Accumulation of Zinc in Degenerating Hippocampal Neurons of ZnT3-Null Mice after Seizures: Evidence against Synaptic Vesicle Origin

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In several brain injury models, zinc accumulates in degenerating neuronal somata. Suggesting that such zinc accumulation may play a causal role in neurodegeneration, zinc chelation attenuates neuronal death. Because histochemically reactive zinc is present in and released from synaptic vesicles of glutamatergic neurons in the forebrain, it was proposed that zinc translocation from presynaptic terminals to postsynaptic neurons may be the mechanism of toxic zinc accumulation. To test this hypothesis, kainate seizure-induced neuronal death was examined in zinc transporter 3 gene (*ZnT3*)-null mice, a strain that completely lacks histochemically reactive zinc in synaptic vesicles. Intraperitoneal injection of kainate induced seizures to a similar degree in wild type and *ZnT3*-null mice. Staining of hippocampal sections with a zinc-specific fluorescent dye, *N*-(6-methoxy-8-quinolyl)-*p*-carboxybenzoylsulfonamide, revealed that zinc accumulated in degenerating CA1 and CA3 neurons in both groups, indicating that zinc originated from sources other than synaptic vesicles. Injection of CaEDTA into the cerebral ventricle almost completely blocked zinc accumulation in *ZnT3*-null mice, suggesting that increases in extracellular zinc concentrations may be a critical event for zinc accumulation. Arguing against the possibility that zinc accumulation results from nonspecific breakdown of zinc-containing proteins, injection of kainate into the cerebellum did not induce zinc accumulation in degenerating granule neurons. Taken together, these results support the existing idea that zinc is released into extracellular space and then enters neurons to exert a cytotoxic effect. However, the origin of zinc is not likely to be synaptic vesicles, because zinc accumulation robustly occurs in *ZnT3*-null mice lacking synaptic vesicle zinc.

Key words: TFL-Zn; neuronal degeneration; zinc transporter; kainate; cerebellum: CaEDTA

In the mammalian forebrain, an abundant pool of zinc is sequestered in synaptic vesicles along with glutamate (Frederickson et al., 1987; Frederickson, 1989; Danscher, 1996; Choi and Koh, 1998; Lee et al., 1999). In contrast to metabolic zinc that is tightly bound to proteins, this vesicular zinc is free or relatively weakly bound and hence readily visualized with zinc-selective fluorescent dyes (Frederickson et al., 1987; Budde et al., 1997; Suh et al., 1999; Lee et al., 2000) or the neo-Timm's stain (Danscher, 1996). After neuronal excitation, synaptic vesicle zinc is released into the synaptic cleft (Assaf and Chung, 1984; Howell et al., 1984), where it may modulate the activity of various ion channels (Peters et al., 1987; Westbrook and Mayer, 1987; Christine and Choi, 1990).

In addition to modulating neuronal transmission, zinc may contribute to neuronal injury in certain pathological conditions. Suggesting this, cytosolic zinc accumulation has been shown to correlate very well with selective neuronal death in ischemia, seizures, and trauma (Frederickson et al., 1988, 1989; Tonder et al., 1990; Koh et al., 1996; Suh et al., 2000). Because of dynamic changes of zinc in the synaptic vesicles, it has been proposed that extracellular release of vesicular zinc and its subsequent uptake by postsynaptic neurons underlie selective neuronal degeneration phenomena (the zinc translocation hypothesis) (Frederickson, 1989; Choi and Koh, 1998; Lee et al., 1999). However, this zinc translocation hypothesis, which specifically implicates synaptic vesicle zinc as the source for the toxic zinc accumulation, has not been directly proven.

Recently, it has been shown that zinc transporter 3 (ZnT3) is present on the membranes of zinc-accumulating synaptic vesicles (Palmiter et al., 1996; Wenzel et al., 1997). Furthermore, ZnT3 is considered essential for the accumulation of vesicular zinc, because knocking out the *ZnT3* gene results in complete disappearance of zinc in synaptic vesicles throughout the brain without affecting other nonvesicular zinc pools in the mouse (Cole et al., 1999). Therefore, *ZnT3*-null mice provide an ideal system for directly testing the zinc translocation hypothesis. In the present

Received Feb. 8, 2000; revised March 20, 2000; accepted March 27, 2000.

This study was supported by Creative Research Initiatives of the Korean Ministry of Science and Technology (J.-Y.K.).

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 0270-6474/00/200001-05$15.00/0

This article is published in *The Journal of Neuroscience*, Rapid Communications Section, which publishes brief, peer-reviewed papers online, not in print. Rapid Communications are posted online approximately one month earlier than they would appear if printed. They are listed in the Table of Contents of the next open issue of JNeurosci. Cite this article as: JNeurosci, 2000, 20:RC79 (1–5). The publication date is the date of posting online at www.jneurosci.org.

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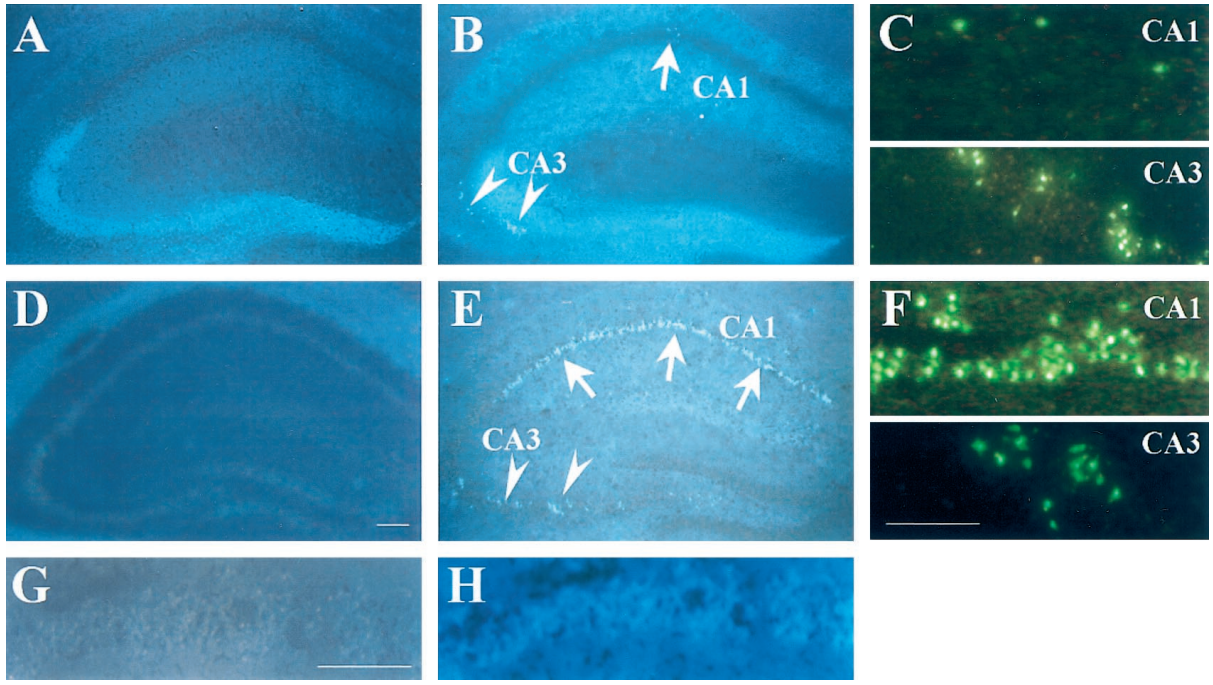

Figure 1. *A–F*, Hippocampi from WT (*A–C*) and *ZnT3*-null (*D–F*) mice stained with TFL-Zn (*A, B, D, E*) and the TUNEL method (*C, F*). *A, D*, Controls; *B, C, E, F*, 24 hr after kainate seizures. The TUNEL staining showed that CA1 neuronal death was greater in ZnT3 null mice (*C, F*). Mice were intraperitoneally injected with kainate (40 mg/kg). Two hours later, seizures were halted by intraperitoneal injection of sodium phenytoin (50 mg/kg). *G, H*, Treatment of hippocampal sections of a kainate-injected *ZnT3*-null mouse with 10 mM dithizone for 10 min completely removed TFL-Zn fluorescence from CA1 neurons (*G*), which was restored by the subsequent incubation in 100 mM $ZnCl_2$ (*H*) but not in $FeCl_2$ or $CuCl_2$ solution (results not shown). Scale bars, 500 μm.

study, this hypothesis was tested in *ZnT3*-null mice by examining zinc accumulation in hippocampal neurons and their death after kainate seizures.

MATERIALS AND METHODS

Animals. *ZnT3*-null mice and their wild-type (WT) littermates were bred and maintained in the facility of University of Ulsan College of Medicine. Animals were allowed free access to food and water at 24 ± 0.5°C and exposed to 12 hr light/dark cycles. All animal experiments were performed according to the Guidelines for Laboratory Animal Care and Use (University of Ulsan). Before all experiments, genotyping for *ZnT3* was performed using the PCR method as described previously (Cole et al., 1999).

Induction of seizures and scoring of seizure severity. Five WT and *ZnT3*-null mice were injected intraperitoneally with 40 mg/kg kainate (Tocris Cookson, Bristol, UK) dissolved in 0.9% normal saline. Separately, 1 μl of 50 mM kainate or a mixture of 100 mM $ZnCl_2$ and 50 mM kainate was injected into the cerebellum of *ZnT3*-null mice. To determine the effect of zinc chelation on kainate-induced seizure and neuronal cell death, 2 μl of 300 mM CaEDTA in saline was given stereotaxically into the lateral ventricle under anesthesia with halothane in a 1:3 mixture of O_2 and N_2O, beginning 30 min before kainate injection. For 2 hr after kainate injection, seizure severity was behaviorally estimated according to the classification of Peng et al. (1997). Two hours after kainate injection, seizures were halted by intraperitoneal injection of sodium phenytoin (50 mg/kg) (Lee et al., 2000). Features of each seizure stage are as follows: stage 1, hypoactivity; stage 2, sedation; stage 3, hyperactivity; stage 4, scratching; stage 5, loss of balance control; stage 6, tremors and generalized convulsions; and stage 7, death.

Tissue preparation and zinc-specific fluorescence staining. 24 hr after kainate injection, brain was harvested, immediately frozen in dry ice, and stored at −70°C. Coronal brain sections (10 μm thick) including the hippocampus were prepared using the cryostat and mounted on prechilled glass slides coated with poly-L-lysine. Unfixed brain sections were stained with *N*-(6-methoxy-8-quinolyl)-*p*-carboxybenzoylsulfonamide (TFL-Zn; K_d, 20 μM; Calbiochem, La Jolla, CA) dissolved in Tris buffer (0.1 mM, pH 8.0) for 90 sec (Budde et al., 1997; Lee et al., 2000). After washing with saline, TFL-stained sections were examined under a fluorescence microscope (excitation, 355–375 nm; dichroic, 380 nm; barrier, 420 nm; Olympus, Tokyo, Japan) and photographed.

Cell death detection by the terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling method. To identify neuronal cell death in the sections, terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling (TUNEL) staining was performed with the *in situ* cell death detection kit, following the manufacture's instruction manual (Boehringer Mannheim, Mannheim, Germany). After fixation in 4% paraformaldehyde, the sections were incubated in 0.3% H_2O_2 and in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). After TUNEL reaction, the sections were examined under a fluorescent microscope and photographed.

Cell counting and acid-fuchsin staining. TFL-Zn fluorescent (zinc-accumulating) neurons in the pyramidal layer of CA1 and CA3 were counted in three coronal sections taken every 150 μm starting 4.2 mm from bregma. Statistical comparisons were made using a two-tailed *t* test. Adjacent brain sections were fixed overnight with 4% paraformaldehyde and stained with 1% acid-fuchsin (Lee et al., 2000). After briefly rinsing in tap water, sections were immersed into acid-fuchsin solution for 30 sec and washed with tap water. Acidophilic (degenerating) neurons in CA1 and CA3 were counted under a bright-field microscope.

RESULTS

Consistent with previous studies (Frederickson et al., 1987; Koh et al., 1996), TFL-Zn staining of the hippocampus of WT mice showed that chelatable zinc was present densely in the mossy fiber terminals and sparsely in other presynaptic fibers, including stratum radiatum of CA1 (Fig. 1*A*). But knocking out the *ZnT3* gene resulted in complete disappearance of zinc in synaptic vesicles throughout the brain (Fig. 1*D*), whereas it does not affect other nonvesicular zinc pools in the mouse (Cole et al., 1999).

In the present study, WT and *ZnT3*-null mice ($n = 5$ each) were intraperitoneally injected with 40 mg/kg kainate, a dose sufficient to produce severe seizures in all mice. Because *ZnT3*-null mice

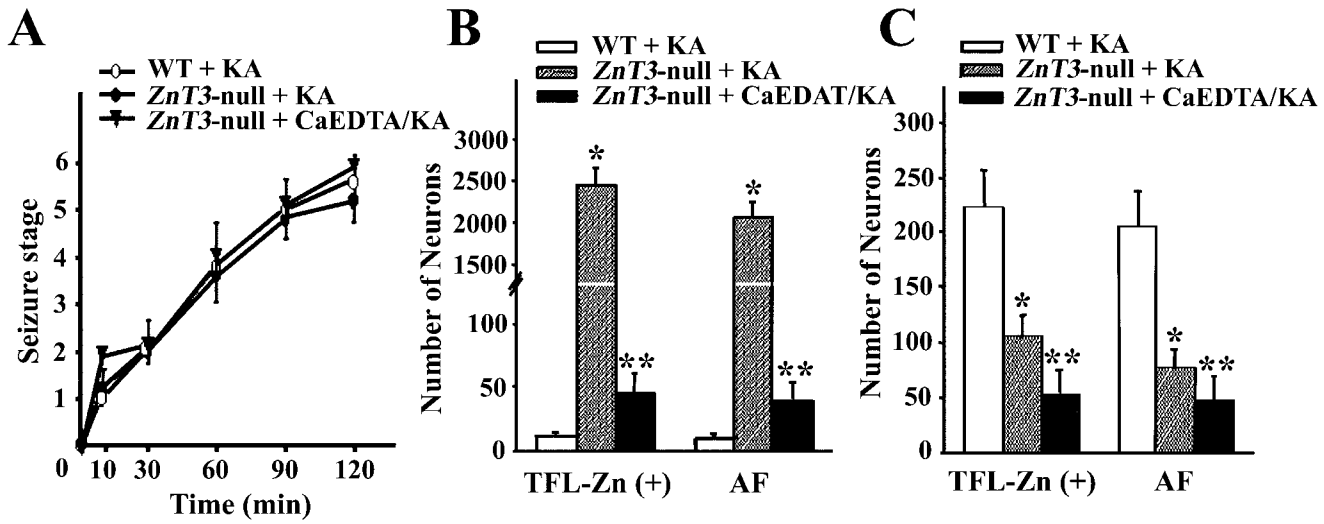

Figure 2. *A*, Time course for the progression of seizure stages (mean ± SD; $n = 5$ each) in WT or *ZnT3*-null mice without or with intraventricular injection of 2 μl of 300 mM CaEDTA. See Materials and Methods for the seizure classification. *B*, *Bars* represent number of TFL-Zn(+) (*right bars*) and acid-fuchsin(+) (*left bars*) neurons (mean + SEM; $n = 5$) in bilateral CA1 in three brain sections (10 μm thick) of WT, *ZnT3*-null mice, and *ZnT3*-null mice injected with CaEDTA 24 hr after kainate injection. Cell count was done on both sides of the hippocampus. *C*, Number of TFL-Zn(+) and acid-fuchsin(+) neurons (mean + SEM; $n = 5$) in bilateral CA3 in the above sections. *Difference from WT + KA; **Difference from ZnT3-null + KA ($p < 0.05$, two-tailed t test).

are more sensitive than WT mice to seizures induced by kainate (Cole et al., 2000), we chose this dose to produce comparable seizures. To lessen the mortality (Lee et al., 2000), seizures in both groups were stopped by intraperitoneally injecting sodium phenytoin 2 hr after kainate injection. Using this method, all mice developed seizures with similar time course and severity, as estimated by the behavioral seizure severity scores of Peng et al. (1997) (Fig. 2*A*).

Staining brain sections of seizure-experienced mice 24 hr later with a zinc-specific fluorescent dye, TFL-Zn, revealed dense zinc accumulation in neuronal cell bodies and somewhat increased TFL-Zn fluorescence in strata radiatum and oriens of not only WT (Fig. 1*B*) but also *ZnT3*-null mice (five of five mice for each; Fig. 1*E*). The TFL-Zn fluorescence of *ZnT3*-null mice was abolished by treatment with the zinc chelator–remover dithizone (Fig. 1*G*). Staining with the TUNEL method or with hematoxylin and eosin (Cole et al., 2000) revealed neuronal death in densely TFL-Zn fluorescent cells in both WT and *ZnT3*-null mice. Interestingly, whereas death of CA3 neurons in *ZnT3*-null mice was less than that in WT mice, death of CA1 neurons in *ZnT3*-null mice was markedly enhanced compared with WT mice (Fig. 1*C,F*). Counting the number of zinc-accumulating neurons and acid-fuchsin-stained neurons in CA1 and CA3 confirmed this impression (Fig. 2*B,C*).

Next, we examined the possibility that cytosolic zinc in WT and *ZnT3*-null mice originates from nonspecific release of zinc from degrading zinc-containing proteins. Arguing against this possibility, injection of kainate into the cerebellum resulted in death, but not TFL-Zn staining, of granule neurons in both WT and *ZnT3*-null mice (Fig. 3A). Only when zinc was given with kainate did TFL-Zn fluorescence appear in most TUNEL(+) cerebellar granule neurons (Fig. 3*B*). These results are consistent with the previous report that zinc is not implicated in kainate-induced granular cell death in the cerebellum (Frederickson et al., 1989).

To further examine whether toxic zinc accumulation originates from outside the neurons, a cell membrane-impermeant zinc chelator was used. Intraventricular injection of CaEDTA had little effect on kainate-induced seizure severity in *ZnT3*-null mice (five of five mice; Fig. 2*A*) but markedly attenuated both zinc accumulation and neuronal death in the hippocampus (Figs. 2, 4), favoring the external origin for toxic zinc accumulation even in *ZnT3*-null mice.

DISCUSSION

Although vesicular zinc is completely absent in *ZnT3*-null mice, dense TFL-Zn fluorescence develops in degenerating hippocampal neurons after kainate-induced seizures. This result indicates that the main origin of zinc responsible for the TFL-Zn fluorescence after brain injury in *ZnT3*-null mice is not the histochemically reactive zinc stored in ZnT3-containing synaptic vesicles. Although the present study cannot completely exclude the possibility that certain adaptations in *ZnT3*-null mice, such as alteration of metallothionein levels or other events related to zinc homeostasis, may underlie the zinc accumulation in degenerating neurons, overall our results suggest the nonvesicular origin of zinc also in WT animals after brain injuries (Frederickson et al., 1988, 1989; Tonder et al., 1990; Koh et al., 1996; Suh et al., 2000).

Where does zinc accumulating in degenerating neurons of *ZnT3*-null mice come from, if not synaptic vesicles? The present study does not provide a specific answer to this question. However, overall it suggests the external origin (i.e., from outside of zinc accumulating neurons) of zinc based on the following results. First, injection of kainate into the cerebellum resulted in death of granule neurons but no TFL-Zn fluorescence in them in either WT or *ZnT3*-null mice, arguing against the possibility that zinc is nonspecifically released from degraded zinc-containing proteins. Only when zinc was given with kainate did the fluorescence appear in the TUNEL(+) granule cells. Corroborating this, Frederickson et al. (1989) reported that zinc is not implicated in kainate-induced neuronal cell death in the cerebellum. These results are consistent with previous findings in cortical culture that excitotoxic, oxidative, or apoptotic injury is not associated with zinc fluorescence unless zinc is added to the exposure medium (Koh et al., 1996). Second, intraventricular injection of a

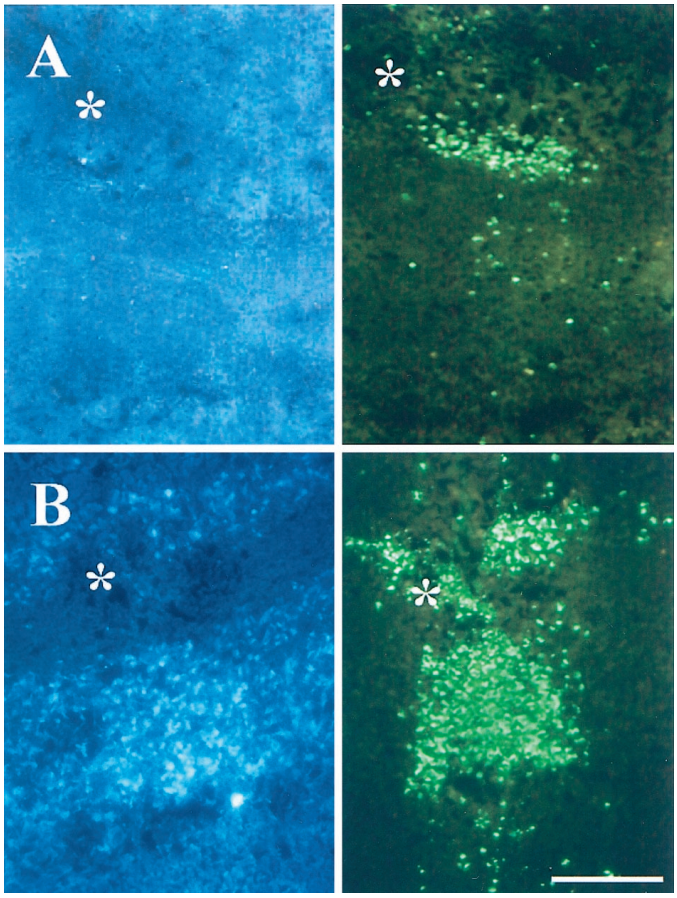

Figure 3. *A*, Cerebellum of a *ZnT3*-null mouse 24 hr after parenchymal injection of kainate (1 μl of 50 mM), TFL-Zn stained and TUNEL stained. Whereas the TUNEL method stained many granule neurons (*green* fluorescence) around the injection site, TFL-Zn stained none. *B*, Cerebellum of a *ZnT3*-null mouse injected with 1 μl of kainate (50 mM) and zinc (100 mM). Most TUNEL(+) granule neurons were stained also with TFL-Zn. *Asterisks* denote injection sites. Scale bar, 500 μm.

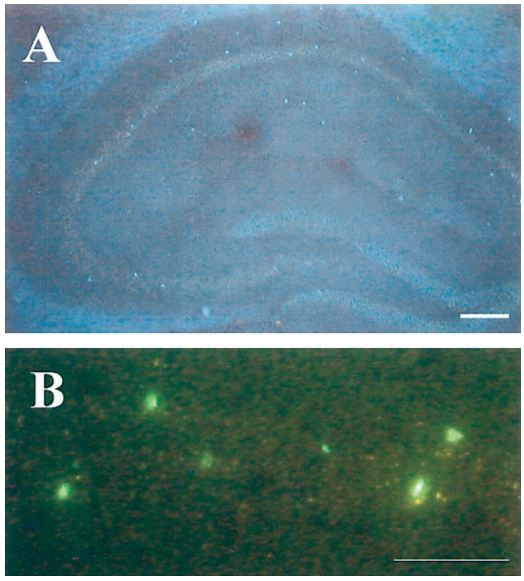

Figure 4. *A*, TFL-Zn fluorescence of hippocampus of a CaEDTA-treated *ZnT3*-null mouse 24 hr after kainate injection. Intraventricular injection of 2 μl of 300 mM CaEDTA markedly attenuated kainate seizure-induced zinc accumulation in CA3 and CA1 neurons. *B*, TUNEL staining of an adjacent brain section of *A*. CaEDTA injection markedly reduced CA1 neuronal death (compare with Fig. 1*F*). Scale bar, 500 μm.

membrane-impermeant chelator, CaEDTA, blocks zinc accumulation and neuronal death after seizures, ischemia, or trauma (Koh et al., 1996; Cuajungco and Lees, 1998a; Lee et al., 2000; Suh et al., 2000). Also in *ZnT3* null mice, CaEDTA nearly completely abolished zinc accumulation and neuronal cell death. These observations favor an external origin of zinc, which implies transient increases in extracellular zinc concentrations.

Although neuronal excitation could release a histochemically invisible, ZnT3-independent pool of zinc, another possibility is that zinc may be released intracellularly and then pumped out by transporters such as zinc-efflux transporter 1 (ZnT1) (Palmiter and Findley, 1995; McMahon and Cousins, 1998), raising local extracellular zinc concentrations. ZnT1 is readily induced in the hippocampus in response to insults such as ischemia (Tsuda et al., 1997). Once released to the extracellular space, the zinc would be available for uptake into vulnerable postsynaptic neurons via open ion channels (Frederickson et al., 1989; Koh et al., 1996). However, although the complete blockade of zinc accumulation and cell death by CaEDTA makes an external origin more likely, the present study cannot completely rule out the alternative possibility that zinc accumulation is an intrinsic event. For example, it seems plausible that hippocampal neurons *in vivo* may have a mechanism for internal release of zinc (Cuajungco and Lees, 1998b), a feature not shared by cortical neurons *in vitro* or cerebellar granule neurons *in vivo*, and that extracellular CaEDTA somehow drives intracellular zinc out of these neurons.

Regardless of which of these possible mechanisms is operational, zinc accumulation is likely a cause rather than a sequel of neuronal death, because its blockade protects against neuronal death. However, contrary to the current zinc translocation hypothesis (Frederickson, 1989; Choi and Koh, 1998; Lee et al., 1999), the present results obtained from *ZnT3*-null mice suggest that synaptic vesicle zinc may not be the principal source of toxic zinc accumulation. Further studies seem warranted to elucidate the detailed dynamics of zinc homeostasis in brain injury.

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