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Review

Synaptically released zinc: Physiological functions and pathological effects

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Abstract

In addition to its familiar role as a component of metalloproteins, zinc is also sequestered in the presynaptic vesicles of a specialized type of neurons called 'zinc-containing' neurons. Here we review the physiological and pathological effects of the release of zinc from these zinc-containing synaptic terminals. The best-established physiological role of synaptically released zinc is the tonic modulation of brain excitability through modulation of amino acid receptors; prominent pathological effects include acceleration of plaque deposition in Alzheimer's disease and exacerbation of excitotoxic neuron injury. Synaptically released zinc functions as a conventional synaptic neurotransmitter or neuromodulator, being released into the cleft, then recycled into the presynaptic terminal. Beyond this, zinc also has the highly unconventional property that it passes into postsynaptic neurons during synaptic events, functioning analogously to calcium in this regard, as a transmembrane neural signal. To stimulate comparisons of zinc signals with calcium signals, we have compiled a list of the important parameters of calcium signals and zinc signals. More speculatively, we hypothesize that zinc signals may loosely mimic phosphate 'signals' in the sense that signal zinc ions may commonly bind to proteins in a lasting manner (i.e., 'zincylating' the proteins) with consequential changes in protein structure and function.

Overview

Although it was Maske (1955) who first discovered 'stainable' (i.e., weakly bound) zinc in the brain (Figure 1), it was actually Turner McLardy who introduced the notion of synaptic zinc. For it was he who realized in the late 1950's that the swath of bright red zinc-dithizonate staining in the hippocampal formation of the brain was exactly coextensive with the peculiar 'mossy' axons that connect the dentate gyrus to ammon's horn (see McLardy 1970). The electronmicrographs of Finn-Mogens Haug (1967) later showed that this peculiar, 'stainable', pool of zinc was located exactly and exclusively in the presynaptic vesicle regions of synaptic terminals (Figure 2).

Two developments since have carried the concept of 'synaptic zinc signals' from neuroscience heresy to dogma (Baranano *et al.* 2001; Weiss & Sensi 2000; Weiss *et al.* 2000). First, Danscher and colleagues have adduced incontrovertible evidence that the stainable zinc in the brain is virtually without exception located in the synaptic vesicles of presynaptic boutons, a finding which has allowed the histochemical methods to be used to study the synaptic pool (Danscher 1996; Franco-Pons *et al.* 2000). Second, Palmiter and his co-workers have identified a specific zinc-transporter protein (ZnT-3) that pumps zinc into vesicles and is co-localized with vesicular zinc, on the membranes of synaptic vesicles in the Golgi apparatus, axons, and presynaptic boutons of zinc-containing CNS neurons (Palmiter *et al.* 1996). Because the ZnT-3 knockouts have no stainable zinc in their presynaptic vesicles (Cole *et al.* 1999), there is essentially no remaining doubt that the stainable zinc in synaptic terminals and the ZnT-3-dependent, presynaptic vesicular zinc pool are one and the same.

In the brain, the only neurons that have vesicular zinc (i.e., stainable zinc) are glutamatergic (Beaulieu

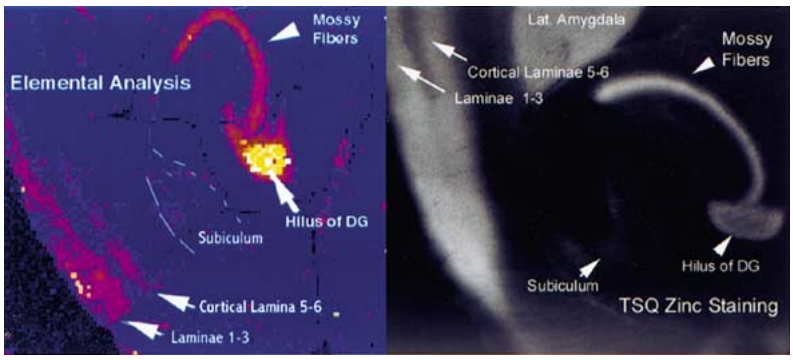

Fig. 1. The left image shows synchrotron-radiation-induced x-ray fluorescence of a rat temporal lobe (horizontal section) (Courtesy of Dr Jane Flinn. Morvan et al. 2000), and the right shows a similar section (somewhat more ventral) stained for free zinc by TSQ. The pseudo color on the left shows total elemental zinc, and the peak intensity (yellow-white) corresponds to approximately 400 ppm (dry weight), with the red (CA3 mossy fibers) corresponding to about 200 ppm. These values for total zinc are in reasonable agreement with prior averages obtained by micro dissection (Frederickson et al. 1983), with the present ($\sim 2 \times$ higher) values presumably more accurate reflections of true peak levels. Background total zinc is about 60 ppm. DG, dentate gyrus; TSQ, toluene-sulfonamide quinoline zinc staining method (Frederickson et al. 1987a).

et al. 1992) (Figure 3). Not all glutamatergic neurons are of this 'zinc-containing' flavor, but all zinc-containing neurons are glutamatergic (Frederickson 1989; Frederickson *et al.* 2000 for review). In the spinal cord of the lamprey (Birinyi *et al.* 2001) and the mouse (Danscher, personal communication), zinc is co-localized with glycine and/or GABA, and spinal vesicular zinc may have different roles than cerebral Zn^{2+} (Kovacks & Larson 1997).

The parcellation of cerebral glutamatergic neurons into the zinc-containing and the non-zinc-containing variety is definitely non-random. As a general rule, the large-neuron, long-axon systems of the brain are non-zinc-containing. Specific examples include all of the first-order sensory fibers of the cranial nerves, most of the thalamocortical fibers of those same, ascending sensory pathways, and essentially all of the long-fiber pathways descending, for example, from the cerebral cortex to the brain stem or spinal cord, or descending pathways from the diencephalon or mesencephalon (reviews in Frederickson *et al.* 2000; Franco-Pons *et al.* 2000; Frederickson & Moncrieff 1994).

Another view of the segregation of zinc-containing versus non-zinc-containing glutamatergic neurons is that almost all of the zinc-containing neuronal somata that have been identified in the brain have been found in either cortical (including allo and isocortex) or amygdalar brain regions. But for a few exceptions (granule neurons in the dorsal cochlear nucleus (Rubio & Juiz 1998), scattered medial thalamic neurons (Long & Frederickson 1994)) the overwhelming preponderance of the zinc-containing neurons of origin are cerebrocortical or amygdalar (Casanovas-Aguila *et al.* 1998; Christensen & Frederickson 1998).

There are some thought provoking changes in the zinc-containing innervation during early brain development. For example, in the hippocampal formation of the newborn rat pup, transient columnar 'patches' of zinc-containing innervation are conspicuous in the CA1-CA3 fields. These patches disappear quickly thereafter, as the entire blank spaces in the neuropil fill in with a quite uniform staining (Frederickson *et al.* 1991). Another example occurs in the striatum, where patches that are destined to become neurochemically distinct from the surrounding 'matrix' of the striatum are first identifiable in the zinc staining, which distinctly labels the patches even on post natal day 1, before the other neurochemical and anatomical markers of the patches have arrived (Vincent & Semba 1989). A third example is found in the lateral geniculate nucleus of the albino rat, where there are transient patches of zinc-containing axonal boutons that correspond to the ingrowing uncrossed retino-geniculate pathway (Figure 4). This last case is even more impressive than the first two because the zinc-containing boutons lose their zinc later in development, leaving the adult LGN without any zinc-containing boutons at all.

In considering these cases one is tempted to speculate that the zinc-containing boutons serve some early pioneer or induction function, laying down a skeletal substrate for later development. Experiential effects on these developmental processes have been identified (Land & Akhtar 1999; see also Dyck & Cynander

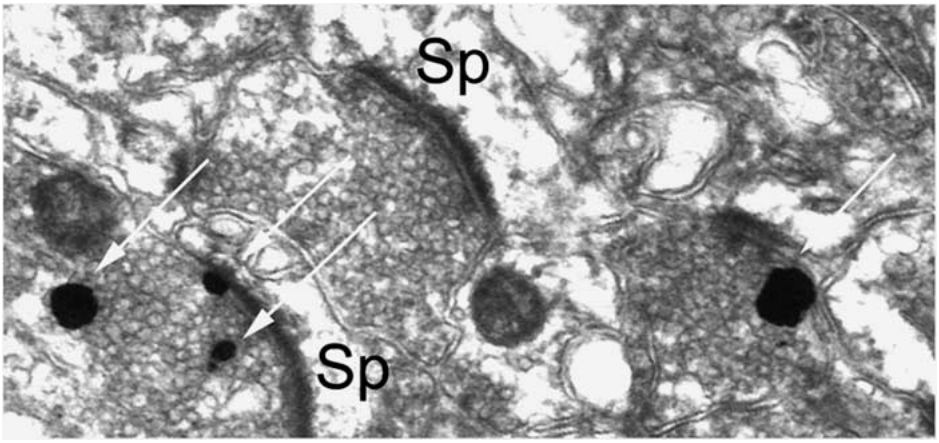

Fig. 2. Timm-Danscher method for tissue metal (which precipitates Zn^{2+} as ZnS, then coats the ZnS with silver) shows silver grains (arrows) amidst the vesicles of Type I presynaptic boutons, contacting spines (Sp). Image from the CA1 field of the rat hippocampus.

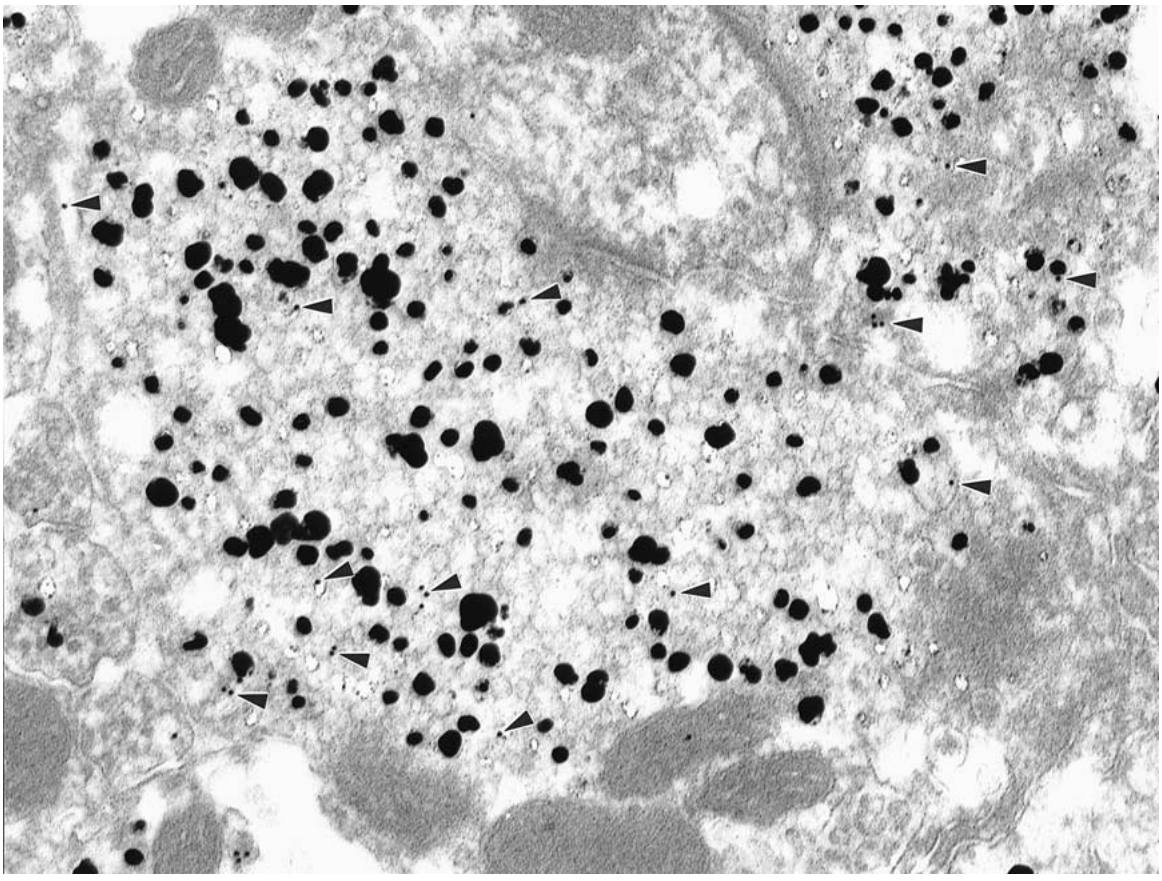

Fig. 3. Timm-Danscher silver staining for zinc shows silver grains amidst vesicles of a mossy-bouton in the rat hippocampus. Colloidal gold dots (in circles) indicate glutamate immunostaining, which co-localizes one-for-one with Timm's in the mouse hippocampus (Courtesy R. Palmiter) ~ 40,000 X.

1993). On the other hand, knockout mice congenitally lacking zinc in their vesicles develop with at least superficially normal brain organization and behavior (Cole *et al.* 2000), so whatever the developmental function of vesicular zinc might be, it would appear that developmental plasticity can compensate.

There are three kinds of Zn^{2+} signals

At the time of this writing, we can identify three distinct classes of zinc signals which we will call: Zn^{2+}-SYN, Zn^{2+}-TRANS , and Zn^{2+}-INT.

Zn^{2+}-SYN

The first of these signals, 'synaptic Zn^{2+}' (Zn^{2+} -SYN) is a conventional, transmitter-like, synaptic signal, between presynaptic bouton and postsynaptic spine of dendrite. The zinc ions for this signal are stored in presynaptic vesicles at concentrations probably reaching low millimolar levels (see calculations in Frederickson 1989; Frederickson *et al.* 2000). Upon the arrival of an action potential at the bouton, the calcium- and impulse-frequency dependent exocytosis of these zinc-filled vesicles produces a rapid 'puff' of essentially free, ionic Zn^{2+} in the extracellular fluid surrounding the boutons. These Zn^{2+} transients, or 'puffs' have rise-times of a few milliseconds, and can reach apparent concentrations of 10–30 μM Zn^{2+} in the extracellular fluid (Li *et al.* 2000, 2001; Thompson *et al.* 2001; Vogt *et al.* 2000). Zn^{2+} 'puffs' or 'flashes' comparable to those seen during synaptic release can also be seen during exocytosis of a similar zinc-rich secretory granule, the insulin-containing beta pancreatic cell (Qian *et al.* 2000), and could presumably be seen during exocytosis of Mast cell granules, salivary cell granules, and that of the dozen-odd other cell types that have zinc-filled secretory granules (Frederickson 1989; Frederickson *et al.* 1987b).

The Zn^{2+}-SYN signal reaches multiple zinc-modulated extracellular zinc-recognition sites on membrane-spanning receptors, pumps, and channels of postsynaptic neurons. The most thoroughly studied of these are the zinc-modulated amino acid receptors, with both the glutamate receptor families and the GABA receptors having diverse and potent responses to the Zn^{2+} ion signal (Smart *et al.* 1994). One of the intriguing examples of the complex and facile nature of these interactions is found at the mossy-fiber to CA3 pyramidal neuron, where GABA, zinc and glutamate are all released, with the possibility arising of differential zinc modulation of both receptors by zinc at the single synapse (Walker *et al.* 2001). The time-varying expression of receptor-ionophore splice variants which have different zinc sensitivity give the nervous system another degree of freedom with respect to the impact of zinc modulation (Chen *et al.* 1997). Ectopic sprouting of zinc-releasing fibers is another potential mechanism of zinc-mediated plasticity (Coulter 2000).

Despite the complex and protean effects of zinc upon amino acid synapses, it appears that the predominant effect of the Zn^{2+}-SYN signal in the brain is a tonic defacilitation at glutamate-gated excitatory ion channel(s). Thus, blocking Zn^{2+}-SYN by chelation tends to be proconvulsive (Mitchell & Barnes 1993), and adding modest concentrations of zinc, anticonvulsive (Morton *et al.* 1990). In ZnT-3 knockout mice (lacking stainable zinc in their vesicles) one of the surprisingly few (Cole *et al.* 2001) defects observed is that the mice have increased susceptibility to kainic-acid induced seizures (Cole *et al.* 2000). The overall electrophysiological impact of the Zn^{2+}-SYN signal on the hippocampal mossy-CA3 glutamatergic synapse was recently described in elegant detail and summarized with the statement that "... the metal ion Zn^{2+} is a neurotransmitter and ... the activity-dependent synaptic release of Zn^{2+} modulates NMDA receptor function. This modulation involves an inhibition mediated by both the high-affinity, voltage-independent binding site and the low-affinity, voltage-dependent binding site of Zn^{2+}" (Vogt *et al.* 2000).

Zn^{2+}-TRANS

The second Zn^{2+} signal that has been observed and characterized in the brain occurs roughly simultaneous with the conventional Zn^{2+}-SYN signal. This signal is analogous to the transmembrane Ca^{2+} signals, and consists of a transmembrane flux of Zn^{2+} from the extracellular milieu, through gated, zinc-permeable channels, and into the somata (Li *et al.* 2001a, b) or dendrites (Suh *et al.* 2001) of postsynaptic neurons (Figure 5). The highest conductance Zn^{2+} channel is the Ca-AK channel (Figure 6), though NMDA-gated Ca^{2+} channels will also pass zinc (Weiss & Sensi 2000; Weiss *et al.* 2000). Because the presynaptic release of Zn^{2+} is the only known source of appreciable amounts of extracellular Zn^{2+}, this signal

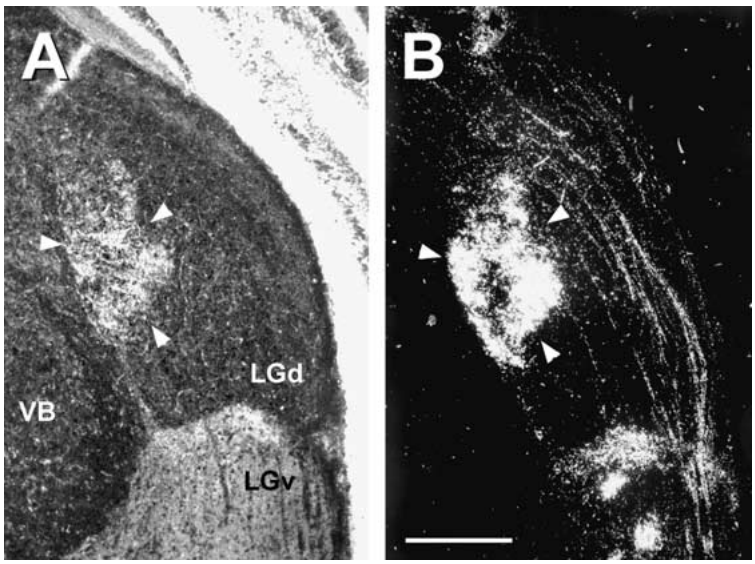

Fig. 4. Zinc staining is present briefly in uncrossed, but not crossed axons innervating the lateral geniculate nucleus of the albino rat. Compare the zinc-staining (A) with the location of the uncrossed axon terminals (B) **A**. Darkfield photomicrograph showing distribution of histochemically reactive zinc in a coronal section through the lateral geniculate nucleus of a postnatal day 15 rat. Synaptic zinc (stained by the Timm-Danscher method, white arrowheads) is localized to a discrete zone in the medial portion of the nucleus. **B**. Section adjacent to that illustrated in A showing distribution of incrossed axon terminals (white arrowheads) that were stained by injection of horseradish peroxidase into the ipsilateral eye. Note that synaptic zinc and uncrossed retinal projections are localized to an identical region of the LGd. This developmental zinc staining disappears in the adult. Calibration bar = 300 mm for A and B. Abbreviations: LGN – lateral geniculate nucleus; LGd – lateral geniculate dorsal; LGv – lateral geniculate ventral; VB – ventrobasal thalamic nucleus. Courtesy Peter Land.

Zn^{2+}-TRANS is both a transynaptic and a transcellular signal. The temporal and spatial characteristics of these Zn^{2+}-TRANS 'flashes', or 'sparks' in neuronal cytosol have not been established, but the rise times can be within a few 10's of milliseconds of the initial stimulation of the zinc-containing presynaptic fiber system (Figure 5) (Li *et al.* 2001a; Suh *et al.* 2001). Whether Zn^{2+}-TRANS signals can, in turn, induce subsequent intracellular zinc mobilization Zn^{2+}-INT in the same way that transmembrane Ca^{2+} fluxes induce mobilization of Ca^{2+} from the SER (or SR) (Melamed-Brook *et al.* 1999; Keizer & Levine 1996) is not presently known.

Zn^{2+}-TRANS signals can be blocked either in the cleft, by bath application of a sufficiently fast and high-affinity chelator, or at the postsynaptic neuron, by the use of channel blockers (e.g., CNQX) that prevent the opening of the dominant postsynaptic Zn^{2+} channel, Ca-AK). Conversely, the Zn^{2+}-TRANS signal can be mimicked by bath application of Zn^{2+} and a suitable zinc ionophore (e.g., pyrithione). John Sarvey and his colleagues have recently shown by both the blockade and the mimicry approaches that the Zn^{2+}-TRANS signal is necessary and (in the presence of glutamate) sufficient to induce LTP of mossy fiber input in CA3 pyramidal neurons (Li *et al.* 2001b).

Zn^{2+}-INT

The third Zn^{2+} signal now under scrutiny is analogous to another class of Ca^{2+} signal, namely, the intracellular Ca^{2+} signal. In the case of calcium, the sarcoplasmic or smooth endoplasmic reticulum are established storage sites for intracellular release of the ion, and the pumps and channels and ligands that mediate the Ca^{2+} movement into and out of those depots are well characterized (Keizer & Levine 1996). With Zn^{2+}, on the other hand, there is (as yet) no candidate organelle for the storage of pools for intracellular release. The neuronal vesicle has no detectable Zn^{2+} until it moves out of the Golgi apparatus and into the orthograde axoplasmic flow down axons (Frederickson & Danscher 1990), and no other zinc-filled organelle is found in healthy neurons (Frederickson *et al.* 1992; Danscher 1996; Fredrickson & Danscher 1990).

No matter from where the intracellular Zn^{2+} signals originate, they are prominent and robust in many types of cells, including neurons. Nitric oxide is one of the more potent inducers of Zn^{2+}-INT signals. In the

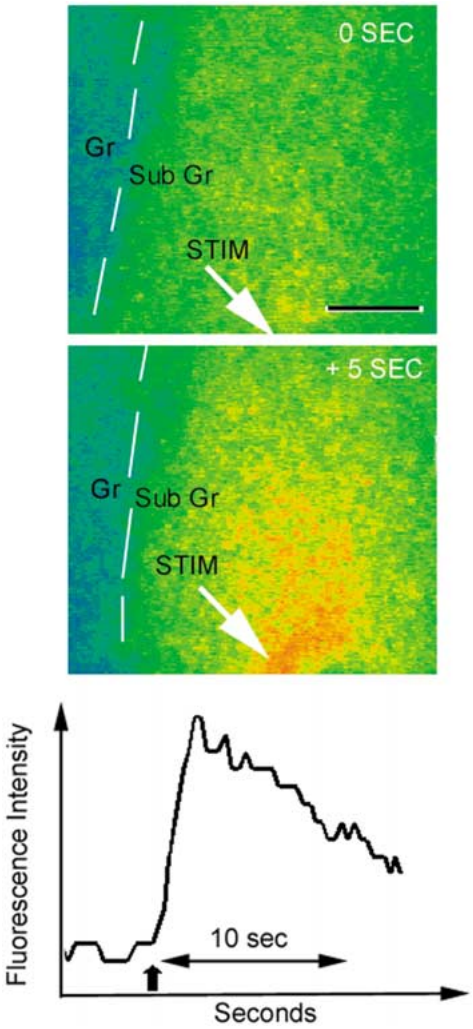

Fig. 5. Intracellular zinc 'puff' is shown in pseudo color, using the membrane-permeable ('trappable') intracellular zinc indicator, Newport green. The electrode tip (STIM) was within the hilus of the dentate gyrus with approximate location of the Granule-Sub-Granule border indicated. Note the 'puff' of zinc at +5 sec after a 5 sec, 100 Hz stimulation. Note rapid rise time of this Zn^{2+}-TRANS fluorescent signal in the quantitative figure at bottom. Quantitative figure reflects changes within pixels located at the center of the 'puff'. Courtesy Yang Li, Chris Hough and John Sarvey. See: Li *et al.* (2001a).

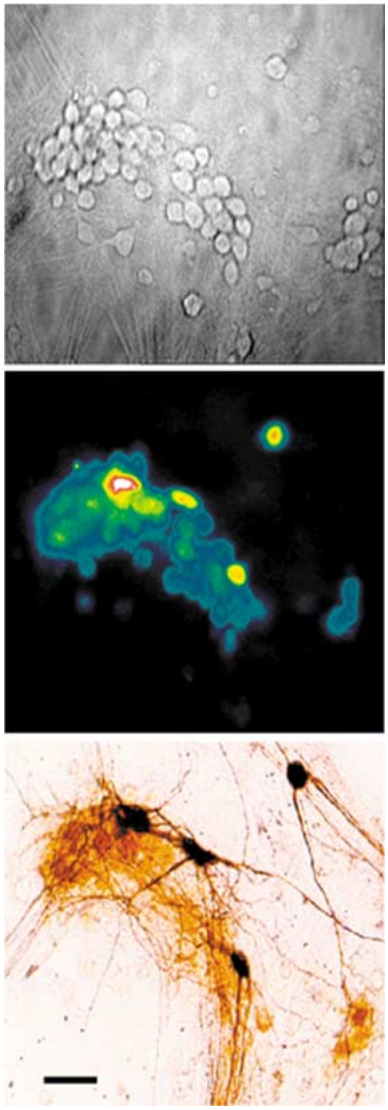

Fig. 6. Neurons that fill with Zn^{2+} during a glutamate + zinc challenge, are the same neurons that express abundant Ca-AK channels. Cultured neurons (top) respond differentially to combined stimulation with zinc and glutamate, with four somata attaining high levels of intracellular zinc (yellow-red-white in the pseudo color, middle panel), and the rest showing only marginal intracellular zinc. Double staining by the cobalt method which labels via the Ca-AK channels (bottom panel) reveals that the 4 zinc-filled neurons each had robust expression of the high-zinc-permeability Ca-AK channels (Courtesy John Weiss & Stefano Sensi; see Weiss & Sensi 2000).

otherwise intact brain, NO* stimulation causes immediate appearance of perikaryal and nuclear zinc staining (Figure 7) (Cuajungco & Lees 1998; Frederickson *et al.* 2001a). In excitotoxicity accompanying seizures, ischemia, hemorrhage, or blunt trauma, similar Zn^{2+} staining occurs within 10's of minutes of the insult (Figure 7) (Frederickson *et al.* 1988, 1989; Prough *et al.* 2001; Suh & Frederickson 2001). Because these Zn^{2+}-INT signals are seen (i) in neurons far from any

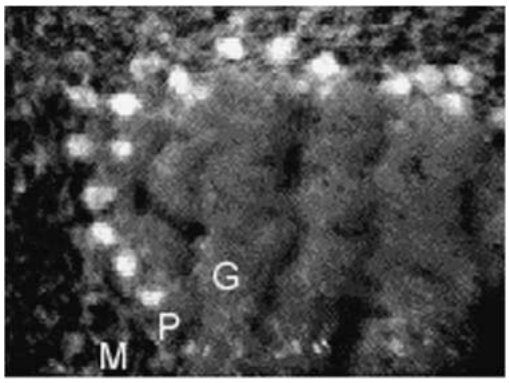

Fig. 7. The cerebellar cortex is normally completely negative for zinc staining throughout the Molecular (M), Purkinje (P) and Granule (G) strata. However, infusion of a NO* donor (spermine NO) *in vivo* causes Purkinje somata to rapidly develop intense TSQ fluorescence for zinc (white spheres).

synaptic zinc source (Figure 6; Frederickson *et al.* 2001a), and are seen (ii) in neurons of mutant mice lacking detectable synaptic zinc (Lee *et al.* 2000), and are seen (iii) in a variety of dissociated cell types in culture (Zangger *et al.* 2001 and references therein), there is little doubt that the intracellular zinc can arise from some still-undiscovered zinc depot within cells. One likely candidate for the source of the Zn^{2+}-INT signals would be zinc-sequestering proteins such as metallothionein, which can release up to 7 zinc ions per molecule upon command. The NO* stimulus has been shown to release zinc from proteinaceous storage sites (including MT) by nitrosylation of ligands (Zangger *et al.* 2001). Other redox and oxidative stimuli can cause a similar Zn^{2+}-INT zinc release (Maret 2000; Ye *et al.* 2001).

The targets and physiological functions of the Zn^{2+}-INT signals are still largely undiscovered. Because the Zn^{2+}-INT signals appear in conditions of biological stress, one focus of research has been on Zn^{2+}-INT control of mitochondrial function and of apoptotic events (Truong-Tran *et al.* 2001; Yoon *et al.* 2000; Hyun *et al.* 2000). One powerful new technology that will doubtless accelerate progress is the development of antibodies for the zinc-binding sites that will distinguish the zinc-site-occupied (zincylated ?) from the zinc-site-unoccupied (dezincylated ?) condition (Herwald *et al.* 2001). One physiological response to a Zn^{2+}-INT signal has been tentatively identified by this antibody strategy (Herwald *et al.* 2001).

Zinc versus calcium

Conventional wisdom holds that zinc and calcium have fundamentally different roles in the brain. Zinc is viewed as an enduring component of proteins and an ion that is sometimes temporarily 'free' (i.e., between proteins) whereas calcium is typically viewed as a 'free' extracellular ion, that is generally bound in the intracellular milieu, (i.e., between signaling duties) in calcium-sequestering proteins that serve to 'send' calcium signals. Thus, the term 'zinc-binding protein' generally refers to any of hundreds of metalloproteins that have zinc tightly bound into more-or-less permanent sites (e.g., zinc-finger motifs; Berg 1990) at critical folding points or active catalytic sites (Vallee & Falchuk 1993). Calcium-binding protein, in contrast generally refers to any of dozens of proteins known to scavenge, store, and release the ion, in the course of calcium signaling events (e.g., Williams 1996; Toutenhoofd & Strehler 2000).

It is now clear that the conventional wisdom about zinc is wrong, at least as concerns the brain. As described above, zinc ions are stored, released, taken up, and translocated across membranes and within cells, and there are myriad proteinaceous targets and receptors specifically tuned to these zinc signals. Furthermore, it is now appreciated that there are zinc-binding proteins that are kinetically-labile, having relatively rapid on- and off-rates (Maret 2000; Auld 1995).

If both zinc and calcium serve as signal ions, then one may ask why there are two such signals and how their differences (and similarities) could be utilized by cells. As Table 1 shows, one salient difference is the fact that extracellular free zinc (unlike extracellular calcium) is essentially absent in the extracellular fluid, with the estimated concentration being below the detection limits for most analytical methods ($\sim$1 pM; see Simons 1991; Powell *et al.* 1999; discussion in Frederickson 1989). Therefore, whereas transmembrane calcium signals can be initiated by merely opening a calcium channel, transmembrane zinc signals require the release of some ion into the extracellular space *and* opening of transmembrane channels. So far, zinc-containing presynaptic vesicles, with mM levels of zinc ions, are the only known source of such extracellular zinc in brain tissue. This means that transmembrane zinc signals are always 'AND' signals, with synaptic release *AND* membrane channel opening both required.

The other notable difference is that there are already dozens of calcium scavenging, sequestering,

Table 1. Comparison of zinc and calcium signal properties.

Attribute	Zinc (Zn^{2+})	Calcium (Ca^{2+})
Approximate ion concentration in deep ocean	1 pM - 1 nM	10 mM
Approximate concentration of free ion extracellularly	not established, but likely 1 pM to 1 nM	22 mM
Approximate concentration of free ion in cytosol	not established, but ~005–10 nM	~50 nM
Approximate concentration of ion in storage (releasable) pools	~3–30 mM in presynaptic vesicles	? low μM in SER ?
Magnitude of transient physiological ion signals	~1000-fold increases in cytosol	~ 1000-fold increases in cytosol
Rise-time of transient physiological signals	1–10 msec	1–10 msec
Specific membrane-bound storage organelles ?	YES: presynaptic vesicles many other secretory granules	YES: SER (& synaptic vesicles) in neurons; SR in muscle
Specific releasable pools ?	YES: presynaptic vesicles; MT	YES: SER, SR
Membrane-spanning gated channels for the ion ?	YES: voltage and ligand gated	YES: voltage and ligand gated
Ion- sequestering proteins ?	YES (few described, eg, metallothionein)	YES (dozens described, eg, calbindin, calmodulin)
Ion-sensitive ion channels ?	YES: (NMDA, AMPA KA, K^+; GABAa,b all modulated by extracellular Zn^{2+})	YES, Na^+, K^+, CA^{++} all modulated by intracellular Ca^{++}
Ion modulated enzymes	YES	YES
Cytotoxic at high intracellular levels	YES 100's nM fatal	YES 100's nM fatal
Number of ion-containing proteins	hundreds of zinc finger and other zinc-containing proteins	many calcium storage and calcium-modulated proteins
References	Li *et al.* 2001a,b; Thomson *et al.* 2000, 2001; Canzoniero *et al.* 1999; Simons 1991; Smart *et al.* 1994; Frederickson *et al.* 2000; Lobner *et al.* 2000; Powell *et al.* 1999; Frederickson 1989; Choi & Koh 1998	Melamed-Brook *et al.* 1999; ZhuGe *et al.* 2000; Keizer & Levine 1996; Brown & MacLeod 2000; Van Assche *et al.* 1996; Wilson 1996

and releasing proteins identified, as well as calcium-modulated signal cascades, whereas only metallothionein (Maret 2000) and the Zn-T zinc pumps (ZnT1, 2, 3) have been established as 'helper proteins' for storing and releasing zinc signals (Pamiter *et al.* 1996). This may be mostly due to a lack of interest in the past: the search for proteins that control the zinc signal pathways is doubtless now beginning.

Pathophysiology

Zinc dysregulation is implicated as a contributing factor in two types of neuropathology: (i) Alzheimer's disease, and (ii) the so-called 'excitotoxicity' which injures neurons after ischemia, hemorrhage, seizures, or mechanical brain traumas.

In broad outline, the evidence implicating zinc is roughly the same in both cases. Thus, in the first place, both conditions are marked by the appearance of vivid,

anomalous, and emblematic staining for zinc in the histochemical centers of the disease processes in the brain (Frederickson *et al.* 1989; Suh *et al.* 2000a, b). In the second place, both conditions can be ameliorated by the simple strategy of treating the brain with a zinc chelator to lower the extracellular Zn^{2+} burden (Koh *et al.* 1996; Frederickson *et al.* 2001; Suh *et al.* 2000; Cherny *et al.* 2001). Third, both conditions can be simulated in certain *in vitro* test models by the addition of excess exogenous zinc to the brain tissue under study (Bush *et al.* 1994a, b; Weiss & Sensi 2000). Fourth, both conditions are plausibly triggered (or accelerated) by the pathological release of an excess of Zn^{2+} into the extracellular milieu from the only known store of such Zn^{2+}, the zinc-containing boutons. Fifth, both conditions tend to strike preferentially in those cerebrocortical regions (hippocampal formation, amygdala, frontal cortex) where the concentration of zinc-containing axonal boutons is highest (reviews in Suh *et al.* 2000a, b; Frederickson *et al.* 1989, 1992, 2000).

Alzheimer's Disease (AD)

One of us (AIB) has developed a model where abnormalities of the zinc homeostatic control mechanisms may explain the pathological features found in AD. The model is based on the tendency of amyloid beta (Aβ a primary constituent of AD plaques and AD angiopathy) to precipitate in the presence of sufficient zinc to occupy its low affinity (micromolar) binding site (Figure 8).

Amyloid beta peptide binds zinc at both high- and low-affinity binding sites (Bush *et al.* 1994b). Zinc concentrations above 300 nM rapidly precipitate synthetic human Aβ1-40 (Bush *et al.* 1994a, b). Interestingly, zinc preserves the α-helical conformation of Aβ1-40 and its complexation is completely reversed with chelation treatment (Huang *et al.* 1997). This result suggests that zinc-induced Aβ aggregation may possibly inhibit its intrinsic neurotoxicity. Meanwhile, rat Aβ1-40 (with substitutions of Arg→Gly, Tyr→Phe and His→Arg at positions 5, 10 and 13, respectively) binds zinc less avidly ($K_A = 3.8\ \mu M$) and is unaffected by zinc at these concentrations, perhaps that is why rats do not form Alzheimer's like plaques (Shivers *et al.* 1988). In the absence of zinc, the solubilities of the rat and the human Aβ species are indistinguishable (Bush *et al.* 1994a).

Zinc-induced Aβ precipitation at pH 7.4 is highly specific for zinc, however, copper Cu^{2+} and iron

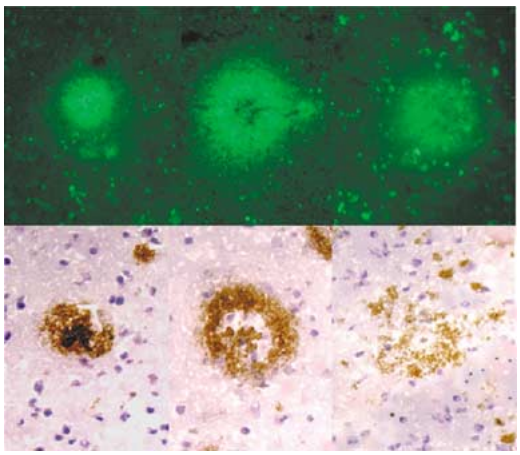

Fig. 8. Autopsy material from patients deceased of Alzheimer's disease show TSQ staining (upper panels) of amyloid plaques; shown, for comparison, stained with AB1-42 immunostaining (lower panels). The six plaques are all from different sections, and have been combined digitally for illustration. Courtesy of Math Cuajungco, Sang Won Suh, Al Rampy, and Zoran Gatalica.

Fe^{2+} can also bind, especially at lower (6.6) pH (Atwood *et al.* 1998). While Cu^{2+} and Fe^{3+} binding to Aβ induces O_2 dependent H_2O_2 production and toxicity (Huang *et al.* 1999a, b), co-incubation with Zn^{2+} (>4:1, Zn:Cu) inhibits H_2O_2 production (Cuajungco *et al.* 2000). Zn^{2+} had previously been reported to protect cell cultures from Aβ toxicity (Lovell *et al.* 1999). Extending this observation, we found that Zn^{2+} rescued primary cortical and human embryonic kidney 293 cells that were exposed to Aβ1-42 (Cuajungco *et al.* 2000). Since plaques contain exceptionally high concentrations of Zn^{2+} ($\approx$1mM, see below), we examined the relationship between oxidation (8-OH guanosine) levels in AD-affected tissue and histological amyloid burden, and found a highly significant negative correlation. Therefore, Zn^{2+} loading into plaques may represent an attempt at protective homeostatic response in AD, where plaques form as the result of a more robust Zn^{2+} antioxidant response to the underlying oxidative attack, and that the Aβ in the plaques has been redox-silenced by the high concentrations of Zn^{2+}, whereas the diffuse and soluble Aβ accumulations within the brain would be a source of H_2O_2 and oxidative damage (McLean *et al.* 1999).

Recently, we have shown that Aβ binds Cu^{2+} and Zn^{2+} through selective binding sites. When synthetic Aβ is co-incubated with excess but equal amounts of Cu^{2+} and Zn^{2+}, $\approx$1.5 equivalents of each metal ion bind to each mole of peptide. Since the affinity of the low and high affinity Cu^{2+} binding sites on Aβ ranges

Table 2. Zinc-binding and zinc-dependent proteins implicated in AD pathology.

Proteins	Zinc effects	Role in Alzheimer's	References
α_2-Macroglobulin	zinc-binding protein	zinc binding triggers Zn+Aβ + α_2-M complex formation	Du *et al.* 1997; Hughes *et al.* 1998
Nerve Growth Factor-β	removal of zinc 'zipper' releases active fragment from zymogen	neurotrophic agent - combats AD advance	Pattison & Dunn 1976a, b; Ross *et al.* 1997
S100β	zinc-containing protein, neurite extension factor	elevated amidst plaques and NFT in AD brains	Griffin *et al.* 1989; Marshak *et al.* 1991
Metallothionein	zinc-sequestering protein	altered in AD brains	Zambenedetti *et al.* 1998; Adlard *et al.* 1999
Alpha- and beta-secretases	cleave proteins at zinc-binding site	cleave APP releasing Aβ	Bush *et al.* 1994
Matrix metalloproteinases	zinc-containing enzymes	can degrade Aβ1-41 and Aβ 1-42	Backstrom *et al.* 1996; Roher *et al.* 1994
Caspase	zinc-modulated enzyme: up- or down-regulated, depending on concentration	modulates apoptosis may modulate AD apoptotic cell death	Cuajungco & Lees 1997; Choi & Koh 1998

from nM- attoM, and since the highest affinity Zn^{2+} site on Aβ is 100 nM, the finding that Cu^{2+} does not compete for all of the available metal binding sites when co-incubated with Zn^{2+} implies that Aβ possesses separate and selective Cu^{2+} and Zn^{2+} binding sites (Atwood *et al.* 2000). Importantly, mildly acidic conditions, representing physiological acidosis (e.g., pH 6.8), abolish Zn^{2+} binding to Aβ, but enhance Cu^{2+} binding to Aβ, so that when Cu^{2+} and Zn^{2+} are co-incubated with Aβ at pH 6.8, $\approx$3.0 equivalents of Cu^{2+} bind to the peptide, but virtually no Zn^{2+} (Atwood *et al.* 1998; Atwood *et al.* 2000). Physiological acidosis may therefore be one mechanism by which Aβ loses the redox-protective Zn^{2+} binding, and may then be liable for inappropriate redox activity.

In keeping with our prediction that Aβ is a zinc metalloprotein, we have now published observations that amyloid plaques in post-mortem AD have a marked enrichment of zinc (to mM levels (Lovell *et al.* 1998)) that is histochemically visible (Figure 8; Suh *et al.* 2000b). Intriguingly, Zn^{2+} is also markedly enriched in the neocortical amyloid plaques of APP transgenic mice (Lee *et al.* 1999), supporting the likelihood that abnormal Zn-Aβ interaction is responsible for plaque formation *in vivo*. The significance of Zn (and Cu) being present in the amyloid mass in the brain in AD is that it may be possible to create therapeutic drugs for AD that safely target the Aβ-Zn interaction. Zn/Cu-selective chelators reverse Zn/Cu-induced aggregation of synthetic Aβ *in vitro* (Huang *et al.* 1997; Atwood *et al.* 1998), inhibit Aβ-mediated H_2O_2 formation (Huang *et al.* 1999a, b; Bush *et al.* 1999), and solubilize Aβ from amyloid deposits in post-mortem AD-affected brain tissue (Cherny *et al.* 1999). Recently, we reported the profound inhibition of Aβ deposition in the pellet phase of brain homogenates (375 μg/g wet weight, $P = 0.0001$) in a blinded study of APP2576 transgenic mice treated orally for 9 weeks with clioquinol, an antibiotic and bioavailable Cu/Zn chelator. This was accompanied by a modest increase in soluble Aβ (1.45% of total cerebral Aβ); but APP, synaptophysin and GFAP levels were unaffected. Behavioral and body weight parameters were significantly more stable in the treated animals, and there was no evidence of systemic metal depletion (Cherny *et al.* 2001). The affinity of clioquinol for Zn is only nanomolar, so therefore while the molecule therapeutically targets the metals that induce Aβ aggregation or redox activity, unlike common chelators (e.g., EDTA, desferrioxamine) clioquinol

does not appear to have sufficient affinity for Zn and Cu to disturb metal-dependent biochemistry.

Beyond the immediate interactions of zinc and amyloid, there are a number of less direct pathways by which zinc dysregulation can affect the rate and severity of the AD pathophysiology. Table 2 lists some examples of zinc-containing and zinc-sensitive protein signals and enzymes that can modify the course of the AD pathology, and would be themselves perturbed in the face of any primary disturbance of zinc homeostasis in the brain.

Excitotoxicity

In conditions of compromised cerebral blood flow and in sustained status epilepticus, the so-called 'excitotoxic' cell injury cascade is triggered in the brain. The release of copious glutamate and consequent depolarization of neurons that constitute excitotoxicity is accompanied by the appearance of very high levels of free Zn^{2+} in the somata of (and only of) the dying neurons (Frederickson *et al.* 1988, 1989; Tonder *et al.* 1990; Suh *et al.* 2000a; Suh & Frederickson 2001; Prough *et al.* 2001) (Figure 9). Because this anomalous, pathological intracellular Zn^{2+} burden can be found in neurons not surrounded by appreciable zinc-containing innervation (Frederickson *et al.* 2001a), and can be found in neurons of knockout mice congenitally-lacking detectable Zn^{2+} in their presynaptic vesicles (Lee *et al.* 2000), it seems certain that there is a Zn^{2+}-INT signal contributing to the excitotoxic zinc signal. As mentioned earlier, nitric oxide and superoxide stimuli mobilizing zinc off proteins such as metallothionein probably contribute part of this Zn^{2+}-INT signal.

At the same time, in the brains of otherwise normal animals, the massive release of glutamate during excitotoxicity is accompanied by an equally massive release of Zn^{2+} from the presynaptic boutons (Frederickson *et al.* 1988, 2001b; Suh *et al.* 2000; Sorensen *et al.* 1998). Therefore it is hard to imagine that there is not a Zn^{2+}-TRANS signal that also contributes to the zinc-loading of neurons during excitotoxic crises.

Regardless of the relative contributions of Zn^{2+}-INT and Zn^{2+}-TRANS, the Zn^{2+} apparently kills cells by entering mitochondria and disrupting function, with both a release of reactive oxidative species and induction of both apoptosis and necrosis (depending on paradigms) and the death of the cell (Weiss *et al.* 2000; Weiss & Sensi 2000; Choi & Koh 1998).

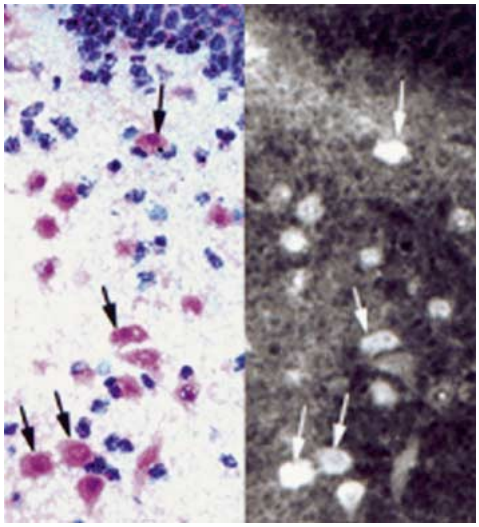

Fig. 9. In the rabbit subjected to brief global ischemia, neurons throughout the cerebrum (arrows) show TSQ staining for zinc (right panel) and (in the corresponding neurons) acidophilic changes indicative of injury/degeneration (left panel). Double-staining of the same section; 1 h post ischemia. Courtesy of Mark Zornow, Don Prough, and Sang Won Suh.

As discovered by Choi and his colleagues, therapeutic administration of an extracellular zinc-specific chelator can reduce excitotoxic cell loss by up to 80% (Koh *et al.* 1994). This has been shown in experimental animals with excitotoxicity induced by ischemia (Koh *et al.* 1994), trauma (Suh *et al.* 2000a), or trauma plus hemorrhage (Prough & Frederickson, unpublished) and (with some mixed results) in some seizure models (Lees *et al.* 1998). In all cases, the neuron sparing is judged by the reduced numbers of neurons showing acidophilic signs of injury up to 24 h after the initial insult. Interestingly, this rescue of neurons by a chelator can be done even if the zinc insult (from exogenous zinc) is applied first then terminated, and the therapeutic zinc chelation (by CaEDTA) is applied some time later. In this latter case, the zinc-staining of cells induced by a prior insult is reversed by the subsequent addition of CaEDTA to the medium (Frederickson *et al.* 2001b). In addition to conventional chelators, such as CaEDTA, the highly-specific zinc-stripped (apo) form of a zinc-containing protein (carbonic anhydrase) is also neuroprotective when administered intraventricularly in a trauma + hemorrhage rat model (Prough & Frederickson, unpublished). Because the on- and off-rates of carbonic anhydrase can be separately modified by genetic engineering (Thompson *et al.* 2000, 2001), this latter

approach may prove useful for design and fabrication of intracerebral zinc buffers.

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