Zinc Is Externalized Rather than Released during Synaptic Transmission

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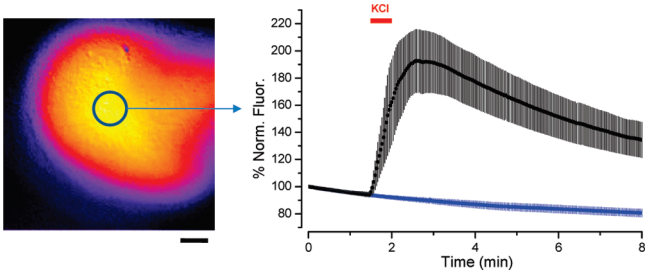
Abstract



The synaptic vesicles of some glutamatergic terminals contain a high concentration of zinc that serves functions that remain obscure. In this publication, we have used the membrane permeant zinc fluophore, ZnAF-2, to determine whether zinc is released during the course of synaptic transmission. Stimulation of the slices either with high potassium or electrically, leads to an increase in fluorescence that long outlasts the stimulus and remains elevated for many minutes. We demonstrate that this response is inconsistent with the free release of zinc but is consistent with the presentation of zinc coordinated to macromolecules within the exocytosed vesicles to the extracellular space; a process we term "externalization". Our data suggests a novel mechanism of synaptic transmission at zinc-rich glutamatergic terminals that distinguishes them from their metal free counterparts.

Keywords: fluorescence, zinc, synaptic, vesicles, hippocampus, glutamatergic

There is abundant evidence to suggest that a subpopulation of glutamatergic terminals in the mammalian brain contain high concentrations of ionic zinc (*1*). Although this has been known for a long time, the precise role of this zinc remains uncertain. The presence of the ion in synaptic vesicles naturally led to the notion that during synaptic transmission zinc is released and acts as a modulator of neurotransmitter receptors (*2*). Although there is some evidence for this hypothesis, it has been difficult to establish unequivocally (*3*).

Fluorimetric probes present a means for detecting and localizing ions in live cells that has been particularly useful in the case of calcium. A great number of zinc probes have been synthesized (*4*), and some have proven valuable in localization pools of weakly chelated zinc; however, their record in sorting out whether zinc is released has been less successful. Part of the problem has been in determining the localization of the probes, since the interpretation of results depends crucially on the correct identification of their location within the cell (*5*).

In this publication, we extend our previous work on the probe ZnAF-2 (*6*), which passes across membranes and localizes in synaptic vesicles, to determine whether zinc is released from synaptic vesicles (*7*). We show that the fluorescence response is inconsistent with zinc release and that, rather than being released, zinc is presented to the extracellular space coordinated to macromolecules on the presynaptic membrane (Figure 1).

Results and Discussion

There is little dispute that certain well-defined neuronal pathways have synaptic vesicles that are filled with ionic zinc; however, whether zinc is released in the course of synaptic transmission remains contentious. In this communication, we have used the fluorimetric zinc probe ZnAF-2 to determine whether zinc is indeed released from hippocampal slices. For the sensor to serve as a reliable reporter in this context, it is necessary to know the location of the probe, minimize experimental artifacts, and understand the probes chemistry. Moreover, to interpret the data, we need an explicit model of the zinc probe in the slice to anticipate what might happen when exocytosis occurs.

We have shown previously that the application of the free-acid form of ZnAF-2 to live hippocampal slices led to the demarcation of the mossy fibers and hilus, which have high levels of vesicular zinc (*3*). We were able to show that slices loaded with ZnAF-2 and then washed with normal saline did not respond to the application of exogenous zinc, confirming the intracellular location of the probe. Because of the probe's intravesicular location, stimulation of synaptic activity should lead to a decrease of fluorescence in the hilus and mossy fibers, if

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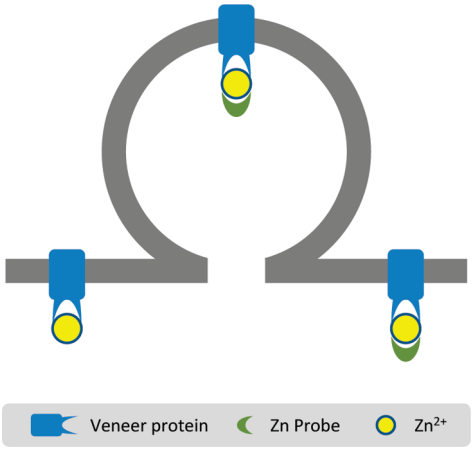

Figure 1. Schematic illustrating zinc externalization from synaptic vesicles.

the probe−zinc complex diffuses out after exocytosis. This should occur much in the same way that the destaining of synaptic terminals preloaded with FM1-43 can be used to monitor neurotransmitter release from presynaptic terminals (*8*). However, contrary to this expectation, synaptic stimulation leads to an increase of fluorescence overlying fluorescent puncta that is sustained for many minutes after the cessation of the stimulus (Figure 2). In what follows, we have attempted to account for the origins of this stimulus-induced increase in fluorescence (abbreviated as SIIF). There are two aspects of the SIIF that require explanation: (1) why the fluorescence increases rather than decreases and (2) why the increase persists for so long after cessation of stimulation.

All experiments have been carried out in the hilus of the dentate gyrus; however, similar results were obtained for the hippocampal mossy fibers and synaptic terminals in the neocortex.

Hippocampal slices derived from ZnT3 knockout mice, which show no signs of vesicular zinc in Timm's stained sections (*9*), exhibit little fluorescence after loading with ZnAF-2 compared with wild-type (wt) animals (Figure 3). The fluorescent intensity of the hilus in wt mice was ∼16 times that of unstained slices, while that of ZnT3 KOs was ∼2.5times. This suggests that the fluorescence induced by ZnAF-2 staining arises from synaptic vesicles, with very little contribution from any other intracellular compartments. Synaptic puncta can be seen in high-power images of rat dentate gyrus with neuronal somata in dark relief (Figure 3c).

Because of the susceptibility of fluorescent ion-sensor experiments to artifacts, we should first inquire whether the SIIF is unrelated to changes of zinc within synaptic vesicles. KCl stimulation of unlabeled slices led to a rapid and short-lived increase in autofluorescence that declined below baseline within a minute. The fact that the time course is so different from the SIIF makes it

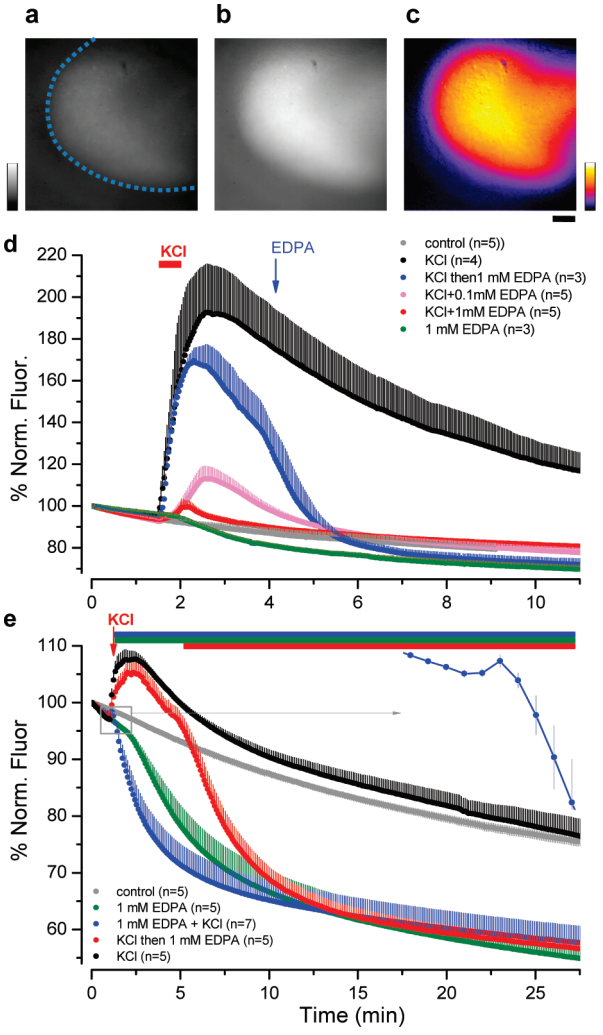

Figure 2. The stimulus-induced increase in fluorescence in young and old hippocampal slices. (a) Fluorescence of the dentate gyrus prior to KCl stimulation. The outer edge of the hilus is demarcated by the dotted line. The cell bodies lie on the concave side of this line. (b) Fluorescence 2 min after stimulation. Gray scale runs from 540 to 770 au. (c) Pseudocolor image of the difference between images a and b. Color scale runs from 35 to 140 au. Scale bar 100 μm. (d) Fluorescence of ZnAF-2 loaded hippocampal slices from young rats: (black circles) 50 mM KCl stimulation (30 s); (blue circles) fluorescence response to 50 mM KCl stimulation followed by 2 min wash and then 1 mM EDPA application; (pink circles) 50 mKCl and 0.1 mM EDPA applied simultaneously; (red circles) 50 mM KCl and 1 mM EDPA applied simultaneously; (green circles) 1 mM EDPA without KCl stimulation; (gray circles) control slices. (e) Fluorescence of ZnAF-2 loaded hippocampal slices from older rats: (black circles) addition of 50 mM KCl for 30 s; (blue circles) 1 mM EDPA and 50 mM KCl simultaneously; (green circles) 1 mM EDPA addition to unstimulated slices; (red circles) 50 mM KCl for 30 s followed by 1 mM EDPA addition after 2 min; (gray circles) control slices. Bars indicate the time and duration of EDPA application. Inset shows a blow-up of the graph close to the simultaneous addition of EDPA and KCl. Sum of two exponential fits to the curves: red curve, $\tau_1 = 174 \pm 2.6$ s, $A_1 = 86.7\% \pm 1.2\%$, $\tau_2 = 3313 \pm 1155$ s, $A_2 = 13.3\% \pm 2.7\%$; black curve, $\tau_1 = 199.3 \pm 24.6$ s, $A_1 = 60.6\% \pm 0.6\%$, $\tau_2 = 2007 \pm 277$ s, $A_2 = 39.4\% \pm 1.8\%$; blue curve, $\tau_1 = 96.1 \pm 0.3$ s, $A_1 = 72.2\% \pm 0.8\%$, $\tau_2 = 1193 \pm 21$ s, $A_2 = 27.7\% \pm 0.1\%$.

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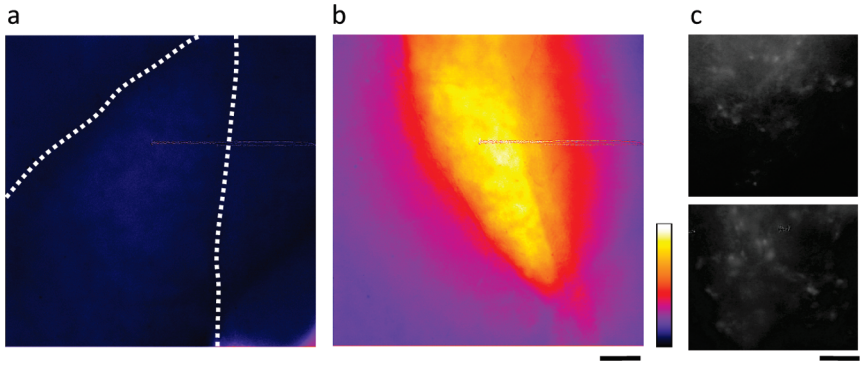

Figure 3. Genetic ablation of the zinc transporter ZnT3 eliminates synaptic zinc and ZnAF-2 fluorescence. Pseudocolor images of the dentate gyrus of mice, (a) ZnT3 KO or (b) wt, after staining with ZnAF-2 (5 μM) for 1 h. The dotted line demarcates the inner edge of the cell body layer. Both images were captured under identical conditions and have been scaled over the same range that runs from 530 to 820 au. Scale bar 100 μm. The horizontal lines in both images result from defects in the CCD chip. (c) Synaptic puncta in the hilus of rat dentate gyrus stained with ZnAF-2. Scale 20 μm.

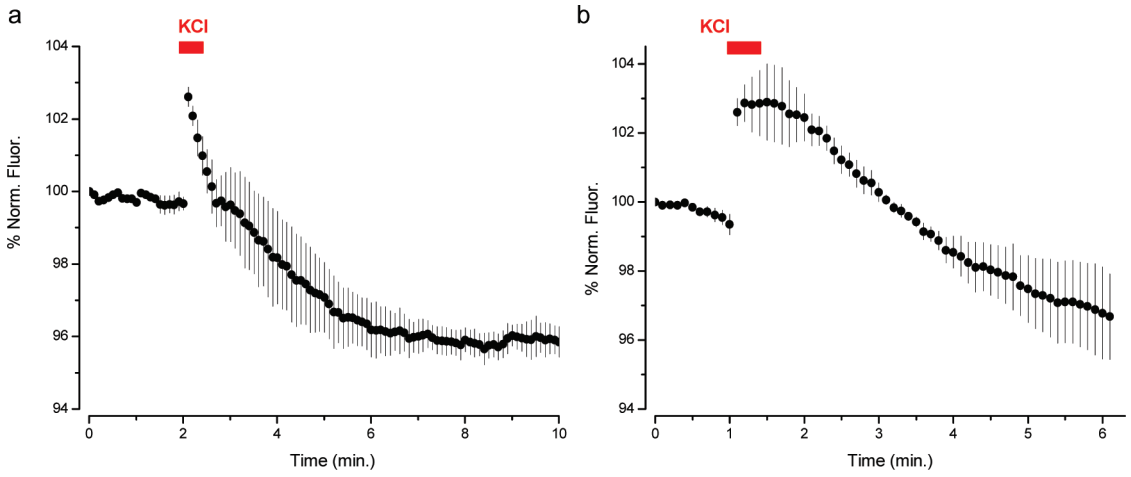

Figure 4. (a) Response of the hilus of unstained hippocampal slices to 50 mM KCl (30 s) (n = 3) and (b) change in emission of fluorescent microspheres embedded in slice to application 50 mM KCl for 30 s (n = 3). Fluorescent microspheres (Dragon Green, Bangs Beads) with diameters of ~1 μm were used.

unlikely that the SIIF arises from changes in autofluorescence (Figure 4a). KCl stimulation also induces alterations in the light scattering properties of slices, which could lead to changes in fluorescence (*10*). High potassium stimulation produces a decrease in scattering as shown by the increase in transmittance of light. This could produce an increase in intensity of a fluorophore, because the number of photons reaching the molecule and the detector might increase. To determine whether changes in scattering have any impact on our results, fluorescent beads were placed ~50 μm below the slice surface by pressure ejection from a glass electrode and imaged during KCl stimulation (Figure 4b). This led to a more rapid increase in fluorescence than the SIIF, but it did not endure for as long as the SIIF. All this makes it rather unlikely that any significant portion of the SIIF results from changes in scattering.

It could be argued that the KCl stimulation is a somewhat unnatural stimulus and might evoke pathological phenomena. Electrical stimulation of the hilus led to an increase in fluorescence that endured for many minutes similar to that induced by KCl stimulation (Figure 5). A SIIF was also elicited by the application of a high sucrose solution (data not shown), a stimulus that is widely used to explore synaptic physiology (*11*). It might also be argued that the high potassium stimulus might lead to irreversible damage. However, repeated KCl applications could elicit SIIFs (Figure 6b).

Although this article is primarily focused on experiments using ZnAF-2, we have also observed phenomenologically similar SIIFs with the following zinc probes: zinquin (*12*), ZP1 (Zinpyr-1), ZP4 (*13*), and ZnAF-2F (*14*), and in slices loaded with FluoZin-3 AM (*15*). Similar responses have been reported by others with AZn2 (*16*). This suggests that the SIIF is not some peculiarity of the chemistry of ZnAF-2 but reflects changes in the intravesicular zinc provoked by synaptic stimulation.

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It is worth noting that in the course of our experiments we found that the zinc ionophore, pyrithione quenches the fluorescence of ZnAF-2 and ZnAF-2F. In cuvette experiments, 5 μM pyrithione quenches the fluorescence of ZnAF-2 (0.5 and 10 μM $ZnSO_4$) by ~60%. Addition of more zinc does not alleviate the quenching, which suggests that pyrithione does not compete with the sensor for zinc. Pyrithione does not quench the fluorescence of FluoZin-3 under similar conditions.

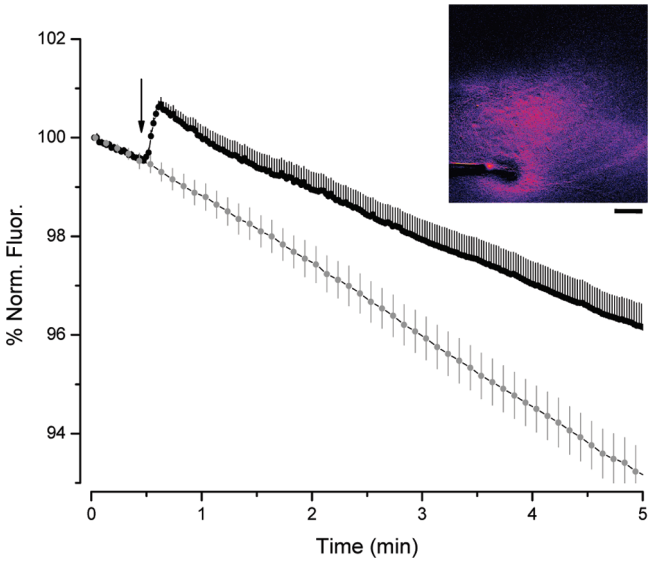

Figure 5. Electrical stimulation induces an SIIF. Response of slices loaded with ZnAF-2 to electrical stimulation (500 pulses, 100 Hz). The arrow indicates the start of the electrical stimulus (n = 8). Inset shows the pseudocolor image of the difference between the fluorescence at the peak of the response and that just before applying the stimulus. Scale bar 100 μm.

When the zinc chelator EDPA (*17*), which has a low affinity for calcium and magnesium, is applied to mature hippocampal slices loaded with ZnAF-2, it leads to the slow quenching of a fraction of the fluorescence. We attribute this to what we have termed the "zinc veneer", a layer of zinc associated with the extracellular face of the plasma membrane (Figure 2e). We postulate that ZnAF-2 forms ternary complexes between zinc and as yet unidentified macromolecules. The application of a membrane-impermeant chelator then abstracts the zinc, leading to a decline in fluorescence. It is important to note that there do not appear to be significant levels of free ZnAF-2 in the extracellular space, since the infusion of zinc does not lead to a rapid increase in fluorescence (*7*).

In our previous publication (*7*), we did not observe as large a decline in response to membrane impermeant chelators as depicted in Figure 2e, suggesting that the veneer increases as the rats age. In experiments on slices derived from rats between the ages of 14 and 18 days, there was little appreciable veneer, yet the slices exhibited a robust SIIF (Figure 2d). The mean fluorescence intensities in the hilus of the dentate gyrus were 139 ± 9 for young rats (14−18 d; n = 17) and 335 ± 25 for old rats (26−31 d; n = 16) (arbitrary units). This is consistent with an increase in veneer in the older rats.

Might some of the EDPA chelatable signal be attributable to probe−zinc complex associated with damaged cells, which would be more prevalent in older animals? If this were so, one would expect the fluorescence to decline more rapidly in older animals than in younger ones. This turns out not to be so (cf. controls in Figure 2d,e). Moreover, one would expect that

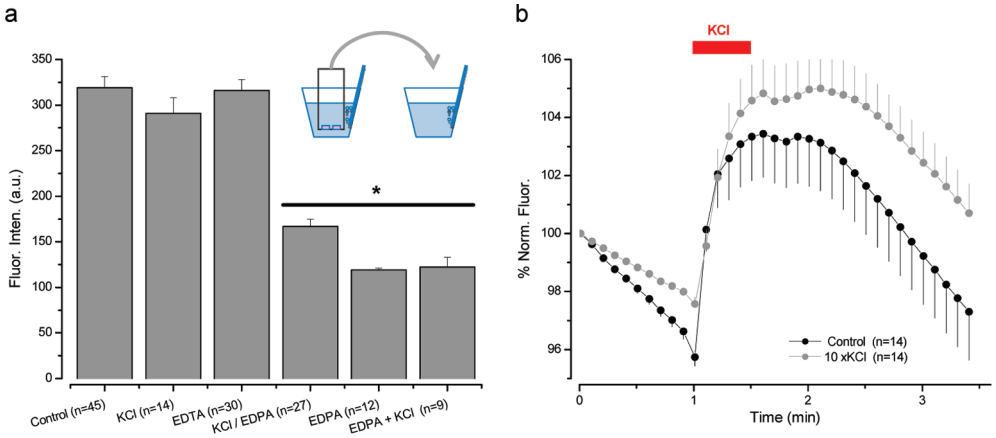

Figure 6. Effect of repeated stimulation on synaptic zinc. (a) The average fluorescent intensity in the hilus of the dentate gyrus for the slices subjected to different stimulation regimes. The slices were held on nylon stocking stretched and glued over the end of a cutoff 15 mL centrifuge tube, suspended in a 15 mL beaker filled with 10 mL of saline, bubbled with carbogen through a microfil needle (WPI Inc.) (see inset). The slices were transported on the net between beakers to change the solution. After these manipulations, the slices were loaded in 10 μM ZnAF-2 for 30 min at room temperature and washed with saline for at least 5 min, and then the fluorescence intensity was measured in the hilus. Only the last three conditions are significantly different from the first three. (b) Response of slices to KCl stimulation (50 mM, 30s); control slices were not stimulated with KCl prior to loading with ZnAF-2. "10xKCl" slices were subjected to ten 30 s KCl stimuli before ZnAF-2 loading (same as "KCl" in panel a).

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Ca-EDTA should lead to a decrease in fluorescence similar to that of EDPA. If the ZnAF-2−zinc complex were free, Ca-EDTA should be able to abstract the zinc from the complex as it does in cuvette experiments. The application of 1 mM Ca-EDTA leads to no acceleration of the loss of fluorescence (data not shown). The EDPA-sensitive ZnAF-2 signal in the dentate gyrus is associated largely with fluorescent puncta in the hilus (Figure 3c). If it were associated with damaged cells, we might expect a more widespread distribution. In all, these results suggest that the fraction of the fluorescence susceptible to EDPA chelation cannot be attributed in any large measure to cell damage.

When hippocampal slices were stimulated with 50 mM KCl for 30 s, then washed for 1.5 min, followed by the addition of 1 mM EDPA, the chelator brought down the fluorescence more rapidly than was the case where EDPA was not applied (Figure 2d,e). This is consistent with the chelator appropriating zinc from the probe when vesicles undergo exocytosis and the probe is exposed to the extracellular space. However, the level of the fluorescence does not decline below that of control slices, making the changes inconsistent with zinc release (Figure 2d,e), since release should lead to a net loss of the fluorescence associated with the synaptic vesicles.

When EDPA was applied to slices before a KCl stimulus, the rising phase of the SIIF was truncated but was nevertheless detectable, and afterward the fluorescence declined monotonically, but again not to levels below that of the EDPA control (Figure 2 e).

In the older animals, it is difficult to distinguish the fraction of fluorescence removed by EDPA with and without stimulation. This is not so in younger animals where there is little detectable veneer and the fluorescence only becomes open to chelation during stimulation (Figure 2d). Here the interpretation is clearer; during exocytosis zinc is externalized and becomes available for chelation, whereas in older animals the separation is less obvious.

These results suggest that during the course of exocytosis zinc is presented to the extracellular space (i.e., externalized, Figure 1), becoming available for chelation; however, with a single KCl stimulus, the decline in fluorescence is insufficient to detect the loss of zinc. To determine whether a net loss of fluorescence, and hence loss of zinc, could be induced, slices were subjected to repeated KCl stimuli. The experiments were performed as follows: a group of slices was subjected to 50 mM KCl for 30 s either in the presence (KCl/EDPA) or absence of 1 mM EDPA (KCl). The slices were then transferred to control saline for 5 min. The stimulus was repeated 10 times, and then the slices were loaded with ZnAF-2 (10 μM) for 30 min and washed with saline, and the fluorescence intensity measured in the hilus. Control experiments were also performed where the slices were subjected to the same manipulations without KCl stimulation (control) (Figure 6a). Further batches of slices were exposed to 1 mM Ca-EDTA during the whole 1 h but not subjected to KCl stimulation (Ca-EDTA), just EDPA (EDPA) without stimulation, and EDPA during the stimulus and rest periods (EDPA+KCl). The average fluorescence values of the EDPA, KCl/EPDA, and EDPA+KCl experiments were significantly less than those of control experiments. However, KCl stimulation alone did not reduce the fluorescence, suggesting that zinc does not diffuse out freely from the slices. These experiments suggest that even repeated stimulation of slices does not lead to a depletion of the vesicular pool. If it did, KCl/EDPA and EDPA+KCl should be less than EDPA, which they are not. All the fluorescence removed can be attributed to the veneer, which appears to be replenished on a slower time scale than the experiments.

Using chelators one can partition the fluorescence that is disclosed by ZnAF-2 into intracellular and extracellular compartments. Application of EDPA removes only extracellular zinc, and the fraction of the fluorescence removed from the total fluorescence can be attributed to the veneer. The membrane permeant chelator TPEN (*18*) chelates zinc both in the veneer and within synaptic vesicles. By this accounting, the zinc associated with synaptic vesicles is ~50% in slices from older rats. Application of TPEN after KCl stimulation leads to slightly more quenching of the fluorescence, which can be attributed to the decline in autofluorescence induced by stimulation (Figure 7a and Figure 4a).

We also determined the effect of the membrane-permeant chelator diethyldithiocarbamate (DEDTC) (*19*) on the ZnAF-2 fluorescence. DEDTC, being a smaller molecule, appears to enter slices more rapidly; however it has a lower affinity than TPEN and results in less quenching than TPEN (Figure 7b). The quenching of the fluorescence by DEDTC on control slices is biphasic with a rapid component of 73±1.3 s and a slower component of 3502±487 s. If, however, slices were stimulated with KCl, DEDTC was far quicker but reached a steady-state similar to that of the unstimulated slices. We suggest that the rate of chelation by DEDTC reflects the tightness of the ternary complexes formed between ZnAF-2, zinc, and synaptic vesicular protein of unknown identity, with stimulation weakening the bonds. The kinetic distinction between the quenching of the fluorescence in control and stimulated slices was not evident in the case of TPEN, perhaps because it is a far more avid chelator than DEDTC.

Our experiments suggest that externalized zinc becomes available for chelation; however this does not account for the rising phase of the SIIF. During the process of exocytosis the intravesicular pH is believed to transition

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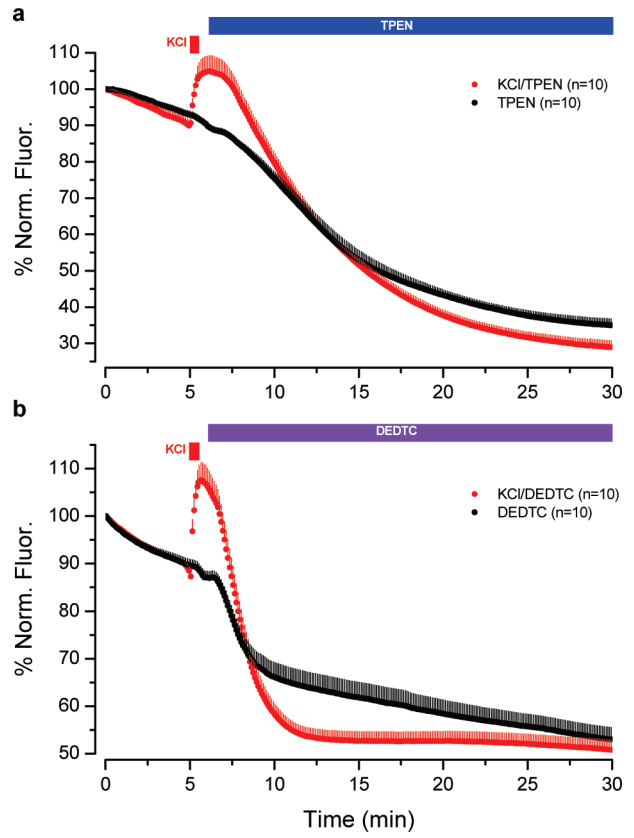

Figure 7. Effect of membrane permeant chelators on the fluorescence of ZnAF-2 loaded hippocampal slices. The fluorescence intensity was measured in the hilus. Slices were superfused with the chelators TPEN (a, 200 μM) or DEDTC (b, 1 mM) with (red) or without (black) the application of 50 mM KCl for 30 s.

from around 5.5 to the extracellular pH, 7.4. Since the fluorescence of most probes is pH-dependent, it is possible that this could lead to a change in fluorescence during stimulation. In separate work (unpublished observations), we have found that surprisingly the pH within zinc-rich vesicles is close to pH 7, and the SIIF cannot be attributed to neutralization of the vesicles during exocytosis.

Conclusions

The release of neurotransmitters by the process of exocytosis can be accounted for by diffusion of the ligand from fused vesicles into the extracellular cleft (*20*). The dissipation of the neurotransmitters is consistent with the chemistry of diffusion and active uptake (*21*). The dynamics can be followed by measurement of changes in the postsynaptic conductance, which reflects the molecules' concentration in the extracellular cleft. The case of zinc is more complicated since a direct measure for its free concentration within the synaptic cleft is as yet unavailable.

The contention of the zinc release hypothesis is rather straightforward, namely, that the zinc within vesicles in the process of exocytosis diffuses freely into the extracellular space where it may then bind to receptors or channels. Others (*22*) and we have found, using the membrane impermeant zinc probe FluoZin-3, that stimulation of the slices leads to a fluorescent increase consistent with zinc being presented to the extracellular space. From our results, we concluded that zinc was not freely released but appeared to be detained on sites in the extracellular space. This conclusion was based on the low levels of extracellular zinc detected even with strong stimuli, the persistence of the response, and the absence of bulk zinc release from slices. It was also based on the knowledge that zinc probes can form ternary complexes (*23*). Moreover, an extracellular zinc probe could remove zinc from externalized sites, giving rise to an apparent release event (*5*). Qian and Noebles (*22*) have found qualitatively similar results but they believe that the balance of evidence lies with zinc release.

In contrast to the experiments with FluoZin-3, our results with ZnAF-2 are difficult to reconcile with the classical zinc release hypothesis, since release of the zinc−probe complex should lead to a monotonic decline in fluorescence on stimulation, whereas synaptic stimulation leads to an increase in fluorescence. In addition, the increase observed on stimulating slices persists for many minutes, contrary to the prediction of free diffusion. It might be argued that the complex sticks to the membrane; however we have shown previously that application of the complex to slices does not adhere (*7*).

It seems to us that there are two ways in which our data could be consistent with zinc release. The first would be if there were residual ZnAF-2 in the extracellular space, so that on release the zinc binds to the probe and gives rise to an increase in fluorescence. As evidence against residual extracellular ZnAF-2, application of a high concentration of zinc to slices preloaded with ZnAF-2, does not generate an increase in fluorescence (*7*). Second, one might argue that there is ZnAF-2 in the postsynaptic cell that detects the flux of zinc into the postsynaptic cell. However, the increases in fluorescence overlie the presynaptic puncta and do not appear to move into a postsynaptic location (*7*).

There are two mechanisms that could potentially account for the rising phase of the SIIF: (1) During exocytosis, a change in the chemical environment of the probe could boost the fluorescence or the probe could diffuse laterally, effectively diluting the fluophore and reducing self-quenching (*24*). (2) Stimulation of the synapses could induce increased activity of the zinc transporter. If this were true, it would have to be only in the vesicles that are undergoing exocytosis, since all of the rising phase of the SIIF can be eliminated by chelators. This can be seen at least in the case of the slices from younger animals, where there is little veneer (Figure 2d). What accounts for the rising phase of the SIIF will have to await further experiments.

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The concentration of ZnAF-2, because it is not an acetoxymethyl ester (AM) derivative, is likely to be less than the 5 μM applied, unless there are mechanisms in the vesicles that concentrate the probe. The concentration of zinc within synaptic vesicles is not known with any degree of certainty, but probably is greater than 0.5 mM, which should saturate the probe. It is therefore unlikely that pumping more zinc into these vesicles will increase the fluorescence. However, if zinc were transported into vesicles containing ZnAF-2 but little zinc, the signal would rise. The increase in transport may simply be triggered by an increase in presynaptic Ca.

Ketterman and Li (*25*) recently reported that stimulating hippocampal slices loaded with ZP1 leads to a monotonic decline of presynaptic fluorescence, consistent with zinc release, paralleled by an increase in postsynaptic fluorescence. We have been unable to reproduce their results; where they report declines in fluorescence, we find increases. In reporting changes in fluorescence in slice experiments it is important to control for movements that may result from stimulation or from solution flow. A sensitive way of doing this is to take the difference between the image at the peak of a response and that prior to stimulation. Movement is easily detected by the peak-valley profiles oriented in the direction of movement.

As yet, we only have an operational definition of the veneer as the component of fluorescence that is open to chelation by the membrane-impermeant chelator EDPA in unstimulated slices. We suspect that the veneer has its origin in a membrane-associated macromolecule that can form ternary complexes with zinc−probe complexes. There is some evidence for this from ultrastructural studies of zinc accumulation on the extracellular face of the presynaptic membrane (*26*). We have postulated that the veneer is supplied by synaptic zinc; however, it is unknown how long it persists. From the repeated KCl stimulation experiments (Figure 6), it appears that the veneer is resupplied only very slowly, that is, if it is depleted, it is not replenished within 30 min. Further experiments are needed to assess how long it takes to be resupplied.

After full exocytosis has occurred, some macromolecules may remain on the presynaptic membrane, constituting what has been termed a pool of "stranded" proteins (*27*). As an example, De Wit et al. (*28*) have recently shown that certain vesicular cargo proteins (e.g., semaphorin 3A) remain in a long-term association with the extracellular face of the presynaptic membrane after exocytosis.

From the repeated KCl stimulation experiments (Figure 6), it seems that the synaptic zinc component is resupplied. We presume that during each bout of stimulation when EDPA is present, that both the veneer and exocytosed zinc are removed. However, the synaptic zinc does not appear to decline. This suggests that zinc is continuously being supplied to the vesicles. It seems likely to us that zinc is transported from the cytoplasmic zinc pool where it is associated with metallothionein and other chelators by ZnT3 into synaptic vesicles.

We have noted before that externalization is not inconsistent with zinc acting as a neuromodulator (*3*). We have proposed that the externalized zinc can, as might be expected from the law of mass action, detach from the veneer. We postulate further that neurons might modulate the strength of the interaction between zinc and the putative veneer protein so that at one extreme it might bind zinc avidly and on the other it might allow relatively free diffusion. Pathological circumstances may also lead to large-scale release of zinc from the veneer and synaptic vesicles.

Only in very rare cases do experimental results provide direct evidence for or against a hypothesis. In drawing an inference from experiments one has to have an explicit model of the biological system and, crucially, the system used to assay it. It may be possible to account for the SIIF in terms of classical release; however, it falls to the proponents of this view to put forward a model consistent with what is known of synaptic release, probe photophysics and chemistry that can account for the SIIF and be subjected to experimental test. If the zinc release hypothesis is to be restored, it will require active refutation of the externalization hypothesis rather than eliding over this competing view.

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 (14−135 day old) were decapitated, and their brains were 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 1 h prior to loading with the zinc indicator. Slices were loaded in ZnAF-2 (5 μM) for at least 1 h.

Slices were stimulated with bipolar tungsten electrodes (5 MΩ; 200−500 μA; A-M Systems) coupled through a stimulus isolation unit (A.M.P.I.) to a DigiData 1322A interface (Axon Instruments). Field potentials were recorded using a patch electrode (1−3 MΩ) filled with physiological saline coupled to an Axoclamp 2B (Axon Instruments). Potentials were digitized by a DigiData 1322A coupled to a personal computer (Dell) using Pclamp 9.0 (Axon Instruments).

The slices were stabilized with a U-shaped stainless steel wire cross-strung with nylon fibers in a temperature controlled

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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 (T.ILL Photonics), passed through a dichroic (Q495lp; Chroma Technology) and then through a filter (HQ530/60; Chroma Technology) onto the faceplate of a Princeton Instruments cooled CCD camera. Data were acquired by the MetaFluor program (Universal Imaging Corporation), and images were analyzed using ImageJ (NIH). No black-level adjustment was applied to images. The fluorescence intensity was expressed as $\% \Delta F/F_0 = \% (F - F_0)/F_0$, where F is the fluorescence intensity and F_0 is the fluorescence intensity at time zero. The dark current of the CCD was subtracted from all intensity measurements. All data are expressed as mean ± SEM.

Reagents

Fluozin-3 was obtained from Invitrogen, zinquin from TefLabs, ZnAF-2 from Alexis, ZP4 from Neurobiotex, and ZP1 from Toronto Research Chemicals; all other chemicals were obtained from Sigma-Aldrich.

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Author Contributions

A.R.K. conceived the project. I.N. performed experiments in Figures 2, 5, 6, and 7. S.M.R. performed some experiments from Figure 2. A.R.K. performed experiments in Figures 2, 3, 4, and 6. I.N., S.M.R., and A.R.K. analyzed the data. A.R.K. wrote the manuscript with input from I.N. and S.M.R.

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Abbreviations

EDPA, ethylenediamine-*N*,*N*′-diacetic-*N*-*N*′-di-β-propionic acid; DEDTC, diethyldithiocarbamate; SIIF, stimulus-induced increase in fluorescence; TPEN, *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine.

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