Studies of Zinc Transport and Its Contribution to Zinc Homeostasis

in Cultured Cortical Neurons

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This dissertation titled

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ABSTRACT

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Zn\(^{2+}\) dyshomeostasis in brain might be involved in the pathogenesis of brain diseases such as Alzheimer’s disease and stroke. Thus, neurons tightly control the level of intracellular free Zn\(^{2+}\) within a narrow window of optimal concentration. In this study, the mechanisms of transporter-mediated Zn\(^{2+}\) extrusion and uptake across the plasma membrane of cultured cortical neurons were studied. Changes in intracellular Zn\(^{2+}\) levels were tracked in individual neurons by microfluorometry using a Zn\(^{2+}\) selective fluorophore, FluoZin3. Zn\(^{2+}\) uptake and efflux was measured by first loading cultured cortical neurons with Zn\(^{2+}\) then reducing extracellular Zn\(^{2+}\) to near zero by addition of EDTA. Studies revealed that the primary means of Zn\(^{2+}\) efflux in cortical neurons required both extracellular Na\(^{+}\) and Ca\(^{2+}\). A Na\(^{+}\), Ca\(^{2+}\)/Zn\(^{2+}\) exchanger mechanism is proposed to extrude Zn\(^{2+}\) at the expense of electrochemical sodium and calcium gradients. ZnT1 (SLC30A1) protein levels were reduced around 40% in cultured cortical neurons (*p<0.001) by vector-based shRNA interference (shRNAi). Reducing ZnT1 expression caused the Zn\(^{2+}\) efflux to decrease compared with the control neurons (*p<0.01), which are consistent with ZnT1’s role as a Zn\(^{2+}\) efflux transporter or at least regulating Zn\(^{2+}\) efflux. In case of Zn\(^{2+}\) uptake, acidosis or alkalosis both inhibited Zn\(^{2+}\) uptake at resting condition. Depolarization induced large Zn\(^{2+}\) uptake in neurons. ZIP1
(SLC39A1) protein levels were reduced around 22% by shRNAi (*p<0.001) and resulted in less Zn\(^{2+}\) uptake (*p<0.05). In addition, effects of intracellular zinc levels on the expression of zinc transporter proteins were studied. Right after hZIP1 mRNA was injected into oocytes, oocytes were incubated with different solutions with different levels zinc. The expression of hZIP1 protein on the membrane of oocytes treated with TPEN was increased compare with control (regular medium plus DMSO). When the oocytes were incubated with 10 µM ZnCl\(_2\), the hZIP1 expression was highly reduced observed by immunostaining.

The findings of my studies can be summarized into three aspects: firstly, a Na\(^{+}\), Ca\(^{2+}\)/Zn\(^{2+}\) exchanger and ZnT1 appear to be separate routes acting to reduce intracellular Zn\(^{2+}\) levels in cultured cortical neurons. Second, a Zn\(^{2+}\), HCO\(_3\)^- symporter mechanism and ZIP1 could uptake Zn\(^{2+}\) into neurons at resting condition. Third, hZIP1 protein expression can be regulated by zinc levels at translational levels.

Approved: _____________________________________________________________

Robert A. Colvin
Professor of Biological Sciences
Dedicated to my parents, Zhongbin Qin and Ximei Chen
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INTRODUCTION

I. Introduction of zinc

Zinc is an essential trace element found in all body tissues and fluids with 85% of whole body zinc in muscle and bone, 11% in the skin and the liver (Calesnick & Dinan, 1988). The prostate gland contains high concentration of zinc (150 µg/g tissue), which is about 2-5 times greater than most other tissues (Costello & Franklin, 1998). Being an essential component of many enzymes, DNA binding proteins and structural proteins, zinc contributes to important biological processes, including DNA synthesis, gene expression, hormone control, enzymatic reactions, and cell proliferation (Beyersmann & Haase, 2001). The important role of zinc in an organism was first reported when it was shown to be required for the growth of *Aspergillus niger* (Raulin, 1869). Zinc is essential for many physiological functions (Yanagisawa 2008, Beyersmann & Haase, 2001) including growth and development, wound healing, skin metabolism, spermatogenesis and oogenesis (Falchuk & Montorzi, 2001), normal thyroid, pancreatic, parathyroid function (Neve, 1992), normal immune function (Rink & Gabriel, 2001), bone mineralization, sense of taste and olfaction (Tomita, 2002), function of central nervous system (Prasad, 1997), retinal function (aid in vitamin A metabolism), synthesis and action of insulin, and lipid metabolism. Zinc deficiency has been shown to cause a series of syndromes which include growth retardation, hair loss, skin problems, immune dysfunction, cognitive impairment, and testicular atrophy (Todd et al., 1934; Brown et
The zinc intake of recommended dietary allowance is 12 mg/day for women and 15 mg/day for men aged 19–30 (Lukaski, 2000). On the other hand, excess zinc would be toxic. Although zinc is not stored in the body and excess intake of zinc would result in less absorption and more excretion, lots of studies showed zinc poisoning (Stefanidou et al., 2006). A minimum risk concentration (MRL) of 0.3 mg/kg/day was recommended for intermediate oral zinc intake (Stefanidou et al., 2006). Prolonged intakes of zinc supplements from 50 mg to 300 mg/day have been involved in leucopaenia, neutropaenia, sideroblastic anaemia, reduced copper concentrations, decreased activity of copper containing enzymes, SOD and ceruloplasmin, damaged lipoprotein metabolism and impaired immune function (Sandstead, 1995). Exposure to very high concentrations of zinc could result in fatal acute lung injury in humans (Homma et al., 1992). Excess intracellular zinc has been reported to be responsible for cell injury and necrosis, especially in the setting of excitotoxicity in the central nervous system (Choi & Koh, 1998).

Extracellular Zn\(^{2+}\) can affect cell signaling indirectly. The idea of zinc sensing receptor (ZnR) was proposed recently (Hershfinkel et al., 2007). The receptor is specifically activated by extracellular Zn\(^{2+}\) at physiological concentrations. ZnR was suggested to appear in organs where dynamic zinc homeostasis needs to be maintained so that extracellular Zn\(^{2+}\) can trigger intracellular signaling pathways to regulate essential cell functions such as proliferation and survival, ion transport and hormone secretion. One example of ZnR is a G protein-coupled, Zn\(^{2+}\)-sensing receptor by studies in colonocytes (Hershfinkel et al., 2001). Extracellular Zn\(^{2+}\) activates this ZnR, inducing
intracellular Ca release, therefore regulating a series of intracellular signal transduction and cell functions including ERK1/2 phosphorylation, Na⁺/H⁺ exchanger activation (Azriel-Tamir et al., 2004) and ATP secretion (Sharir & Hershfinkel, 2005). However, the exact gene of such a ZnR is uncharacterized.

II. Zinc plays an important role in the brain

A. Zn²⁺ in synaptic transmission

The zinc concentration in the brain increases with growth after birth (Sawashita et al. 1997). Zinc concentration is around 8 μg/g wet brain tissue in infants and more than 10 μg/g wet brain tissue (less than 15 μg/g) in adults (Markesbery et al., 1984). Zinc levels were shown to be highest in the hippocampus and caudate nucleus and lowest in the middle frontal lobe and globus pallidus (Markesbery et al., 1984). In the brain, approximately 90% of the total zinc is bound to zincproteins (Frederickson 1989). The unique feature of brain zinc is that it is also concentrated in the presynaptic vesicles and histochemically reactive, which can be called free Zn²⁺ (by Timm’s sulfide-silver staining method) (Haug 1973; Frederickson 1989). The vesicular Zn²⁺ was highly concentrated in the hippocampus, amygdale and paralimbic regions. In the hippocampus, the mossy fibers extend from the dentate granule neurons to the CA3 pyramidal neurons, which projects to the apical dendrites of the CA1 pyramidal neurons. Zn²⁺ is enriched in the axons of mossy fibers, CA3 and CA1 pyramidal neurons (Kandel et al., 2000, Li et al., 2001; Daumas et al., 2004). Zn²⁺ concentrations in the vesicles in the giant boutons of hippocampal mossy fibers were estimated to be 300–350 μM (Frederickson et al., 1983).
$\text{Zn}^{2+}$ was suggested to act like a neurotransmitter during synaptic transmission (Frederickson et al., 2005). Lots of studies have shown the evidence of $\text{Zn}^{2+}$ release. Electrical stimulation of hippocampal granule cells induces a calcium-dependent release of $\text{Zn}^{2+}$ (Howell et al., 1984). A calcium-dependent release of $\text{Zn}^{2+}$ from hippocampal slices was also demonstrated after stimulation with high K$^+$ (Assaf & Chung, 1984). Kainate was also demonstrated to induce $\text{Zn}^{2+}$ release from hippocampal mossy fibers (Frederickson et al., 1988). Moreover, $\text{Zn}^{2+}$ release was also found in the amygdala stimulation with high K$^+$ (Takeda et al., 1999). It was also demonstrated that presynaptic stimulation of hippocampal mossy fibers caused $\text{Zn}^{2+}$ release from terminal vesicles into the surrounding milieu (Li et al., 2001, Ueno et al., 2002). A recent study showed direct observation of decreasing vesicular $\text{Zn}^{2+}$ from presynaptic axonal boutons in the stratum lucidum of CA3 when induced by depolarization (Ketterman & Li, 2008). The $\text{Zn}^{2+}$ released by excitation is released with glutamate (Howell et al., 1984). Additionally, exogenous $\text{Zn}^{2+}$ was shown to be taken up into mossy fiber neuropil by electrical stimulation (Howell et al., 1984). Recent data provide further evidence that synaptically released $\text{Zn}^{2+}$ can be taken up into presynaptic terminals (Ketterman & Li, 2008).

Increase of extracellular $\text{Zn}^{2+}$ might affect the membrane receptors and channels. $\text{Zn}^{2+}$ can inhibit the activity of GABA receptor and the $\text{Zn}^{2+}$ binding site in the GABA receptor was identified by site-directed mutagenesis (Hoise et al., 2003). Synaptic $\text{Zn}^{2+}$ was shown to be responsible for the function of glycinergic neurotransmission. Low (<10 $\mu$M) concentrations of $\text{Zn}^{2+}$ enhance glycinergic currents, whereas higher concentrations (>10 $\mu$M) have an inhibitory effect (Laube et al., 2000, Hirzel et al., 2006). $\text{Zn}^{2+}$ was also
shown to inhibit NMDA receptor-mediated channels in cultured neurons (Christine & Choi 1990; Legendre & Westbrook, 1990). A most recent paper showed that Zn$^{2+}$ could activate the receptor tyrosine kinase TrkB and potentiate hippocampal mossy fiber-CA3 pyramid synapses (Huang et al., 2008). Thus, Zn$^{2+}$ function is similar to neurotransmitters which are stored in membrane-enclosed synaptic vesicles and released by exocytosis. Evidence exists that synaptically released Zn$^{2+}$ binds to gated ion channels and activates postsynaptic cells (Hershfinkel et al., 2001, Colvin et al., 2003).

**B. Zinc is involved in brain injury**

Intracellular free Zn$^{2+}$ levels in neurons are tightly regulated, and in particular large increases in intracellular free Zn$^{2+}$ are toxic (Sensi et al., 1997; Manev et al., 1997). Experimental evidence supports the hypothesis that selective neuronal death following transient brain ischemia, which occurs during cardiac arrest and resuscitation or as a result of cerebral thombosis, is mediated, at least in part, by toxicity resulting from large amounts of glutamate and Zn$^{2+}$ that are co-released presynaptically (Choi et al., 1998; Koh et al., 1996). After transient forebrain ischemia in rats, free Zn$^{2+}$ accumulated in degenerating neurons in the hippocampal hilus and CA1, cerebral cortex, thalamus, striatum and amygdale, and neurodegeneration followed. Intraventricular injection of Zn$^{2+}$ chelators could prevent this damage (Koh et al., 1996). A 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., 1989; Suh et al., 2001). Free intracellular Zn$^{2+}$ concentrations reaching as high as 400-600 nM
have been observed (Canzoniero et al., 1999). Rapid Zn\(^{2+}\) influx through Ca\(^{2+}\) permeable AMPA/kainate (Ca-A/K) channels triggers the generation of reactive oxygen species, which are toxic to neurons (Weiss & Sensi, 2000). More recent studies provide evidence that cytosolic protein bound Zn\(^{2+}\) can be released by oxidation, causing increases in free intracellular Zn\(^{2+}\) and increased Zn\(^{2+}\) release (Cuajungco et al., 1998; Aizenman et al., 2000; Frederickson et al., 2002) and this Zn\(^{2+}\) contributes to the toxic Zn\(^{2+}\) rise as well.

In addition, Zn\(^{2+}\) metabolism and homeostasis might be related to brain aging and to the onset and development of age-related neurodegenerative diseases (Doraiswamy & Finefrock et al., 2004; Mocchegiani et al., 2001). In the aging mouse models, total Zn\(^{2+}\) concentration in the synaptic vesicles of the mossy fiber pathway is low (Saito et al., 2000). Zn\(^{2+}\) may act as a neuromodulator at excitatory synapses and might play a significant role in the response to stress, in the process of myelination and in maintaining brain compensatory capacity (Takeda, 2000). Zn\(^{2+}\) has been suggested to complex in the histidine rich region of the amyloid-\(\beta\) (A\(\beta\)) peptide during its aggregation, indicating this metal might be involved in the pathogenesis of Alzheimer’s disease (Maynard 2005).

Zn\(^{2+}\) chelators, such as clioquinol will cause the dispersion of A\(\beta\) plaques (Nitzan 2003).

III. Zinc homeostasis is maintained through membrane transporters

Because of its important and complicated function in the brain, the cellular free Zn\(^{2+}\) in the neuron should be maintained at optimal physiological levels for its proper
function. Extensive evidence suggests that neuronal $\text{Zn}^{2+}$ homeostasis is regulated by membrane transport proteins; cytosolic metal-binding proteins such as metallothionens; and sequestration into intracellular organelles (Colvin et al., 2003). The role of membrane transporters in regulating $\text{Zn}^{2+}$ homeostasis in neurons is of great interest to scientists. These transporters act as the “doors” on the membrane to $\text{Zn}^{2+}$ and might be activated in different conditions. Until now two families of membrane proteins in eukaryotes have been identified as $\text{Zn}^{2+}$ transporters: the SLC30 family that moves $\text{Zn}^{2+}$ out of cell or into intracellular vesicles and the SLC39 family that imports $\text{Zn}^{2+}$ into cell (Harris, 2002).

A. SLC 30 family

The SLC30 family, previously called the cation diffusion facilitator (CDF) family, is thought to confer $\text{Zn}^{2+}$ resistance and $\text{Zn}^{2+}$ is the only metal known to be transported by mammalian SLC30 family (Palmiter & Huang, 2004). At least nine members are recognized in mammals and their important functions have been indicated by a series of knockout and mutation experiments in mice. SLC30A1, the only member of this family located primarily on the plasma membrane, is suggested to lower intracellular $\text{Zn}^{2+}$ concentration and increase resistance to $\text{Zn}^{2+}$ toxicity (Palmiter & Huang, 2004). Homozygous knockout of the SLC30A1 (ZnT1) gene is embryonic lethal in mice, suggesting ZnT1 is essential for normal embryonic development (Andrews et al., 2004). SLC30A3 (ZnT3) is suggested to be required for transport of synaptic $\text{Zn}^{2+}$ because loss of SLC30A3 function in mice prevents accumulation of $\text{Zn}^{2+}$ in synaptic vesicles (Cole et al., 1999). SLC30A2 (ZnT2) and SLC30A4 (ZnT4) were found to be localized in
intracellular compartments and confer $\text{Zn}^{2+}$ resistance (Palmiter et al., 1996; Murgia et al., 1999; Michalczyk et al., 2002). Nonfunctional mouse SLC30A4 results in $\text{Zn}^{2+}$-deficient lethal milk syndrome (Huang & Gitschier, 1997). SLC30A5 (ZnT5) was detected in Golgi and SLC30A5 knockout mice display bone abnormalities and heart failure (Inoue et al., 2002). SLC30A5 and SLC30A6 (ZnT6) are thought to functionally interact as a complex (Ishihara et al., 2006). SLC30A7 (ZnT7) was reported to be localized in the Golgi (Kirschke & Huang, 2003) and might play an important role in dietary $\text{Zn}^{2+}$ absorption and fat accumulation (Huang et al., 2007). SLC30A8 (ZnT8) was suggested to be a pancreas-specific $\text{Zn}^{2+}$ transporter and colocalized with insulin in cultured pancreatic β-cells and might regulate insulin secretion (Chimienti et al., 2006). Recently, this transporter has been shown to be a major autoantigen in human type 1 diabetes (Wenzlau et al., 2007). In addition, a polymorphism of SLC30A8 in humans was identified to increase type 2 diabetes risk (Sladek et al., 2007).

Studies of the transport mechanisms of SLC30A family members have begun, although a transport mechanism for each family member has not been fully clarified. The bacterial homologs YiiP (Lu & Fu, 2007) and ZitB (Chao & Fu, 2004) were shown to be $\text{Zn}^{2+}/\text{H}^+$ antiporters. A recent study of the structure of YiiP showed that it is a homodimer, the cytoplasmic regions of which are closely associated with their transmembrane domains separated, providing a “Y” shape. Four $\text{Zn}^{2+}$ ions sites were identified per subunit that might be required to stabilize the subunit structure (Lu & Fu, 2007). In the case of mammalian SLC30A proteins, the subcellular localization of most mammalian SLC30A family members to acidic cellular compartments suggests that they
might also act as secondary active transporters such as a Zn$^{2+}$/H$^+$ exchanger. For example, the function of SLC30A5 was reported to be pH dependent, increasing extracellular pH would induce Zn$^{2+}$ uptake by this protein (Cragg et al., 2002). As for the plasma membrane localized SLC30A1 protein, it is unclear if the proton gradients across the plasma membrane would be sufficient to catalyze Zn$^{2+}$ extrusion. Two conflicting hypotheses have been proposed to explain the physiological function of SLC30A1. Several studies (Palmiter & Findley, 1995; Kim et al., 2000) have provided evidence that SLC30A1 when expressed at the plasma membrane mediates a net Zn$^{2+}$ efflux, particularly when intracellular levels of Zn$^{2+}$ are elevated. Both in vivo and in vitro studies indicated that the expression of the SLC30A1 gene was regulated by Zn$^{2+}$. In rats, dietary supplements of Zn$^{2+}$ induced SLC30A1 mRNA levels in the small intestine, liver and kidney (Liuzzi et al., 2001). In mouse Hepa cells, Zn$^{2+}$ treatment increased SLC30A1 mRNA levels, while Zn$^{2+}$ chelator treatment reduced the levels of SLC30A1 mRNA (Langmade et al., 2000). Also, the transcription factor MTF1 which is regulated by intracellular Zn$^{2+}$ concentration could bind to the SLC30A1 promoter and be involved in regulating the expression of this protein (Langmade et al., 2000). An intriguing report suggests that SLC30A1 might additionally act as a blocker of L-type calcium channels (LTCC) to inhibit Zn$^{2+}$ influx. This study showed that coexpression of SLC30A1 with L-type calcium channel (LTCC) caused 3-fold reduction in the rate of Zn$^{2+}$ influx compared with only LTCC transfected cells (Segal et al., 2004).
B. SLC39 family

SLC39 proteins are members of the ZIP family of metal ion transporters which stands for Zrt-, Irt-like protein, in charge of transporting metal ions from the cell exterior or lumen of intracellular organelles into the cytoplasm (Eide, 2004). At least 15 SLC39-related proteins were found to be encoded by the human genome. Studies of SLC39A1, SLC39A2, and SLC39A4, encoding the proteins hZIP1, hZIP2, and hZIP4, have indicated roles in Zn\(^{2+}\) uptake across the plasma membrane of various cell types (Eide, 2004). Genetic studies have specifically suggested SLC39A4 in the uptake of dietary zinc into intestinal enterocytes. The patients with acrodermatitis enteropathica, a genetic disease of zinc deficiency, have mutations in gene hZIP4 (Dufner-Beattie et al., 2003; Wang et al., 2004b) ZIP5 is also a Zn\(^{2+}\) uptake transporter that is specific for Zn\(^{2+}\) over other potential metal ion substrates (Wang et al., 2004c). ZIP6 was reported to regulate Zn\(^{2+}\) uptake in neuroblastoma cells under resting but not depolarizing conditions (Chowanadisai et al., 2008). Studies of mechanisms of SLC39 proteins suggest that they might be metal/bicarbonate symporters. hZIP2 is stimulated by increased HCO\(_3^-\) levels, suggesting that Zn\(^{2+}\) uptake occurs via a Zn\(^{2+}\)/HCO\(_3^-\) symport mechanism (Gaither & Eide, 2000). Studies showed that ZIP8 and ZIP14 could use HCO\(_3^-\) gradients across the plasma membrane as the driving force for manganese, cadmium and Zn\(^{2+}\) uptake (He et al., 2006; Liu et al., 2008; Girijashanker et al., 2008).
C. Other mechanisms

Other mechanisms of cellular Zn\(^{2+}\) efflux and influx have been reported, but the transporter protein responsible has not been identified in all cases. For Zn\(^{2+}\) efflux, cation gradients have been reported as driving force to extrude Zn\(^{2+}\) out of cells. It has been observed that Zn\(^{2+}\) can be extruded from mammalian cells by a reversible Na\(^+/\)Zn\(^{2+}\) exchange mechanism which utilizes sodium electrochemical gradients maintained by the Na\(^+/\)K\(^+\) ATPase (Ohana et al., 2004). Additionally, a Ca\(^{2+}/\)Zn\(^{2+}\) exchanger was suggested in human red blood cells, which could catalyze the uphill removal of Zn\(^{2+}\) driven by the inward Ca\(^{2+}\) gradient mediated by the Ca\(^{2+}\) pump (Simons, 1991). Finally, Zn\(^{2+}\) ATPase pumps have been discovered in bacteria and Arabidopsis (Sharma et al., 2000; Eren et al., 2004); however, no evidence exists for a mammalian Zn\(^{2+}\) ATPase.

For Zn\(^{2+}\) uptake, [Zn(HCO\(_3\))\(_2\)Cl\(^-\)] (Torrubia & Garay, 1989), ZnCO\(_3\)Cl\(^-\) or [Zn(HCO\(_3\))Cl·OH\(^-\)] (Kalfakakou & Simons, 1990) anion carrier mechanisms were proposed because the Zn\(^{2+}\) uptake into human erythrocytes was stimulated by bicarbonate and chloride, and inhibited by the band 3 anion exchange inhibitor 4,4'-diiodothiocyanatostilbene-2,2'-disulphonic acid (DIDS) (Torrubia & Garay, 1989; Kalfakakou & Simons, 1990). Bicarbonate also has been reported to stimulate Zn\(^{2+}\) uptake in human fibroblasts (Vazquez & Grider, 1995). In neurons, a proton/Zn\(^{2+}\) exchanger was suggested as a mechanism. Intracellular acidification could induce \(^{65}\)Zn\(^{2+}\) uptake and extracellular acidification could inhibit \(^{65}\)Zn\(^{2+}\) uptake in cortical neurons (Colvin, 2002). In addition, \(^{65}\)Zn\(^{2+}\) uptake was induced in PC12 cells preloaded with protons (Balaji & Colvin, 2005). Studies in cultured neurons have indicated that Zn\(^{2+}\) can
enter neurons through voltage-sensitive Ca\(^{2+}\) channels (VSCCs), NMDA channels, or Ca\(^{2+}\)-permeable AMPA/kainate (Ca-A/K) channels (Yin and Weiss, 1995; Sensi et al., 1997; Kerchner et al., 2000). Among these routes, Ca-A/K channels have the greatest permeability to Zn\(^{2+}\) (Yin and Weiss, 1995), and NMDA channels have least permeability to Zn\(^{2+}\) (Westbrook and Mayer, 1987; Yin et al., 2002). Recent studies also found that Zn\(^{2+}\) could be uptake into CA3 pyramidal cells via activation of AMPA/kainate receptors (Takeda et al., 2007).

D. Zinc transporters in pathological conditions

Intracellular zinc homeostasis would be disturbed if the expression or activities of zinc transporters are altered and impaired. Studies have indicated that zinc transporters might be involved in different diseases.

a) Zinc transporters and cancer

The ZIP transporter family was found to be associated with cancers. ZIP4 was shown to be related to the pathogenesis and progression of pancreatic cancers (Li et al., 2007). The protein levels of ZIP4 were greatly higher in pancreatic adenocarcinoma specimens compared with the surrounding normal tissues. ZIP4 mRNA expression was significantly higher in human pancreatic cancer cells than control (human pancreatic ductal epithelium, HPDE) cells. Overexpression of ZIP4 caused pancreatic tumor growth (Li et al., 2007). In human prostate cancer, ZIP1, ZIP2 and ZIP3 proteins were largely reduced (Franklin et al., 2005; Desouki et al., 2007). Human ZIP1 was identified to be a
Zinc transporter to uptake zinc into prostate cells (Franklin et al., 2003). Studies also found that ZIP1 expression in prostate glandular epithelium was low in African-American males as compared to Caucasian males, which might account for the higher susceptibility of African-Americans to prostate cancer (Rishi et al., 2003). The gene expression and protein levels of ZIP1 were reduced in malignant prostate glands compared to nonmalignant glands (Franklin et al., 2005). ZIP1 was suggested to function as a prostate tumor suppressor by accumulating zinc to induce tumor cell apoptosis and other tumor suppressor effects (Costello & Franklin, 2006).

ZIP6 was found to be regulated by estrogen and involved in estrogen-receptor-positive and the subsequent spread to the regional lymph nodes (Manning et al., 1994; Schneider et al., 2006). Recently, ZIP6 was identified to act as a reliable marker of luminal A type clinical breast cancer (Perou et al., 2000). In addition, ZIP10 was demonstrated to be associated with the metastasis of breast cancer to the lymph node (Kagara et al., 2007).

**b) Zinc transporters and Alzheimer’s disease**

Previous studies presented the relation of zinc transporters to Alzheimer’s disease (AD). Beta-amyloid peptide (Aβ) containing senile plaques (SP) and neurofibrillary tangles are the pathological features of AD. High extracellular zinc concentration can initiate the deposition of Aβ and lead to the formation of SP. ZnT1 expression was shown to be reduced in the hippocampus/parahippocampal gyrus of subjects with mild cognitive impairment and increased in early and late stage AD patients; while ZnT4 and ZnT6
proteins were increased significantly (Lovell et al., 2005; Smith et al., 2006). ZnT6 levels were higher with disruption of trans-Golgi network in AD subjects (Lovell et al., 2006). Knockdown of ZnT3 caused 50% reduction of Aβ proteins deposition in TG2576 mice (Lee et al., 2002). Further studies indicated that ZnT1, ZnT3-7 were abundantly expressed in the Aβ plaques of the AD patient brains and APP/PS1 transgenic mouse brain (Zhang et al., 2008a; Zhang et al., 2008b). In the APP/PS1 transgenic mouse brain, ZnT1 and ZnT4 were expressed all over the Aβ plaques. ZnT3, ZnT5 and ZnT6 were found abundantly in the degenerating neurites in the peripheral regions of the senile plaques; whereas ZnT7 was focused in the center of the plaques. For ZnT3, it had high expression in amyloid angiopathic vessels. The different location and expression of ZnT transporter family members in Aβ plaques suggests that they might play different roles in the aggregation and organization of Aβ plaque.

c) Zinc transporters and other diseases

Zinc transporters were demonstrated to be related to other diseases. ZIP4 was defective in patients of acrodermatitis enteropathica (AE) who have severe zinc deficiency because of reduced zinc absorption in the duodenum and jejunum (Wang et al., 2001). Recently, new discoveries have linked ZIP8 with diabetes. Type 1 diabetes is caused by an autoimmune response leading to death of islet β cells, resulting in lack of insulin production. Type 2 diabetes is caused by desruption of the signaling events after activation of the insulin receptor (Chausmer, 1998). ZnT8 was revealed to be a major autoantigen in type 1 diabetes (Wenzlau et al., 2008). Genome-wide association studies
showed that Arg325Trp polymorphism in ZnT8 is associated with type 2 diabetes and reduced insulin release (Boesgaard et al., 2008). Zinc was clinically linked to asthma and zinc deficiency worsens lung function. In animal models of allergic airway inflammation, increased expression of ZIP1 and ZIP14 and decreased expression of ZIP4 and ZnT4 were observed during acute inflammation (Lang et al. 2007).

IV. Regulation of zinc transporters by zinc levels

As transporters that can act to maintain intracellular Zn\(^{2+}\) homeostasis, ZnT and ZIP proteins have been found to be regulated by Zn\(^{2+}\) levels from various aspects, including transcription and intracellular protein trafficking. Current studies show that ZnT proteins were regulated mostly at the transcriptional level. When free intracellular Zn\(^{2+}\) levels are elevated, the Zn\(^{2+}\) will bind to metal response element-binding transcription factor MTF-1. MTF-1 will then bind the metal response element on the DNA to promote the transcription of ZnT1 (Langmade, 2000). It was reported that ZnT1 and ZnT2 mRNAs were markedly greater when a supplemental Zn\(^{2+}\) intake was provided; while ZnT4 was refractory to changes in Zn\(^{2+}\) intake (Liuzzi et al., 2001).

As for ZIP proteins, they can be regulated at different levels. At the transcriptional level, ZIP2 gene was sensitive to Zn\(^{2+}\) and its mRNA levels rise significantly when Zn\(^{2+}\) is depleted in human monocytic/macrophage THP-1 cells; while the abundance of ZIP1, ZIP3 and ZIP4 mRNA were not affected greatly (Cousins et al., 2003). Another study found that the dietary zinc deficiency causes a marked increase in the accumulation of ZIP4 mRNA in intestines and embryonic visceral yolk sac, and
injection of zinc or increasing dietary zinc rapidly reduces its amount (Dufner-Beattie et al., 2003). It was also reported that the ZIP1, ZIP2 and ZIP3 mRNAs were not regulated by dietary zinc in the intestine and visceral endoderm (Dufner-Beattie et al., 2003). Additional studies indicated that the localization of several ZIP proteins could be controlled at the post-translational level. The accumulation of ZIP4 protein at the apical surface of enterocytes and visceral endoderm cells could be regulated by Zn$^{2+}$ (Dufner-Beattie et al., 2003). In transfected HEK293 cells, both mouse and human ZIP4 protein levels were increased at the plasma membrane by TPEN and the rapid endocytosis of the transporters were stimulated when cells were cultured in Zn$^{2+}$-rich medium (Kim et al., 2004). When expressed in transfected cells, both mZIP1 and mZIP3 were largely present in intracellular organelles when Zn$^{2+}$ is rich and they rapidly transit to the plasma membrane when Zn$^{2+}$ is deficient because the endocytosis rate was decreased (Wang et al., 2004a). In contrast, the distribution of mZIP5 was not affected by TPEN or Zn$^{2+}$ treatment in transfected HEK293 cells (Wang et al., 2004c). A recent paper reported that ZIP5 could be regulated by Zn$^{2+}$ at the translational level and Zn$^{2+}$ deficiency might stall the ZIP5 on the polysomes (Weaver et al., 2007). Previous studies have suggested that the expression of hZIP1 could be regulated at post-translational trafficking level and the di-leucine sorting signal, ETRALL144–149 was required for hZIP1 internalization (Huang & Kirschke, 2007).
V. Overview of dissertation research

Although many studies have been conducted regarding the function and mechanisms underlying Zn$^{2+}$ homeostasis, very little is known about this topic in neurons. The purpose of my study is to systematically characterize the various transporter-mediated Zn$^{2+}$ efflux and influx mechanisms active in cultured cortical neurons. These studies are of great significance. The process of Zn$^{2+}$ homeostasis is important for neuron survival, especially after synaptic activity and influx of Zn$^{2+}$ during brain injury such as ischemia and stroke (Frederickson et al., 2006; Lee et al., 2006).

In addition, changes in brain Zn$^{2+}$ levels are observed in Alzheimer’s disease (AD) (Doraiswamy & Finefrock, 2004). AD is an irreversible brain disorder with progressive memory loss and there is no known cure. More than 4.5 million Americans are estimated to have AD and by 2050; the number could eventually increase to 13.2 million. As discussed before, Zn$^{2+}$ transporter protein levels are altered in AD (Lovel et al., 2005; Lovel et al., 2006), but it is not known how such changes affect neuronal Zn$^{2+}$ levels. Elucidation of the association of Zn$^{2+}$ transporter function with neuronal Zn$^{2+}$ homeostasis would provide clues to understanding the role of Zn$^{2+}$ in the pathogenesis of AD or other brain diseases. The following questions were proposed to study the roles of Zn$^{2+}$ transporters in neuronal Zn$^{2+}$ homeostasis.

1. What is/are the Zn$^{2+}$ efflux mechanism(s) in neurons?
   1) What is the primary Zn$^{2+}$ efflux mechanism activated when intracellular Zn$^{2+}$ are increased such that intracellular levels can be maintained at low concentration (less than or equal to 1 nM)?
2) What is the role of ZnT1 in reducing intracellular Zn\textsuperscript{2+} in neurons? To remove Zn\textsuperscript{2+} or block Zn\textsuperscript{2+} influx?

2. What is the Zn\textsuperscript{2+} uptake mechanism in neurons?
   1) Will Zn\textsuperscript{2+} uptake be affected by extracellular pH?
   2) Will ZIP1 be involved in Zn\textsuperscript{2+} uptake in neurons?
   3) Will depolarization affect Zn\textsuperscript{2+} uptake?

3. How are the zinc transporters ZnT1 and ZIP1 regulated by Zn\textsuperscript{2+}?
   1) Will the protein levels of ZnT1 and ZIP1 be modulated by Zn\textsuperscript{2+} levels?
   2) Since the mRNA levels of ZnT1 were reported to be sensitive to Zn\textsuperscript{2+} levels, whereas ZIP1 transcription was not affected by Zn\textsuperscript{2+}; will zinc affect ZIP1 at the translational level or the post transcriptional level?
MATERIALS AND METHODS

1. Primary cortical neuron culture

A pregnant rat (E17) was euthanized with CO₂. The fetuses were removed from the uterine horns of the pregnant rat. The fetal heads were then separated from the body and placed in a Petri dish containing cold 70% ethanol under the dissecting microscope. Using forceps, the two cortical lobes were separated from the cerebellum, olfactory bulb, hypothalamus and meninges. The cortices cut into quarters were then placed into a sterile tube containing Hank’s Balanced Saline Solution (HBSS) (without Ca²⁺ and Mg²⁺), and three milliliters of 0.1% Trypsin in HBSS is added to the tube to dissociate the cells from one another, gently inverted and allowed to stand for 15 minutes. The trypsin solution was removed and the cells were washed with 6-7 ml of fresh HBSS (Appendix A). The HBSS wash is removed and 3 ml of 0.1% soybean trypsin inhibitor was then added to the cells. The cells were triturated by pipetting up and down through a series of pipettes with fire polished increasingly narrow openings. Cell density was then checked with a 1:10 dilution of the sample in a hemocytometer. About 10 μl to 50 μl of the cell suspension was added to each well of cell culture plates containing 12 mm round glass coverslips coated with polyethyleneimine coating solution (Appendix A). In 24 well plate, 0.5 ml MEM+ was already added to each well; in 96 well plate, 100 μl was already added to each well so that the cell density was kept low or high. Low density cells indicate that individual cells clearly discernable without significantly overlap of the processes (Fig.1A). High density cells mean that neurons were nearly confluent, much overlap of process (Fig.1B). Cells were then allowed to attach to the coverslip for 3-6 hours at 37
°C, and then the media is changed to 0.5 ml of fresh MEM+ and incubated overnight. After one day, neurons were treated for 48 hours with 1 µM Ara-C to inhibit the proliferation of non-neuronal cells. Neurons were maintained in Neurobasal medium (Gibco BRL) with B27 supplement (Gibco BRL) and L-glutamine.

Figure 1. Representative pictures showing the low density and high density of neuron cultures.

(A) Low density of neurons loaded with FluoZin3 and incubated with 10 µM ZnCl₂ for 30 minutes. (B) High density of neurons loaded with FluoZin3 and incubated with 10 µM ZnCl₂ for 30 minutes.

2. Measurement of Zn²⁺ efflux in cultured cortical neurons

Microfluorometry was used so that changes in intracellular free Zn²⁺ could be tracked in individual neurons. Intracellular free Zn²⁺ concentration was monitored using a Zn²⁺ specific fluorophore, FluoZin3 (Kd(Zn²⁺)~15nM, Invitrogen). Cultured neurons were
preloaded with 10 µM FluoZin3 in Locke’s buffer (Appendix A) for 30 minutes (Lin et al., 2007). The neurons grown on glass coverslips were then placed in a perfusion chamber (Warner Inst., model RC-30HV), so that the buffers could be changed and the effects could be observed in real time (Lin et al., 2007). The rate of perfusion was held at approximately 0.5ml per minute for all buffers. The neurons were washed with low calcium Locke’s buffer (Appendix A) for 5 minutes and perfused with 10 µM ZnCl₂ solutions for 30 minutes to increase intracellular Zn²⁺ concentrations. These conditions were chosen because they result in high levels of Zn²⁺ uptake (Colvin et al. 2008). The Zn²⁺ containing buffer was then switched to buffer (without Zn²⁺ added) containing 100 µM EDTA (free extracellular Zn²⁺ is nominally 0) to inhibit Zn²⁺ influx. Finally, the metal selective membrane permeable chelator TPEN (50 µM) was added to indicate if the fluorescence changes were from Zn²⁺. The low calcium concentration minimizes the potential effects of calcium influx on FluoZin3 fluorescence (Colvin et al., 2008). A conventional epifluorescence microscope (Nikon, Diaphot 300) equipped with either Nikon 20x or 60x objectives (Plan Apo/0.75, Plan Apo/1.40 oil DIC, respectively) and FITC-HYQ filter (Chroma) was used to capture fluorescent images using a CCD camera (Spot, RT ES, model9.1 Monochrome w/IR-6). When neurons were perfused with Locke’s buffer as a control condition, we observed a small time dependent increase in fluorescence that was resistant to TPEN reversal. We observed that the fluorescence increase was related to the duration of exposure to and intensity of the excitation light. Therefore, in all experiments, neurons were exposed to excitation light for the shortest
time possible and a neutral density filter was used to reduce the photoactivation effect to negligible amounts.

Each experiment was replicated several times usually using different preparations of cortical neurons (n equals the number times the experiment was replicated, not the number of neurons used for data analysis). Changes in intracellular FluoZin3 fluorescence was quantified using standard image analysis software tools (MetaMorph). On each coverslip, three to ten neurons were randomly selected and the soma of each cell was traced and the intensity of the fluorescence within the trace was determined. The trace outline was then copied onto all subsequent images of the experiment and the intensity determined. In some experiments the number of replicates was large and the average fluorescence value on each coverslip was used for data analysis. The fluorescence of individual cells was normalized to starting fluorescence (F/F₀) (Fig.1) and was plotted against time using Prism software (Graphpad, La Jolla, CA). The Zn²⁺ efflux rate was calculated as ΔF/s = (F₀-Fₜ)/time (F₀ is the maximum fluorescence intensity after 30 minutes of Zn²⁺ incubation).

3. $^{65}$Zn²⁺ efflux assay

Neuron cultures plated on coverslips were washed with Locke’s buffer once. Then Locke’s buffer containing $^{65}$Zn²⁺ (0.4 μCi/ml, Brookhaven labs) plus nonradioactive Zn²⁺ ([total Zn²⁺] =10 μM) was applied to the neurons (Colvin et al., 2005) and temperature was maintained at 37 °C by water bath. After 30 minutes of incubation period, the solution was switched to Locke’s buffer plus EDTA for efflux measurement. After
various times (0, 5, 30 min), cells were washed three times with the same solutions, and the cultures were placed on ice then frozen. The plates were scraped by adding Chelex treated 100 mM HEPES (pH 7.4). Protein levels were determined by BioRad assay and the radioactivity was determined in a scintillation counter. The radioactivity counted by the scintillation counter was recorded as CPM (counts per minute).

4. \( \text{Zn}^{2+} \) measurement

The radioactivity of \( \text{Zn}^{2+} \) was measured by scintillation counter. The standards for the experiment were obtained by sampling 10 µl of the 10 µM stock solution three times. The average CPM of the 10 µl standard (3 samples from the stock) was calculated. The concentration of the stock (10 µM) was converted to nM/10 µl which gives a value of 100 nM/10 µl. This is further corrected for the amount of protein to obtain values as nM/mg. The amount of \( \text{Zn}^{2+} \) remaining in the cells was measured after lysing the cells by freezing and thawing. The cells that were frozen in the – 80 °C freezer were thawed at room temperature, and then placed into a clean culture dish. A uniform protein suspension was obtained by pipetting up and down. 200 µl of the cell suspension was transferred to each scintillation vial, and 3 ml scintillation fluid was added. One determination was done for each coverslip or oocyte. The vials were counted in the scintillation counter using protocol 5 for \( \text{Zn}^{2+} \).
5. BioRad protein assay

The quantity of $^{65}\text{Zn}^{2+}$ (in nanomoles) remained in neurons was corrected for the amount of protein on the coverslips. After thawing the frozen coverslips at room temperature, 20 µl of the cell suspension was added to 780 µl of dH$_2$O in a tube. Two replications were made for each coverslip. 200 µl of BioRad reagent was added and vortexed well. CaryWin UV Simple Reads program and the Spectrophotometer (Cary 50 Probe UV visible, Varian) at wavelength 595.0 nm were used. A blank (200 µl of BioRad reagent added to 800 µl dH$_2$O) was read and the program zeroed, then the standards 5 µl, 10 µl, 15 µl, 20 µl and 25 µl made from 1 mg/ml Bovine Serum Albumin were read. Again a blank was read, and then the samples were read in duplicate. Using Graphpad Prism 4.0 program, linear regression was performed to obtain the protein concentration. $^{65}\text{Zn}^{2+}$ in nM/mg/min was calculated by dividing $^{65}\text{Zn}^{2+}$ in nM/min by the protein concentrations.

6. Reduction of the expression of ZnT1 and ZIP1 by shRNA method

Selection of target sequence

The RNA interference (RNAi) technique was used to reduce the level of ZnT1 and ZIP1 in cultured neurons. The RNAi-Ready pSiren-DNR-DsRed Express vector (Clontech) was used to construct a shRNAi plasmid which could generate specific RNA fragments when transfected into cells. This plasmid contains a constitutively expressed red fluorescent protein (DsRed) which can be used as a marker to identify the ZnT1 or ZIP1 knockdown cells. Three different ZnT1 shRNAi plasmids were constructed using
three different targets (Table 1). ZnT1 RNAi1 and ZnT1 RNAi2 were first constructed by Dustin Thomas (http://www.ohiolink.edu/etd/view.cgi?acc_num=ohiou1163544193)

Table 1.
The Potential ZnT1 RNAi Target Sequences.

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<th>Name</th>
<th>Target Position (NM_022853)</th>
<th>Target Sequences</th>
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<tbody>
<tr>
<td>ZnT1 RNAi1:</td>
<td>380-398</td>
<td>5’-GAACACATTCCGGCTGGATC-3’</td>
</tr>
<tr>
<td>ZnT1 RNAi2:</td>
<td>1,181-1,201</td>
<td>5’-GGAGTCCGCTCTCATTCTTCT-3’</td>
</tr>
<tr>
<td>ZnT1 RNAiQ:</td>
<td>1,429-1,449</td>
<td>5’-TTCGCTAGCGTAGGCTCTA-3’</td>
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</table>

I constructed the third plasmid ZnT1 RNAiQ using the target sequence which has been shown to have 70% knockdown effect for ZnT1 (Ohana et al., 2006), but failed to obtain this high knockdown effects in neurons (Fig. 2). Cotransfection with two plasmids targeting different ZnT1 ORF (open reading frame) sequences was also tried, but failed to increase the knockdown efficiency (Fig. 2). Since the ZnT1 RNAi1 had no knockdown effect and cotransfection can not ensure the same neurons are transfected by both plasmids, the total knockdown effects were then be reduced.
Figure 2. Immunofluorescence of the ZnT1 protein in neurons transfected with nonsense plasmid and shRNAi plasmid targeting different ZnT1 ORF sequences.

The ZnT1 fluorescence staining of transfected neurons was normalized to the average of non-transfected neurons within the same field. (Nonsense: n= 7, ZnT1RNAi2: n=18, ZnT1 RNAiQ: n=7, contransfect: n=14. compared with nonsense control, *p<0.05, t-test, the error bar denotes the standard error)

The ZIP1 shRNAi plasmid was constructed using the same strategy as ZnT1. According to the manual instructions (Clontech), the rat ZIP1 gene sequence was analyzed in GenBank with the accession number NM_001134577. A suitable target was determined by locating a sequence in the gene that is located after the first 100 base pairs of the mRNA sequence. Criteria for the target include about 50% GC content, no runs of more than 3 continuous T residues and no homology to any sequence on other genes.
Rational siRNA Design software was used to help find RNAi targets (http://ihome.ust.hk/~bokcmho/siRNA/siRNA.html). Four different targets were selected because the effects of RNA interference are not predictable (Table 2.)

**Table 2.**

*The Potential ZIP1 RNAi Target Sequences.*

<table>
<thead>
<tr>
<th>Name</th>
<th>Target Position (NM_001134577)</th>
<th>Target Sequences</th>
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<tr>
<td>ZIP RNAiQ1:</td>
<td>915-933</td>
<td>5’-CTGGCACCTTCTCTATAT-3’</td>
</tr>
<tr>
<td>ZIP RNAiQ2:</td>
<td>991-1009</td>
<td>5’-GGTCATTCTGCTCCTAGCA-3’</td>
</tr>
<tr>
<td>ZIP RNAiQ3:</td>
<td>458-477</td>
<td>5’-AGATCACACTGGCTTATAA-3’</td>
</tr>
<tr>
<td>ZIP RNAiQ4:</td>
<td>223-241</td>
<td>5’-CTGTGTGCTTCGAAGGTC-3’</td>
</tr>
</tbody>
</table>

After performing a BLAST search with the sequences, it was determined none of the targets contain significant homology to other genes. After initial trials of knockdown verification, target 2 was determined to yield the highest protein knockdown and subsequently used for all experimentation (Fig.3).
Figure 3. Immunofluorescence of the ZIP1 protein in neurons transfected with nonsense plasmid and shRNAi plasmids targeting different ZIP1 ORF sequences.

The ZIP1 fluorescence staining of transfected neurons was normalized to the average of non-transfected neurons within the same field. (nonsense: n=9, ZIP1Q2:n=24, ZIP1Q3: n=8, ZIP1Q4: n=5, compared to nonsense control, ***p<0.001, *p<0.05, t-test, the error bar denotes the standard error.)
Construction of shRNAi plasmid

The designed oligonucleotide contains the target sequence, the hairpin loop region and the antisense sequence as well as the flanking regions that contain the restriction sites for insertion, an XhoI restriction site for insertion verification and a run of T residues as the U6 stop sequence (Fig. 4). Oligonucleotides were synthesized by Invitrogen. The complementary sequence to the target was synthesized to create a double stranded oligo for ligation into a plasmid. The 5′ region of the oligo contains a GATC overhang which is compatible to the BamHI site on the RNAi-Ready pSiren-DNR-DsRed Express plasmid as well as a 5′ AATT overhang on the complementary strand which is compatible with the EcoRI restriction site also on the plasmid.

The RNAi-Ready pSiren-DNR-DsRed Express from Clontech arrives linearized by BamHI and EcoRI. Before ligation, the two oligonucleotides were first annealed together by resuspending each purified oligonucleotide in TE buffer to a concentration of 100 µM. The complimentary oligos were then mixed in a 1:1 ratio, which yields a final concentration of 50µM (assuming 100% theoretical annealing). The mixture was then ligated according to manufacturer’s instruction. When this constructed plasmid is transfected into cells, the short hairpin loop RNA can be generated for gene silencing effects (Fig. 4)
**Figure 4. Schematic design of shRNAi plasmid of rat ZIP1 and shRNA (short hairpin loop RNA) production.**

The target sequences with restriction sites were inserted into the RNAi-Ready pSiren-DNR-DsRed Express vector. When this plasmid is transfected into cells, shRNAi can be generated by using endogenous RNApolIII. (The vector is from Clontech: http://www.clontech.com/images/pt/PT3739-1.pdf)

**Selection of optimal transfection condition**

Transfection in primary neurons is always difficult. I tried different conditions to compare the transfection effect. First, the best transfection time was selected by transfecting neurons with plasmid expressing red fluorescent proteins on different days after plating neurons (DIV). When neurons are transfected two days after plating, the cells were damaged a lot (Fig.5A). If neurons were transfected on the 7th day, most
transfected cells were non-neuronal cells (Fig. 5D). The best time for transfection was from three to six days after plating (Fig. 5B&C).

Figure 5. Transfection of neurons on different days.

The transfected neurons were indicated by pSiren-DNR-DsRed protein. (A-C) Representative merged images of TRITC and DIC showing both transfected and nontransfected neurons with transfection after 2 DIV (A), 3 DIV (B), and 4-6 DIV (C). (D) The TRITC image indicates transfected cells transfected on 7th day. Pictures were taken three days after transfection.
By using different transfection reagents and different DNA/reagent ratios, I tried to find the best transfection efficiency to transfect cultured cortical neurons. The transfection reagents tested were lipofectamine2000 (Invitrogen), LipofectamineLTX (Invitrogen) and Metafectene pro. Five or sixth days after plating cortical neurons, medium was changed to fresh neurobasal medium (400ul per well in 24 well palte). If using Lipfectamine2000 and Metafectene pro, DNA and reagent were added to the opitimMEM up to 50 µl separately. Five minutes later, these two solutions were mixed and incubated for 20 to30 minutes. If using Lipsecamine LTX, PLUS reagent was added with DNA (1:1) in opitimMEM solution. Five to fifteen minutes later, 0.75 to 1.75 µl of LipofectamineLTX was added into the diluted DNA solution up to 100 µl and incubated at room temperature for 25 minutes to form DNA-LipofectamineLTX complexes. The mixed solutions were added to cells. Two days later, the transfected cells were counted (i.e. expressing red fluorescent proteins) using a conventional epifluorescence microscope (Nikon, Diaphot 300) equipped with a Nikon 20× objective and TRITC filter (Chroma). On each coverslips, five images were randomly selected. The transfection ratio was calculated by dividing the transfected cells from the total cells counted from these images.

When using Metafectene pro (Table 5), the transfected cells did not have long axons, indicating they might be non-neuronal cells. The transfected cells were aggregated on the edge of coverslips, suggesting they might be the amplification of glia cells. Thus Metafectene pro might not be a good choice to transfect neurons. When using Lipofectamine2000 and LipofectamineLTX, the transfected cells had long axons and
round cell body and they showed the same efficiency of tranfection. LipofectamineLTX is more expensive (Table 3 & Table 4); therefore, Lipofectamine2000 was chosen as the transfection reagent. The appropriate DNA to lipofectamine2000 ratio was 0.5 µg to 2 µg (Table 3).

Table 3.

*The Transfection Efficiency with Different Lipofectamine2000 to DNA Ratios.*

<table>
<thead>
<tr>
<th>DNA (µg)</th>
<th>Lipofectamine2000 (µg)</th>
<th>2</th>
<th>4</th>
<th>6</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>0.82%</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>4</td>
<td>N.D.</td>
<td>Cell dead</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>3.17%</td>
<td></td>
</tr>
<tr>
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<td>1.42%</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>N.D.</td>
<td>0.84%</td>
<td></td>
</tr>
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<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
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<td>1.3%</td>
<td>N.D.</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>0.62%</td>
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</tr>
</tbody>
</table>
Table 4.
*The Transfection Efficiency with Different LipofectamineLTX to DNA Ratios.*

<table>
<thead>
<tr>
<th>DNA (µg)</th>
<th>LipofectamineLTX (µg)</th>
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<th>1.75</th>
<th>2.25</th>
<th>3</th>
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<tbody>
<tr>
<td>0.5</td>
<td>1</td>
<td>0.32%</td>
<td>1.28%</td>
<td>1.44%</td>
<td>3.54%</td>
</tr>
<tr>
<td>0.75</td>
<td>1.5</td>
<td>0.93%</td>
<td>1.9%</td>
<td>1.48%(toxic)</td>
<td>1.29% (toxic)</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.61%</td>
<td>0.56%</td>
<td>4.7% (toxic)</td>
<td>2.3% (toxic)</td>
</tr>
</tbody>
</table>

Table 5.
*The Transfection Efficiency with Different Metafectene to DNA Ratios.*

<table>
<thead>
<tr>
<th>DNA (ug)</th>
<th>Metafectene (ug)</th>
<th>0.5</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
<th>2.5</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.48%</td>
<td>0.79%</td>
<td>0.9%</td>
<td>Cell dead</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>3</td>
<td>4.5</td>
<td>6</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>1%</td>
<td>1.79%</td>
<td>0.83%</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>1.5</td>
<td>0.65%</td>
<td>1%</td>
<td>1.79%</td>
<td>0.83%</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>


In detail, 2 µg Lipofectamine2000 (Invitrogen) was added to Opti-Mem Plus (Gibco) to a final volume of 50 µl and incubated at 25°C for 5 minutes. Also, 0.5 µg DNA was added to Lipofectamine2000 to a final volume of 50 µl and incubated for 5 minutes at 25°C. After the 5 minute incubation, the Lipofectamine2000 solution was added to the DNA solution and let stand for 20 minutes at 25°C. The complete mixture was then added to one well of a 24 well plate containing a cortical cell coated cover slip and 400 µl of Neurobasal media (Gibco) containing B27 supplement and L-Glutamine. The media was then replaced with fresh Neurobasal media containing B27 and L-Glutamine 6 hours after transfection.

7. ZnT1 and ZIP1 protein knockdown verified by immunofluorescence

To verify reduction of protein expression in RNAi transfected cells, immunofluorescence was performed. Seventy-two hours after transfection, the cells were fixed in 4% paraformaldehyde (Fisher), then permeabilized in 0.1% Triton X-100 (Sigma). The cells were then incubated with a 1:100 dilution of rabbit anti-rat ZnT1 primary antibodies (obtained from Dr.Liping Huang) and 1:1000 dilution of rabbit anti-rat ZIP1 primary antibodies with BSA/NGS (Appendix A) (obtained from Dr. Glen Andrews) for one hour. After three washes of PBS, a DAPI (1:333) and secondary antibody, Alexa-fluor 488 anti-rabbit (1:2000, Molecular Probes) were then bound to the primary by incubation for one hour. The coverslips were finally mounted onto glass slides (VWR) using Anti-Fade Reagent (Molecular Probes) and observed with
fluorescence microscopy. A ZnT6 antibody (obtained from Dr. Liping Huang) was used to show the specificity of ZnT1 knockdown effects.

A dichroic FITC filter (Chroma 41001) was used to analyze the ZnT1 and ZIP1 expression levels. Using the Spot Imaging software, all post-processing of images was turned off, 12 bit images were gathered and all FITC images were saved as 16-bit TIFF images to maintain the highest quality. DAPI (Chroma 31000) was used to verify healthy neurons with diffuse nuclear staining; apoptotic and necrotic cells exhibiting punctate or no nuclear staining were deleted from analysis. A TRITC filter (Chroma 41002) was used to identify successful transfected cells through the production of the red fluorescent protein DsRed transcribed from the plasmid. The immunostaining intensity of the cells through the FITC filter was measured and analyzed. Individual transfected cells on the same coverslip were compared to the average of all the non-transfected cells to obtain a ratio. The non-sense plasmid transfected cells were treated identically to the RNAi knockdown plasmid transfected cells.

Control runs were also performed that omitted addition of the primary antibody. This control was to observe any background fluorescence the secondary antibody created. The control background fluorescence was deducted from the fluorescence intensities of ZnT1, ZnT6 and ZIP1 staining cells. Since the transfected cells showed a little higher fluorescence intensities than nontransfected cells because of bleeding through of the red fluorescent protein, the data of fluorescence intensities of transfected cells were normalized to those of nontransfected cells so that the ratio of fluorescence intensities of nonsense transfected cells to nontransfected cells is 1 [normalized fluorescence ratio= (F
shRNAi transfected \( F_{\text{nontransfected in shRNAi}} \) × \( ( \text{Average of } F_{\text{nontransfект in nonsense}} \) \text{/ Average of } F_{\text{nonsense transfected}} \).}

8. **Construction of pTLN-hZIP1 expression vector**

To overexpress hZIP1 protein in oocytes, pTLN-hZIP1 plasmid was constructed. The entire open reading frame sequence of hZIP1 was obtained by PCR using the pCMV-hZIP1 (a kind gift from Dr. Eide) as a template. The forward primer contains XbaI and Kozak sequence: (5’-TTTTTCTAGAGCCGCACCATGGGGCCCTGGGGAGAGCCAGA-3’). The reverse primer contains XhoI sites and epitope HA (YPYDVPDYA) sequences: (5’-TTTTTCTCGAGCTAGGCGTAGTCGGGCACGTCGTAGGGGTAGATTTGGATGA AGAGCAGGCCAGTGA-3’). The PCR reaction mixture contained: 5 µl 5×GC cDNA PCR reaction buffer, 2.5 µl GC Melt (5 M), 1 µl pCMV-hZIP1 template, 1 µl forward primer (10 µM), 2 reverse primer (10 µM), 0.5 µl dNTP (10 mM), 1.5 µl Taq and 12.5 µl dH20. PCR cycle RAC5C was chosen to use: 1) 94°C 5 min. 2) 94°C 1 min 30 seconds 3) 68°C 2 min 4) 72°C 5 min 5) to 2 cycle 31 times 6) 72°C 10 min 7) 4°C.

The resulting PCR product was digested with XbaI and XhoI and subsequently inserted into the custom oocyte expression plasmid pTLN (a kind gift from Dr. Romero). The constructed expression plasmids were verified by sequencing from Ohio State University (http://www.biosci.ohio-state.edu/~pmgf/index.htm). Na/K ATPase-HA expression vector (a kind gift from Yanli Ding) was used for control.
9. cRNA synthesis

The constructed plasmid pTLN–hZIP1 was linearized using the restriction endonuclease Swal. Complementary RNA was transcribed beginning at the SP6 promoter upstream of the hZIP1 cDNA using mMESSAGE mMachine high yield capped RNA transcription kit (Ambion). cRNA integrity was checked by 1.2% formaldehyde agarose gel (Appendix A) electrophoresis. RNA was diluted in 5x loading buffer (Appendix A) and incubated at 65°C in the thermal cycler for 10 minutes, then chill on ice, and loaded onto the equilibrated formaldehyde gel. Gel was run at 50–70 V/cm in 1x FA gel running buffer (Appendix A). Ethidium Bromide was used to stain RNA. The RNA concentration was determined using UV spectrophotometric analysis. RNA samples were diluted at 1:1000 and placed into a quartz cuvette. The absorbance at 260 nm was read and converted to concentration by the formula: RNA concentration (µg/ml) = (OD 260) x (dilution factor) x (40 µg RNA/ml)/(1 OD 260 unit).

10. Animal surgery and oocyte extraction

Oocytes were extracted from Xenopus frog (Nasco). The recovery tank was placed at an angle against the wall and contained only enough 0.2% tricaine (Sigma A-5040) to cover the frog. The Xenopus was kept in tricaine solution for 3-5 minutes with nose above the fluid level. The frog was checked for every 2 minutes for anesthesia by pinching the toe to see if she reacts. Xenopus was placed on ice and covered with ice for 5 minutes. Then frog was transferred to ice pan and covered with ice, except for head. While the frog’s sides were slightly squeezed together, a diagonal cut about 1.5 cm was
made above the leg on the low belly. A cut through was made through the skin and muscle layers and the oocytes should be in view. The area was rinsed with 50% penicillin/streptomycin solution (Appendix A). The ovary lobes were taken out using the angled forceps. Two or three batches of oocytes was cut off and placed into a 50 ml tube with about 10-15 ml of Ca\(^{2+}\) free ND96 (Appendix A). When oocytes were removed, the abdomen was rinsed with 50% penicillin/streptomycin. The muscle layer and the skin were sewed with suture. The frog was rinsed with recovery water and to remove of all the residual tricaine, then the frog was placed into a recovery tank with a little water to keep wet, but do not allow the nose to submerge. Frog recovered 30-60 minutes later. After recovery, fresh water was changed in the recovery tank several times to get rid of debris. The recovery tank was then filled with 0.1 M NaCl to recover the frog for 2 hours and minimize stress.

11. Injecting RNA into oocytes

The extracted oocytes were dissociated in Ca\(^{2+}\) free ND96 solution (Appendix A) and rinsed twice. The ovarian lobes were cut open to create flat sheets of oocytes in membrane and cut open into strips. Oocytes were placed into a 50 ml tube and agitated in 20 ml Ca\(^{2+}\) free ND96 solution using several solution changes. Once oocytes had been “loosened” from the surrounding connective tissue, they were treated with 1mg/ml type sterile A collagenase (Boehringer Mannheim # 1088785) in Ca\(^{2+}\) free ND96 buffer for 20-40 min. Check oocytes for dispersion and rinse with 2-3 changes of Ca\(^{2+}\) free ND96. Finally, oocytes were washed with 20-30 ml ND96 solution for 4-5 times. Oocytes were
then incubated in sterile fresh OR3 (Appendix A) in an incubator at 15°C. On the second day, healthy oocytes were selected for injection. The oocytes were placed in a dish with sterile OR3 solution on a 0.5 mm nylon or polypropylene mesh and visualized under a dissecting microscope. Injections were performed using micropipettes held by a micromanipulator. The injection pipettes were filled with paraffin oil, and RNA was then pulled up into the pipette. The Drummond ‘Nanoject’ (#3-000-203) was used to deliver 46-50 nl of solution (1-20 ng RNA) to each oocyte. A microprocessor controlled version of Nanoject (# 3-000-203-XV) was used to motorize injection of variable volumes (4.6 to 73.6 nanoliters), and thereby improves the reproducibility and speed of injection. 30 oocytes need 1-1.5 ul RNA. The site of injection in the oocyte was in the transitional zone, where the animal and vegetal poles meet. Oocytes were microinjected with usually 23 nl × 2 of solution containing RNA. Control oocytes were injected with the same amount of water.

12. Immunofluorescence staining

The expression of hZIP1 in oocytes was tracked by immunofluorescence staining. Oocytes were fixed in 4% paraformaldehyde for 1 hour. They were then incubated with 100 mM glycine for 30 minutes and incubated in 30% sucrose overnight at 4 °C. The fixed oocytes were then moved into Tissue-Tek (O.C.T compound, Sakura) and frozen in liquid nitrogen. The oocyte samples were then stored in -80 °C until use. Oocytes were sectioned using a cryostat into slices of 10-20 mm thickness that were collected on microscope coverslips (precoated with 0.01% polylysine). The oocyte slices were
incubated in block buffer (Appendix A) for 1 hour and then incubated with monoclonal anti-HA antibody (Sigma) overnight at 4 °C. After several wash with PBS (Appendix A), samples were incubated with goat anti-mouse IgG (Alexa Fluor® 546) for 1 hour. All incubations took place at ambient temperature. As a negative control, oocytes were incubated with the same conditions except the primary antibody was replaced by block solution. After mounting, slides were stored in the dark at 4°C until observation. The immunofluorescence can be observed directly by epifluorescence. A conventional epifluorescence microscope (Nikon, Diaphot 300) equipped with a Nikon 20× objective and FITC-HYQ filter (Chroma) was used to acquire fluorescent images using a CCD camera (Spot, RT ES, model 9.1 Monochrome w/IR-6). Filter TRITC was used to take pictures of HA staining. The average fluorescence intensities of HA was measured (F) by outlining the fluorescence on the cell membrane. The similar region on the control oocytes (water injection) was outlined and its fluorescence was recorded (Fc). The expression of hZIP1 protein was indicated by the relative fluorescence which is the result of subtracting Fc from F.

13. \(^{65}\text{Zn}^{2+}\) uptake assay in oocytes

Function of hZIP1 expressed in oocytes was verified by \(^{65}\text{Zn}^{2+}\) uptake assay. A stock solution of \(^{65}\text{Zn}^{2+}\) (Brookhaven labs) was made at approximately 0.2 μCi/ml. Additional nonradioactive ZnCl\(_2\) was added to make a 30μM concentration. In each condition, five to ten oocytes were mixed with 0.5ml oocyte reaction buffer (Appendix A) in a well of 24 well plate containing \(^{65}\text{Zn}^{2+}\) for 1.5 hours at room temperature. Uptake
reactions was stopped by the addition of 3ml/well oocyte ice-cold wash solution (Appendix A) and washed four times. Individual oocytes were then transferred into clean scintillation vials and lysed by 10% SDS. The radioactivity was determined in a scintillation counter. Since total Zn\textsuperscript{2+} added to the buffers were known (\textsuperscript{65}Zn\textsuperscript{2+} plus nonradioactive Zn\textsuperscript{2+}), aliquots of the uptake buffer were then counted for radioactivity to determine the specific activity of \textsuperscript{65}Zn\textsuperscript{2+}. Using this value, the counts associated with individual oocytes were converted to nanomoles of Zn\textsuperscript{2+} per oocyte (Colvin et al., 2005).

**14. Alamar blue test**

After neurons were treated with ZnCl\textsubscript{2} and TPEN, NB media was removed and 500 µl Alamar Blue (1:10 dilution in Neurobasal medium, AbD, CA) was added to each well and incubated for 6 hrs at 37\textdegree{}C. After 6 hrs, 200 µl of each solution was transferred to a 96 well plate. Fluorescent intensity of the media was determined using a fluorescence spectrometer (FluoMax-3) at 560 nm excitation and 580 nm emission. The neuronal survival (%) was calculated by dividing the fluorescence intensity with different treatment from that with just NB incubation.

**15. Immunostaining of K562 cells or cortical neurons**

K562 cells or cortical neurons were placed on coverslips and treated with different conditions. For K562 cells, the 24 well cell plate containing coverslips previously coated with Poly-D-Lysine were incubated with \(5 \times 10^4\) K562 cells for 2 hours. Then the cells were treated with 1 μM TPEN and the control cells were treated with the
same amount of DMSO for 1 hour. For neurons, cells were plated on coverslips as described before and were treated with TPEN or ZnCl₂ or transfection.

The coverslips containing K562 cells or cortical neurons were washed with Neurobasal medium and removed to 4% paraformaldehyde in Locke’s pH7.4 buffer for 8 min. The cover slips were then washed through 2 PBS baths. They were then incubated in 0.1% Triton-X 100 for 1 min and again washed through 2 PBS baths. The coverslips were removed from the final PBS bath to a humid Petri dish where they were incubated for 1 hour with 20 µl primary antibody (ZIP1 or ZnT1 IgG) for 1 hour. After the incubation, cells were then washed through 3 fresh PBS baths and returned to the humid Petri dish. They were incubated, again for one hour, with Alexa 488 1:1000 and DAPI 1:333 in NGS/BSA (Appendix A). After the incubation they were again washed through three fresh PBS baths. The coverslips were then attached to microscope slides with 6.5 µl ProlongAntifade reagent. This was allowed to dry for 30 min before being sealed with clear fingernail polish. The slides were viewed 12 hours later after being stored at -20°C.

16. Fluorescein uptake

Cortical neurons were placed in 96 well plates. Three to five days later, the normal NB medium was changed to medium containing fluorescein with different ions or chemical reagents for certain time. The fluorescence intensity then was then measured in a fluorescence spectrometer (FluoMax-3) at 492 nm excitation and 516 nm emission.
17. **Measurement of intracellular trace elements in oocytes**

Oocytes were washed with Chelex-PBS and one oocyte was placed into one tube with desiccation overnight. Then 75 µl concentrated HNO₃ was added to each tube overnight. The dissolved solution was heated for 20 minutes. The volume was then brought 1 ml with 1% HNO₃. Measurements were taken using an UltraMass 700 (Varian Inc.) ICP-AES according to the instrument manuals. A total of seven metals were analyzed using the ICP-OES including aluminum, calcium, copper, iron, magnesium, manganese and zinc.

18. **Data Analysis.**

Since the transfection efficiency is different for different coverslips and experiments, the average data of the transfected cells on each coverslips were calculated (n) for data analysis. In other conditions, the n represents the number of neurons used. Statistical analysis was carried out to indicate if the experimental treatments had significant effects. Student’s t test, two-way analysis of variance (ANOVA) test and one-way ANOVA with Tukey's Multiple Comparison Test embedded in Prism were used to analyze data.
RESULTS

Part one: Mechanisms of Zn$^{2+}$ efflux in cultured cortical neurons

A Na$^+$/Zn$^{2+}$ exchange mechanism was suggested to extrude Zn$^2+$ from mammalian cells by using sodium electrochemical gradients (Ohana et al., 2004). In addition, a Ca$^{2+}$/Zn$^{2+}$ exchanger was suggested in human red blood cells, which could catalyze the uphill removal of Zn$^{2+}$ driven by the inward Ca$^{2+}$ gradient mediated by the Ca$^{2+}$ pump (Simons, 1991). Based on these studies, the first hypothesis in this part was proposed: sodium and calcium gradients can be used for Zn$^{2+}$ efflux in neurons.

Since ZnT1 was found to confer zinc resistance in 1995 (Palmiter & Findley, 1995), its exact role to confer zinc resistance is controversial. One opinion is that ZnT1 mediates a net Zn$^{2+}$ efflux by overexpression of ZnT1 in BHK cells (Palmiter & Findley, 1995) or PC12 cells (Kim et al., 2000). Another research group argued that ZnT1 acts as a blocker of L-type calcium channels (LTCC) to inhibit Zn$^{2+}$ influx (Segal et al., 2004). The latter study only recorded the Zn$^{2+}$ efflux in two minutes, which might account for why they failed to get more Zn$^{2+}$ efflux when overexpressing ZnT1 in HEK293 cells. I proposed my second hypothesis in this part: ZnT1 expression levels will correlate with Zn$^{2+}$ efflux in neurons.

I. Characterization of changes in intracellular free Zn$^{2+}$ levels using FluoZin3 loaded cortical neurons.

FluoZin3 loaded cortical neurons were perfused with 10 μM ZnCl$_2$ for 30 minutes, and then switched to a 100 μM EDTA solution, which reduced free extracellular
Zn\(^{2+}\) to near zero, without significantly affecting extracellular free calcium or magnesium concentrations. Thus, in the presence of 100 μM EDTA unopposed efflux of Zn\(^{2+}\) should be observed.

When neurons were incubated in 10 μM Zn\(_{\text{Cl}_2}\) for 30 minutes, the FluoZin3 fluorescence intensity increased with time (Fig.6B&E). When the perfusion solution was switched to 100 μM EDTA, the fluorescence intensity decreased with time (Fig.6C&E). FluoZin3 fluorescence intensity dropped sharply with the addition of 50 μM TPEN, indicating that the fluorescence was likely generated from changes in intracellular free Zn\(^{2+}\) (Fig.6D&E). The reduced fluorescence after addition of EDTA could be explained by either of two situations: Zn\(^{2+}\) flowing out of neurons or being sequestered into intracellular compartments. The \(^{65}\text{Zn}^{2+}\) efflux assay (Colvin et al., 2005) was conducted to verify that the reduced fluorescence was associated with net Zn\(^{2+}\) efflux under the above conditions. When neurons were incubated with \(^{65}\text{Zn}^{2+}\), and then perfused with 100 μM EDTA solutions, the total cellular \(^{65}\text{Zn}^{2+}\) content was reduced (Fig.6F), showing a net efflux of Zn\(^{2+}\) did occur during this period. The slope of \(^{65}\text{Zn}^{2+}\) efflux was smaller than the slope of Zn\(^{2+}\) efflux observed by FluoZin3 (Fig. 6E vs 6F), which is caused by the difference of these two methods. Using FluoZin3, the intracellular fluorescence intensities were normalized to the starting fluorescence, the change in this ratio does not equate to the net Zn\(^{2+}\) efflux measured by \(^{65}\text{Zn}^{2+}\) assay. In addition, high magnification (60×) images were captured after neurons were incubated with 10 μM ZnCl\(_2\) for 30 minutes (Fig.6B) and then after 30 minutes with 100 μM EDTA (Fig.6C). The
intracellular FluoZin3 fluorescence was uniform and widespread after EDTA treatment, thus no evidence was observed of Zn$^{2+}$ sequestration into intracellular compartments.

When neurons were perfused with Locke’s buffer as a control condition, fluorescence slightly increased (Fig.6E). We observed that the fluorescence increase was related to the time of exposure to excitation light. Thus, neurons were exposed to light for the shortest time possible during all experiments to reduce this photoactivation effect.
Figure 6 Continued

**Figure 6. Zn\(^{2+}\) flux tracked by FluoZin3 microfluorometry (pH8).**

(A) Cortical neurons were first loaded with FluoZin3 for 30 minutes. (B) Then neurons were incubated with 10 µM ZnCl\(_2\) for 30 minutes. (Inset) high magnification of image obtained by a 60x objective. (C) The neurons were then perfused with 100 µM EDTA for 30 minutes to inhibit Zn\(^{2+}\) influx. (Inset) high magnification of image obtained by a 60x objective showed that the fluorescence was widespread and uniform. (D) Finally neurons were exposed to 50 µM TPEN. (E) Normalized fluorescence (F/F\(_0\)) changes plotted as a function of time (▲). After 20 minutes of EDTA treatment, Zn\(^{2+}\) fluorescence was significantly reduced compared to the start of EDTA perfusion (n=35 neurons form coverslips from 3 rats, *p <0.05, t-test). After adding TPEN for 5 minutes, the fluorescence signal was further lowered (***p <0.001, t-test). Neurons perfused with Locke’s buffer (●) showed a small photoactivation effect (n=15 neurons from 3 coverslips from 1 rat). (F) \(^{65}\text{Zn}\) efflux measured after \(^{65}\text{Zn}\) preloaded neurons were exposed to 100 µM EDTA. After 30 minutes of EDTA incubation, \(^{65}\text{Zn}\) was lower than the coverslips without EDTA treatment (n=3 coverslips, *p <0.05, t-test), indicating that the decreased fluorescence observed in (C) and (E) was accompanied by a net Zn\(^{2+}\) efflux. The error bar denotes the standard error.

II. Sodium/calcium dependent Zn\(^{2+}\) efflux in neurons

A. Zn\(^{2+}\) efflux depends on extracellular sodium

FluoZin3 loaded cortical neurons were perfused with 10 µM ZnCl\(_2\) for 30 minutes, and then switched to a 100 µM EDTA solution with or without sodium. After the 30 minute EDTA exposure, the fluorescence of neurons in solutions without sodium (sodium was replaced by equal molar NMG to maintain the same ionic strength and osmolarity) was reduced to only 88% of the fluorescence measured just before perfusion with EDTA was begun. Fluorescence of neurons perfused with sodium decreased to approximately 40% in solutions containing sodium (Fig.7A, *** p <0.001).
The sodium dependent efflux rate was calculated by subtracting the normalized fluorescence ($F/F_0$) of neurons in sodium solution from the neurons in sodium free solutions at the same time point (Fig. 7B). These results suggested that the presence of extracellular sodium significantly enhanced $Zn^{2+}$ efflux in cortical neurons. The nonspecific channel blocker lanthanum blocked the sodium dependent $Zn^{2+}$ efflux, suggesting that the observed efflux was not caused by a nonspecific membrane leak but by a transporter or channel mechanism at the plasma membrane.

**Figure 7. Sodium dependent $Zn^{2+}$ efflux in cortical neurons (pH 8).**

(A) shows $Zn^{2+}$ efflux traces of neurons with (▲) or without sodium (■). When the external sodium was replaced by NMG, $Zn^{2+}$ efflux was greatly reduced (With Na: n= 45 cells from 9 coverslips from 3 rats; Without Na: n= 65 cells from 13 coverslips from 3 rats; $p<0.001$, two-way ANOVA). The sodium dependent $Zn^{2+}$ efflux could be blocked by the non-specific channel inhibitor lanthanum (10 µM). (B) shows the rate of sodium dependent $Zn^{2+}$ efflux obtained from (A) $[\Delta(F/F_0) = (F/F_0)_{0Na} - (F/F_0)_{Na}]$. The $Zn^{2+}$ efflux rate did not change with time. Solutions were made in low calcium (0.5mM) Locke’s buffer. The error bar denotes the standard error.
B. \(\text{Zn}^{2+}\) efflux is increased only when the sodium gradient is inwardly directed

The sodium dependence of \(\text{Zn}^{2+}\) efflux suggested two possible mechanisms: either sodium binding to activate a \(\text{Zn}^{2+}\) transporter or a \(\text{Na}^+/\text{Zn}^{2+}\) exchanger mechanism. To investigate the concentration dependence of the sodium effect, experiments were first conducted by comparing \(\text{Zn}^{2+}\) efflux rate \(\frac{\Delta F}{s} = \frac{(F_0 - F_t)}{\text{time}}\) of neurons perfused in different extracellular sodium concentrations. It was found that the \(\text{Zn}^{2+}\) efflux rate was increased with increasing extracellular sodium concentration (Fig. 8A). To determine the need for an inwardly directed sodium gradient, the \(\text{Na}^+/\text{K}^+\) ATPase blocker ouabain was used to reduce the sodium gradients while maintaining extracellular sodium at physiological levels. The \(\text{Na}^+/\text{K}^+\) ATPase in rat brain has three inhibitory sites for ouabain, and two have high and one has low affinity for ouabain (Berrebi-Bertrand et al., 1990). Here two concentrations of ouabain were applied. The high concentration (100 \(\mu\text{M}\)) ensured that the \(\text{Na}^+/\text{K}^+\) ATPase was blocked completely. And the low concentration 10 \(\mu\text{M}\) was used to observe the inhibition without interference from possible nonspecific actions of ouabain on other transporters.

The results showed that sodium dependent \(\text{Zn}^{2+}\) efflux could be blocked by 10 \(\mu\text{M}\) ouabain (Fig. 8B, \(*** p < 0.001\)), and 100 \(\mu\text{M}\) ouabain actually resulted in an overall increase in fluorescence. Since the extracellular \(\text{Zn}^{2+}\) concentrations were near zero during EDTA perfusion, the increased fluorescence might be the result of \(\text{Ca}^{2+}\) influx mediated by reversal of the \(\text{Na}^+/\text{Ca}^{2+}\) exchanger (a common explanation for the actions of ouabain on cardiovascular contractility). When calcium was removed from the EDTA solution, adding 100 \(\mu\text{M}\) ouabain no longer caused the fluorescence to rise (Fig. 8C, \(*** p\)).
<0.001), suggesting that Ca\(^{2+}\) influx is, at least in part, responsible for the increased fluorescence.
Figure 8. Zn\textsuperscript{2+} efflux is activated by extracellular sodium in cortical neurons (pH8).

(A) Zn\textsuperscript{2+} efflux rate was calculated (ΔF/s) in different extracellular sodium concentrations (n= 15 neurons from 3 coverslips of 2 rats for each concentration). The reduced sodium was replaced by NMG to keep the same ionic strength and osmolarity. (B) Zn\textsuperscript{2+} efflux was blocked by co-incubation with different concentrations of ouabain (n=25 neurons from 3 coverslips of 2 rats for each treatment, p <0.001, two-way ANOVA). Solutions were made in low calcium (0.5mM) Locke’s buffer. (C) Effect of ouabain on Zn\textsuperscript{2+} efflux with calcium or without Ca\textsuperscript{2+} (n= 20 neurons from 4 coverslips of 2 rat for each treatment, p <0.001, two-way ANOVA). After removing Ca\textsuperscript{2+}, a net increase in fluorescence was not observed, suggesting that the increased fluorescence intensity observed after addition of ouabain might partially be caused by Ca\textsuperscript{2+} uptake. The error bar denotes the standard error.
Next, the sodium gradients were reversed by preincubating neurons with 100 µM ouabain. After 30 minutes of pretreatment with ouabain, intracellular sodium concentrations would presumably be increased, then neurons were exposed to 10 µM ZnCl₂ without extracellular sodium and Zn²⁺ uptake was measured. Under this condition, the sodium concentration gradients across the plasma membrane were reversed and the fluorescence measurement showed Zn²⁺ uptake was greatly increased compared with conditions where ouabain pretreatment did not occur (Fig.9, *** *p* <0.001). The increased Zn²⁺ uptake was likely the result of reversed Na⁺/Zn²⁺ exchange. On the other hand, the Zn²⁺ uptake observed under control conditions (pretreatment with Locke’s buffer) might be via mechanisms other than Na⁺/Zn²⁺ exchange since it appeared to be unaffected by changes in extracellular sodium concentration.
Figure 9. Zn$^{2+}$ uptake was activated by increased intracellular Na$^+$ in the absence of extracellular Na$^+$ (pH8).

Cortical neurons preloaded with FluoZin3 were treated with (▲) or without (■) 100 µM ouabain, then exposed to 10 µM ZnCl$_2$ without sodium (no ouabain). Pretreatment with ouabain caused greater Zn$^{2+}$ uptake presumably because the outwardly directed sodium gradients were increased (control: n=25 neurons from 5 coverslips of 2 rats, ouabain: n=35 neurons from 7 coverslips of 2 rats $p<0.001$, two-way ANOVA, The error bar denotes the standard error). Solutions were made in low calcium (0.5mM) Locke’s buffer.
C. Zn\textsuperscript{2+} efflux was increased by increasing extracellular calcium, but this effect was dependent on extracellular pH.

The calcium gradient is another means that could be used to drive net Zn\textsuperscript{2+} removal, although extracellular calcium is much smaller than sodium. In addition, the mechanism of the proposed Na\textsuperscript{+}/Zn\textsuperscript{2+} exchanger is very similar to that of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, both using the sodium gradients to drive cation efflux. To address if this proposed Na\textsuperscript{+}/Zn\textsuperscript{2+} exchanger is the same protein as a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, effects of different concentrations of extracellular calcium on Zn\textsuperscript{2+} efflux were investigated. Previous studies indicated that the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was most efficient at extracellular pH 7.6 and would be inhibited by extracellular alkalization under pathophysiological conditions (Egger & Niggli, 2000). Effects of calcium on Zn\textsuperscript{2+} efflux were investigated at pH 7.6 (Fig.10A) and pH 8 (Fig.10B). If the observed sodium gradient dependent Zn\textsuperscript{2+} efflux was caused by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, increasing extracellular calcium should inhibit Zn\textsuperscript{2+} efflux. The results showed that Zn\textsuperscript{2+} efflux in the presence of sodium at pH 7.6 was decreased by removing extracellular calcium (Fig.10A, *** \(p < 0.001\)), whereas at pH 8 removing extracellular calcium had no effect on Zn\textsuperscript{2+} efflux (Fig.10B). These data suggest that Zn\textsuperscript{2+} efflux was not mediated by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (because increasing extracellular calcium only increased Zn\textsuperscript{2+} efflux) and that there might be a Ca\textsuperscript{2+}/Zn\textsuperscript{2+} exchange mechanism in neurons. In addition, the effects of extracellular calcium were dependent on extracellular pH, disappearing at pH 8.
Figure 10. Effects of decreasing extracellular calcium on Zn$^{2+}$ efflux at either pH 7.6 or 8.

(A) Zn$^{2+}$ efflux with different extracellular calcium concentrations at pH 7.6 (0: ■, 0.5 mM: ▼, 2.5 mM: ▲) was recorded. Removing external calcium greatly decreased the rate of Zn$^{2+}$ efflux (n=20 neurons from 4 coverslips of 2 rats for each concentration, $p<0.001$, two-way ANOVA). (B) Zn$^{2+}$ efflux with different extracellular calcium concentrations at pH 8 (0: ■, 0.5 mM: ▼, 2.5 mM: ▲). No significant differences were observed comparing Zn$^{2+}$ efflux with different extracellular calcium concentrations at pH 8 (0 Ca; n=9 coverslips/45 neurons from 3 rats; 0.5 mM Ca/2.5 mM Ca: n=20 neurons from 4 coverslips of 2 rats). Solutions were made in Locke’s buffer containing sodium. The error bar denotes the standard error.
D. The effect of extracellular sodium on Zn\textsuperscript{2+} efflux was independent of extracellular pH.

Since the actions of extracellular calcium were shown to depend on extracellular pH, we next studied if extracellular pH had any effect on sodium dependent Zn\textsuperscript{2+} efflux. At either pH 7.6 or 8, replacing extracellular sodium with NMG reduced Zn\textsuperscript{2+} efflux to the same extent. In addition, the rate of Zn\textsuperscript{2+} efflux in the presence of sodium was nearly identical at either pH 7.6 or pH 8 (Fig. 11). These results suggest that the sodium dependence of Zn\textsuperscript{2+} efflux was not affected by extracellular pH, whereas, the calcium dependence was. The effects of extracellular pH on Zn\textsuperscript{2+} efflux were investigated and no significant difference was found (Fig. 12).
Figure 11. Effects of extracellular pH on the sodium dependence of Zn$^{2+}$ efflux.

Zn$^{2+}$ efflux with or without extracellular sodium at pH 7.6 (■▲) or pH 8 (○△). At both pH 7.6 (n=30 neurons from 6 coverslips of 2 rats, $p<0.001$, two-way ANOVA) and pH 8 (n=15 neurons from 3 coverslips of 1 rat, $p<0.001$, two-way ANOVA), adding extracellular sodium induced Zn$^{2+}$ efflux independent of pH. Solutions were made in low calcium (0.5 mM) Locke’s buffer. The error bar denotes the standard error.
Figure 12. Effect of extracellular pH on Zn$^{2+}$ efflux.

Zn$^{2+}$ efflux were measured at pH7.2 (■), pH8 (▲) and pH9 (▼) and no differences were detected among them (For each condition: n=15 cells from 3 coverslips of 2 rats, $p = 0.8270$, two-way ANOVA). The error bar denotes the standard error.

E. Evidence that the actions of extracellular sodium and calcium on Zn$^{2+}$ efflux were codependent.

Since Zn$^{2+}$ efflux could be increased by increasing either extracellular sodium or calcium, a possible interaction between sodium and calcium was investigated. First, using buffers nominally free of calcium the sodium dependence of Zn$^{2+}$ efflux was determined.
Results showed that when extracellular calcium was absent at pH 7.6, Zn\(^{2+}\) efflux was reduced and what efflux remained was largely independent of extracellular Na\(^+\) concentration (Fig. 13A), suggesting that the Na\(^+\)/Zn\(^{2+}\) exchanger requires extracellular calcium to function.

Next, extracellular sodium was replaced by NMG and the calcium dependence of Zn\(^{2+}\) efflux (at pH 7.6) was determined. Results showed that increasing extracellular calcium could induce more Zn\(^{2+}\) efflux in the absence of extracellular Na\(^+\) (Fig. 13B), but the level of calcium-dependent Zn\(^{2+}\) efflux was much smaller than when extracellular Na\(^+\) was present (compare Fig. 13B vs. Fig. 10A). These data indicate that the rate of Zn\(^{2+}\) efflux was codependent on extracellular sodium and calcium, suggesting the same exchanger protein could mediate both effects.
Figure 13. Codependence of sodium and calcium for Zn$^{2+}$ efflux in cortical neurons.

(A) Sodium dependent Zn$^{2+}$ efflux without extracellular calcium at pH 7.6. When external calcium was removed, the Zn$^{2+}$ efflux with sodium (▲) was only slightly induced compared to that without sodium (■) (0 Na: n=25 neurons from 5 coverslips of 2 rats; Na: n=20 neurons from 4 coverslips of 2 rats, $p<0.05$, two-way ANOVA). (B) Calcium dependent Zn$^{2+}$ efflux without extracellular sodium at pH 7.6. The effect of increasing extracellular calcium (0: ■, 0.5 mM: ▼, 2.5 mM: ▲) on Zn$^{2+}$ efflux was blunted in the absence of sodium (0 Ca: n=25 neurons from 5 coverslips of 2 rats, 0.5 mM Ca: n=30 neurons from 6 coverslips of 2 rats; 2.5 mM Ca: n=15 neurons from 3 coverslips of 1 rat, $p<0.05$, two-way ANOVA). The error bar denotes the standard error.

To investigate the correlation of extracellular sodium and calcium in case of Zn$^{2+}$ efflux, effects of extracellular sodium and calcium on Zn$^{2+}$ uptake were studied. Neurons preloaded with FluoZin3 were incubated with 10 µM ZnCl$_2$ with different concentrations of calcium for 30 minutes. When extracellular sodium was present, increasing extracellular calcium reduced Zn$^{2+}$ uptake (Fig.14A, $p<0.001$). This phenomenon agrees
with the Na\(^+\), Ca\(^{2+}\)/Zn\(^{2+}\) exchanger mechanism. When extracellular sodium is present, the Na\(^+\), Ca\(^{2+}\)/Zn\(^{2+}\) exchanger triggers Zn\(^{2+}\) efflux and the more extracellular calcium, the more Zn\(^{2+}\) extruding out of cells and less Zn\(^{2+}\) retained inside neurons. While extracellular sodium was replaced by NMG, increasing extracellular calcium had no effects on Zn\(^{2+}\) uptake (Fig.14B). These results provided further evidence that Zn\(^{2+}\) efflux was most efficient when both extracellular sodium and calcium were present. In the control, neurons were incubated with Locke’s buffer without extracellular sodium for 30 minutes. Whether extracellular calcium was present or not, the fluorescences changes under these two conditions were both small (Fig.14B), suggesting that the calcium influx (if any) when extracellular sodium was absent was not big enough to be detected by FluoZin3. These data ruled out the possibility that the higher Zn\(^{2+}\) uptake observed in higher extracellular calcium and zero extracellular sodium conditions was caused by greater Ca\(^{2+}\) influx because the Ca\(^{2+}\)/Na\(^+\) exchanger was reversed.
Figure 14. Calcium effects on Zn\(^{2+}\) uptake.

(A) When extracellular sodium was present, increasing extracellular calcium to 0.5 mM reduced Zn\(^{2+}\) uptake significantly (\(p<0.001\)). The Zn\(^{2+}\) uptake was decreased with higher extracellular calcium (■: 0Ca, ▲: 0.5 mM Ca, ▼: 2.5 mM Ca, ●: 10 mM Ca, in all four conditions: n=30 cells from 6 coverslips of 2 rats). (B) When extracellular sodium was absent, changing extracellular calcium did not affect Zn\(^{2+}\) uptake (0 Ca: n=30 cells from 3 coverslips of 1 rat; 2.5 mM Ca: n=20 cells from 2 coverslips of 1 rat; \(p=0.6451\), two-way ANOVA). In the control, the fluorescence changes in zero sodium Locke’s buffer without (▼) or with (●) extracellular Ca\(^{2+}\) were both small (in both control conditions: n=20 cells from 2 coverslips of 1 rat). The error bar denotes the standard error.
F. Zn$^{2+}$ efflux was not affected by extracellular magnesium concentration.

Since Zn$^{2+}$ efflux could be modulated by both calcium and sodium, magnesium was investigated to see if it could affect Zn$^{2+}$ efflux. Results showed that at either pH 7.6 or 8, removing external magnesium did not affect Zn$^{2+}$ efflux significantly (Fig.15A&B).

**Figure 15. Effects of extracellular magnesium on Zn$^{2+}$ efflux.**

(A) At pH 7.6, Zn$^{2+}$ efflux with (▲) or without (■) extracellular magnesium. (B) At pH8, Zn$^{2+}$ efflux with (▲) or without (■) extracellular magnesium. In either case, removing extracellular magnesium did not affect Zn$^{2+}$ efflux (n=30 neurons from 3 coverslips of 1 rat for each condition, $p = 0.1427$ at pH 7.6; $p = 0.1265$ at pH 8, two-way ANOVA). Solutions were made in low calcium (0.5 mM) Locke’s buffer. The error bar denotes the standard error.
III. Contribution of ZnT1 to Zn\textsuperscript{2+} efflux

A. Verification of reduction in ZnT1 expression in cultured neurons

To study the role of ZnT1 in Zn\textsuperscript{2+} efflux, an RNAi strategy was used to reduce ZnT1 protein expression. Since the transfection ratio of primary cortical neurons is very low, vector-based RNA interference (RNAi) was conducted so that individual transfected neurons could be identified. Neurons expressing DsRed were compared to neighboring non-transfected neurons in the same field to assess changes in ZnT1 protein expression (Fig.16A-D). ZnT1 protein levels were monitored in individual neurons by immunofluorescence. Nonsense plasmids which cannot produce specific RNA fragments were used as a negative control (Fig.16E-F). Compared to the mean of the non-transfected cells, RNAi knockdown neurons showed a 40% lower fluorescence intensity (Fig.16I, *** \( p < 0.001 \)). Nonsense plasmid transfected cells showed no difference in intensity when compared to nontransfected neurons.
**Figure 16 continued**

**Figure 16. Immunofluorescence of the ZnT1 protein in ZnT1 knockdown neurons.**

A) DAPI staining showing nuclei of healthy cells used for analysis. B) Image of DsRed expressing neuron, TRITC filter. C) FITC filter showing ZnT1 immunofluorescence. The arrow indicates ZnT1 knockdown neuron. D) Triple merged image of DAPI, TRITC and FITC images. The knockdown neuron shows a much lower fluorescence signal due to the reduction of ZnT1 levels. E-H) DAPI staining, DsRed expression, ZnT1 immunofluorescence and triple merged image of neurons transfected with nonsense plasmids. Size bar=10 µm. I) The ZnT1 fluorescence staining of transfected neurons was normalized to the average of non-transfected neurons within the same field. Neurons transfected by shRNAi exhibited a 40% lower fluorescence intensity compared to nonsense transfected neurons when analyzed with t-test (nonsense: n=8 coverslips/20 transfected cells; shRNAi: n=11 coverslips/40 transfected cells, p < 0.001, t-test). The error bar denotes the standard error.
To address the question whether expression of ZnT1 shRNAi had additional off-target effects, experiments were conducted to investigate the expression of another Zn\(^{2+}\) transporter ZnT6 (the protein sequences have 14.2% identity to ZnT1 protein sequences; no significant similarity between the target sequence and ZnT6 open reading frame). Immunofluorescence staining was conducted in cortical neurons transfected with nonsense plasmid and ZnT1 shRNAi plasmid using anti-ZnT6 antibody. The results showed that there was no difference between neurons expressing ZnT1 shRNAi and nonsense transfected neurons when individual cells were compared to the non-transfected cells in the same field (Fig.17).

**Figure 17. Immunofluorescence of the ZnT6 protein in ZnT1 knockdown neurons.**

Transfected cells were stained with anti-ZnT6 primary antibodies to ensure the specificity of ZnT1 knockdown. No significant difference was observed between nonsense transfected cells (n=5 transfected cells from 1 coverslip) and knockdown cells (n=5 transfected cells from 1 coverslip) (p=0.224, t-test). The error bar denotes the standard error.
B. Effect of ZnT1 knockdown on Zn\textsuperscript{2+} efflux

Zn\textsuperscript{2+} efflux was induced in neurons as described above (pH8, low calcium Locke’s buffer) with sodium. After the 30 minute EDTA exposure, the fluorescence of ZnT1 knockdown neurons was reduced to approximately 73% of the fluorescence at the start of the EDTA perfusion, while the fluorescence of nonsense plasmid transfected neurons was reduced to approximately 48% (Fig. 18 ** \( p < 0.01 \)). There were no significant differences in fluorescence traces among the nonsense transfected neurons and nontransfected neurons (data not shown).

![Figure 18. Zn\textsuperscript{2+} efflux mediated by ZnT1.](image)

Zn\textsuperscript{2+} efflux traces in neurons transfected (▲) with ZnT1 shRNAi plasmid and nonsense plasmid (■) (nonsense: n=9 coverslips/19 transfected cells from 3 rats; shRNAi: n=8 coverslips/20 transfected cells from 3 rats, \( p < 0.01 \), two-way ANOVA). The error bar denotes the standard error.
C. Is ZnT1 dependent Zn\(^{2+}\) efflux active in neurons when sodium dependent Zn\(^{2+}\) efflux is blocked?

Cultured neurons were perfused with 10 µM ZnCl\(_2\) for 30 minutes, and then switched to a 100 µM EDTA solution without sodium, which blocked the Na\(^+\)/Zn\(^{2+}\) exchanger. After the 30 minute EDTA exposure, the fluorescence intensity of ZnT1 shRNAi transfected neurons kept bright, while the fluorescence of nonsense plasmid transfected neurons was reduced to around 88% of the fluorescence of the start of the EDTA perfusion (Fig. 19 * \(p < 0.05\)). These results showed that ZnT1 dependent Zn\(^{2+}\) efflux was not relying on extracellular sodium.
**Figure 19. Zn\textsuperscript{2+} efflux mediated by ZnT1 in the absence of sodium.**

After neurons were loaded with 10µM ZnCl\textsubscript{2} for 30 minutes, perfusion was switched to a Na-free (NMG) buffer containing 100 µM EDTA. Zn\textsuperscript{2+} efflux traces in neurons transfected with ZnT1 shRNAi plasmid (▼) or nonsense plasmid (■) (n=13 coverslips with 25 neurons from 2 rats, \(p<0.05\), two-way ANOVA). The error bar denotes the standard error.

To further compare the function of ZnT1 in Zn\textsuperscript{2+} efflux with sodium and without sodium, the ZnT1 dependent Zn\textsuperscript{2+} efflux \([\Delta(F/F_0)]\) was plotted as a function of time with or without extracellular sodium (Fig.20A). The data show that the Zn\textsuperscript{2+} efflux slope decreased with time, which was different from the slopes of the sodium dependent Zn\textsuperscript{2+} efflux, which remained nearly constant with time (Fig.7B). The slope of ZnT1 dependent Zn\textsuperscript{2+} efflux in the first 10 minutes of EDTA perfusion was larger than the next 20 minutes.
(Fig.20B), suggesting that the activity of ZnT1 might rely on net Zn\textsuperscript{2+} concentration gradients.

![Figure 20. Comparison of Zn\textsuperscript{2+} efflux with or without sodium.](image)

(A) ZnT1 dependent Zn\textsuperscript{2+} efflux, transformed from Fig.11 and Fig.12
\[\Delta(F/F_0) = (F/F_0)_{RNAi} - (F/F_0)_{nonsense}\]. ZnT1 dependent Zn\textsuperscript{2+} efflux with sodium or without sodium overlaps. (B) The ZnT1 dependent Zn\textsuperscript{2+} efflux rate during different time periods (0-10min vs 10-30min). When sodium was present, the Zn\textsuperscript{2+} efflux rate was higher in the first 10 minutes than the next 20 minutes (n=8 from 3 rats, \(p<0.05\), t-test). When sodium was absent, the Zn\textsuperscript{2+} efflux rate was higher in the first 10 minutes than the next 20 minutes (n=13 from 2 rats, \(p<0.05\), t-test). The error bar denotes the standard error.

**Part two: Zn\textsuperscript{2+} uptake in cultured cortical neurons**

Previous studies have suggested that a Zn\textsuperscript{2+}, HCO\textsubscript{3}\textsuperscript{-} symporter mechanism might be used for Zn\textsuperscript{2+} uptake. Bicarbonate was shown to stimulate Zn\textsuperscript{2+} uptake into human erythrocytes (Torrubia & Garay, 1989; Kalfakakou & Simons, 1990) and human
fibroblasts (Vazquez & Grider, 1995). ZIP2, ZIP8 and ZIP14 were found to use HCO$_3^-$ gradients across the plasma membrane as the driving force for manganese, cadmium and Zn$^{2+}$ uptake (Gaither & Eide, 2000; He et al., 2006; Liu et al., 2008; Girijashanker et al., 2008). In our lab, a proton/Zn$^{2+}$ exchanger was suggested for Zn$^{2+}$ uptake (Colvin, 2002; Balaji & Colvin, 2005). The Cd$^{2+}$ uptake by ZIP8 and ZIP14 using Cd$^{2+}$, HCO$_3^-$ symporter mechanism was maximal at pH 7.5 (He et al., 2006; Girijashanker et al., 2008). My first hypothesis proposed in this part is: Zn$^{2+}$ uptake in cortical neurons is maximal around pH7.5.

Lots of Zn$^{2+}$ influx occurs in the postsynaptic cell after ischemia followed by neuronal death. Depolarization was shown to induce $^{65}$Zn$^{2+}$ influx into cortical neurons. My second hypothesis is: Depolarization causes Zn$^{2+}$ uptake into neurons, which can be measured by Fluozin3.

Zn$^{2+}$ uptake was observed by hZIP1 when this protein was overexpressed in K562 cells (Gaither & Eide, 2001). However, the mechanism of this protein is different from hZIP2, ZIP8 and ZIP14. The function of this protein cannot be stimulated by bicarbonate. It is unknown if it is involved in Zn$^{2+}$ uptake in neurons. My third hypothesis in this part is: ZIP1 mediates Zn$^{2+}$ uptake in neurons.

I. Effects of extracellular pH on Zn$^{2+}$ uptake

To find a proper pH range to study the pH effect, control experiments were conducted. FluoZin3 loaded cortical neurons were perfused with Locke’s buffer from pH6 to pH8 for 30 minutes. At pH6, the Zn$^{2+}$ fluorescence increased with time; while
fluorescence kept constant at pH7 and pH8 (Fig.21A). To eliminate the minimal Zn\(^{2+}\) in solutions, FluoZin3 loaded cortical neurons were perfused with 100 µM EDTA buffer from pH6 to pH9 for 30 minutes. From pH7-8, Zn\(^{2+}\) fluorescence decreased slightly with time; while the Zn\(^{2+}\) fluorescence increased at pH6 (Fig.21B). These data suggested that acid conditions like pH6 might induce Zn\(^{2+}\) fluorescence to rise, perhaps due to intracellular Zn\(^{2+}\) release. For this reason, pH6.8 to pH9 was selected. Neurons preloaded with FluoZin3 were incubated with 10 µM ZnCl\(_2\) for 30 minutes, Zn\(^{2+}\) uptake was increased with time (Fig.21C). The Zn\(^{2+}\) uptake rate was highest around pH7.4 to pH8.2, and both lower and higher extracellular pH caused the Zn\(^{2+}\) uptake to reduce (Fig.21D).
Figure 21. pH effect on Zn$^{2+}$ uptake.

Neurons loaded with FluoZin3 were incubated in different pH Low Ca$^{2+}$ Locke’s buffer (■: pH 6 n= 20 neurons from 4 coverslips of 2 rats; ▲: pH 7 n=10 neurons from 2 coverslips; ▼: pH 8 n=10 neurons from 2 coverslips;) for 30 minutes. Acid buffer induced the Zn$^{2+}$ fluorescence to increase. (B) Neurons loaded with FluoZin3 were incubated in different pH EDTA buffer (■: pH 6; ▲: pH 7; ▼: pH 8; ●: pH 9) for 30 minutes (in all four conditions, n= 10 neurons from 1 coverslips;). Acid buffer induced the Zn$^{2+}$ fluorescence to increase. (C) Neurons loaded with FluoZin3 were incubated with different pH 10 µM ZnCl$_2$ (■: pH 6.8, n= 25 cells from 5 coverslips of 2 rats; ▲: pH 7, n= 15 cells from 3 coverslips of 2 rats; ▼: pH 7.4, n= 35 cells from 7 coverslips of 2 rats; ●: pH 8.2, n= 25 cells from 5 coverslips of 2 rats; △: pH 9, n= 25 cells from 5 coverslips of 2 rats). (D) The Zn$^{2+}$ uptake rate changed with pH ($\Delta F=F_{30\text{min}}-F_0$). The error bar denotes the standard error.
II. Zn\(^{2+}\) uptake in neurons when depolarized

Under normal physiological conditions, intracellular free Zn\(^{2+}\) levels are maintained at low levels by the Na\(^+\), Ca\(^{2+}\)/Zn\(^{2+}\) exchanger as showed by previous results in this dissertation. Under pathological conditions such as ischemia, lots of Zn\(^{2+}\) enters neurons, followed by neuronal death. Membrane depolarization has been shown to induce a rise in intracellular Zn\(^{2+}\) by TSQ fluorescence (Weiss et al., 1993) and \(^{65}\)Zn\(^{2+}\) assay (Sheline et al., 2002). Here, I investigated if depolarization could induce an increase in intracellular free Zn\(^{2+}\) that could be detected by changes in FluoZin3 fluorescence in cortical neurons. High extracellular potassium (60 mM KCl) was used to depolarize neurons. After long incubation with high KCl solutions, the neurons would begin to die so that toxic Zn\(^{2+}\) would be released from subcellular compartments (personal communication with Dr. Yang V Li). For this reason, intracellular zinc changes only in the first five minutes were compared here. In the control, neurons loaded with FluoZin3 were incubated with Locke’s buffer where 60 mM KCl replaced an equal amount of NaCl. The fluorescence traces with or without extracellular calcium showed no differences (Fig.22C), suggesting that the induced Ca\(^{2+}\) influx by depolarization was not big enough to be detected by FluoZin3. In these control conditions, the intracellular fluorescences increased with time (Fig.22C), indicating intracellular Zn\(^{2+}\) release or uptake of adventitious Zn\(^{2+}\) in Locke’s buffer. Low concentration of extracellular ZnCl\(_2\) (10 µM) resulted in fluorescence changes in the first five minutes that were almost the same as in the absence of added Zn\(^{2+}\), which suggests no net Zn\(^{2+}\) uptake was induced by
depolarization when extracellular Zn\(^{2+}\) is low. The increased fluorescence with long time incubation might be caused by intracellular released as a result of neuroanl injury. In contrast, when extracellular ZnCl\(_2\) concentrations was high (100 µM), depolariziton induced measureable Zn\(^{2+}\) uptake immediately within 5 minutes (Fig.22B). After 5 minutes, the Zn\(^{2+}\) uptake rates were the same between resting and depolarized neurons. At almost 30 minutes, the fluorescence ratio reached the same level, indicating that the FluoZin3 dye might be saturated. These results showed that large Zn\(^{2+}\) influx occured quickly (the first 5 minutes) when neurons were depolarized, possibly by the voltage-dependent calcium channels.
Figure 22. Zn\textsuperscript{2+} uptake at depolarization.

(A) Neurons loaded with FluoZin3 were incubated in 10 µM ZnCl\textsubscript{2} with (▲, n=20 neurons from 4 coverslips) or without (■) 60 mM K\textsuperscript{+} (n=48 neurons from 5 coverslips from 1 rat, p=0.4419, two-way ANOVA for first 10 minutes). (B) Neurons loaded with FluoZin3 were incubated in 100 µM ZnCl\textsubscript{2} with (▲) or without (■) 60 mM K\textsuperscript{+} (n=20 neurons from 4 coverslips from 1 rat, p<0.001, two-way ANOVA for first 10 minutes). Depolarization induced high amount of Zn\textsuperscript{2+} uptake at short time. (C) Neurons loaded with FluoZin3 were incubated with high K\textsuperscript{+} Locke’s buffer with (▲) or without (■) extracellular calcium for 30 minutes (n=20 from 1 rat). In control, neurons preloaded with FluoZin3 were incubated with 0.5 mM Locke’s buffer (n=30 from 1 rat) (▼). The error bar denotes the standard error.
III. Contribution of ZIP1 to Zn$^{2+}$ uptake

A. Verification of reduction in ZIP1 expression in cultured neurons

To study the role of ZIP1 in Zn$^{2+}$ uptake, the same method was used as to study ZnT1 by shRNAi. Neurons expressing DsRed were compared to neighboring non-transfected neurons in the same field to assess changes in ZIP1 protein expression (Fig. 23A-D). ZnT1 protein levels were monitored in individual neurons by immunofluorescence. Nonsense plasmids which cannot produce specific RNA fragments were used as a negative control (Fig. 23E-F). Compared to the mean of the non-transfected cells, RNAi knockdown neurons showed a 22% lower fluorescence intensity (Fig. 23I, \( p < 0.001 \)). Nonsense plasmid transfected cells showed no difference in intensity when compared to nontransfected neurons.
Figure 23 continued

**Figure 23. Immunofluorescence of the ZIP1 protein in ZIP1 knockdown neurons.**

A) DAPI staining showing nuclei of healthy cells used for analysis. B) Image of DsRed expressing neuron, TRITC filter. C) FITC filter showing ZnT1 immunofluorescence. The arrow indicates ZIP1 knockdown neuron. D) Triple merged image of DAPI, TRITC and FITC images. The knockdown neuron shows a lower fluorescence signal due to the reduction of ZIP1 levels. E-H) DAPI staining, DsRed expression, ZIP1 immunofluorescence and triple merged image of neurons transfected with nonsense plasmids. Size bar=10 µm. I) The ZIP1 fluorescence staining of transfected neurons was normalized to the average of non-transfected neurons within the same field. Neurons transfected by shRNAi exhibited a 22% lower fluorescence intensity compared to nonsense transfected neurons when analyzed with t-test (nonsense: n=6 coverslips/32 transfected cells from 2 rats; shRNAi: n=4 coverslips/20 transfected cells from 2 rats, $p < 0.001$, t-test). The error bar denotes the standard error.

**B. Zn$^{2+}$ uptake in ZIP1 knockdown neurons**

Zn$^{2+}$ uptake was induced in neurons (pH7.4, low calcium Locke’s buffer) with sodium. Due to the variation in different coverslips with different FluoZin3 incubation,
the fluorescence changes were different for nontrasfected neurons from different coverslips (Fig. 24A&C). For this reason, the percentile inhibition was calculated by dividing the fluorescence changes (Ft/F0) of transfected neurons to that of nontransfected neurons (Fig. 24B). After the 30 minutes of 10 µM ZnCl2 exposure, Zn^{2+} uptake in nonsense transfected neurons was reduced 10% than nontransfected neurons; while for ZIP1 shRNAi transfected neurons, Zn^{2+} uptake was reduced 34% than nontransfected neurons (Fig. 24B, p<0.05). For nonsense transfected coverslips, there is no difference between the fluorescence ratio at 30 minutes of transfected neurons and nontransfected neurons; while shRNAi trasfected neurons showed greatly lower fluorescences than nontransfected neurons from the same coverslips (Fig. 24C, p < 0.01).
**Figure 24. Zn$^{2+}$ uptake mediated by ZIP1.**

Neurons were incubated with FluoZin3. (A) The Zn$^{2+}$ uptake traces in neurons transfected (▲) with ZIP1 shRNAi plasmid and nonsense plasmid (■) (nonsense: n=8 coverslips/17 transfected cells; shRNAi: n=8 coverslips/23 transfected cells). (B) Percentile inhibition of Zn$^{2+}$ uptake in nonsense transfected neurons (■) and ZIP1 shRNAi transfected neurons (▲) after 30 minutes of ZnCl$_2$ incubation (n= 8, p <0.01, t test). (C) Comparison of Zn$^{2+}$ fluorescence ratio after 30 minutes of Zn$^{2+}$ incubation between transfected and nontransfected plasmid. There is no significant different difference between nonsense transfected and nontransfected neurons on the same coverslips (nontransfected: n=8 coverslips/115 cells; nonsense: n=8 coverslips/17 transfected cells, p = 0.512, t-test). For shRNAi transfected neurons, the fluorescence ratio was greatly lower than the nontransfected cells (nontransfected: n=8 coverslips/ 115 cells; shRNAi: n=8 coverslips/ 23 transfected cells, p < 0.01, t-test). The error bar denotes the standard error (from 2 rats).
Part three: Regulation of the expression of Zn\(^{2+}\) transporter proteins by Zn\(^{2+}\) levels

ZnT1 was found to be regulated by Zn\(^{2+}\) at transcriptional levels. When free intracellular Zn\(^{2+}\) levels are elevated, Zn\(^{2+}\) binds to metal response element-binding transcription factor MTF-1, which then promotes the transcription of ZnT1 (Langmade, 2000). It was reported that ZnT1 mRNAs were markedly greater when a supplemental Zn\(^{2+}\) intake was provided (Liuzzi et al., 2001). Since the ZnT1 mRNA was affected by Zn\(^{2+}\) levels, I proposed my first hypothesis in this part: ZnT1 protein levels would be regulated by intracellular Zn\(^{2+}\) levels.

ZIP1 mRNA amount was insensitive to changes in zinc levels in THP-1 cells (Cousins et al., 2003) and the intestine and visceral endoderm (Dufner-Beattie et al., 2003). It was suggested that ZIP1 can be regulated at post transcriptional levels by trafficking protein to and from the plasma membrane. It is thought that ZIP1 proteins are largely sequestered in intracellular organelles when cells are Zn\(^{2+}\) replete and then ZIP1 proteins rapidly are transported to the plasma membrane when Zn\(^{2+}\) is deficient (Wang et al., 2004a; Huang and Kirschke, 2007). No studies indicate that ZIP1 protein expression can be regulated at the translational level. I proposed my second hypothesis in this part: ZIP1 mRNA translation is regulated by intracellular Zn\(^{2+}\) levels.
I. **Effects of Zn\(^{2+}\) on the protein levels of Zn\(^{2+}\) transporters ZnT1 and ZIP.**

Too much or too little Zn\(^{2+}\) were both toxic to neurons. Neurons were treated with different concentrations of TPEN overnight (24 hours) and the cell viability was assayed by alamar blue. Neurons were still healthy when treated with TPEN up to 5 µM (Fig. 25A). Neurons were incubated with different concentrations of ZnCl\(_2\) overnight. When ZnCl\(_2\) in the medium was higher than 150 µM, the cell viability decreased with increasing amount of ZnCl\(_2\) from 150-300 µM (Fig. 25B). Further experiments showed that long term (5-11 days) incubation of 1 µM TPEN and 100 µM ZnCl\(_2\) in neural basal media would not affect neuronal survival (used for ZnT1 and ZIP1 staining).
**Figure 25. Effects of Zn$^{2+}$ levels on neuronal survival.**

(A) Changes of neuronal survival with different concentrations of TPEN. Neurons were healthy in TPEN less than 5 µM for 24 hours. (B) Changes of neuronal survival with different concentrations of ZnCl$_2$. Neurons were healthy in ZnCl$_2$ when less than 150 µM for 24 hours. (n= 4 wells for each concentration of TPEN or ZnCl$_2$). The error bar denotes the standard error.
Cortical neurons were extracted from fetal rats and placed in culture plates, the medium was changed to fresh sterile neural basal with or without 1 µM TPEN. Half medium in each well was changed with fresh medium every three days to ensure that degraded TPEN was renewed. After 11 days of incubation, the ZIP1 and ZnT1 levels were measured by immunofluorescence staining. Experiments were repeated in two rats. Due to the variation in different sets of staining, the protein fluorescence was normalized to control cells (regular Neuralbasal medium) that were stained at the same time. Results showed that TPEN treatment caused overall ZIP1 levels to slightly increase ($p < 0.05$); while overall ZnT1 levels decreased with TPEN incubation ($p < 0.01$) (Fig.26). The DAPI and ZIP1 staining indicated that the neurons were healthy after 11 days of treatment with 1 µM TPEN (Fig.27).
**Figure 26. Effects of TPEN on Zn$^{2+}$ transporter expression.**

After 11 days treatment of neurons with TPEN, ZIP1 levels were increased (control: n = 85 neurons from 3 coverslips of 2 rats; TPEN: n = 55 neurons from 2 coverslips of 2 rats, $p < 0.05$, t-test); while ZnT1 levels were reduced (for both control and TPEN treatment: n = 55 neurons from 2 coverslips of two rats, $p < 0.01$, t-test). The error bar denotes the standard error.
Figure 27. Effects of long term treatment of TPEN on neuronal survival

(A) The DAPI staining showed mostly healthy neurons after 11 days treatment with TPEN. (B) ZIP1 staining showed normal cell morphology after 11 days treatment with TPEN. Experiments were repeated with 3 coverslips from 2 rats.
II. Evidence for the regulation of hZIP1 mRNA translation by intracellular Zn$^{2+}$.

A. Cell surface expression of hZIP1

An immunofluorescence assay was used to confirm hZIP1 protein expression and indicate the localization of expressed hZIP1 in *Xenopus* oocytes. Oocytes were injected with hZIP1-HA cRNA as described in methods. After six days oocytes were processed for immunofluorescence assay as described in methods. Oocytes exhibited a bright immunofluorescent signal at the plasma membrane (Fig.28A). There was also scattered weak immunofluorescence in the cytoplasm. Control oocytes which were injected with an equal amount of water showed no fluorescence (Fig.28B).

![Figure 28. Immunostaining of hZIP1 on oocytes by anti-HA antibody.](image)
A) Oocyte injected with hZIP1-HA cRNA. B) Oocyte injected with water.
B. Functional studies of hZIP1 expressed on oocytes

$^{65}$Zn$^{2+}$ uptake in *Xenopus* oocytes. Oocytes injected with hZIP1 cRNA (15ng or 30ng) or water 7 days later were incubated in ND96 buffer containing $^{65}$Zn$^{2+}$. $^{65}$Zn$^{2+}$ uptake was determined in the presence 30 µM ZnCl$_2$ for 1.5 hours. $^{65}$Zn$^{2+}$ uptake of oocytes injected with hZIP1 RNA was significantly greater than that of control oocytes (Fig.29A, **p <0.01), suggesting the hZIP1 expressed in oocytes functions as a Zn$^{2+}$ transporter. Since there were different absolute amounts of $^{65}$Zn$^{2+}$ uptake in different batches of oocytes used in different experiments, the ratio of $^{65}$Zn$^{2+}$ uptake in hZIP1 overexpressed oocytes to that in water injected oocytes (same batch/ same experiment) was used to compare the $^{65}$Zn$^{2+}$ uptake levels at different times after cRNA injection. Results showed that $^{65}$Zn$^{2+}$ uptake increased with time after cRNA injection and reached a peak after five days (Fig.29B).
Figure 29. $^{65}$Zn$^{2+}$ uptake of oocytes overexpressing hZIP1.

(A) $^{65}$Zn$^{2+}$ uptake of oocytes that had been injected with cRNA 7 days previously (water: n = 10, 15ng RNA: n = 5, 30 ng RNA: n = 5, ** $p < 0.01$ compared to water, t-test. The error bar denotes the standard error.) (B) $^{65}$Zn$^{2+}$ uptake ratio of oocytes that had been injected with cRNA for different times. $^{65}$Zn$^{2+}$ uptake was increased with time after hZIP1 RNA injection. The same letter indicated that no significant difference between the two groups. n = 5 oocytes for each time point, one-way ANOVA with Tukey's multiple comparison test. The error bar denotes the standard error.)

C. Acute changes in intracellular Zn$^{2+}$ appear to be without effect on hZIP1 trafficking

Oocytes were injected with hZIP1 cRNA and 6 days later were transferred to regular medium, medium containing 10μM TPEN or 10μM ZnCl$_2$ for 8 hours. After 6 days, hZIP1 protein was expressed; treatments of zero Zn$^{2+}$ or high concentration of Zn$^{2+}$ did not affect hZIP1 trafficking. Immunofluorescence of hZIP1 indicates that hZIP1 proteins were still primarily localized on the plasma membrane (Fig.30B&C). In another experiment set, the different Zn$^{2+}$ treatments were conducted for 24 hours (at 6 days after
of hZIP1 cRNA injection). There were still no observable changes in hZIP1 immunofluorescence localization (data not shown).

Figure 30. Effect of acute changes in Zn\(^{2+}\) (8 hours) on expression of hZIP1-HA in oocytes.

Oocytes injected with hZIP1 cRNA were grown in regular medium. After 6 days, medium was changed to regular medium containing DMSO (A), 10 μM TPEN (B) or 10 μM ZnCl\(_2\) (C). Different Zn\(^{2+}\) treatments did not affect the pattern of hZIP1 immunofluorescence. The representative picture was taken from three oocytes for each condition.
**D. Effect of long term changes in intracellular Zn\(^{2+}\) on hZIP1cRNA translation**

Immediately after oocytes were injected with hZIP1 cRNA, oocytes were transferred to regular medium, medium containing 10 μM TPEN or 10 μM ZnCl\(_2\) for 6 days (Fig.31A-C). Treatments with TPEN (zero Zn\(^{2+}\)) increased expression of hZIP1 (Fig.31B). Oocytes incubated with high concentration of Zn\(^{2+}\) (10 μM ZnCl\(_2\)) exhibited little or no fluorescence (Fig.31C), indicating hZIP1 proteins were not expressed. Oocytes injected with Na/K ATPase-HA cRNA were used as negative control (Fig.31D-F). It can be seen that treatment with either zero Zn\(^{2+}\) or high levels of Zn\(^{2+}\) had no effect on the expression of the ATPase (Fig.31E&F). The fluorescence of expressed proteins was measured. TPEN treatment for six days caused the expression of hZIP1 proteins to enhance higher significantly than zinc treatment (Fig.32A). Under the same incubation condition, no differences were obtained for ATPase expression between different incubation (Fig.32B)
Figure 31. Effects of Zn$^{2+}$ deficiency or high levels of Zn$^{2+}$ (6 days) on expression of hZIP1-HA in oocytes.

Oocytes injected with hZIP1 cRNA were grown in regular medium containing DMSO (A), 10 µM TPEN (B) or 10 µM ZnCl$_2$ (C) for 6 days. Treatments with TPEN (zero Zn$^{2+}$) increased expression of hZIP1 (B) and 10 µM ZnCl$_2$ brings out little or no ZIP1 protein expression (C). Representative pictures were taken from 3-9 oocytes of 2 frogs. Oocytes injected with Na/K ATPase-HA cRNA were used as a negative control and incubated in DMSO (D), 10 µM TPEN (E) or 10 µM ZnCl$_2$ (F) respectively for 6 days. Representative pictures were taken from 6-7 oocytes of 1 frog.
Figure 32. Effects of Zn$^{2+}$ deficiency or high levels of Zn$^{2+}$ (6 days) on expression of hZIP1-HA protein in oocytes.

(A) The fluorescence of hZIP1 protein expressed in oocytes incubated with OR3 medium, 10 µM TPEN or 10 µM ZnCl$_2$ for 6 days. TPEN treatment increased higher expression of hZIP1, while ZnCl$_2$ treatment reduced hZIP1 expression, although the differences are not significant. There were significant differences in hZIP1 protein levels between TPEN and ZnCl$_2$ treatment as evidenced by differences in fluorescence intensity measured at the membrane surface. (OR3: n=3, TPEN/ZnCl$_2$: n =9 from two frogs, ***p<0.001, one way ANOVA with Tukey's multiple comparison test). (B) The fluorescence of ATPase protein expressed in oocytes incubated with 10 µM TPEN or 10 µM ZnCl$_2$ for 6 days. No significant differences were observed between the different treatments. (OR3: n=2, TPEN: n =7, ZnCl$_2$: n =6). The error bar denotes the standard error.
E. Effect of various treatments on total metal contents of oocytes

Next, total metal levels of oocytes were studied by ICP-OES. After oocytes were extracted and recovered overnight, they were injected with water and transferred to regular medium, medium containing 10 μM TPEN or 10 μM ZnCl₂ respectively for 6 days. TPEN treatment caused the total cellular zinc to decrease slightly, whereas ZnCl₂ treatment caused the total cellular zinc to rise slightly (Fig.33). The different trace elements were also measured under different treatment and no significant effects were obtained (Fig.34). The intracellular trace elements levels in oocytes treated with regular medium (OR3 + DMSO) are listed (Table 6). In one oocyte, the amount of magnesium was largest (17.88±0.63 17 nmol/oocyte), and cadmium amounts were smallest (0.0045±0.0080 nmol/oocyte).
Table 6.
Amounts of Trace Elements in Oocytes Injected with Water for 6 Days.

<table>
<thead>
<tr>
<th>Trace element</th>
<th>Al</th>
<th>Ca</th>
<th>Cd</th>
<th>Cu</th>
<th>Fe</th>
<th>Mg</th>
<th>Mn</th>
<th>Zn</th>
</tr>
</thead>
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<tr>
<td>Total metal content (nmol/oocyte) Mean ± SE</td>
<td>0.35±0.25 7.58±2.77 0.0045±0.0080 0.26±0.058 1.116±0.88 17.88±0.63 0.062±0.0058 0.72±0.061</td>
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Figure 33. Effects of Zn$^{2+}$ deficiency or high levels of Zn$^{2+}$ (6 days) treatment on total zinc content in oocytes.

Oocytes injected with water were grown in regular medium containing DMSO (n = 5), 10 µM TPEN (n = 8) or 10 µM ZnCl$_2$ (n = 4) for 6 days. Treatments with TPEN (zero Zn$^{2+}$) slightly reduced intracellular zinc, whereas incubation with 10 µM ZnCl$_2$ slightly increase zinc levels in oocytes. The error bar denotes the standard error.
Figure 34. Effects of Zn$^{2+}$ deficiency or high levels of Zn$^{2+}$ (6 days) treatment on total trace element contents in oocytes.

Oocytes injected with water were grown in regular medium containing DMSO (n = 5), 10 µM TPEN (n = 8) or 10 µM ZnCl$_2$ (n = 4) for 6 days. The error bar denotes the standard error.
III. Effects of intracellular Zn$^{2+}$ on the protein levels of Zn$^{2+}$ transporters ZnT1 and ZIP in K562 cells.

K562 erythroleukemia cells have been used to study Zn$^{2+}$ transporters a lot. It was found that hZIP1 mediates Zn$^{2+}$ uptake when it is overexpressed in K562 cells (Gaither&Eide, 2001). In addition, the characteristics of Zn$^{2+}$ transport in wild type cells was found to be the same as overexpressing K562 cells, suggesting that it is the major endogenous Zn$^{2+}$ uptake transporter in K562 cells (Gaither&Eide, 2001). For ZnT1, the response of its mRNA levels to TPEN treatment in K562 cells is slow compared to other Zn$^{2+}$ transporters (Liuzzi&Cousins, 2004). Thus, I used K562 cells to study the expression of hZIP1 and ZnT1 proteins in response to acute exposure to changing Zn$^{2+}$ levles. K562 cells were incubated with or without 10 µM TPEN for 1 hour, ZnT1 and ZIP1 levels were measured by immunofluorescence staining. After TPEN treatment, ZnT1 protein levels were slightly reduced (Fig.35A vs Fig.35B); while no differences were observed for ZIP1 expression and location after TPEN treatment (Fig.35C vs Fig.35D).
Figure 35. Effects of TPEN (10 µM) on Zn\textsuperscript{2+} transporters in K562 cells.
ZnT1 staining of K562 cells treated with DMSO (A) and TPEN (B) for 1 hour. ZIP1 staining of K562 cells treated with DMSO (C) and TPEN (D) for 1 hour.
Part four: Additional results

1. Effects of carbenoxolone on Zn\textsuperscript{2+} efflux.

Carbenoxolone (CBX) is commonly used to block gap junction hemichannels in cultured cells (Bruzzone et al., 2005), but it is also known that carbenoxolone has many nonspecific actions on ion channels and transporters (Vessey et al., 2004). My studies found that Zn\textsuperscript{2+} efflux could be blocked by carbenoxolone.

Results showed that 5 µM CBX significantly reduced Zn\textsuperscript{2+} efflux and 100 µM CBX could block Zn\textsuperscript{2+} efflux completely (Fig.36A&B). There are two types of gap junction hemichannels in the brain, connexin and pannexin. Connexin is sensitive to extracellular calcium and removing extracellular calcium can open connexin hemichannels spontaneously. However, removing extracellular calcium would inhibit Zn\textsuperscript{2+} efflux in neurons (Fig.10), indicating that connexin hemichannels are not a pathway for Zn\textsuperscript{2+} efflux. Pannexin is very sensitive to CBX (with an IC\textsubscript{50} of ~ 5 µM) (Bruzzone et al., 2005), which was different from that observed for the effects of CBX on Zn\textsuperscript{2+} efflux (5 µM only blocked 30% Zn\textsuperscript{2+} efflux). These comparisons suggested that Zn\textsuperscript{2+} efflux although inhibited by CBX was not the result of Zn\textsuperscript{2+} permeation of open hemichannels.
Figure 36. Effect of carbenoxolone on Zn$^{2+}$ efflux.

A) After neurons were loaded with Zn$^{2+}$ solutions, neurons were perfused with EDTA buffer containing 0 µM CBX (■), 1 µM CBX (●), 5 µM CBX (▲), 50 µM CBX (▼), 100 µM CBX (▲). Zn$^{2+}$ efflux was increasingly reduced with higher concentrations of CBX. Solutions were made in 0.5 mM Locke’s buffer (pH8). B) Shows the effect of increasing concentrations of CBX on the calculated $\Delta F/F_0$ ($\Delta F = F_0 - F_{30\text{min}}$). Compared with control condition without CBX, when CBX was higher than 5 µM, the Zn$^{2+}$ efflux was significantly inhibited (n = 10 cells from 2 coverslips for each concentration from 1 rat, t-test). The error bar denotes the standard error.

Two plausible mechanisms exist for the actions of CBX on Zn$^{2+}$ efflux: the first would be a direct action on the Na$^+$/Zn$^{2+}$ exchanger; the second explanation would be that CBX inhibits the Na/K ATPase. To solve this problem, the Na$^+$/Zn$^{2+}$ exchanger was reversed by pretreatment with ouabain. After pretreatment with ouabain, the Na$^+$/K$^+$ ATPase was blocked, and then CBX was applied to see if it still could block reversed Na$^+$/Zn$^{2+}$ exchanger. Two plausible mechanisms exist for the actions of CBX on Zn$^{2+}$ efflux: the first would be a direct action on the Na$^+$/Zn$^{2+}$ exchanger; the second explanation would be that CBX inhibits the Na/K ATPase. To solve this problem, the Na$^+$/Zn$^{2+}$ exchanger was reversed by pretreatment with ouabain. After pretreatment with
ouabain, the Na\(^+\)/K\(^+\) ATPase was blocked, and then CBX was applied to see if it still could block reversed Na\(^+\)/Zn\(^{2+}\) exchanger.

Neurons were pretreated with 100 µM ouabain, and then switched to Zn\(^{2+}\) solutions without sodium with or without 100 µM CBX. Application of CBX inhibited Zn\(^{2+}\) uptake (Fig.37A), indicating that CBX could directly act on Na\(^+\)/Zn\(^{2+}\) exchanger. When the neurons were pretreated with 100 µM CBX for 30 minutes, then switched to 10µM ZnCl\(_2\) without sodium, the Zn\(^{2+}\) uptake was not changed compared with the control condition which was pretreated with Locke’ buffer (Fig.37B). This result indicated that pretreatment of CBX would not reverse the sodium gradient by inhibiting Na\(^+\)/K\(^+\) ATPase. Previous results have showed that carbenoxolone might inhibit the activity of Na\(^+\)/K\(^+\) ATPase, but its inhibitory activity (IC\(_{50}\): 0.4 mM) was smaller than ouabain (IC\(_{50}\): 0.9 µM) (Zhou et al., 1996). The data suggested that 100 µM CBX is not enough to inhibit Na\(^+\)/K\(^+\) ATPase, so the inhibition on Zn\(^{2+}\) efflux caused by CBX would be a direct inhibition.
Figure 37. Effect of carbenoxolone on Zn$^{2+}$ uptake.

(A) After pretreatment with ouabain, neurons were exposed to 10 µM ZnCl$_2$ solutions without sodium (replaced by NMG) with (▲) or without CBX (■) to induce Zn$^{2+}$ uptake. CBX could inhibit the Zn$^{2+}$ uptake caused by reversed sodium gradients. (preouabain to 0Na: n = 45 neurons from 9 coverslips from 3 rats, preouabain to 0Na+CBX: n = 35 cells from 7 coverslips from 3 rats. p <0.01, two-way ANOVA) (B) Cortical neurons were treated with (●) or without (▼) 100 µM CBX, then exposed to 10 µM ZnCl$_2$ without sodium. No difference was observed for Zn$^{2+}$ uptake when neurons were pretreated with CBX or Locke’s buffer (in both conditions, n = 30 neurons from 6 coverslips from 3 rats, p = 0.7684, two-way ANOVA). Solutions were made in 0.5 mM Locke’s buffer (pH 8). Solutions were made in 0.5 mM Locke’s buffer (pH 8). The error bar denotes the standard error.

However, in control experiments where extracellular sodium was replaced by NMG so that the sodium-dependent Zn$^{2+}$ efflux was blocked, inclusion of CBX caused a further inhibition of Zn$^{2+}$ efflux, as evidenced by a large increase in the FluoZin3 fluorescence (Fig.38). These results indicated that the effects of CBX do not depend on
extracellular sodium. Therefore, the effects of CBX on sodium-dependent Zn\textsuperscript{2+} efflux (Fig.36&37) might be caused by a release of sequestered intracellular Zn\textsuperscript{2+}.

**Figure 38.** Effect of carbenoxolone on Zn\textsuperscript{2+} efflux when extracellular sodium was absent.

Neurons were incubated with 10 \( \mu \)M ZnCl\textsubscript{2} for 30 minutes, then switched to 100 \( \mu \)M EDTA without sodium with (▲) or without (■) CBX or with sodium with (●) or without (▼) CBX for 30 minutes. In both conditions, adding CBX caused intracellular fluorescence to rise. For all these four conditions, \( n = 10 \) neurons from 2 coverslips from 1 rat. The error bar denotes the standard error.
II. Involvement of Zn$^{2+}$ in fluorescein uptake through hemichannels.

A recent study discovered that ischemia could open hemichannels in cortical neurons (Thompson et al., 2006). To study if Zn$^{2+}$ is involved in the functions of hemichannels, fluorescein uptake was conducted. Neurons extracted from fetal rats were placed in precoated 96 well plates.

Three days later, the neural basal medium was changed to Locke’s buffer containing fluorescein with EDTA, zero calcium, 0.5 mM calcium, 1.5 mM calcium, EDTA plus CBX, 10 µM ZnCl$_2$, and 100 µM LaCl$_3$ respectively for 30 and 60 minutes. Results indicated that adding calcium, CBX, 10 µM ZnCl$_2$, and 100 µM LaCl$_3$ could significantly reduced fluorescein uptake compared with in zero calcium Locke’s buffer (Fig.39). Among them, the inhibition by Zn$^{2+}$ was greatest (Fig.39). The fluorescein uptake was higher in 30 minutes than 60 minutes incubation, suggesting longer time of incubation might cause photobleach, resulting in less fluorescence values detected.
**Figure 39. Effects on fluorescein uptake by different reagents.**

(A) Fluorescein uptake studied in Locke’s buffer containing fluorescein with EDTA, zero calcium, 0.5mM calcium, 1.5 mM calcium, EDTA plus CBX, 10 µM ZnCl₂, and lanthanum respectively for 30 minutes. (B) Fluorescein uptake studied in Locke’s buffer containing fluorescein with EDTA, zero calcium, 0.5mM calcium, 1.5 mM calcium, EDTA plus CBX, 10 µM ZnCl₂, and lanthanum respectively for 60 minutes. (Each bar represents the mean ± SE of n= 6 ; one way ANOVA with Tukey’s multiple comparison test, same letter indicates no significant differences existed between the two groups.) F represents the fluorescence intensities measured at 492 nm excitation and 516 nm emission.

Other factors were tested to indicate if they could affect fluorescein uptake in neurons. Previous studies showed that ATP could chelate extracellular calcium and improved dye uptake in macrophage system (Li et al., 1996). In this study, 5 mM cAMP, cGMP and Forskolin were added to 0 Ca²⁺ Locke’s buffer to study the effects of nucleotide on dye uptake. In addition, to indicate if NO could be involved into hemicannels, 1 µM fresh spermine NONOate (Calbiochem) was also added to the dye uptake solution. Three days later after plating neurons, the neural basal medium was
changed to zero calcium Locke’s buffer containing fluorescein with spermine NONOate, spermine NONOate plus CBX, cAMP, cGMP, 10 µM ZnCl₂, CBX, zero calcium, and forskolin (to raise cAMP levels) respectively for 30 minutes. Compared with zero calcium condition, all the reagents could reduce fluorescein uptake, with Zn²⁺ having the greatest effect (Fig.40). However, there are no significant differences among these reagents.

Figure 40. Effects on fluorescein uptake by different reagents.
Fluorescein uptake studied in Locke’s buffer containing fluorescein with NO, NO plus CBX, cAMP, cGMP, 10 µM ZnCl₂, CBX, zero calcium, and forskolin respectively for 30 minutes. (Each bar represents the mean ± SE of n= 6 ; one way ANOVA with Tukey’s multiple comparison test, same letter indicates no significant differences existed between the two groups.) F represents the fluorescence intensities measured at 492 nm excitation and 516 nm emission.
DISCUSSION

I. Significance of studies

Zn$^{2+}$ homeostasis is thought to be tightly regulated in neurons to maintain optimal free Zn$^{2+}$ concentrations. Around 400–600 nM is the toxic threshold of free Zn$^{2+}$ concentration that results in neuron death (Canzoniero et al., 1999). The development of clinically useful therapies aimed at preventing ischemia induced Zn$^{2+}$ toxicity will require a complete understanding of cellular Zn$^{2+}$ homeostatic mechanisms in the brain. Extensive evidence suggests that neuronal Zn$^{2+}$ homeostasis is regulated by membrane transport proteins: the ZIP transporters (SLC39 gene family) mediate Zn$^{2+}$ influx and the ZnT (SLC30 gene family) mediate Zn$^{3+}$ efflux; cytosolic metal-binding proteins such as metallothionens; and sequestration in intracellular organelles (see Colvin et al., 2003 for a review). The unique feature of Zn$^{2+}$ homeostasis in neurons is the existence of a significant pool of compartmentalized Zn$^{2+}$ inside synaptic vesicles of excitatory glutamatergic neurons (Colvin et al., 2003). These neurons are the source of the co-released glutamate and Zn$^{2+}$ during excitotoxic stimulation. In cultured cortical neurons, 22.6% were glutamatergic after 7 DIV (Dooley et al., 1997). All these homeostatic mechanisms are potential therapeutic targets to block toxic increases in intracellular free Zn$^{2+}$. For example, an investigational compound DP-b99, which is a lipophilic metal chelator, has proven efficacious as a neuroprotectant against brain injury in humans caused by stroke or bypass surgery (Frederickson et al., 2005). Among these homeostatic mechanisms, the transporter mediated pathways for Zn$^{2+}$ efflux and Zn$^{2+}$ influx are of
specific interest because the characterization of its transport mechanism(s) might provide possible therapeutic approaches to limit Zn$^{2+}$ entry.

Studies in this dissertation provide evidence of the observable transporter-mediated Zn$^{2+}$ efflux and uptake mechanisms across the plasma membrane in neurons systematically. In addition, regulation of Zn$^{2+}$ transporters by Zn$^{2+}$ levels was investigated. Elucidation of the functional mechanisms of these Zn$^{2+}$ transporters will be useful in better understanding Zn$^{2+}$ dyshomeostasis associated with Alzheimer’s disease and stroke. The new data also provides new clues to help in the development of therapeutic agents and drug targets for treatment of these diseases.

II. Zn$^{2+}$ efflux

The demonstration of the existence of Na$^+$/Zn$^{2+}$ exchange and Ca$^{2+}$/Zn$^{2+}$ exchange mediating Zn$^{2+}$ efflux are not new (Ohana et al., 2004; Simons, 1991). However, in my study, I first presented that cultured cortical neurons can utilize both the electrochemical Na$^+$ and Ca$^{2+}$ gradients to drive Zn$^{2+}$ extrusion and the extracellular Na$^+$ and Ca$^{2+}$ might be correlated. As a control, I studied also the effects of removing external Mg$^{2+}$ on Zn$^{2+}$ efflux and found that this cation cannot modulate Zn$^{2+}$ efflux, suggesting that dependence on Na$^+$ and Ca$^{2+}$ is specific. Other metal cations such as Fe$^{2+}$ were not tested because of their potential toxicity. We also demonstrated the codependence of extracellular sodium and calcium for Zn$^{2+}$ efflux. The activity of Na$^+$/Zn$^{2+}$ exchange was reduced without extracellular calcium and the activity of Ca$^{2+}$/Zn$^{2+}$ exchange was reduced without extracellular sodium, suggesting that sodium and calcium have synergistic effects on Zn$^{2+}$
efflux and thus a singular exchanger mechanism that uses both the sodium and calcium gradients to mediate Zn\(^{2+}\) efflux might exist. In an indirect experiment, I found that reducing extracellular Ca\(^{2+}\) could reduce Zn\(^{2+}\) uptake and this phenomenon was inhibited by removing extracellular Na\(^{+}\). All these data indicated that extracellular Ca\(^{2+}\) and Na\(^{+}\) were both required for Zn\(^{2+}\) efflux. This mechanism was activated by addition of extracellular calcium, suggesting that it is distinct from the Na\(^{+}/Ca^{2+}\) exchanger, as others have shown (Ohana et al., 2004). Since the Ca\(^{2+}\) gradients were maintained by Na\(^{+}/Ca^{2+}\) exchanger and Ca\(^{2+}\) pump, it is possible that this Zn\(^{2+}\) efflux mechanism was caused by Zn\(^{2+}/Ca^{2+}\) exchanger directly and affected by Na\(^{+}/Ca^{2+}\) exchanger indirectly. However, previous studies reported that the Na\(^{+}/Ca^{2+}\) exchanger inhibitor KB-R7943 failed to inhibit sodium-dependent Zn\(^{2+}\) efflux (Ohana et al., 2004). Also, I found the pH effects on sodium dependence and calcium dependence are different, which ruled out that the dependence of sodium was achieved by extracellular calcium through Na\(^{+}/Ca^{2+}\) exchanger. Therefore, we proposed that a new Na\(^{+},Ca^{2+}/Zn^{2+}\) exchange mechanism for Zn\(^{2+}\) efflux in cortical neurons.

The sodium dependence of Zn\(^{2+}\) efflux was not affected by pH\(_{o}\), whereas the calcium dependence was, suggesting that Ca\(^{2+}\) binding to the exchanger is pH sensitive in the range between 7.6 to 8. At pH 8 the exchanger appears to function largely independent of extracellular calcium concentrations. In brain slices, the extracellular pH was shown to change as much as 0.4 pH units during electrical stimulation (Chesler, 1990). Thus, the physiological sensitivity of Zn\(^{2+}\) efflux to extracellular calcium might be related to neuron activity. Acidosis is a common pathological feature of brain ischemia.
(Siesjo, 1988). We were careful in the present study to maintain extracellular pH within a narrow range between pH 7.6 to 8. Under these conditions, intracellular pH is largely unaffected (Pedersen et al., 1998). At lower extracellular pH, intracellular pH is rapidly reduced in cultured neurons and we observed a rise in FluoZin3 fluorescence (data not shown), which would complicate the interpretation of our Zn\(^{2+}\) efflux data. Similar effects were reported previously that extracellular acidification (pH 6) impaired normal intracellular Zn\(^{2+}\) homeostasis in cultured cortical neurons. (Sensi et al. 2003). Thus, we have no evidence for the effects of extracellular acidosis on the Zn\(^{2+}\) efflux mechanisms studied here.

The Na\(^+/\)K\(^+\) ATPase inhibitor ouabain appears to inhibit Na\(^+\), Ca\(^{2+}/\)Zn\(^{2+}\) exchange and Zn\(^{2+}\) efflux by increasing intracellular sodium concentration. Under normal physiological conditions, the Na\(^+\) gradients across the neuronal plasma membrane are maintained by the Na\(^+/\)K\(^+\) ATPase, ensuring a high activity of Na\(^+\), Ca\(^{2+}/\)Zn\(^{2+}\) exchangers. Excitotoxic stimulation, which occurs during stroke could reduce Na\(^+/\)K\(^+\) ATPase activities (Lees, 1991), which might attenuate the Na\(^+\), Ca\(^{2+}/\)Zn\(^{2+}\) exchanger function, contributing to toxic free Zn\(^{2+}\) accumulation in neurons. It has been reported that ischemic injury caused the translocation of intracellular Zn\(^{2+}\) from presynaptic neurons to postsynaptic neurons, and subsequent neuron degeneration (Galasso & Dyck, 2007; Frederickson et al., 2005). Also, previous studies showed that depolarization could significantly induce Zn\(^{2+}\) uptake (Sheline et al., 2002; Sensi et al., 1999). Depolarization might open sodium channels, which would reduce the sodium gradient dependent Zn\(^{2+}\) efflux, further contributing to the free Zn\(^{2+}\) overload in neurons. In addition, abundant
evidence has shown that ischemia induces increases of intracellular Ca\(^{2+}\) in neurons (Choi 1988; Lipton 1999), which might further decrease the activity of Na\(^{+}\), Ca\(^{2+}/\)Zn\(^{2+}\) exchange mediating Zn\(^{2+}\) efflux. Increasing intracellular Na\(^{+}\) and Ca\(^{2+}\) concentrations might decrease or even reverse the Na\(^{+}\), Ca\(^{2+}/\)Zn\(^{2+}\) exchange activity (which might uptake Zn\(^{2+}\) into neurons), just when it is needed to rid the neuron of a Zn\(^{2+}\) load. However, other Zn\(^{2+}\) efflux mechanisms could be activated under these conditions.

One of these possible mechanisms is ZnT1 which is a facilitated diffusion transporter moving Zn\(^{2+}\) according to the Zn\(^{2+}\) gradient. ZnT1 has been shown to confer Zn\(^{2+}\) resistance in many cells and tissues (Cousins & McMahon, 2000). However, there has been a debate as to the function of ZnT1. Some studies have indicated that ZnT1 can promote direct Zn\(^{2+}\) efflux (Palmiter & Findley, 1995; Kim et al, 2000). PC12 cells preloaded with 20 \(\mu\text{M} \) \(^{65}\text{Zn}^{2+}\) for 90 minutes were tested for Zn\(^{2+}\) efflux for 2 hours. Cells overexpressing ZnT1 showed higher Zn\(^{2+}\) efflux rates than control cells (Kim et al, 2000). However, other studies have argued that ZnT1 is a Zn\(^{2+}\) influx channel LTCC blocker (Segal et al., 2004; Ohana et al., 2006). ZnT1 itself could not contribute to Zn\(^{2+}\) efflux because HEK293 cells overexpressing ZnT1 showed the same Zn\(^{2+}\) efflux trace as the control cells by Fura-2 measurement (Segal et al., 2004). Our studies used shRNAi method to reduce endogenously expressed ZnT1 to study the role of this protein in cultured cortical neurons. The results showed that cortical neurons with reduced levels of ZnT1 showed slower Zn\(^{2+}\) efflux rates compared with nonsense transfected cells. The ZnT1-mediated Zn\(^{2+}\) efflux was greatest during the first 10 minutes after addition of EDTA, consistent with previous data in PC12 cells (Kim et al, 2000) and suggesting
ZnT1-mediated efflux might be very sensitive to changes in intracellular Zn\(^{2+}\) concentration. Under resting physiological conditions, intracellular free Zn\(^{2+}\) is very low (Colvin et al., 2008) and ZnT1-mediated efflux would not be strongly activated. However, under certain conditions such as ischemia, intracellular Zn\(^{2+}\) is increased and ZnT1-mediated efflux would be greater. It was reported that the expression of ZnT1 correlated to the levels of free Zn\(^{2+}\) in specific brain regions and neuron types (Sekler et al., 2002). The hippocampus CA3 regions with higher levels of ZnT1 were more resistant to ischemia than CA1 neurons that showed less immunostaining of ZnT1. Additionally, ZnT1 was induced by transient ischemia of the forebrain (Cousins & McMahon, 2000; Tsuda et al., 1997). Combining all previous data and our results, we can conclude that the function of ZnT1 is related to intracellular free Zn\(^{2+}\) levels and might play a critical role for protection during ischemia.

We observed that when the Na\(^{+}\), Ca\(^{2+}\)/Zn\(^{2+}\) exchanger was blocked by removing extracellular sodium and the ZnT1 expression was reduced by shRNAi, intracellular Zn\(^{2+}\) levels increased when Zn\(^{2+}\) preloaded neurons were switched to zero Zn\(^{2+}\) solutions. This finding suggested that under zero Zn\(^{2+}\) conditions, Zn\(^{2+}\) release was occurring from intracellular compartments not being balanced by Zn\(^{2+}\) efflux because Zn\(^{2+}\) efflux routes were largely disrupted. Also, ZnT1 might be responsible for trafficking Zn\(^{2+}\) into intracellular organelles since it has intracellular localization shown by immunostaining. Thus, when ZnT1 expression was reduced, net Zn\(^{2+}\) release from intracellular stores might be increased.
III. Zn$^{2+}$ uptake

Previous studies have showed that the Zn$^{2+}$, HCO$_3^-$ symporter (Gaither & Eide, 2000) and Zn$^{2+}$/H$^+$ antiporter (Colvin, 2002) might be two possible mechanisms for Zn$^{2+}$ uptake. I studied the effects of extracellular pH on Zn$^{2+}$ uptake using broad pH ranges from 6.8 to 9, trying to discover if these two mechanisms might be the same. My data showed that from pH6.8 to pH8, Zn$^{2+}$ uptake was increased with higher extracellular pH and reached a peak at pH8; while Zn$^{2+}$ uptake was decreased again at pH9 in cortical neurons. These data suggested that the Zn$^{2+}$ uptake was most efficient at pH7.4 to 8 which is close to physiological pH. The bicarbonate buffering system maintains a stable pH in the brain. When the pH changes too far outside of the physiological range in direction, acidosis or alkalosis are induced which would affect proper brain function in various aspects. Previous results showed that high extracellular pH increases and low pH decreases brain excitability (Bonnet et al., 2000). The effects caused by pH might be related to synaptic transmitter release and conductance of ionic channels. Acidosis in cytoplasm induced noradrenalin, serotonin and dopamine to release from synaptosomes (Cannizzaro et al., 2003; Pittaluga et al., 2005). Extracellular acidosis inhibited and alkalosis increased Na$^+$ currents in rat hippocampal neurons (Tombaugh & Somjen, 1996). Intracellular acidosis inhibited excitability of locus coeruleus neurons from rat brain slices by the opening of an inward rectifier potassium channel (Pineda & Aghajanian, 1997). Cytoplasmic protons were showed to inhibit the sodium/calcium exchanger (Doering & Lederer, 1993). Extracellular alkalosis activated high-voltage
activated (HVA) calcium channels (types L,N, P,Q, and R) and acidosis inhibited these channels (Tombaugh & Somjen, 1996).

A series of studies have shown that hypoxia/anoxia caused acidosis in neurons. Hypoxia decreased intracellular pH of CA1 pyramidal cells in mouse brain slices and the pH was recovered with reoxygenation (Fujiwara et al., 1992). More complicated pH changes were reported in cultured postnatal rat hippocampal neurons (Diarra et al., 1999). Anoxia evoked a triphasic change in intracellular pH consisting of an initial fall in pH, a subsequent increase in pH in the continued absence of O₂ and a further alkalinization upon the return to normoxia, with subsequent recovery towards normal pH levels. When extracellular pH was reduced or raised from the normal range, the bicarbonate buffer system might regulate to maintain proper pH levels (Fig.41). At low pH, excess protons bind HCO₃⁻ and convert to carbon dioxide (CO₂); whereas at high pH, excess OH⁻ bind HCO₃⁻ and convert to CO₃²⁻ (Fig.41). Acidosis and alkalosis might both cause extracellular HCO₃⁻ concentration to drop, resulting in lower Zn²⁺ uptake, which would reduce the Zn²⁺ toxicity in ischemia or anoxia.

\[
\begin{align*}
\text{CO}_3^{2-} + 2 \text{H}_2\text{O} & \rightleftharpoons \text{HCO}_3^- + \text{H}_3\text{O}^- + \text{OH}^- & \rightleftharpoons \text{H}_2\text{CO}_3 + 2 \text{OH}^- \\
\text{H}_2\text{CO}_3 + 2 \text{H}_2\text{O} & \rightleftharpoons \text{HCO}_3^- + \text{H}_3\text{O}^- + \text{H}_2\text{O} & \rightleftharpoons \text{CO}_3^{2-} + 2 \text{H}_3\text{O}^+
\end{align*}
\]

**Figure 41. The equilibrium reactions of bicarbonate buffering system.**

Since ZIP2, ZIP8, and ZIP14 were found to be Zn²⁺, HCO₃⁻ symporters, they might be the major Zn²⁺ uptake transporters across the plasma membrane of cortical
neurons. In case of the ZIP1 transporter, its function cannot be stimulated by extracellular bicarbonate (Gaither & Eide, 2001). Using the hZIP1 oocyte expression system, we found that $^{65}\text{Zn}^{2+}$ uptake by hZIP1 was lower at pH7.5 than at pH6 (Fig.42), suggesting that hZIP1 utilize an unknown mechanism to uptake $\text{Zn}^{2+}$ different from $\text{Zn}^{2+}$, $\text{HCO}_3^-$ symporters in which both lower and higher pH would inhibit $\text{Zn}^{2+}$ uptake. Reducing ZIP1 gene expression in cortical neurons reduced $\text{Zn}^{2+}$ uptake, indicating ZIP1 could uptake $\text{Zn}^{2+}$ into neurons. How these different ZIP proteins work with each other to uptake $\text{Zn}^{2+}$ into neurons needs further study.

Figure 42. pH effects on $^{65}\text{Zn}^{2+}$ uptake of ZIP1.

The $\text{Zn}^{2+}$ uptake caused by hZIP1 was calculated by subtracting the $\text{Zn}^{65}$ uptake in water injected oocytes from that in hZIP1-HA cRNA injected oocytes. The $\text{Zn}^{2+}$ uptake was higher at pH6 ($n = 25$ from 4 frogs) than at pH 7.5 ($n = 24$ from 4 frogs), but the difference was not significant ($p = 0.4022$, t-test, the error bar indicates the standard error).
Our data also showed that large amounts of Zn\(^{2+}\) influx occurred actually with depolarization. Previous studies have reported \(^{65}\text{Zn}\)^{2+} influx was induced by depolarization in neurons (Sheline, 2002) and I used microfluorometry to conduct similar experiments and obtained the same results. Low concentrations (10 µM) and high concentrations (100 µM) of ZnCl\(_2\) were both used to study depolarization induced Zn\(^{2+}\) influx. We found that in the first five minutes after depolarization, only high concentrations of Zn\(^{2+}\) showed depolarization-induced Zn\(^{2+}\) influx sensed by FluoZin3. The extracellular concentration of Zn\(^{2+}\) after intense neuronal activity may reach several hundred micromolar (Assaf & Chung, 1984). Depolarization in neurons was performed by exposure to 60 mM KCl because the EC\(_{50}\) for K\(^+\)-induced \(^{65}\text{Zn}\)^{2+} accumulation was 22.5 mM so that complete depolarization could be achieved with 60 mM KCl (Sheline, 2002). This level of depolarization was observed after spreading depression (SD) and severe hypoxia. When pyramidal neurons were depolarized at the SD-like levels, extracellular K\(^+\) was increased by 47 mM and extracellular Na\(^+\) was dropped by 91 mM (Muller & Somjen, 2000). Computer simulation also confirmed that SD can be induced by increasing extracellular K\(^+\) concentrations (Somjen, 2001).

IV. Regulation of Zn\(^{2+}\) transporters by Zn\(^{2+}\) levels

Most previous studies regarding the regulation of the expression of Zn\(^{2+}\) transporters were based on either cellular or animal experiments. For example, several studies demonstrated the relationship between the Zn\(^{2+}\) status and regulation of Zn\(^{2+}\) transporters using cultured cells (Cousins et al., 2003, Devergnas et al., 2004 and Andree
Expression of various Zn$^{2+}$ transporter genes was shown to be dependent on dietary Zn$^{2+}$ availability in rats (Liuzzi et al., 2001 and Pfaffl & Windisch, 2003). For those studies using cell lines to express the exogenous proteins by transfection, there are many technological challenges to overcome. The tranfection efficiency might be low. Also, it is hard to distinguish if effects are caused at transcriptional, translational or post-translational levels. This paper was the first to use a heterologous expression system—Xenopus oocyte—to study this problem which eliminates the transcriptional actions. The oocyte expression system has been used to overexpress and study many membrane transporters (Bianchi and Driscoll, 2006). By injecting a certain amount of cRNA of hZIP1, the possible interference of transcriptional regulation by Zn$^{2+}$ was avoided because the cRNA for hZIP1-HA expression was fixed. Also, negative control experiments were done by overpressing Na$^{+}$/K$^{+}$ ATPase. It was found that the expression levels of Na$^{+}$/K$^{+}$ ATPase was not affected by Zn$^{2+}$ status, indicating that the effects of Zn$^{2+}$ on ZIP1 translation was specific.

As the first member found in this protein family, ZIP1 is expressed widely in different tissues and cell types (Eide, 2004). In transfected K562 cells, hZIP1 was found to be localized to the plasma membrane (Gaither and Eide, 2001); while in adherent cells (Milon et al., 2001), it is localized mainly to the endoplasmic reticulum. Regulation of hZIP1 protein expression is of great significance. Studies have found that hZIP1 regulation is involved in prostate cancer: its gene expression and protein were greatly down-regulated and Zn$^{2+}$ was depleted in adenocarcinomatous glands (Franklin et al., 2005). Regulation of ZIP1 by Zn$^{2+}$ abundance was reported at post-translational
trafficking level (Wang et al., 2004a). It was found that the protein levels of ZIP1 in human prostate epithelial cells were reduced by Zn\(^{2+}\), which was not associated with transcriptional regulation (Huang et al., 2006). Studies also indicated that disruption of a functional di-leucine signal in ZIP1 inhibited the endocytosis of ZIP1 and caused an accumulation of ZIP1 protein on the cell surface (Huang and Kirschke, 2007). These data suggested that ZIP1 travels through the plasma membrane to lysosomes for degradation when cellular Zn\(^{2+}\) is high. However, our data indicated that when the ZIP1 protein was expressed, its localization was not affected by Zn\(^{2+}\) abundance. This finding conflicts with the previous reports. Firstly, the observed trafficking regulation might be a result of translational regulation. Second, the expression and regulation of endogenous proteins in oocytes and cell lines might be different. Third, since the cRNA injected into oocytes only contain coding sequences, the untranslated regions (UTR) that might be involved into regulation of protein turnover and location were removed.

Our data was the first to show that ZIP1 was regulated by Zn\(^{2+}\) at the translational level. I used Xenopus oocyte expression system which is well known for its advantage for *in vitro* translation studies. Since the oocyte has the ability to correctly assemble proteins composed of individual subunits and also make the appropriate posttranslational processing of the protein (Lane, 1983; Soreq, 1985), the membrane protein hZIP1 can be successfully expressed on the oocyte membrane. Although it is unknown if oocytes and neurons share the same translational regulation mechanism, this study would provide clues of the possible of regulation mechanisms for ZIP1 in neurons. We found that when Zn\(^{2+}\) was deficient (TPEN treatment), the translation of ZIP1 cRNA was increased and
greater amounts of the protein were found on the oocyte surface; whereas the translation of ZIP1 cRNA was reduced by increased Zn\textsuperscript{2+} treatment and the expression of the protein on the oocyte surface was reduced. This regulation was achieved at the translational level because the ZIP1 cRNA was obviously controlled at a constant level. ICP studies showed that the TPEN treatment did not cause significant reduction of intracellular zinc levels, suggesting the response of ZIP1 translation to zinc is sensitive. When the zinc levels were decreased at a certain normal range, the zinc transporters could be modulated accordingly to maintain the intracellular zinc homeostasis for normal biological functions.

Translational regulation mechanisms are not new. Previous studies have reported that the iron transporters IREG can be modulated by iron at translational levels (McKie et al., 2000; Crichton et al., 2002). Iron uptake proteins such as the transferring receptor and DCT1/DMT1 could be regulated in response to iron by mRNA targeting (Theil, 2000). These protein genes contain non-coding sequences that can be transcribed to mature mRNA which is called IRE which can be recognized by IRP (iron regulatory proteins). IREs contain hairpin loop structures with an internal loop/bulge so that its interaction with IRP could be regulated by iron. When iron is low, IRE/IRP is formed to trigger protein synthesis; whereas protein synthesis stops when iron is high. Studies also demonstrated that ZIP5 mRNA remains associated with polysomes during Zn\textsuperscript{2+} deficiency (Weaver et al., 2007). All these data suggested that translational control might also play an important role in modulation of metal ion transporter levels. The regulation of hZIP1 translation by Zn\textsuperscript{2+} might depend on cRNA stability. Since we only injected the
coding cRNA to oocytes in this study, the same mechanism as IRE/IRP regulation was ruled out. It is very likely that the Zn\(^{2+}\) sensing of hZIP1 expression uses translation stall mechanisms, the same as suggested for ZIP5.

V. Additional results

The opening of hemichannels in cortical neurons was demonstrated by uptake of 5(6)-carboxyfluorescein into cells from the culture medium when extracellular calcium levels were reduced (Li et al., 1996). I used the same dye to study the effects of Zn\(^{2+}\) and other reagents on fluorescein uptake in neurons. Fluorescein uptake through hemichannels in cortical neurons could be blocked by 10 µM ZnCl\(_2\) significantly, suggesting that Zn\(^{2+}\) flux could be conducted by hemichannels or Zn binds to hemichannels and inhibits their function. Previous results have showed that 10 µM ZnCl\(_2\) enhanced hemichannel currents and 1 mM ZnCl\(_2\) suppressed hemichannel currents in oocytes overexpressing Cx35, a connexin expressed in neurons of the vertebrate retina (Chappell et al., 2003). Chappell studied the overexpressed hemichannel which needs high concentration of ZnCl\(_2\) to inhibit currents; whereas I just tested the endogenous hemichannel properties and much lower concentrations of ZnCl\(_2\) apparently blocked these channels. It is also possible that the inhibition by ZnCl\(_2\) was a result of nonspecific effects (same as lanthanum).

Studies have revealed the relationship of hemichannels with ischemia. It was found that ischemia could open neuronal gap junction hemichannel (Thompson et al., 2006). Ischemia induced hemichannels in cardiomyocytes to open transiently which was
involved in reperfusion injury following brief ischemia (Shintani-Ishida et al., 2007).

Therefore, two interesting hypotheses could be proposed. First, large amounts of Zn$^{2+}$ flux observed during ischemia (Frederickson et al., 1989; Suh et al., 2001) might be through hemichannels. Or, Zn$^{2+}$ would inhibit hemichannel opening during ischemia, which would initiate a self-protection mechanism (such as ischemic preconditioning). Further detailed studies are needed to elaborate these hypotheses.

VI. Methodology

In this dissertation, microfluorometry was used to study Zn$^{2+}$ efflux and uptake. The advantage of this method compared with $^{65}$Zn$^{2+}$ and spectrofluorometry is that changes in single neurons could be observed over time, ensuring the same neurons were observed after various treatments. This avoids the possibility that the Zn$^{2+}$ signal was reduced only because neurons are flushed away or die with long incubation. To measure the intracellular free Zn$^{2+}$ concentration changes, we used FluoZin3 which is highly selective for Zn$^{2+}$ and has a high affinity required to monitor low nanomolar changes in intracellular free Zn$^{2+}$. The affinity of FluoZin3 for calcium is much less than Zn$^{2+}$, but its sensitivity to biologically relevant changes in intracellular free calcium is controversial (Devinney et al., 2005; Martin et al., 2006; Zhao et al., 2008; Molecular Probes Handbook - http://probes.invitrogen.com/handbook/). It has been noted previously that even low affinity fluorophores can produce surprisingly robust intracellular fluorescence partly due to high intracellular concentrations of the fluorophore (Dineley et al., 2002). In our experiments, we used low calcium Locke’s buffer (0.5 mM Ca$^{2+}$) and presumably
intracellular calcium concentrations were maintained at low levels by the Ca\(^{2+}\) ATPase and Na\(^{+}/Ca^{2+}\) exchanger. Therefore, fluorescence signals measured here are most likely due to changes in intracellular free Zn\(^{2+}\) concentrations. However, when ouabain was applied, the sodium gradients were ruined, resulting in reversal of the Na\(^{+}/Ca^{2+}\) exchanger with apparently large influx of Ca\(^{2+}\). It appeared that under these conditions intracellular calcium concentrations reached levels sufficient to bind to FluoZin3 or that Ca\(^{2+}\) influx caused release of sequestered Zn\(^{2+}\). Other control experiments were also conducted by replacing extracellular sodium with NMG (Fig. 9B) or adding high potassium (60 mM KCl) (Fig. 17C) in Locke’s buffer with 2.5mM calcium. Results showed that at these conditions, the Ca\(^{2+}\) influx did not reach to level large enough to be detected by FluoZin3.

In this study, cultured cortical neurons were used to study the potential Zn\(^{2+}\) flux mechanisms in the brain. It is known that primary neuronal cultures cannot exactly replicate the intact adult nervous system (Choi, 1990) and they are different in connectivity, 3-dimensional architecture and maturity. But many studies on primary neuron culture have suggested that this model can be used to study various aspects of neuronal properties, including the behavior of transmitter chemistry (Thomas, 1986), neurotransmitter receptors (Hertz, 1984; Schousboe, 1985), membrane channels (Thibault et al., 1993), and membrane transporters (Sheline, 2000). It was also reported that long term culture of cortical neurons could be a model for neuronal development, aging and death (Lesuisse & Martin, 2002). The advantage of using primary neuron culture is that the environment can be manipulated precisely and neurons can be isolated from other
types of cells (such as glia) so that the specific response of neurons in case of cellular and molecular mechanisms can be identified. In this paper, the Zn$^{2+}$ efflux and Zn$^{2+}$ influx mechanisms were specifically studied in individual neurons.

In this study, the reduction of neuronal ZnT1 and ZIP1 expression was accomplished using shRNAi. Transfection of primary cultured neurons is always a challenge because of the low transfection efficiency and high toxicity. The conventional techniques such as Ca$^{2+}$ transfection usually show 1-5% transfection rate in primary neurons (Washbourne & McAllister, 2002). For cationic lipid-based methods in primary neurons, the transfer rate is in the range of 2.4 to 5% (Wiesenhofer & Humpel, 2000). Electroporation based techniques can increase the transfection efficiencies to 30-70%, but only 10% of the neurons might survive (Junghans et al., 2004). Lentiviral-based gene transfer techniques can have low toxicity, but still exhibit at best 50% transfection rate and is more time-consuming (Bender et al., 2007). For these reasons, we combined microfluorometry with expression based shRNAi to track individual neurons that had been transfected with shRNAi plasmids. This method allowed us to track the intracellular Zn$^{2+}$ concentration changes in the transfected neurons without the interference of untransfected cells. Hence, the results are more reliable and ensured observed changes were caused by gene silencing.
VII. Limitation of studies

I provide several significant findings through systematically studying the $\text{Zn}^{2+}$ homeostasis using molecular and cellular methods. However, a few questions can not be explained very well due to the limitation of the techniques used. First, the transfection efficiency in neurons is still very low, restricting the amount of quantification that can be done to measure knockdown. Second, the specificity of ZnT1 and ZIP1 primary antibody could be low, which might underestimate the knockdown effects. In addition, since the transfection ratio is low, only immunofluorescence staining could be used to verify the knockdown effects. Thirdly, low pH (pH6) would induce intracellular $\text{Zn}^{2+}$ release which caused FluoZin3 fluorescence to rise, preventing the studies to discover the relationships of acidosis to $\text{Zn}^{2+}$ flux. Fourth, the hZIP1 protein was successfully expressed in oocytes. However, $^{65}\text{Zn}^{2+}$ assay is hard to study the function of membrane transporters by oocyte expression systems because the variation between different oocytes from different batches and frogs is big. The electrophysiological studies can overcome this challenge because it could be used to record currents using the same oocyte with different treatments. Unfortunately, the mediated $\text{Zn}^{2+}$ currents through hZIP1 are too small (around nA, Fig.43) to be reliably measured. This presents a big challenge to conduct electrophysiological studies using this hZIP1 overexpressed oocyte system.
I = \frac{Q}{t} = \frac{(0.02 \times 10^{-9} \times 2 \times 10^6)}{(1.5 \times 60 \times 60)} = 7.4 \text{ nA}

**Figure 43. Conversion of ion flux to current in a single oocyte.**

In 1.5 hour (1.5 X 60 X 60 seconds), the net $^{65}$Zn$^{2+}$ uptake was 0.02 nmoles. One mole of electrons is known as a faraday and a faraday equals around $10^{-6}$ (96485.3399) coulombs. Zn$^{2+}$ ion carries two negative charges. Then the predicted current is around 7.4 nA. The error bar denotes the standard error.

**VIII. Summary**

1. A Na$^+$, Ca$^{2+}$/Zn$^{2+}$ exchanger may exist in cortical neurons that could be the primary mechanism responsible for maintaining low intracellular free Zn$^{2+}$ concentrations under resting conditions (Fig.44A). While in pathological conditions such as ischemia, ZnT1 might be activated by increased intracellular Zn$^{2+}$ concentrations (Fig.44B). Acidosis is a common pathological feature of brain ischemia (Siesjo, 1988). Currently, we have no evidence for the effects of acidosis on the Zn$^{2+}$ efflux mechanisms studied here. Further studies are required to find the gene encoding neuronal Na$^+$, Ca$^{2+}$/Zn$^{2+}$ exchangers and how these exchangers and ZnT1 can protect neurons from Zn$^{2+}$ toxicity during ischemia.
2. At resting conditions, Zn$^{2+}$ uptake in cortical neurons was affected by extracellular pH. When extracellular pH was reduced or raised from the normal range pH 7.4-8, Zn$^{2+}$ uptake was inhibited, which agrees with a Zn$^{2+}$, HCO$_3^-$ symporter mechanism reported for ZIP2, ZIP8 and ZIP14. ZIP1 also may play a role to uptake Zn$^{2+}$ into neurons, but the effect on the function of ZIP1 is different. Compared to pH 7.5, Zn$^{2+}$ uptake by hZIP1 is lower at lower pH 6, suggesting that ZIP1 might use a different, yet undefined transport mechanism.

3. The data in this dissertation demonstrated that hZIP1 protein expression can be modulated at the translational level. Zn$^{2+}$ deficiency caused more ZIP1 mRNA to be translated into proteins; and Zn$^{2+}$ repletion decreased the translation of ZIP1, either by degrading ZIP1 mRNA or stalling ZIP1 mRNA on polysomes. This finding could provide clues for understanding the biological roles of hZIP1. The detailed mechanisms of this regulation and how it can be involved in prostate cancer need further studies.
Figure 44. Models of Zn\textsuperscript{2+} efflux and uptake under physiological and pathological conditions.

(A) Under physiological conditions, Na\textsuperscript{+}, Ca\textsuperscript{2+}/Zn\textsuperscript{2+} exchanger is the major transporter to extrude Zn\textsuperscript{2+} out of neurons. ZIP1 is involved in Zn\textsuperscript{2+} uptake when Zn levels are low; while other ZIP1 family members are also probably involved in Zn\textsuperscript{2+} uptake. (B) Under pathological condition, intracellular free Zn\textsuperscript{2+} is increased by Zn\textsuperscript{2+} influx through voltage-dependent channels and intracellular Zn\textsuperscript{2+} release. ZnT1 is activated to remove Zn\textsuperscript{2+} out of neurons.
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APPENDIX A: BUFFERS

1. **Hank’s Balanced Salt Solution (HBSS)**

   One bag of HBSS powder (Sigma. H2387-1L) is dissolved in 950ml Mili-Q H$_2$O, then 0.35g of NaHCO$_3$ is added and the pH adjusted to 7.25. The volume is brought up to 1000ml with Mili-Q H$_2$O. The solution is sterilized by membrane filtration. Finally 0.5ml gentamycin (10mg/ml) is added to 500ml HBSS. The solution is stored at 0-4°C.

2. **Minimal Essential Medium**

   One bag of MEM powder (Sigma. MO769-1L) is dissolved in 950 ml Mili-Q H$_2$O. Then 2.2 grams of NaHCO$_3$ and 10 grams of glucose are added and the pH adjusted to 7.25. The volume is brought to 1000 ml. The solution is sterilized by membrane filtration. 1ml of 10 mg/ml gentamycin (0.5 ml to 500 ml) is added. The solution is stored at 0-4°C.

3. **MEM+**

   To make 200 ml MEM+, 20 ml sterile heat-inactivated fetal bovine serum (final concentration 10%), 22 mg of UV treated pyruvic acid (1 mM) (0.022 g), 32 mg L-glutamine and 220 mg KCl are mixed together, then MEM is added to bring the volume to 200 ml. The solution is sterilized by membrane filtration using an Acro 50A filter (0.2 µm, Gelman Sciences. Cat. No. 4260). The solution is stored at 0-4°C.

4. **Neurobasal Medium**
To make 200 ml Neurobasal medium, 14.6 mg L-Glutamine and 4 ml B-27 supplement (2%) was added to 46 ml warmed Neurobasal Medium (GIBCO. 21103-049) to the tube and mix gently. Once the L-glutamine is completely dissolved, the solution was filtered with a snap-cap syringe filter into a sterile glass bottle. 150ml Neurobasal was then added to get a final volume of 500 ml. The solution is stored at 0-4°C for up to two weeks.

5. Lockes’ Buffer
This 5x solution has the composition 770mM NaCl, 28mM KCl, 11.5mM CaCl₂, 5mM MgCl₂, 25mM HEPES and 50mM Glucose, pH7.4.

6. Low Ca²⁺ Locke’s buffer (5×)
This 5x solution has the composition 770mM NaCl, 28mM KCl, 2.5mM CaCl₂, 5mM MgCl₂, 25mM HEPES and 50mM Glucose, pH8

7. Na⁺ free Low Ca²⁺ Locke’s buffer (5×)
One liter Na⁺ free Low Ca²⁺ Locke’s buffer (5×) contains: NMG (770mM):150.31g, KCl(28mM) : 2.09g, CaCl₂(2.5mM) : 367.4mg, HEPES (25mM) : 6g, Glucose(50mM) : 9.01g, MgCl₂ (5mM) : 1.0165g, pH8.

8. 1.2% Formaldehyde Gel
To prepare Formaldehyde Gel (1.2% agarose), mix the following in a specified conical flask: 1.2 g agarose, 10 ml 10x FA gel buffer (buffer 9). RNase-free water is added to
bring volume to 100 ml. The mixture is heated to melt agarose. When the solution
temperature is reduced to a holdable degree, 1.44 ml of 37% formaldehyde is added and
mixed thoroughly. The solution is poured into the specified gel mold in the fume hood.
Insert the specified combs. Prior to running the gel, equilibrate in 1x FA gel running
buffer for at least 30 min. Ethidium Bromide (0.5 µg/ml) is used to stain RNA.

9. **10x FA Gel buffer**

This solution contains: 200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free
acid), 50 mM sodium acetate, 10 mM EDTA and pH is adjusted to 7.0 with NaOH.

10. **1x FA Gel Running Buffer**

This solution contains 100 ml 10x FA gel buffer and 20 ml 37% formaldehyde, RNase-
free water is added to bring solution to 1 liter.

11. **5x RNA Loading Buffer**

This solution contains 8 µl saturated aqueous Orange G solution, 40 µl 0.5 M EDTA, pH
8.0, 360 µl 37% formaldehyde, 1 ml 100% glycerol, 1542 µl formamide, 2 ml 10 x FA
Gel Buffer, RNase-free water to 5 ml.

12. **0.2% tricaine (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma
A-5040)**
2g tricaine and 5ml 1M HEPES was added to 1 liter nanopure Millipore water. The pH was adjusted to 7.5.

13. Recovery water
11.69 g NaCl was dissolved in 4 liter of tap H₂O.

14. penicillin/streptomycin solution (Sigma P-0781)
The frozen penicillin/streptomycin solution (Sigma P-0781) was melted and mixed with Mili-Q H₂O at 1:1 ratio.

15. Ca²⁺ free ND96
This solution contains 96mM NaCl, 2mM KCl, 1mM MgCl₂, 5mM HEPES. Osmolarity should be 195-200 mOsm (adjust with NaCl 10 to 20mg at a time). Sterile filter with 0.22- 0.45 μm filter and store at 4°C.

16. ND96
This solution contains 96mM NaCl, 2mM KCl, 1mM MgCl₂, 5mM HEPES, 1.8mM CaCl₂ and 2.5mM Na pyruvate. Osmolarity should be 195-200 mOsm (adjust with NaCl 10 to 20mg at a time). Sterile filter with 0.22- 0.45 μm filter and store at 4°C.

17. OR3
In 2 liters of OR3, 1 pack powdered Leibovitz L-15 media with L-glutamine (Gibco-BRL #41300-039), 100 ml penicillin/streptomycin solution in 0.9% NaCl (Sigma P-0781) and 10 ml 1M HEPES was added to 1800 ml H₂O. The pH was adjusted to 7.5 with NaOH. Osmolarity should be 195-200 mOsm (adjust with NaCl 10 to 20 mg at a time). Sterile filter with 0.22-0.45 μm filter and store at 4°C.

18. Collagenase type A (Boehringer Mannheim # 1088793)
Prepare solution just before needed at 1 mg/ml in Ca²⁺ free ND96 buffer. Filter using a syringe filter (0.22 or 0.45 μm) and pour into a sterile 50 ml tube.

19. Oocyte reaction buffer
This solution contains 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.5. Osmolarity should be 195-200 mOsm (adjust with NaCl 10 to 20 mg at a time).

20. Oocyte ice-cold wash solution
This solution contains 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, 0.1 mM EDTA, pH 7.5.

21. BSA/NGS
To make 10 ml BSA/NGS solution, 1 ml 10×PBS, 10 mg BSA and 0.4 ml NGS were mixed together. Mili-Q H₂O was added to bring the volume to 10 ml.
**22. PBS 10x (Dulbecco’s Phosphate Buffered Saline)**

One bottle of DPBS (VWR. 55-031-PB) was dissolved into 950 ml Mili-Q H₂O in a 1000 ml beaker and pH was adjusted to 7.4. The solution volume was brought to 1 liter.

**23. polyethyleneimine coating solution**

First, Borate Buffer 10x (for plate coating, 500 ml) was made by filling a 500 ml beaker with about 450 ml Mili-Q H₂O. Then 1.55 g boric acid and 2.37 g borax were added, the pH was adjusted to 8.2, the volume was brought to 500 ml with water stored in a glass bottle and stored in 4 °C. To make 50 ml of coating solution, 50 µl of the polyethylenimine (SIGMA, P-3143 50% solution) was added to 5 ml borate buffer and mixed completely. Mili-Q H₂O was added to bring volume to 50 ml. The coating solution should be made fresh just before coating the cell culture plates.

**24. Block solution**

To make 100 ml block solution, 10 ml goat serum, 1 g BSA, 10 ml and 0.1 ml Triton-X were mixed together, the volume was brought to 100 ml by Mili-Q H₂O.

**25. Borate Buffer**

To make 500 ml borate buffer, 1.55 g boric acid and 2.37 g borax were mixed well and added to 450 ml Milli-Q water in a 1000 ml beaker with gently stir until dissolved. The pH was adjusted to 8.2 with 1N NaOH or HCl, the volume was brought to 500 ml by
Mili-Q H₂O.

26. PBST

0.5 ml Triton-x was added to 50ml 10×PBS, and the volume was brought to 500 ml by Mili-Q H₂O.
APPENDIX B: ABBREVIATION

NMG- N-methyl-d-glucamine
TPEN- N,N,N',N'-Tetrakis-(2-pyridylmethyl)- Ethylenediamine
GABA- Gamma-aminobutyric acid
EDTA- ethylenediaminetetraacetic acid
Aβ – amyloid beta plaque
dsRNA – double stranded RNA
HBSS – Hank’s balanced salt solution
LTCC – L-type calcium channel
MEM – minimal essential medium
miRNA – micro RNA
MTF – metal response element-binding protein
NB – neurobasal medium
PBS – phosphate buffered saline
RNAi – RNA interference
RNApolIII – RNA polymerase III
shRNA – short hairpin RNA
siRNA – small interfering RNA
SLC – solute linked carrier
ZIP – zrt-, irt-like protein
ZnT – zinc transport protein