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A startling role for synaptic zinc

Alan R. Kay¹, Jacques Neyton² and Pierre Paoletti²

¹ Biological Sciences, University of Iowa, Iowa City, Iowa, USA

² Laboratoire de Neurobiologie, CNRS UMR 8544, Ecole Normale Supérieure, 46 rue d'Ulm, 75005 Paris, France

It has long been known that the synaptic vesicles of certain glutamatergic terminals, as well as some inhibitory terminals, are richly supplied with zinc ions, yet the functional role of this pool of zinc in synaptic transmission has remained elusive. In this issue of *Neuron*, Hirzel et al. provide the first *in vivo* evidence that endogenous zinc is required for proper functioning of neuronal circuitry in the brain stem and spinal cord. They show that knock-in mice carrying a point mutation which eliminates zinc potentiation of α 1-containing glycine receptors develop severe sensorimotor deficits characteristic of impaired glycinergic neurotransmission.

Zinc is an indispensable element in the molecular economy of all cells. While most zinc ions are trapped within proteins, as structural or catalytic co-factors, in the brain there is a pool of zinc that is less tightly bound and that can be revealed by histochemical techniques, such as the Timm's stain. The distribution of this potentially mobilizable zinc is remarkable for two reasons: first, it is mainly restricted to higher brain regions (viz. neocortex, hippocampus, striatum and amygdala); second, at the ultrastructural level, histochemically reactive zinc is localised almost exclusively within synaptic vesicles of a subset of glutamatergic axon terminals, where it is accumulated by the vesicular zinc transporter ZnT3 (Frederickson et al., 2005). However, there are notable exceptions to these rules since recently some staining of "chelatable" zinc has also been found in certain inhibitory axon terminals of the cerebellum and spinal cord (Danscher and Stoltenberg, 2005).

Most of the key players in fast synaptic transmission are highly sensitive to extracellular zinc (Smart et al., 2004; Frederickson et al., 2005). This is the case for both the neurotransmitter receptors and transporters of excitatory and inhibitory transmission and, depending on the nature of the target, zinc may either boost or depress the synaptic response. Zinc is a potent inhibitor of NMDA and GABA_A receptors as well as glutamate and GABA transporters. In contrast, at glycine receptors, zinc displays a biphasic effect, potentiating at submicromolar concentrations and inhibiting at submillimolar concentrations.

The abundance of potential synaptic targets and the location of zinc in synaptic vesicles all seem to point to a role for zinc as a modulator of synaptic transmission (Vogt et al., 2000). However, despite clear indications that endogenous zinc is involved in excitotoxicity under pathological conditions (Choi and Koh, 1998), there has been no clear demonstration of a role for zinc in synaptic transmission under physiological conditions. Moreover, two recent findings have cast some doubt upon the physiological relevance of zinc modulation. Knocking-out the ZnT3 gene leads to the total disappearance of histochemical reactive zinc, yet results in no marked phenotype other than a moderate increase in susceptibility to epileptic seizures (Cole et al., 2000). Furthermore, whether zinc is indeed elevated in the synaptic cleft during neuronal activity has been challenged by experiments using zinc-sensitive fluorescent indicators, which suggest that zinc may after exocytosis stick to the presynaptic membrane and not diffuse freely in the cleft (Kay, 2006).

In this context, the paper by Hirzel et al. in this issue of *Neuron*, will certainly revive the flagging hopes of zincologists. This work provides the first demonstration that interfering with zinc modulation of a synaptic pathway leads to a significant alteration in the phenotype of the animal. The authors used an original approach that circumvents most of the bugaboos encountered in previous attempts to pin down the role of zinc. Rather than modifying zinc levels with zinc chelators or genetic manipulations, which necessarily produce pleiotropic effects, Hirzel et al. chose to modify a specific synaptic zinc target, the glycine receptor, which mediates synaptic inhibition in the brain stem and spinal cord. Given the strong association of zinc with glutamatergic terminals, picking this particular target was risky but Hirzel et al. clearly show that it paid off. The authors produced a knock-in (KI) mouse by introducing in the gene coding for the murine $\alpha 1$ glycine receptor subunit a point mutation (D80A)

known from previous studies on recombinant receptors to suppress high-affinity zinc potentiation. Around P12, when the adult $\alpha 1$ GlyR subunit replaces the neonatal $\alpha 2$ subunit, KI mice develop both motor and sensory deficits typical of impaired glycinergic transmission: inducible tremor, delayed righting reflex, abnormal gait, increase in electro-retinogram b-wave amplitude and an enhanced acoustic startle response. This behavior is similar to a genetic disorder in human infants termed hyperekplexia (startle disease), which is characterized by an exaggerated startle reflex, with ensuing stiffness and marked brain-stem reflexes.

At the cellular level, the authors carefully verified that the mutation selectively eliminated the high-affinity zinc potentiation of glycine receptors without affecting their glycine sensitivity, expression level and synaptic targeting. They further compared inhibitory transmission in wild-type (wt) and KI animals by recording in brain stem slices spontaneous IPSCs from hypoglossal motoneurons. The striking observation was that at P14-P16, when $\alpha 1$ GlyR subunit dominates, both the IPSC amplitude and decay are reduced in KI compared to wt animals. These results clearly indicate that in wt animals, endogenous zinc effectively binds to and potentiates synaptic glycine receptors by increasing receptor sensitivity to the agonist. Undoubtedly this work is important, since it is the first demonstration that zinc acts as an endogenous modulator of synaptic transmission.

Exciting as these results are, there are of course many questions that remain, e.g.: how much zinc is seen by the receptors? Does zinc act in a tonic or phasic fashion? Where does it come from? Surprisingly, Hirzel et al. found that application of exogenous zinc at low μM concentrations to wt slices did not increase the amplitude of glycinergic IPSCs, suggesting that in slices the zinc potentiating site on GlyRs is persistently saturated. Consistent with this interpretation, the authors found that application of the chelator tricine depressed the IPSCs in wt but not KI animals. However, the authors' conclusion that the levels of ambient zinc are in the μM range is more questionable. This relies on the estimated affinity of the zinc potentiating site on GlyRs. Using buffered zinc solutions with controlled free zinc concentrations, other groups have measured zinc affinities of native and cloned glycine receptors more than one order of magnitude higher than that reported by Hirzel et al. (Suwa et al., 2001; Miller et al., 2005). Therefore, synaptic zinc levels in the 10-100 nM range may be sufficient to prevent potentiation by exogenously applied zinc.

There are two ways in which we can imagine zinc modulating synaptic transmission (see Figure 1). Firstly, phasically, where synaptic zinc diffuses freely into the synaptic cleft after exocytosis and then binds to post- or pre-synaptic receptors and transporters. Zinc modulation would then wane rapidly as the zinc diffuses away and is transported into cells. Secondly, tonically, where zinc is bound to proteins within the synaptic vesicle and is externalized on exocytosis but not much diffuses into the cleft. Under this scenario, a few zinc ions could be supplied to an extracellular layer of zinc, termed the 'veneer' (Kay, 2006), where the metal stays associated with proteins on the pre- and post-synaptic membranes, including the neurotransmitter receptors. The degree of tonic zinc modulation would be determined by the number of zinc ions in the veneer, which accumulate slowly and progressively with synaptic activity and declines as zinc is removed from the veneer. High enough tonic zinc would explain the absence of effect seen by Hirzel et al. upon application of exogenous zinc. However, these results are also compatible with a phasic mechanism assuming that during normal synaptic activity zinc transiently reaches concentrations high enough to saturate the GlyR zinc binding site. Thus, whether the modulation occurs via the phasic rise of zinc or through regulation of the tonic level of extracellular zinc remains wide open; clearly more experiments are needed. This question applies also to two other major neurotransmitter receptors involved in fast neurotransmission, NMDA and GABA_A receptors, since both receptor families harbour zinc binding sites of high-sensitivity (nM affinity; Paoletti et al., 1997; Hosie et al., 2003).

What is the source of zinc at a glycinergic synapse? One possibility could be that glycinergic presynaptic vesicles accumulate zinc as do glutamatergic and some GABAergic terminals. Looking for ZnT3 expression in glycinergic neurons and analyzing zinc modulation of glycinergic transmission in ZnT3^{-/-} animals should help clarifying this point, although the fact that ZnT3 KOs do not show hyperekplexia makes it unlikely that ZnT3 loads vesicles associated with GlyR modulation. There is certainly no shortage of potential zinc transporters that could do the job, as numerous genes have been identified that code for proteins transporting zinc into intracellular compartments (Palmiter and Huang, 2004). Spillover of zinc from neighbouring synapses, likely glutamatergic, may provide an alternative source of zinc (Kodirov et al., 2006 and see Figure 1).

The work of Hirzel et al. puts a nice dent in the zinc armour, which held firm for more than 50 years, with their demonstration that this ion acts as a physiological neuromodulator of glycinergic transmission. Oddly enough, zinc makes its début as a synaptic neuromodulator not where one would have expected, at the zinc-enriched glutamatergic synapses of the forebrain, but rather at glycinergic synapses in the hindbrain. We have little doubt that this initial success by inhibitory “outsiders” will trigger excitement in the glutamatergic transmission field, where the paradox of the co-existence of an activator (glutamate) and an inhibitor (zinc) remains unsolved.

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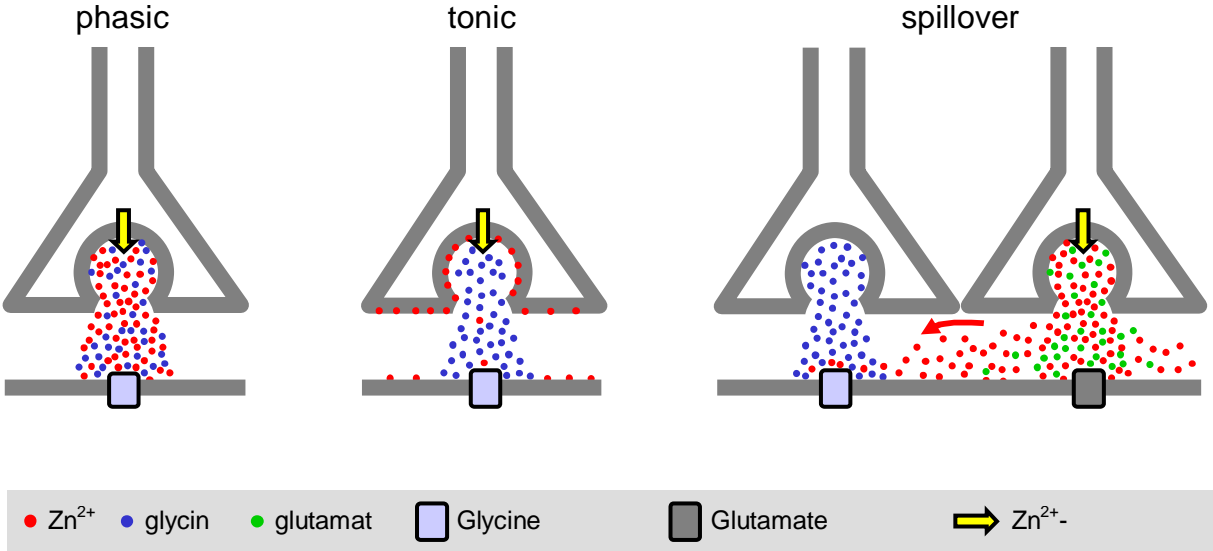


Figure 1: Possible mechanisms for zinc modulation of glycinergic