 1994). As it would not be surprising if Zn^{2+} enters and leaves the mitochondria by mechanisms similar to or via Ca^{2+} pathways, an understanding of Ca^{2+} transport provides a framework for potentially understanding Zn^{2+} transport by mitochondria. Mitochondria show low permeability to cations when lacking a source of energy. However, when mitochondria are actively respiring, a mitochondrial membrane potential is generated that is negative with respect to the cytosol. Many studies have confirmed that it is the mitochondrial electrochemical gradient for Ca^{2+} (dominated by the inside negative membrane potential), which provides the driving force for the electrogenic uptake of Ca^{2+}. Convincing evidence for the cotransport of other ions in association with Ca^{2+} uptake (although other ions can and certainly do move when Ca^{2+} is transported) does not exist. Thus, the transporter has been named the mitochondrial Ca^{2+} uniporter.

What is the evidence that the Ca^{2+} uniporter could mediate Zn^{2+} uptake? First, it is thought that the Ca^{2+} uniporter is quite nonspecific, mediating the transport of other divalent cations such as Mg^{2+}, Sr^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, Pb^{2+}, and lanthanides. Only a handful of studies has reported the uptake of Zn^{2+} by isolated mitochondria, and in each case, Zn^{2+} transport has characteristics similar to that of the Ca^{2+} uniporter. The first study, conducted 35 years ago (Brierley and Knight, 1967), used isolated heart mitochondria and showed that the uptake of Zn^{2+} was dependent on respiration and was associated with the loss of mitochondrial K^+ and Mg^{2+} when they were not present in the extramitochondrial media. Mg^{2+} blocked the uptake of Zn^{2+} and Mg^{2+} is known to block mitochondrial Ca^{2+} uptake (Gunter and Pfeiffer, 1990). A difficulty with these early studies of mitochondrial Zn^{2+} uptake is the excessively large (>100 μM) concentrations of Zn^{2+} used. A more recent study (Saris and Niva, 1994) uses lower concentrations of Zn^{2+} and provides additional evidence of similarities between Zn^{2+} and Ca^{2+} uptake in mitochondria. Zn^{2+} inhibited Ca^{2+} uptake at concentrations of < 10 μM by lowering the membrane potential. Zn^{2+} uptake (measured as a decrease in extramitochondrial Zn^{2+}) by respiring mitochondria was inhibited by the relatively specific inhibitor of the calcium uniporter ruthenium red. Thus, it is well established that isolated mitochondria can take up Zn^{2+} (when extramitochondrial Zn^{2+} is quite high). The Ca^{2+} uniporter appears to mediate Zn^{2+} uptake under these conditions. However, it is entirely uncertain how Zn^{2+} exits mitochondria. This is an important missing piece of information; for if mitochondria are to function as Zn^{2+} stores, rapid influx and efflux must take place. Another problem arises when considering the apparent affinity for Zn^{2+} uptake, which is reported to be in the micromolar range. This would suggest that mitochondria could function as steady state regulators of intracellular free Zn^{2+} concentrations only when intracellular Zn^{2+} was near or just below 1 μM. This fact is difficult to reconcile with the many studies that show that intracellular free Zn^{2+} concentrations are much lower than this (see Section 2.1). Thus, although Zn^{2+} entry into mitochondria is demonstrated when pathological free Zn^{2+} concentrations exist and evidence suggests the existence of endogenous mitochondrial Zn^{2+}, it is much less certain how Zn^{2+} enters and exits the mitochondria (Dineley et al., 2003). An intriguing alternative to the transporter model is suggested by studies that show metallothionein localization to liver mitochondria (Ye et al., 2001). In this model, metallothionein bound with Zn^{2+} crosses the mitochondrial membrane. Endogenous mitochondrial Zn^{2+} would be complexed with metallothionein and Zn^{2+} release could occur by reversal of this process. However, the import of metallothionein is tissue-specific and it is not known if it occurs in brain mitochondria. We will now move on to a more in depth discussion of Zn^{2+} transporters and their role(s) in neuronal Zn^{2+} homeostasis.

3. Zinc transporters: several different zinc transporter families have been identified; each family probably has a unique transport mechanism, function, and cellular location

In eukaryotic organisms, three families of metal cation transporters have now been identified and well characterized and others are certainly yet to be discovered. Most studies of the identification and functional characterization of these transporters have not been done in neurons, but what we do know from functional studies and expression analysis in brain suggests that these transporters are present in neurons and function in ways similar to those better characterized in other eukaryotic organisms or cell types. The three families are the following: ZIP—an acronym for ‘‘Zrt-Irt like proteins’’ first discovered in *Saccharomyces cerevisiae* (Zrt) and *Arabidopsis thalina* (Irt) (Zhao and Eide, 1996; Eide et

al., 1996); CDF—an acronym for "cation diffusion facilitator" a gene that confers metal resistance to many eukaryotic cell types (Palmiter and Findley, 1995); and lastly, DMT—an acronym for "divalent metal transporter" that was shown to be highly homologous to the human natural resistance associated macrophage protein, Nramp (Gunshin et al., 1997).

3.1. Structure predictions for the metal transporters

This topic has been reviewed recently (Gaither and Eide, 2001a; Taylor and Nicholson, 2003), so it will be discussed only briefly here. The ZIP family of zinc transporters are predicted to have a membrane topology consisting of eight transmembrane spanning domains with the amino and carboxy terminals both extracytoplasmic and a long variable cytoplasmic loop connecting transmembrane spanning domains 3 and 4 (see Fig. 6). Within this variable region is a histidine-rich sequence, which is thought to be a metal binding domain, although this has not been shown by direct experimental evidence. Transmembrane domains 4 and 5 are highly conserved among ZIP family members. These domains are highly amphipathic and contain histidine residues that have been mutated with resulting loss of function (Rogers et al., 2000). The loop between transmembrane domains 2 and 3 faces the extracellular surface and has been predicted to be involved in metal ion selectivity (Rogers et al., 2000).

The CDF family of zinc transporters are mostly predicted to have six transmembrane domains with intracellular amino and carboxy terminals (Gaither and Eide, 2001a) (see Fig. 6). However, exceptions have been documented. For example, the recently reported hZTL1 (Cragg et al., 2002), a human apical intestinal Zn^{2+} transporter, is predicted to have 12 transmembrane domains with extracellular amino and carboxy terminals. The greatest homology among CDF family members occurs in transmembrane domains 1 through 4. Like the ZIP proteins, CDF family members are predicted to have a large histidine-rich intracellular loop.

DMT/Nramp is predicted to have 12 transmembrane domains with intracellular amino and carboxy terminals and predicted glycosylation sites in the extracellular loop between transmembrane domains 7 and 8 (Gunshin et al., 1997). Unlike the ZIP and CDF family members, a histidine-rich intracellular loop is not present. However, the cytoplasmic loop between transmembrane domains 8 and 9 contains a consensus transport motif.

3.2. ZIP family transport mechanism and function in neurons

ZIP family members have been identified in most eukaryotic organisms (for review, see Gaither and Eide, 2001a). However, the transport mechanism(s) of mammalian ZIP proteins have been only partially characterized. The most progress to this end has been reported by Gaither and Eide (2000, 2001b) using K562 erytholeukemia cells overexpressing the human genes hZIP1 or hZIP2. These studies provide convincing evidence that the ZIP proteins mediate Zn^{2+} influx. Zn^{2+} uptake is stimulated by increasing extracellular Zn^{2+} with an apparent affinity of 3 μM. Zn^{2+} uptake is inhibited by other divalent metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{2+}, and Cd^{2+}, but is unaffected by

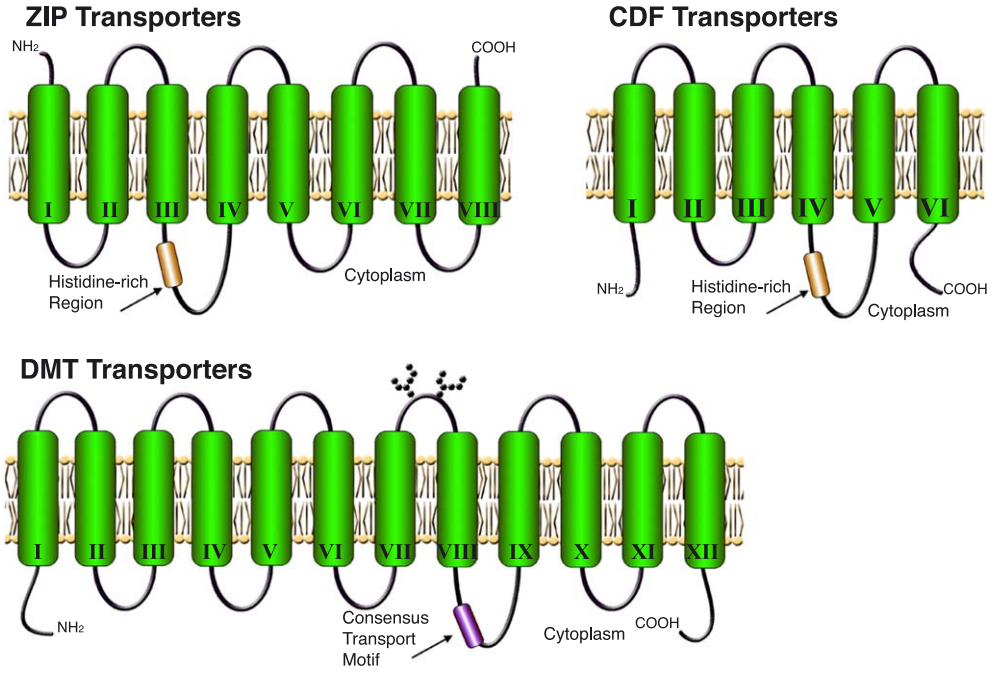

Fig. 6. Predicted membrane topology of metal ion transporters. Adapted from Gunshin et al. (1997) and Gaither and Eide (2001a).

Ca^{2+} and Mg^{2+}. Interestingly, metal ion inhibition differs when comparing hZIP1 and hZIP2. Both transporters are inhibited by Cd^{2+}, Cu^{2+}, and Fe^{2+}, whereas only hZIP1 is inhibited by Ni^{2+} and only hZIP2 is inhibited by Mn^{2+} and Co^{2+}. Zinc uptake mediated by either hZIP1 or hZIP2 is energy independent and is unaffected by cellular ATP depletion. Both transporters show no dependence on extracellular monvalent cations such as Na^{+} or K^{+}, nor does membrane potential appear to affect Zn^{2+} uptake. However, extracellular pH does affect Zn^{2+} uptake mediated by hZIP2 (but not hZIP1) and the pH effect could be mimicked by the addition of bicarbonate suggesting that bicarbonate ion may participate in the uptake mechanism, perhaps in a Zn^{2+}/bicarbonate cotransport mechanism. The hZIP4 gene (a member of the LIV-1 subfamily of ZIP zinc transporters—termed LZT family of proteins, see Taylor and Nicholson, 2003 for a review) is thought to be responsible for a rare inherited condition acrodermatitis enteropathica, which results from reduced intestinal uptake of dietary Zn^{2+} (Wang et al., 2002a). This finding provides additional support for the notion that ZIP proteins are localized to the plasma membrane and are involved in Zn^{2+} uptake.

Are ZIP family members expressed and active in neurons? Although direct evidence is lacking at present, the answer is most likely yes. It has been established in studies using reverse transcriptase-polymerase chain reaction that hZIP1 mRNA is abundant in brain (Gaither and Eide, 2001b) and LIV-1 (a member of the LZT family of proteins) mRNA also occurs in brain (Taylor and Nicholson, 2003). Several functional properties of $^{65}Zn^{2+}$ uptake measured in cortical neurons in primary culture mirror those of hZIP1 (Colvin, 2002). For example, rat cortical neurons in primary culture show a robust uptake of $^{65}Zn^{2+}$ when placed in solutions containing micromolar concentrations of Zn^{2+} (see Fig. 7). $^{65}Zn^{2+}$ uptake is inhibited by a wide variety of transition elements but is unaffected by changes in Ca^{2+} or Mg^{2+} concentration, all findings reminiscent of ZIP family transporters (Colvin et al., 2000; Colvin, 2002). In addition, $^{65}Zn^{2+}$ uptake by cortical neurons is not affected by cellular ATP depletion and does not appear to show a requirement for extracellular monovalent cations. However, differences do exist. The profile of metal inhibition of $^{65}Zn^{2+}$ uptake in rat cortical neurons does not match that observed with either the hZIP1 or 2 homologs (Colvin, 2002). $^{65}Zn^{2+}$ uptake in rat cortical neurons is pH-dependent being inhibited when extracellular pH is reduced (see Fig. 8). Although hZIP2 shows a dependence on pH (Gaither and Eide, 2000), this effect is explained by a requirement for bicarbonate ion. No such requirement for bicarbonate is seen in rat cortical neurons (Colvin, 2002). Thus, $^{65}Zn^{2+}$ uptake in rat cortical neurons shows many characteristics similar to ZIP family transporters, but the metal ion inhibition and pH dependence are clearly different. These findings suggest that species differences in mechanism may exist in ZIP transporters or that yet to be discovered Zn^{2+} transporters are active in rat cortical neurons.

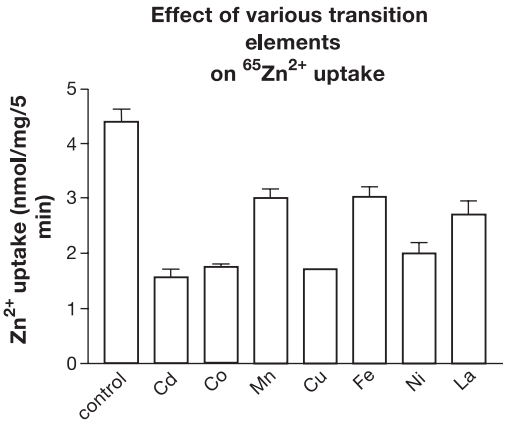

Fig. 7. $^{65}Zn^{2+}$ uptake was measured in rat cortical neurons in primary culture. The total concentration of Zn^{2+} added was 30 μM; the various transition elements were added at a concentration of 300 μM (as chloride salts). Reprinted with permission from Colvin (2002).

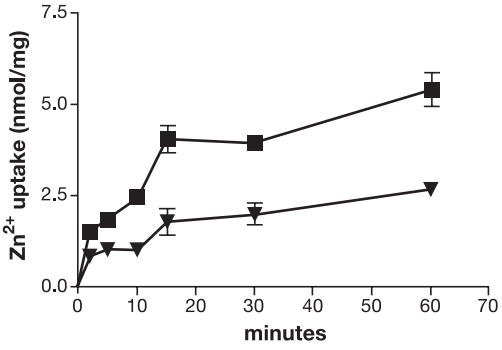

Fig. 8. Time course of $^{65}Zn^{2+}$ uptake by rat cortical neurons exposed to 30 μM Zn^{2+}. (■) pH 7.4, (▼) pH 6. Reprinted with permission from Colvin (2002).

3.3. CDF family transport mechanism and function in neurons

Like the ZIP family, CDF family members are found at all phylogenetic levels in eukaryotic organisms (Gaither and Eide, 2001a). The function, tissue location and mechanism of Zn^{2+} transport by CDF family members are best studied for the mammalian protein ZnT-1 and its several homologs. Palmiter and Findley (1995) first identified ZnT-1 using a molecular genetics approach, in which mutated cultured cells with altered Zn^{2+} homeostasis could be identified and then rescued by transformation with cDNA expression libraries. ZnT-1 was shown to be localized to the plasma membrane and presumably transported Zn^{2+} out of the cell. However, functional studies have not delineated a clear mechanism of transport. Overexpression of ZnT-1 increased both Zn^{2+} influx and efflux and extracellular Zn^{2+} was shown to stimulate Zn^{2+} efflux. No dependence of Zn^{2+} transport on extracellular ions (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, or Cl^{-}) was noted, no nucleotide binding sites were found in the protein sequence, and inhibition of oxidative phosphor-

ylation by cyanide or azide did not affect transport. Finally, ZnT-1 appeared highly specific for Zn^{2+}, as Zn^{2+} efflux was not inhibited by Cd^{2+}. hZTL1 is another CDF family member homologous with ZnT-1 and is expressed at the apical enterocyte membrane. hZTL1 likely complements the activity of hZIP4 (Cragg et al., 2002) to absorb dietary Zn^{2+}. It is unique among the ZnT family members because it is proposed to be involved in Zn^{2+} uptake rather than Zn^{2+} efflux. When hZTL1 was expressed in *Xenopus laevis* oocytes, Zn^{2+} uptake across the plasma membrane increased. Zinc uptake was increased by increasing Zn^{2+} concentration in the micromolar range and was inhibited by increasing extracellular acidity. hZTL1 mRNA is abundant in many tissues including brain (Cragg et al., 2002).

The remaining ZnT family members are preferentially localized to intracellular membranes where they have more limited, tissue-specific expression, probably related to physiological intracellular Zn^{2+} sequestration (for review, see McMahon and Cousins, 1998a). The first to be characterized, ZnT-2, was shown to mediate sequestration of Zn^{2+} into acidic cytoplasmic vesicles (when overexpressed in cells lacking metallothionein and ZnT-1) when cells were exposed to high extracellular Zn^{2+} (Palmiter et al., 1996a). A proton gradient was not required for Zn^{2+} sequestration, as inhibition of the H-ATPase responsible for generating the proton gradient did not affect Zn^{2+} sequestration. ZnT-3 is selectively expressed and is only abundant in brain and testis (Palmiter et al., 1996b). In the brain, ZnT-3 is thought to be responsible for the sequestration of Zn^{2+} into glutamatergic synaptic vesicles. Using mouse brain, the protein is found in the same regions (hippocampus and cerebral cortex) that show the highest levels of histochemically reactive Zn^{2+}. Electron microscopy shows localization of ZnT-3 to synaptic vesicles that stain positively for Zn^{2+} (Wenzel et al., 1997). Finally, it was shown that sequestration of Zn^{2+} into synaptic vesicles requires ZnT-3 in the vesicle membrane, as disruption of this gene in mice eliminated histochemically reactive Zn^{2+} in the synaptic vesicles of these animals (Cole et al., 1999). Disruption of the ZnT-3 gene did not affect histochemically reactive Zn^{2+} in the secretory granules of other tissues (e.g., pancreatic beta-islet cells), suggesting that other ZnT family members are responsible for Zn^{2+} sequestration in these tissues (see ZnT-5 below).

Although expressed in the brain, ZnT-4 is best characterized as involved in the transport of Zn^{2+} into milk during lactation (Kelleher and Lonnerdal, 2002). The lethal milk mutant has a nonsense mutation at arginine codon 297 in the ZnT-4 gene, which likely explains the reduction of Zn^{2+} in the milk of these mice (Huang and Gitschier, 1997). ZnT-4 is highly expressed in the luminal cells of the ducts and alveoli where it has a vesicular, cytoplasmic distribution (Michalczyk et al., 2002), consistent with its role in the addition of Zn^{2+} to the milk of lactating mothers. Similarly, ZnT-5 appears to play a specific role in the accumulation of Zn^{2+} into the secretory vesicles of pancreatic beta cells. ZnT-5 mRNA is found in most tissues but is most highly expressed in the pancreas and is associated with the secretory granules of pancreatic β cells (Kambe et al., 2002). Unlike the other CDF family members characterized to date (see Fig. 6), ZnT-5 is predicted to have 15 membrane spanning domains and is nearly twice as big as other ZnT family members. Both ZnT-6 and ZnT-7 appear to be localized to the golgi apparatus and cytoplasmic vesicles (Huang et al., 2002; Kirschke and Huang, 2003). ZnT-6 protein was most abundant in brain and lung (Huang et al., 2002), while ZnT-7 was most abundant in lung and proximal small intestine (Kirschke and Huang, 2003).

Although functional characterization of ZnT family members consistently show that these proteins are involved in a highly specific Zn^{2+} efflux (directed out of the cell or into cytoplasmic organelles), are transcriptionally regulated by cellular Zn^{2+} status in ways that would be predicted for a Zn^{2+} transporter (see Section 4.1), and rescue several mutant cell types deficient in Zn^{2+} transport, convincing direct mechanistic data of Zn^{2+} transport is still lacking for most of the ZnT family members (but see above—experiments with hZTL1). Thus, we cannot unequivocally assign ZnT proteins as Zn^{2+} transporters; they may act as a necessary component of a protein complex that is ultimately responsible for Zn^{2+} transport. For example, when ZnT-3 was overexpressed in BHK cells (Wenzel et al., 1997), it was associated with vesicles, but did not facilitate Zn^{2+} sequestration as ZnT-2 did. Similarly, ZnT-7 when expressed in several yeast strains mutant in Zn^{2+} transport was unable to augment or rescue the Zn^{2+} phenotypes. Analysis of ZnT-1 mutants (Palmiter and Findley, 1995) showed that when a truncated form of the protein (with the first transmembrane domain deleted) was expressed in wild-type cells, they became Zn^{2+}-sensitive, suggesting a dominant negative effect. Thus, such experiments suggest that ZnT-1 proteins might function as multimers or in association with a larger complex of proteins involved in regulation of Zn^{2+} transport.

Are ZnT family members expressed and active in neurons? As with the ZIP family members, direct evidence is lacking at present, but the answer again is most likely yes. Many ZnT family members are expressed abundantly in the brain: ZnT-1, -3, -4, -6, and hZTL1, suggesting that in neurons Zn^{2+} efflux transporters should localize to both the plasma membrane and intracellular membranes such as the golgi apparatus. Convincing evidence places ZnT-3 in the membrane of many glutamatergic synaptic vesicles being required for Zn^{2+} sequestration (see above). Evidence of neuronal Zn^{2+} efflux comes from studies showing depletion of synaptic vesicle Zn^{2+} in vivo after dietary Zn^{2+} deficiency (Takeda et al., 2003) or after perfusion with the extracellular chelator CaEDTA (Frederickson et al., 2002b). These data support the contention that steady state Zn^{2+} efflux is occurring always and not just as a response to increases in intracellular free Zn^{2+}. Direct observation of carrier-mediated Zn^{2+} efflux from neurons is provided by

the analysis of $^{65}Zn^{2+}$ transport in rat brain using plasma membrane vesicles (Colvin et al., 2000). In these studies, $^{65}Zn^{2+}$ release from preloaded vesicles was stimulated by increasing extravesicular proton concentration (see Fig. 9). It seems likely that ZnT-1 mediates or at least contributes to observed Zn^{2+} efflux in neurons. It should be noted that, as ZnT-1 is ubiquitously expressed, its role zinc homeostasis also must be universal. Thus, it is not surprising that homozygous knockout of ZnT-1 is embryonic lethal (Andrews, 2001). It is much less certain what roles ZnT-4, -6, or hZTL1 might have in neurons. As can be appreciated from the above discussion, the role(s) played by the various ZnT family members in Zn^{2+} transport and homeostasis in the brain is widespread but poorly understood.

3.4. DMT family transport mechanism and function in neurons

The phylogenetic distribution of DMT1/2 genes has not been studied as extensively as ZIP and CDF family members, although human (Nramp1/2—Gunshin et al., 1997), rat (Fleming et al., 1998), and yeast homologues (Smf1p—Sacher et al., 2001) have been functionally characterized. The transport mechanism of DMT1/Nramp2 has been carefully studied (Gunshin et al., 1997). The heterologous expression of DMT1 in *X. laevis* oocytes results in the generation of large inward currents when Fe^{2+} and several other divalent metal ions including Zn^{2+} were added extracellularly. Fe^{2+}-induced currents were enhanced in acidic pH and Fe^{2+} uptake was associated with intracellular acidification of the oocyte. Thus, Fe^{2+} uptake was shown to be proton coupled and Hill analysis suggested a stoichiometry of $1Fe^{2+}$:$1H^{+}$. The expression of DMT1 in the central nervous system neurons is widespread (Fleming et al., 1998; Wang et al., 2002b). Based on the work of Gunshin et al. (1997), it was assumed that zinc would be transported by DMT1/Nramp2. However, several studies (Tandy et al., 2000; Sacher et al., 2001) have failed to show zinc uptake in *X. laevis* oocytes expressing DMT1/Nramp2 and now it seems unlikely that DMT1/Nramp2 are Zn^{2+} transporters. An important function of DMT1/Nramp2 is apical membrane iron uptake in the duodenum (Tandy et al., 2000). In the brain, it may be involved in iron movement across the blood–brain barrier, as it is localized to astrocytic endfeet (Wang et al., 2001).

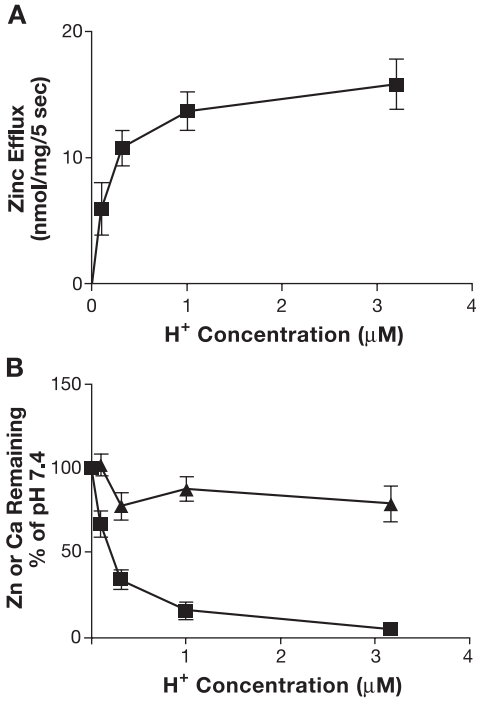

Fig. 9. Effect of increasing $[H^{+}]$ on $^{65}Zn^{2+}$ and $^{45}Ca^{2+}$ efflux from rat brain plasma membrane vesicles preloaded with $^{65}Zn^{2+}$ (■) or $^{45}Ca^{2+}$ (▲). Vesicles were allowed to accumulate $^{65}Zn^{2+}$ or $^{45}Ca^{2+}$ and then were diluted into buffer of different pH to initiate efflux. (A) Dependence of $^{65}Zn^{2+}$ efflux on extravesicular $[H^{+}]$. (B) In this experiment, vesicular $^{65}Zn^{2+}$ or $^{45}Ca^{2+}$ content after dilution is expressed as a percentage of vesicular content prior to dilution. It is shown that the effect of increasing $[H^{+}]$ is specific for $^{65}Zn^{2+}$. Reprinted with permission from Colvin et al. (2000).

4. Is the expression and trafficking of Zn^{2+} transporters regulated by Zn^{2+} status in the brain? Is it a mechanism of Zn^{2+} homeostasis used by neurons?

4.1. ZnT transporters

Whereas ZnT-1 has ubiquitous expression (including the brain), the other ZnT family members show tissue-specific expression. We know little about the factors that control the tissue-specific transcriptional regulation of ZnT family members. It appears that ZnT-1, -3, -4, -5, -6, and hZTL1 are expressed in the brain. The transcriptional regulation and the control of the cellular localization of the various ZnT transporters expressed in the brain are probably complex, but by analogy with simpler eukaryotes, cellular Zn^{2+} status should necessarily be an important regulator of these processes. Change in the expression or cellular trafficking of ZnT transporters is an obvious mechanism by which neurons could regulate cellular Zn^{2+} load and maintain intracellular free Zn^{2+} concentrations within the narrow window optimal for growth and survival (see Fig. 1). What is the Zn^{2+} sensor inside cells and more specifically in neurons? Recent work from the laboratory of Langmade et al. (2000) and Andrews (2001) identified a zinc finger domain containing metal-response element-binding factor-1 (MTF-1), which acts as a cellular sensor of Zn^{2+} status in the mouse. MTF-1 binds to Zn^{2+} when intracellular free Zn^{2+} is high, translocates to the nucleus and then activates the transcription of the metal homeostatic genes metallothionein and ZnT-1. Both proteins then act to lower intracellular free Zn^{2+} levels by either Zn^{2+} chelation (metallothionein) or Zn^{2+} extrusion (ZnT-1) (see Section 2.4). The most complete studies of transcriptional regulation of Zn^{2+} trans-

porters have been performed by Cousins and co-workers by studying the effects of changes in dietary Zn^{2+} on ZnT expression in the intestine (Davis et al., 1998; McMahon and Cousins, 1998b; Liuzzi et al., 2001; Cousins et al., 2003). They noted that ZnT-1, -2, and -4 are differentially regulated by intracellular Zn^{2+}. Whereas ZnT-1 and -2 levels were directly related to changes in cellular Zn^{2+} (either increases or decreases), ZnT-4 expression was little affected by cellular Zn^{2+} levels. Unfortunately, evidence that alterations in dietary Zn^{2+} affect the expression of ZnT transporters in the brain (but as discussed in Section 4.2, brain Zn^{2+} levels are resistant to dietary Zn^{2+} deficiency) does not exist. ZnT-1 expression in adult mouse brain (Sekler et al., 2002) and ZnT-3 expression in developing mouse brain (Valente and Auladell, 2002) were generally found in areas that stain heavily for histochemically reactive Zn^{2+} (synaptic vesicle Zn^{2+}). One exception was noted: ZnT-1 immunoreactivity was found in cerebellar Purkinje cells, areas of the adult brain devoid of synaptic vesicle Zn^{2+} (Sekler et al., 2002). Thus, ZnT-1 and -3 expression in the brain may be primarily constitutive, but ZnT-1 does appear to be regulated, at least in part, by cellular Zn^{2+}.

Certain pathological conditions (e.g., ischemia—see Section 2.3) are thought to cause large increases in extracellular Zn^{2+} (at least locally) with corresponding large increases in intracellular free Zn^{2+}. ZnT-1 expression was specifically induced in pyramidal neurons of the CA1 region of the hippocampus in gerbils after transient forebrain ischemia (Tsuda et al., 1997). In addition, these studies showed upregulation of ZnT-1 mRNA after exposure of primary hippocampal cultures to large increases in extracellular Zn^{2+}. It is interesting that ZnT-6 protein trafficking from the trans-Golgi network to cytoplasmic vesicles was shown to be stimulated by large increases in extracellular Zn^{2+} (Huang et al., 2002). Whether this change in cellular location of the ZnT-6 protein occurs in neurons exposed to high extracellular Zn^{2+} concentrations is unknown.

We know virtually nothing about transcriptional control of other ZnT family members expressed in the brain.

4.2. ZIP transporters

Unlike the many studies with ZnT proteins, few studies have examined the cellular location and transcriptional control by Zn^{2+} of ZIP family members in mammals. One study (Costello et al., 1999) shows that acute treatment of a human prostate malignant cell line with Zn^{2+}, reduced hZIP1 mRNA levels (but was not reproduced by others—Milon et al., 2001). Other studies have shown changes in Zn^{2+} uptake kinetics in cells pretreated with Zn^{2+} (Reeves et al., 2001) or an increase in Zn^{2+} uptake in brain capillary endothelial cells as a result of culture in Zn^{2+}-deficient media (McClung and Bobilya, 1999; Lehmann et al., 2002). However, such results could be the result of changes in either Zn^{2+} influx or efflux pathways. More extensive studies have been performed with yeast, and the story that is evolving (Gaither and Eide, 2001a) suggests that ZIP transporter expression is not under the control of Zn^{2+} status when intracellular Zn^{2+} concentrations are normal. Thus, it appears the ZIP transporters are constitutively expressed and are the cell's primary means of Zn^{2+} uptake for basal cellular Zn^{2+} requirements. When environmental Zn^{2+} is depleted and intracellular levels in yeast are decreased, expression of ZIP transporters is induced (Zhao and Eide, 1996; Zhao and Eide, 1997). Although similar transcriptional control of ZIP transporters in mammals might exist, it should be noted that yeast normally experience wide swings in environmental metal availability and corresponding changes in intracellular Zn^{2+} that require highly responsive mechanisms to upregulate these transporters. In contrast, it appears that in higher eukaryotic organisms the first line of defense against dietary Zn^{2+} deficiency is to upregulate intestinal absorption (McMahon and Cousins, 1998b; Liuzzi et al., 2001). In addition, tissues like the brain can upregulate Zn^{2+} import from the blood across the blood–brain barrier in response to Zn^{2+} status (McClung and Bobilya, 1999; Lehmann et al., 2002). Thus, the concentration of Zn^{2+} in the brain and most likely intracellular free Zn^{2+} is quite resistant to even severe dietary Zn^{2+} deficiency (Wallwork et al., 1983). It would not be surprising then if ZIP transporters in the brain are constitutively expressed and are under little transcriptional regulation related to Zn^{2+} status. There is no evidence that ZIP transporters are downregulated by high intracellular Zn^{2+} levels, rather, as discussed above, ZnT protein levels are likely to be upregulated under these conditions. Clearly, transcriptional control of mammalian ZIP transporters is an area that sorely needs more attention. The cellular location of ZIP transporters is presumed to be at the plasma membrane, where Zn^{2+} uptake must necessarily occur, and has been shown for K562 erythroleukemia cells (Gaither and Eide, 2000, 2001b). However, evidence of an intracellular location for hZIP1 in epithelial cells under certain culture conditions has been presented (Milon et al., 2001).

5. Summary

A better understanding of neuronal Zn^{2+} homeostasis and Zn^{2+} transporters will be critical for defining not only the role(s) played by Zn^{2+} in cell signaling, but also the role Zn^{2+} plays in neurodegenerative human diseases. Much has been learned in the last 10 years, but much more is still to be discovered. Now is an exciting time to be involved in Zn^{2+} research. This review presents a working model of neuronal Zn^{2+} homeostasis, which is most notable for the number of question marks contained within. Zn^{2+} transporters must play an integral role in Zn^{2+} homeostasis, but our understanding is currently limited by a poor knowledge of their mechanism(s) and transcriptional regulation. It seems probable that new Zn^{2+} transporters will be discovered in the future. Although the picture presented in this review is

complex and incomplete, we can be certain that more research will continue to advance our understanding of Zn^{2+} homeostasis and transport in the brain.

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