Zinc Transport in the Brain: Routes of Zinc Influx and Efflux in Neurons[1,2]

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ABSTRACT Studies of the routes of entry and exit for zinc in different tissues and cell types have shown that zinc can use several pathways of exit or entry. In neurons, known pathways include (*1*) presynaptic release along with glutamate when synaptic vesicles empty their contents into the synaptic cleft, (*2*) voltage-gated L-type Ca^{2+} channels and glutamate-gated channels that provide an entry route when cells are depolarized and that mediate extracellular zinc toxicity and (*3*) a plasma membrane transporter potentially present in all neurons important for cellular zinc homeostasis. The least understood of these pathways, in terms of mechanism, is the transporter pathway. The kinetics of zinc uptake in cultured neurons under resting conditions are consistent with and suggest the existence of a saturable transporter in the plasma membrane. The proteins responsible for plasma membrane zinc transport have not yet been definitely identified. Likely candidates include two proteins identified by molecular cloning termed zinc transporter 1 and divalent cation transporter DCT1. Both proteins have been shown to be expressed in the brain, but only DCT1 is clearly demonstrated to be a transport protein, whereas zinc transporter 1 may only modulate zinc transport in association with as-yet-unidentified proteins. Understanding the mechanism and neuromodulation of plasma membrane zinc transport will be an important first step toward a complete understanding of neuronal zinc homeostasis. J. Nutr. 130: 1484S—1487S, 2000.

KEY WORDS: • *zinc* • *ion transport* • *heavy metal ions* • *trace elements* • *metal transporters* • *rat*

Large amounts of zinc are present in the brain, yet very little is actually known about the molecular events underlying cellular zinc homeostasis and the functional role played by zinc in synaptic signaling (for several excellent recent reviews, see Choi and Koh 1998, Cuajungco and Lees 1997, Huang 1997, McMahon and Cousins 1998, Reyes 1996). Most of that zinc is tightly bound to metalloenzymes where zinc is involved as a cofactor in important functions such as gene expression or enzymatic reactions. However, a considerable amount, estimated to be as much as 10% of total brain zinc, is in a highly localized pool that can be detected by histochemical stains for metal ions. The chelatable pool is localized to synaptic vesicles, may be released on excitation and may play a role in modulation of synaptic signaling. Most cells, and neurons are no exception, must maintain cellular zinc content within a very narrow window, because low zinc levels inhibit cell growth and division and can lead to cell death and because high zinc levels are toxic. This presents the neuron with a very difficult problem to solve. Each neuron must be able to juggle the requirement for zinc and its constant turnover (which is particularly true of neurons, which presumably release zinc stored in vesicles on excitation) with the need to maintain zinc levels within narrow limits. It will not be surprising, therefore, if neurons in particular have a large complement of zinc homeostatic mechanisms at their disposal. It is known that neurons contain a ubiquitous zinc binding protein called metallothionein III (Erickson et al. 1997) that is thought to be very important in buffering cytoplasmic levels of zinc. When expressed in cultured cells, metallothionein can confer resistance to extracellular zinc toxicity (Palmiter 1998). Zinc is also known to be sequestered in synaptic vesicles, and this process may be stimulated when neurons are grown in zinc-containing media (Palmiter et al. 1996a). Just as important as these homeostatic processes is the control of zinc influx and efflux across the plasma membrane. Zinc-specific or nonspecific divalent cation transporters, or both, and ion channels are thought to provide entry and exit routes for zinc. The focus of this report is to describe what is known about the mechanisms of zinc entry and exit from neurons.

Histochemically detectable zinc

Released zinc may play a neuromodulatory role. Approximately 10% of the total brain zinc is in a histochemically reactive chelatable pool (Fredrickson et al. 1983). Particularly

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in hippocampal mossy fibers, this zinc is localized to the lumen of glutamate containing synaptic vesicles. When these fibers are stimulated by K^+ depolarization, kainic acid treatment or electrically, it is possible to detect zinc release and the depletion of the histochemically reactive zinc pool (Aniksztein et al. 1987, Assaf and Chung 1984, Charton et al. 1985, Howell et al. 1984). Although not proven, it is presumed that such zinc release is the result of synaptic vesicle fusion with the presynaptic membrane. Large amounts of zinc are thought to be released along with glutamate, so it has been hypothesized that zinc could play an important neuromodulatory role at glutamatergic synapses. In support of this notion, micromolar concentrations of zinc have been shown to inhibit *N*-methyl-D-aspartate (NMDA)[4] receptors (Chen et al. 1997, Paoletti et al. 1997, Traynelis et al. 1998). Two modes of inhibition exist that are either voltage dependent or voltage independent. The NR1 subunit appears to play a critical role in determining the sensitivity to zinc. In addition, zinc blocks γ-aminobutyric $acid_A$ receptor currents (Berger et al. 1998, Fisher and MacDonald 1998), the glutamate transporter EAAT1 (Vandenberg et al. 1998) and the dopamine transporter (Norregaard et al. 1998). Released zinc is then taken back up into neurons to replenish depleted synaptic vesicle stores (Howell et al. 1984, Perez-Clausell and Danscher 1986). The process of neuronal uptake of zinc is mediated by a saturable transporter in the plasma membrane (see later) with a zinc affinity in the range of ≤ 20 μmol/l (Colvin 1998a, Colvin et al. 2000, Howell et al. 1984, Wensink et al. 1988).

Depolarized neurons exhibit rapid zinc uptake that can be associated with excitotoxicity. Cortical neurons in primary culture have provided a useful model system in which to study depolarization-induced zinc entry. This type of movement of zinc across the plasma membrane may have immense pathophysiological significance, because this is thought to be the means by which zinc contributes to excitotoxicity (Koh et al. 1996). Studies have shown that when cells incubated in high concentrations of zinc are excited with glutamate, zinc rapidly enters the cell, and intracellular concentrations of zinc increase significantly and can reach levels that are toxic (Koh and Choi 1994, Sensi et al. 1997). Depolarization-induced zinc influx is probably mediated primarily by L-type dihydropyridine-sensitive Ca^{2+} channels, because K^+-stimulated neuronal $^{65}Zn^{2+}$ uptake and toxicity are blocked by nimodipine, verapamil or 10 μmol/l Gd^{3+} and only partially blocked by ω-conotoxin GVIA (Sensi et al. 1997). Increasing extracellular Ca^{2+} to 10–20 mmol/l reduced zinc-induced neuronal death consistent with a competition between zinc and calcium for entry routes (Koh and Choi 1994). Zinc-induced neurotoxicity showed sensitivity to NMDA channel antagonists (Koh and Choi 1994), suggesting that zinc entry can also occur via glutamate-activated channels. The application of either NMDA or kainate to cortical cells in the presence of 300 μmol/l zinc resulted in a rapid elevation in intracellular zinc. Glutamate agonist–induced zinc uptake was blocked by the addition of the corresponding antagonists [i.e., D2-amino-5-phosphonopentanoate and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide. It should be noted, however, that entry through Ca^{2+} channels also contributes to the rise in intracellular zinc after kainate exposure (Sensi et al. 1997). Using the fluorescent dye mag-Fura-5, cell body zinc was estimated to reach a maximum of 50 nmol/l under these conditions. A small apparent Na^+/Zn^{2+} exchange current was observed in cortical neurons when extracellular Na^+ was held at zero (Sensi et al. 1997). This exchange current was completely inhibited by 300 μmol/l benzamil. These data suggest that zinc may substitute for Ca^{2+} in the Na^+/Ca^{2+} exchange transport mechanism. However, micromolar concentrations of zinc inhibit $^{45}Ca^{2+}$ transport by the Na^+/Ca^{2+} exchanger in a manner that is not consistent with competitive kinetics (Colvin 1998b).

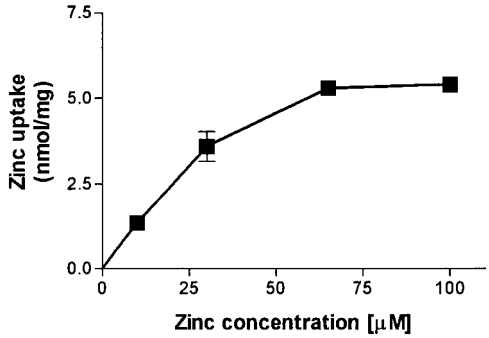

FIGURE 1 $^{65}Zn^{2+}$ uptake was measured in cortical neurons in primary culture 4 d after plating. Uptake assays were performed in 1 ml of modified Locke's solution containing 154 mmol/l NaCl, 5.6 mmol/l KCl, 5.0 mmol/l HEPES, pH 7.4 and 10 mmol/l glucose containing varying concentrations of $ZnCl_2$ and $^{65}Zn^{2+}$ (4 μCi/ml). After 15 min at 37°C, the medium was removed, and 2 ml of ice-cold stop solution was added that contained modified Locke's solution (without zinc) as given earlier with 5 mmol/l La^{3+}. The cells were washed three times with 2 ml of wash buffer containing modified Locke's solution (without zinc) and 1 mmol/l EGTA. Cells were lysed in 0.5 N NaOH, and all lysates were assayed for $^{65}Zn^{2+}$ by gamma counting. Proteins were determined according to the Lowry method. Parallel experiments were performed with the addition of 5 mmol/l La^{3+}, and these values were subtracted from total $^{65}Zn^{2+}$ uptake to obtain the values plotted. Each point represents the mean and ± SE of duplicate determinations.

Resting neurons slowly accumulate zinc. Zinc uptake in resting cultured neurons probably represents transporter-mediated zinc uptake. Resting zinc uptake was measured in cultured cortical neurons by incubating neurons in a physiological buffer (modified Lock's buffer) containing various concentrations of $^{65}Zn^{2+}$. These studies show that cultured neurons slowly accumulate $^{65}Zn^{2+}$ under these conditions and that a steady state $^{65}Zn^{2+}$ content is obtained after several hours of incubation. Shown in **Figure 1** is the concentration dependence of $^{65}Zn^{2+}$ uptake measured in cultured neurons that had been exposed to $^{65}Zn^{2+}$-containing buffers for 15 min at 37°C. Zinc uptake clearly shows saturation kinetics, and the apparent K_m value is 10–20 μmol/l. Lanthanum (maximal effect seen at 5 mmol/l) inhibits a portion of $^{65}Zn^{2+}$ uptake. The data presented in Figure 1 represent La^{3+}-sensitive $^{65}Zn^{2+}$ uptake. The nature of the La^{3+}-insensitive $^{65}Zn^{2+}$ uptake is still unclear. **Figure 2** shows the time course of $^{65}Zn^{2+}$ uptake in the presence of 5 mmol/l La^{3+}. Over the range of zinc concentrations used (10–65 μmol/l) in these studies, a substantial amount of the total uptake of $^{65}Zn^{2+}$ is La^{3+} insensitive. However, a comparison of Figures 1 & 2 shows that important differences exist between La^{3+}-sensitive and - insensitive $^{65}Zn^{2+}$ uptake. Figure 2 shows that La^{3+}-insensitive uptake was very rapid and nearly complete within the first time point assayed (15 min). This finding contrasts with that seen for La^{3+}-sensitive $^{65}Zn^{2+}$ uptake, where uptake continues for several hours. Another important difference is that over the concentration range studied (10–65 μmol/l), La^{3+}-insensitive $^{65}Zn^{2+}$ uptake appears to be nearly saturated. The above results provide evidence for a binding site for $^{65}Zn^{2+}$ in cultured neurons with submicromolar affinity. This binding site may be on the cell surface, as the rapid time course argues against cellular accumu-

[4] Abbreviations used: NMDA, *N*-methyl-D-aspartate; ZnT, zinc transporter.

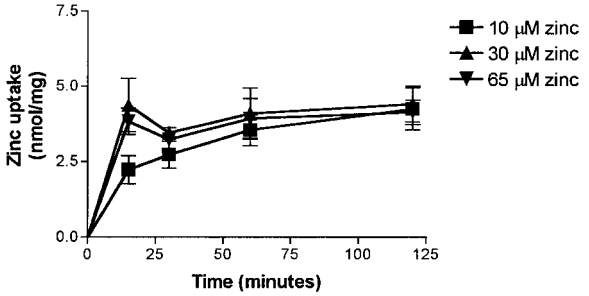

FIGURE 2 Cortical neurons in primary culture were incubated at 37°C for 15, 30, 60 and 120 min in 1 ml of modified Locke's solution described in the legend to Figure 1 except 5 mmol/l La^{3+} was included. The concentrations of $ZnCl_2$ used were 10, 30 and 65 μmol/l. Each point represents the mean ± SE of triplicate determinations.

lation. In the uptake assay, the cells are washed three times with 1 mmol/l EGTA before cells are lysed and $^{65}Zn^{2+}$ content is determined, so if La^{3+}-insensitive $^{65}Zn^{2+}$ uptake represents surface binding, it would have to be occluded in some way. The addition of the heavy metal chelator *o*-phenanthroline to the uptake reaction blocked nearly all of the La^{3+}-insensitive $^{65}Zn^{2+}$ uptake (**Fig. 3**).

The La^{3+}-sensitive $^{65}Zn^{2+}$ uptake was inhibited by lowering buffer pH. This is shown in Figure 3. The incubation of cultured neurons in pH 5.0 buffer resulted in a nearly complete inhibition of the La^{3+}-sensitive $^{65}Zn^{2+}$ uptake. In contrast, the La^{3+}-insensitive $^{65}Zn^{2+}$ uptake was not inhibited by incubation in pH 5.0 buffer. Raising buffer pH to 9.0 had no significant effect on La^{3+}-sensitive $^{65}Zn^{2+}$ uptake. This finding is in contrast to studies of $^{65}Zn^{2+}$ uptake by purified plasma membrane vesicles from rat brain (Colvin 1998a) (Colvin et al. 2000), where it was shown that increasing buffer pH enhanced $^{65}Zn^{2+}$ uptake. The results show that although most characteristics of $^{65}Zn^{2+}$ uptake in vesicles are present in the cellular uptake assay, important differences do exist.

To test the effect of various channel blockers on $^{65}Zn^{2+}$ uptake, purified plasma membrane vesicles were used and the results of these experiments are shown in **Figure 4**. Appropriate blockers were chosen to block all the known routes of depolarization-induced zinc uptake (see earlier). None of these blockers had any effect on resting $^{65}Zn^{2+}$ accumulation (either La^{3+} sensitive or insensitive). These findings support the notion that a distinct plasma membrane transporter is mediating $^{65}Zn^{2+}$ uptake under these conditions.

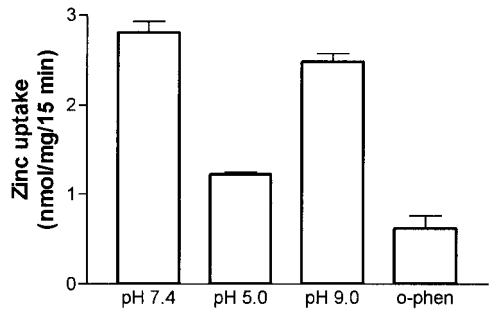

FIGURE 3 $^{65}Zn^{2+}$ uptake was assayed as described in the legend to Figure 1 without the addition of lanthanum. The cells were incubated in 1 ml of modified Locke's solution (Fig. 1) adjusted to the pH indicated containing 20 μmol/l $ZnCl_2$ for 15 min. The concentration of *o*-phenanthroline was 1 mmol/l. Each point represents the mean ± SE of duplicate determinations.

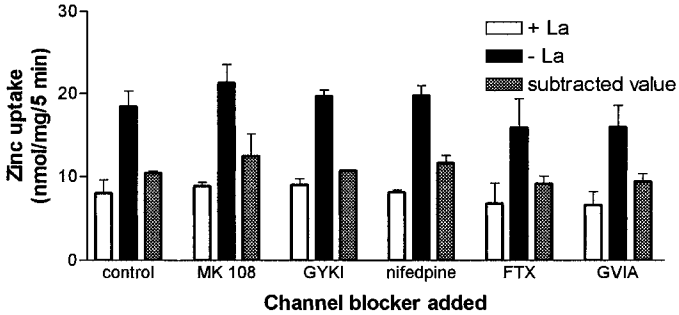

FIGURE 4 $^{65}Zn^{2+}$ uptake was measured, and plasma membrane vesicles were purified from rat brain. Vesicles were incubated in 137 mmol/l KCl and 10 mmol/l HEPES, pH 7.4, for 5 min at 37°C. The concentration of $ZnCl_2/^{65}Zn^{2+}$ was 20 μmol/l. Parallel experiments were performed that included 1 mmol/l La^{3+} from the start of the reaction. To stop the reaction, La^{3+}-containing tubes were placed on ice. To stop the reaction in tubes without La^{3+}, ice-cold La^{3+} was added to a final concentration of 1 mmol/l. Vesicles were immediately filtered on GFC filters and washed four times with an ice-cold buffer containing 137 mmol/l choline Cl, 1 mmol/l EGTA and 10 mmol/l HEPES, pH 7.4. Each blocker was tested for its effects at the following concentrations: MK-801, 100 μmol/l; GYKI 52466, 100 μmol/l; nifedipine, 10 μmol/l; FTX-3.3, 1 μmol/l; and ω-conotoxin GVIA, 1 μmol/l. Each point represents the mean ± SE of duplicate determinations.

Cloned cDNAs code for proteins that can influence zinc transport across the plasma membrane. The first demonstration of a cloned gene that codes for a protein that is likely to transport zinc across the plasma membrane was achieved only recently by Gunshin et al. (1997). They used the heterologous expression system of *Xenopus* oocytes to isolate a nonspecific divalent cation transporter they termed DCT1. In oocytes injected with DCT1 mRNA, 50 μmol/l Fe^{2+} induced a large inward current enhanced by lowering extracellular pH from 7.4 to 5.5. Large inward currents were obtained when Zn^{2+}, Mn^{2+}, Cu^{2+}, Co^{2+} or Cd^{2+} was used, and lesser currents were obtained when Ni^{2+} and Pb^{2+} were used. Pre–steady state currents suggested that H^+ may bind to the transporter. Steady state currents were half-maximal at Fe^{2+} = 2 μmol/l. $^{55}Fe^{2+}$ uptake in NaCl-containing medium was the same as that seen in $NaNO_3$, NaSCN or choline Cl. The application of 50 μmol/l Fe^{2+} at pH 5.5 induced a faster and larger intracellular acidification. These results suggest that DCT1-mediated transport of Fe^{2+} is H^+ coupled in a symport mechanism. The H^+ effect was half-maximal at 1.3 μmol/l, and the Hill coefficient for both Fe^{2+} and H^+ was $\approx$ 1, suggesting a transport stoichiometry of 1:1. DCT1 is a large protein and is predicted to have 12 transmembrane-spanning domains.

A second possible plasma membrane zinc transporter (ZnT-1) has been cloned (Palmiter and Findley 1995). This protein is one member of a family of proteins (Palmiter et al. 1996a, 1996b, Huang and Gitschier 1997; for a review, see McMahon and Cousins 1998) in which the other members of the family are localized to intracellular vesicular membranes. ZnT-1 is a smaller protein than DCT1 and is predicted to have only six transmembrane-spanning domains. Although the expression of ZnT-1 in cultured cells clearly has effects on zinc influx and efflux, it has never been directly demonstrated to transport zinc. The expression of ZnT-1 increased the rate of zinc influx and efflux from cells to the same extent. The efflux of $^{65}Zn^{2+}$ from cells expressing ZnT-1 was increased in a dose-dependent manner by increasing extracellular zinc over a range of 1–50 μmol/l. Extracellular zinc had no effect on the rate of zinc efflux from cells presumably lacking ZnT-1. Vari-

ous inhibitors (10 mmol/l NaN or KCN) had no effect on zinc influx or efflux. Cd^{2+} (10 μmol/l) and iodoacetate (1 mmol/l) inhibited influx by 35% but had little effect on the rate of zinc efflux. There was no effect of changing the ionic composition of the extracellular media (e.g., K^+, tetramethylammonium and Na^+ all gave similar rates of transport; likewise, similar transport rates were obtained in either acetate or Cl^-) on zinc efflux. The removal of Ca^{2+} and Mg^{2+} had no effect on the rate of transport.

Future studies

To arrive at a complete description of neuronal zinc homeostasis, a better understanding of the various zinc homeostatic mechanisms available to neurons is needed. These studies must include an investigation of the interaction and cellular modulation of the processes involved in zinc homeostasis. Particularly lacking is an adequate understanding of the mechanisms of neuronal plasma membrane transport. There are many important questions related to plasma membrane zinc transport that are at present unanswered. Are there separate zinc influx and efflux transporters, or can a single transporter mediate both influx and efflux? How does plasma membrane transport regulate intracellular zinc content? How are intracellular or extracellular zinc levels sensed, and what second messengers are used? Are cells able to regulate zinc influx and efflux in response to changes in either intracellular or extracellular zinc content or concentrations? Hopefully, future biochemical and genetic studies will provide the answers to these intriguing questions.

Note added in proof: Recently the molecular cloning and functional expression of a human homolog of the ZRT1 and ZRT2 genes of *Saccharomyces cerevisiae* have been reported [Gaither, L.A. & Eide, D.J. (2000) Functional expression of the human hZIP2 zinc transporter. J. Biol. Chem. 275: 5560–5564]. When expressed in K562 erythroleukemia cells, the protein was localized to the plasma membrane and demonstrated saturable zinc uptake activity (K_m = 3 μmol/l). Zinc uptake was inhibited when other transition elements were added or when the solution pH was lowered to 5.5.

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