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Evidence for an Extracellular Zinc-Veneer in Rodent Brains from Experiments with Zn-Ionophores and ZnT3 Knockouts

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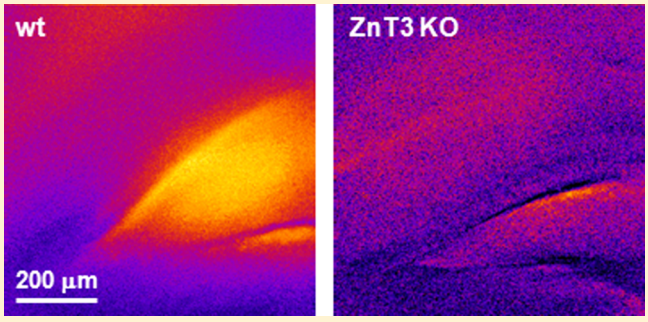
ABSTRACT: Ionic zinc is found at a high concentration in some glutamatergic vesicles of the mammalian brain. Ionic zinc is also found chelated to macromolecules in the extracellular space, constituting what has been called the "zinc veneer". In this communication we show that the zinc ionophore, pyrithione, can be used to demonstrate the presence of the veneer. Application of pyrithione without added ionic zinc to rodent hippocampal slices mobilizes extracellular zinc, which can be detected intracellularly by the zinc probe FluoZin-3. In addition, we show that ZnT3 null mice, which lack the transporter responsible for stocking synaptic vesicles, nevertheless do have a zinc veneer, albeit diminished compared to wild type animals. The presence of the zinc veneer in ZnT3 null mice may account for the absence of any marked deficit in these animals.



KEYWORDS: *Zinc, Synaptic, Ionophore, Hippocampus*

Work spanning a number of decades has shown that the synaptic vesicles in some glutamatergic terminals are stocked with zinc.[1] A combination of chemical and physical techniques has been used to demonstrate that the zinc in the vesicles is in a loosely chelated form.[2] This culminated in the finding that the protein ZnT3 is essential for loading zinc into the vesicles.[3] Although there is some evidence that zinc may act as neuromodulator, its precise role remains obscure.[4]

Free transition metal concentrations are kept very low within cells.[5] This prevents the mismetalation of proteins, restricts the possibility of metals precipitating proteins and metals entering into free radical reactions. For the case of copper which is redox active, and hence needs to be kept under tight control, this is very well understood.[6] Less is known about zinc but it is becoming clear that it has a free metal concentration of about ~0.5 nM [7] and is controlled by the interplay between zinc influx, efflux, and buffering (Figure 1).

The extracellular space in the brain, which constitutes ~20% of total volume, is an important pathway for the passage of molecules.[8] It is believed that zinc is released during the course of synaptic transmission by certain glutamatergic terminals and that there is a transient elevation in the free metal concentration in the extracellular space, approaching somewhere between 1 and 300 μM.[1] However, recent work from our lab has called into question whether synaptic zinc is indeed released in an unfettered form.[9]

What are the levels of zinc in quiescent periods in the extracellular space? We have found evidence for what we have termed the "zinc veneer", that is zinc loosely associated with the extracellular aspect of neurons and probably glia.[10] Application of the membrane impermeant zinc-sensitive fluorescent probe FluoZin-3, led to the increase of fluorescence overlying the

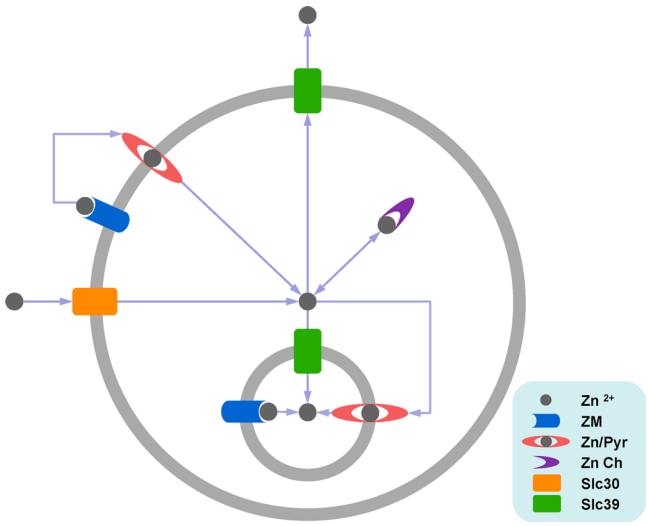


Figure 1. Diagram illustrating processes involved in homeostasis in a typical cell, zinc ionophores and the zinc veneer. Intracellular compartments of all kinds are represented by the interior circle. Zn Ch, represents any proteins or ligands that bind zinc, including exogenous probes. Transporters carrying zinc into the cell belong to the Slc39 family, while those carrying zinc out of the cell belong to the Scl30 family.

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mossy fibers and hilus in unstimulated slices. This fluorescence was sensitive to the application of the membrane impermeant chelator EDPA.[10] This suggests that the fluorescence can be attributed to zinc-probe complex in the extracellular space. We proposed that zinc is coordinated to the membrane through extracellular macromolecules and can form ternary complexes with the probe. Further substantiation of this hypothesis came from experiments with the zinc probe ZnAF-2, which was believed to be membrane impermeant, but which we demonstrated to be membrane permeant.[11] We showed that ZnAF-2 made evident a veneer that was low in neonates and that grew with time.[9]

In this communication, we provide further evidence for the existence of the zinc veneer using zinc ionophores. Ionophores are natural or synthetic molecules that can partition into biological membranes and shuttle ions across the membrane because they can form complexes that shield the ions from the hydrophobic environment of the membrane (Figure 1).[12]

RESULTS

Our strategy to reveal the existence of a weakly chelated layer of zinc in the extracellular space was to use zinc ionophores to mobilize extracellular zinc and monitor its influx into cells with the zinc-sensitive fluorescent probe FluoZin-3. We have used the zinc-ionophore, pyrithione (Pyr), which forms a 2:1 (ligand:metal) hydrophobic complex and shuttles zinc across membranes down a concentration gradient.[13]

To follow the influx of zinc into cells we loaded brain slices with the acetoxymethyl (AM) ester form of the zinc-sensitive indicator FluoZin-3 [14] that leads to accumulation of the probe intracellularly. Application of the membrane impermeant chelator Ca-EDTA [15] leads to a small reduction in the signal confirming that most of the signal arises from intracellularly entrapped probe (Figure 3). In a previous publication we have provided evidence that the increases registered by intracellular FluoZin-3 arise primarily from intracellular compartments rather than from the cytoplasm.[16] However, in this communication we are only interested in whether zinc gets into cells, and not in its precise localization.

To test the efficacy of this procedure Zn/Pyr was applied to hippocampal slices loaded with FluoZin-3 AM for 5 min and then monitored for up to an hour. This led to an increase in fluorescence that outlasted the Zn/Pyr application (Figure 2). Application of the membrane impermeant chelator Ca-EDTA after washing off the Zn/Pyr did not diminish the fluorescence (data not shown). All of this suggests that entrapped FluoZin-3 can serve as an effective sensor for the influx of zinc induced by pyrithione.

To determine the source of zinc we have relied on the clearly delineated anatomy of the hippocampus and the arrangement of zinc-rich terminals.[17] The principal cells of the dentate gyrus, the granule cells, are organized into layers, the granule cell layer (GCL, demarcated with dashed lines in figures) that separates two layers of neuropil, the molecular layer (ML) and the hilus (Figure 2); the later contains the zinc-rich terminals of the granule cells.

We reasoned that if pyrithione in the absence of exogenous zinc was applied to slices it should, if chelatable zinc was indeed outside cells, lead to an increase in intracellular zinc. This is indeed what happens, with the application of 20 μM pyrithione for 5 min leading to an increase in fluorescence (Figure 3). However from this experiment alone it could be argued that the zinc is either a contaminant of the pyrithione or of the saline solution. Treating the pyrithione with chelator beads (BAPTA beads, Molecular Probes) did not abolish this, excluding zinc contamination of the pyrithione as the source of the metal.

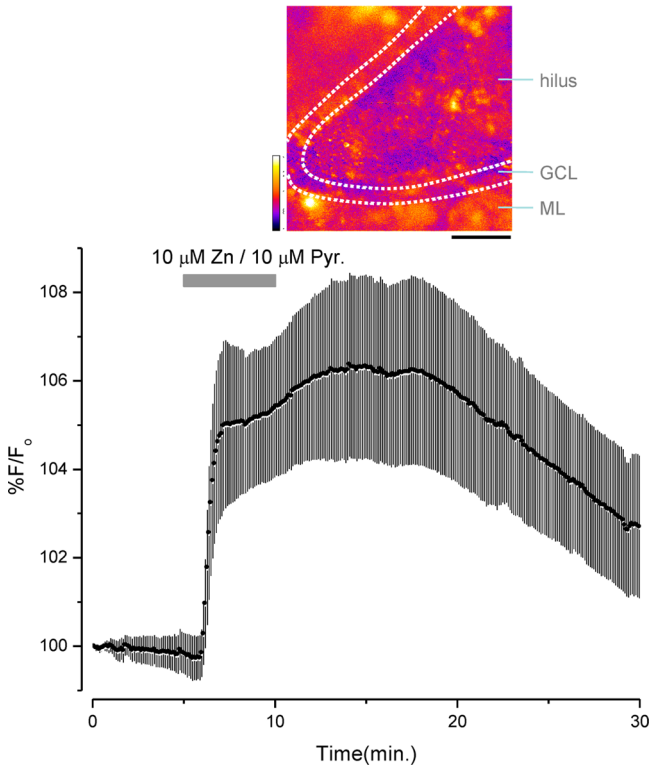

Figure 2. Detecting the influx of zinc into FluoZin-3 loaded hippocampal slice after exposure to Zn/Pyr (n = 4). The pseudocolored inset is a difference image between the peak of the response and the time prior to Zn/Pyr application. The dashed lines demarcate the granule cell layer (GCL). ML, molecular layer. The pseudocolored scale runs from 1 to 30. Scale bar 200 μm.

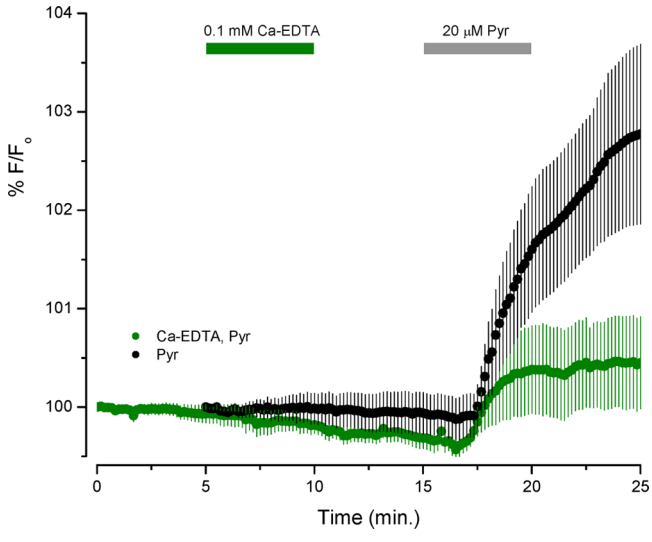

Figure 3. Pretreatment of neocortical slices loaded with FluoZin-3 with Ca-EDTA leads to a diminution of the zinc influx induced by pyrithione. The green points represent experiments where 0.1 mM Ca-EDTA was applied for 5 min, followed by a wash period of 5 min and then the application of 20 μM pyrithione for 5 min (n = 6). The black points represent experiments where only 20 μM pyrithione was applied (n = 9).

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Moreover, no zinc could be detected with FluoZin-3 in pyrithione solutions (data not shown).

To exclude the saline as the source of the zinc the following three stage experiment was performed: neocortical slices were exposed to 100 μM Ca-EDTA for 5 min to chelate zinc in the veneer, this was followed by a 5 min wash with control saline to remove the Ca-EDTA; and only then was pyrithione applied. Control slices, which were not exposed to Ca-EDTA showed a greater increase in fluorescence (Figure 3). This experiment confirms that pyrithione picks up zinc in the extracellular space rather than from the perfusing saline. If the zinc were derived from the saline it would be resupplied during the wash phase and the application of pyrithione should lead to equal increases under both conditions. This experiment also suggests that it is unlikely that pyrithione acts by liberating zinc from intracellular sites.

Pyrithione is commonly used to induce the influx of zinc into cells;[13] however, since it is an ionophore it should also facilitate zinc efflux from cells if an outward directed gradient is created by chelating zinc in the extracellular space. If pyrithione and Ca-EDTA are applied simultaneously to slices, there is first an increase in fluorescence followed by a decrease below baseline, which is consistent with pyrithione inducing an efflux after the zinc from the veneer has been chelated (Figure 4a). But what about the increase in fluorescence? We suggest that it occurs in the following way: Ca-EDTA binds zinc rather slowly, as it has to wait for calcium to dissociate.[18] Immediately after the application of Ca-EDTA/Pyr, pyrithione appropriates zinc from the veneer and carries it down its concentration gradient into the cell. Later when Ca-EDTA has abstracted zinc from the veneer, the gradient reverses and now zinc can flow out of the cell.

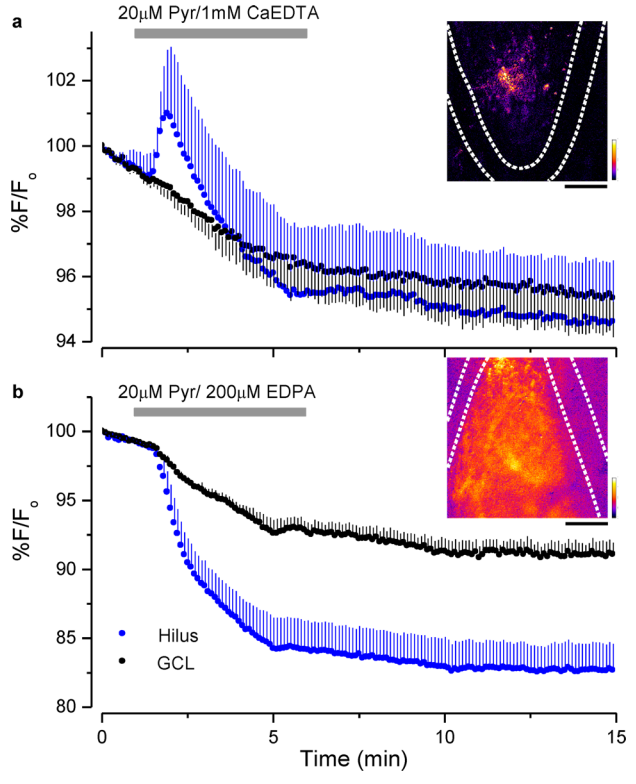

Figure 4. Coapplication of pyrithione and chelators (a) Simultaneous application of pyrithione and Ca-EDTA because of the slow zinc chelation by the latter, allows pyrithione to pick up zinc in the veneer. Inset: pseudocolor difference image: peak of the response minus that just prior to the application of Ca-EDTA/pyr. Color scale bar, 2−16 (b) EDPA rapidly abstracts zinc and allows zinc to flow down its concentration gradient out of the slice. Inset: pseudocolor difference image: Fluorescence at 12 min minus that prior to the application of EDPA/Pyr. Color scale bar 0−19. The dashed lines demarcate the GCL. Scale bars 200 μm.

How can we confirm this scenario? We have previously shown that the impermeant chelator EDPA binds zinc more rapidly than Ca-EDTA.[10] If pyrithione is applied together with EDPA, the increase in fluorescence is eliminated and the fluorescence declines monotonically (Figure 4b). This is consistent with EDPA chelating zinc from the veneer before pyrithione has time to carry it into the cell. EDPA is also more effective in removing zinc from the veneer than Ca-EDTA and hence leads to a more pronounced dip in fluorescence than does Ca-EDTA.

In the case of Ca-EDTA the decline of fluorescence is slower, consistent with its slower binding kinetics. If pyrithione and Ca-EDTA are applied for a longer period (~40 min) it also leads to an undershoot of the fluorescence (data not shown).

The coapplication of pyrithione and chelators provides further confirmation of the veneer. The increase of fluorescence induced by Ca-EDTA/Pyr overlies the hilus and not the cell bodies, which confirms that pyrithione picks up zinc in the hilus consistent with the existence of a veneer overlying the hilus, and not from the solution (Figure 4a and inset). Similarly in the EDPA/Pyr experiments the decline in fluorescence is more marked over the hilus than the GCL (Figure 4b and inset).

We have shown previously that in slices loaded with FluoZin-3 that stimulation with high KCl, which depolarizes neurons, leads to an increase in the fluorescence overlying areas exhibiting high levels of fluorescence.[19] Stimulation leads to the externalization of zinc within synaptic vesicles, where the zinc bound to macromolecules within vesicles is presented to the extracellular space but not freely released. On stimulating slices with KCl an increase in fluorescence is observed largely overlying the hilus. When the KCl is washed off there is some decline in fluorescence, which is accelerated by the application of EDPA (Figure 5). This suggests that after stimulation synaptic zinc becomes part of the zinc veneer, which is then open to chelation by a membrane impermeant chelator.

It seems likely that ZnT3 null mice, which do not have synaptic zinc, might have a zinc veneer, since the putative macromolecule is postulated to be distinct from ZnT3. To test this hypothesis we quantified the zinc veneer in mouse hippocampal slices using FluoZin-3 free acid, which is membrane impermeant.[10] Slices were exposed to 2 μM FluoZin-3 and 50 μM Ca-EDTA (to remove trace zinc in the solutions) and then to 1 mM EDPA to remove the extracellular zinc. The extracellular fluorescence attributable to the veneer is then quantified as the percentage of EDPA chelatable fluorescence (ECF, see Methods). The results of these experiments are shown in Figure 6. The ECF in ZnT3 null mice is significantly lower than that in age matched wild type mice. However the veneer is still present in the hilus and area CA1, as can be seen if one compares it to the ECF of the granule cell layer (Figure 6c).

DISCUSSION

Other than the purported transient elevations in the extracellular space, not much thought seems to have been given to zinc concentrations in the extracellular space. In this

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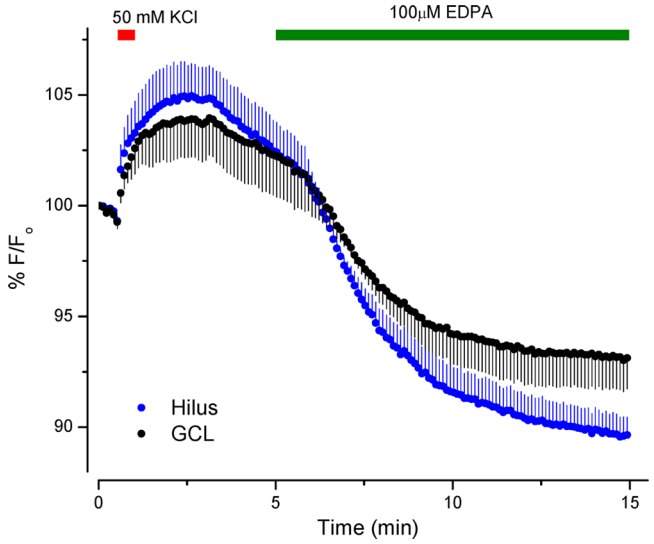

Figure 5. Potassium stimulation of hippocampal slices loaded with FluoZin-3 AM leads to an increase in fluorescence overlying the hilus that is chelated by EDPA.

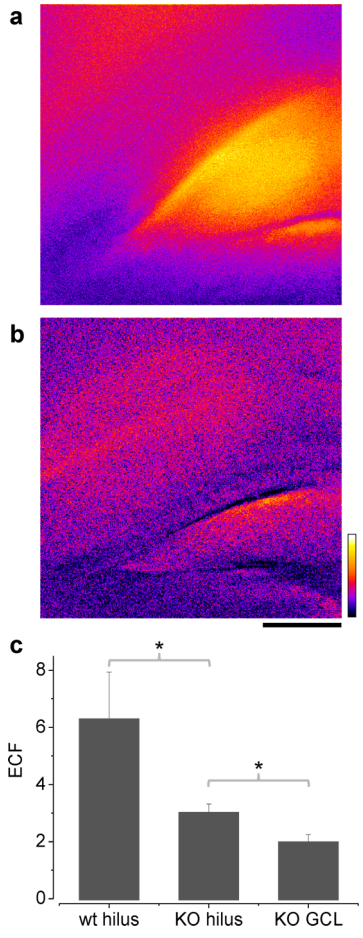

Figure 6. Visualizing and quantifying the synaptic veneer in wt and ZnT3 null mice. Pseudocolor difference images of the dentate gyrus of mouse hippocampi (control = EDPA) (a) wild-type (color scale 0–33) (b) ZnT3 null (color scale 0–13). Scale bar 200 μm. (c) Quantification of the veneer. EDPA chelatable fluorescence (ECF) in the wt hilus (n = 6), ZnT3 KO hilus (n = 8) and ZnT3 KO GCL (n = 8). * $p < 0.05$.

communication we have shown that it is possible to detect chelatable zinc in the extracellular space with a combination of an intracellular zinc probe, zinc-ionophores and chelators. Our experiments confirm that there is weakly chelated zinc in the extracellular space that can be mobilized by the application of ionophores.

Ionophores can serve as passive shuttles for ions moving across membranes and have found widespread use in experimental biology to equilibrate ions across the membrane. For example A23187 is used to equilibrate calcium across membranes for calibrating calcium probes.[20] Pyrithione was first used by Forbes et al. [13] as a means to induce the passage of zinc into cells and since then has been widely used. Other compounds that act as ionophores are: clioquinol,[21] diiodoquin,[22] maltol [23] and Zinquin.[24]

There is a growing recognition that ionophores may be useful as clinical agents. For example it has been suggested that clioquinol and its congeners may be of value in clearing metal from amyloid plaques in Alzheimer's disease.[25] It is important to realize that ionophores only allow metals to follow their concentration gradients. Application of a Zn-ionophore will only lead to a decrease of intracellular zinc if (a) the extracellular zinc concentration is very low or (b) if the ionophore is coapplied with an impermeant chelator.

To serve as an ionophore, a compound should have a zinc-binding site, the capacity to form 2:1 complexes and a hydrophobic external aspect. Since these features are quite common, we anticipate that there may be a number of unsuspected zinc ionophores in the current pharmacopeia. Moreover, their ionophoric action could play a role in their pharmacological activity.

Since most of the fluorescence evident in FluoZin-3 AM labeled slices arises from intracellular vesicles, our experiments suggest that pyrithione is able to partition into the vesicular membrane. In the presence of extracellular EDPA/Pyr, the zinc then flows from the vesicles into the cytoplasm and thence into the extracellular space where it is bound by the EDPA. Unfortunately, there do not appear to be any zinc-ionophores that are restricted to the plasma membrane. Our prediction would be that, if such ionophores were available then on the application of the ionophore and Ca-EDTA the fluorescence would decline far more slowly than in the case of pyrithione/Ca-EDTA. This would be so because little efflux from synaptic vesicles into the cytoplasm would occur and because there is little free zinc in the cytoplasm, therefore little would exit through the plasma membrane ionophore.

The zinc veneer is, as the name suggests, a rather fleeting phenomenon and it would be nice to have evidence from other techniques for its existence. One of the earliest methods for visualizing synaptic zinc is the Timm's stain where the metal precipitate formed by the treatment of tissue with disulfide can be viewed after silver intensification. Electron microscopy of Timm's stained hippocampal slices reveals zinc within synaptic vesicles, but also on the extracellular face of presynaptic membranes,[26] consistent with the existence of the zinc veneer. We were also able to detect the veneer with the fluorescent zinc probe ZnAF-2, which we postulate forms ternary complexes with zinc and the putative veneer macromolecule. The formation of ternary complexes with the zinc probes Zinquin and TSQ has recently been demonstrated by Petering's group.[27,28]

We have proposed that the zinc veneer arises from zinc-coordinated to macromolecules (VM) (Figure 1) expressed on

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the extracellular aspect of cells. Furthermore, we have suggested that these macromolecules reach the plasma membrane via exocytosis, externalizing but not releasing zinc. This may seem a rather fruitless exercise, however the coordinated zinc may serve to form ternary complexes with molecules in the extracellular space and the ternary complexes so formed may serve to transduce signals within the cell.[4]

Our experiments have shown that the veneer persists in ZnT3 null mice, which may explain why these animals do not have any marked abnormality.[3] The presence of the veneer in ZnT3 null mice suggest that VMs may pick up extracellular zinc in passage, bound to proteins or ligands or that VMs are upregulated. We would like to suggest that the long sought role of synaptic zinc may only become manifest when the molecular identity of the VM is revealed.

METHODS

All animal procedures were in accordance with the NIH Guide for the Care and use of Laboratory Animals and approved by the Institutional Animal Care and Use committee of the University of Iowa. Male Sprague–Dawley rats (16–65 day old), c57bl or ZnT3 null mice (50–65 day old) were decapitated, the brain removed and placed in ice-cold normal saline containing (in mM); 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.3 $MgSO_4$, 25 $NaHCO_3$, 1.25 NaH_2PO_4, and 25 glucose, bubbled with 95% O_2-5% CO_2. Slices were cut at a thickness of 400 μm on a McIlwain tissue chopper, and held in an interface chamber at room temperature for at least one hour prior to loading with the zinc indicator. Slices were loaded in FluoZin-3 AM (5 μM) for at least one hour.

The slices were stabilized with a U-shaped stainless steel wire cross-strung with nylon fibers in a temperature-controlled chamber (RC-27 L; Warner Instruments). Images were acquired on an Olympus Optical BX50WI upright microscope. Illumination was provided by a monochromator set at 480 nm (TILL Photonics), passed through a dichroic (T495lp; Chroma Technology) and then through a filter (ET525/50 m; Chroma Technology) onto the faceplate of a Princeton Instruments cooled CCD camera. Images were acquired with the MetaFluor program (Universal Imaging Corporation) and analyzed using ImageJ (NIH). The fluorescence intensity was expressed as % F/F_o, where F is the fluorescence intensity and F_o the fluorescence intensity at time zero. The dark current of the CCD was subtracted from all intensity measurements. To quantify the zinc veneer we measured what we termed the ECF (EDPA chelatable fluorescence) $\%(F_a - F_b)/F_b$, where F_a is the fluorescence measured in the presence of 2 μM FluoZin-3 and 50 μM Ca-EDTA (to chelate contaminating zinc in the saline) and F_b is the fluorescence measured after the further addition of 1 mM EDPA. All data are expressed as mean ± SEM. Student's t tests were performed using Excel (Microsoft). Statistical significance is determined by $p < 0.05$.

Reagents. Fluozin-3 (Invitrogen), all other chemicals were obtained from Sigma-Aldrich.

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Author Contributions

I.N. and S.M.R. contributed equally to the manuscript. A.R.K. conceived the project. I.N. performed experiments in Figures 1, 3, and 4. S.M.R. performed the experiments in Figure 2. A.R.K. performed experiments in Figure 5, and J.Z. performed the fluorimeter experiments. I.N., S.M.R., and A.R.K. analyzed the data. A.R.K. wrote the manuscript with input from I.N., SMR and J.Z.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

EDPA, ethylenediamine-*N*,*N*′-diacetic-*N*–*N*′-di-β-propionic acid; GCL, granule cell layer; ML, molecular layer; Pyr, pyrithione; VM, veneer macromolecule

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