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Is Zinc a Neuromodulator?

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Abstract

The vesicles of certain glutamatergic terminals in the mammalian forebrain are replete with ionic zinc. It is believed that during synaptic transmission zinc is released, binds to receptors on the pre- or postsynaptic membranes, and hence acts as a neuromodulator. Although exogenous zinc modulates a wide variety of channels, whether synaptic zinc transits across the synaptic cleft and alters the response of channels has been difficult to establish. We will review the evidence for zinc as a neuromodulator and propose diagnostic criteria for establishing whether it is indeed one. Moreover, we will delineate alternative ways in which zinc might act at synapses.

Introduction

Synaptic zinc was first identified more than 50 years ago through a histochemical stain, which renders visible the arc of the mossy fibers and the hilus in the hippocampus (1). This can be even more vividly seen in Timm's stains of the hippocampus, an image that has served to tantalize neurobiologists for years, yet even now the precise role of vesicular zinc remains enigmatic. Zinc is found in the synaptic vesicles of about 50% of glutamatergic synapses (2). For example, the mossy fibers in the hippocampus contain zinc, whereas others like the thalamocortical projections do not. Why this division in glutamatergic terminals occurs is a central question in zinc neurobiology. Although most synaptic zinc appears to be localized in glutamatergic terminals, zinc does also localize in some cases with γ -aminobutyric acid (GABA) and glycine (3).

More than 10 years ago, Palmiter's group demonstrated that a single protein, ZnT3 [also known as Slc30a3; see <http://www.bioparadigms.org/slc/intro.asp> for information about the solute carrier (SLC) family], appears to be essential for loading zinc into synaptic vesicles (4). However, deletion of the encoding gene, which eliminates all histochemically evident zinc in the parenchyma of the brain, led to no obvious defects in behavior or physiology, leaving neurobiologists puzzled over the metal's role in synaptic transmission (5).

A number of functions have been suggested for synaptic zinc, but perhaps the most enduring one is that of a neuromodulator (3). This implies that during exocytosis, zinc diffuses into the synaptic cleft and then binds to receptors or channels on the post- or presynaptic membrane. This zinc-release hypothesis has, however, proved difficult to confirm. Many studies have shown that exogenous zinc affects channels, receptors, and transporters (6,7). Indeed, it is difficult to find a channel that is not influenced by micromolar amounts of zinc. However, the conjunction of these observations with the presence of presynaptic zinc does not prove that zinc is a neuromodulator. There is little dispute that high concentrations of weakly chelated

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zinc are present in synaptic vesicles. However, whether it is released in a simple unimpeded fashion during exocytosis and how, if at all, it might exert a postsynaptic effect remain in doubt (8).

In the contentious field of zinc neurobiology, there are four issues on which there might be some consensus: (i) Zinc is found in some synaptic vesicles, of particular neural pathways, at a relatively high concentration and appears not to be tightly associated with macromolecules (9); (ii) traumatic insults or epileptic seizures can lead to the disappearance of synaptic zinc (10); (iii) sometime after such traumatic episodes, the concentration of free zinc may be elevated in the cytoplasm (11,12); and (iv) many ion channels are modulated by zinc (6,7). Together, these four phenomena seem to indicate that zinc is released during normal synaptic transmission or trauma; however, this is not necessarily so. For example, after trauma, synaptic zinc stores might decline through the movement of zinc from the vesicles directly into the cytoplasm of the terminal, where it may be bound by endogenous chelators. This could occur, for example, if the energy gradient needed to power the uptake of zinc into vesicles collapses. However, the somatic cytoplasmic free zinc may originate from the release of zinc from metallothioneins (13), which are small cysteine-rich proteins in the cytoplasm that can bind up to seven transition-metal ions (14,15). Furthermore, there is an increasing body of evidence suggesting that intracellular zinc accumulations arise from intracellular stores such as mitochondria (16,17).

Here, we will focus on efforts to prove that zinc is a neuromodulator, but we will not review the role of zinc in long-term potentiation (LTP), which has been covered thoroughly by others (18,19). However, it is worth noting that Huang *et al.* recently found evidence for the involvement of zinc in hippocampal mossy fiber LTP acting through the receptor tyrosine kinase, TrkB (20).

Criteria for a Modulator

What needs to be done to confirm that zinc is a neuromodulator? In the case of neurotransmitters, candidate molecules have to submit to a rigorous examination of their credentials before being elevated to the level of a bona fide neurotransmitter. The criteria are (i) presynaptic location, (ii) release upon stimulation, (iii) production of a postsynaptic effect, and (iv) blockade by an antagonist. The second and fourth criteria are stumbling blocks in the case for zinc as a modulator.

To prove that a ligand interacts with a channel or receptor, it is necessary to be able to interrupt its action. Typically, this is done with antagonists. Although metals like cadmium or lanthanides may act as antagonists of zinc, their actions are too widespread—that is, they bind to many targets and are not limited to synapses—to provide an unequivocal answer. It is here where chelators literally jump into the breach, because they can potentially intercept and capture zinc if it moves through the synaptic cleft. To illustrate how chelators can be used to address the question of whether zinc is a neuromodulator, we take a detour into the chemistry of chelators.

Metal Chelators

The classical divalent ion chelators have been enormously important in advancing our knowledge about the role of divalent ions in cell biology. In particular, the hexadentate chelators EDTA and EGTA are indispensable tools in the life sciences (Fig. 1).

Divalent ion chelators bind metals with lesser or higher degrees of affinity through the formation of two or more coordination bonds. The avidity with which a given chelator binds a given metal ion depends on the number of coordination bonds and the nature of both the

ligating groups and the metal. An enormous number of compounds have been assessed for their capacities to form chelates, because of their scientific and commercial importance. For chelators that form a 1:1 complex with metals, the affinity is inversely proportional to the dissociation constant. For synthetic chelators, the affinity of a given chelator typically follows the Irving-Williams series, which is $\text{Mn}^{2+} < \text{Fe}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} > \text{Zn}^{2+}$, and all chelators have higher affinities for the aforementioned metals than for Ca^{2+} and Mg^{2+} (21). Ionic radius seems to be the primary determinant of the series, with small radii allowing tighter interactions. The series holds true for chelators with “open” topologies similar to those shown in Fig. 1, but is not valid for toroidal structures, such as crown ethers and the like. The upshot is that it is difficult to find a chelator specific for a particular metal.

Two large curated databases that tabulate the dissociation constants of chelators are the NIST (22) and the SC-database (23). It is important to determine the protonation of the chelator at the pH at which it will be used. To obtain a measure of the affinity of a chelator for a particular metal, one should calculate the effective dissociation constant from the measured dissociation and protonation constants [see, for example, (24,25)].

From the vantage point of cell biology, there are essentially two classes of chelators—membrane-permeant and -impermeant (Fig. 1). Membrane-impermeant chelators like EDTA, EGTA, BAPTA, tricine (26), and EDPA (27) can, if sufficiently rapid, capture zinc in transit in the extracellular space before it binds to a receptor and can remove zinc from fixed binding sites on the extracellular surface of the plasma membrane or on the extracellular matrix. In addition to binding extracellular zinc, membrane-permeant chelators, such as TPEN or DEDTC (Fig. 1), can chelate zinc within the cytoplasm and other cellular compartments and can strip zinc from intracellular proteins. Although a clear division can be made between permeant and impermeant chelators, within a class there are likely to be differences between chelators. For example, one chelator might be able to access a very tight spot in a protein and remove the metal, whereas another may be too bulky to gain access. Yet others may exert an effect on the redox chemistry of the cell. For example, if EDTA chelates iron, the complex can set into motion the Fenton reaction, which generates hydroxyl radicals from peroxide and superoxide (28). In some cases, compounds that were developed for purposes other than chelation but that also bind divalent ions may exert their effects in a biological system through chelation instead of the originally intended chemistry. One example is the reducing agent dithiothreitol (29).

Some agents, such as pyrithione, act as zinc ionophores, chelating zinc, then ferrying it across membranes and allowing it to diffuse into the cytoplasm (30). In contrast, some chelators, like DEDTC, bind zinc and form uncharged complexes that then partition into membranes without releasing zinc into the cytoplasm and thus do not serve as ionophores, perhaps because these complexes are more stable than those formed by pyrithione.

The widely used chelator EDTA has a high affinity for Ca^{2+} and Mg^{2+} , but it has a more than a million-fold higher affinity for Zn^{2+} . In a solution containing 1 mM EDTA with 3 mM Ca^{2+} , the chelator will be saturated with Ca^{2+} . If a few micromoles of Zn^{2+} are introduced into this system, they will bind to the EDTA, displacing Ca^{2+} . However, the rate of Zn^{2+} chelation is sluggish because it is limited by the slow off-rate for Ca^{2+} and the vast excess of Ca^{2+} (19, 27). Nevertheless, if the speed of chelation is not an issue, EDTA prebound to Ca^{2+} can be used as an effective transition-metal chelator in tissue because it does not perturb the all-important Ca^{2+} and Mg^{2+} concentrations.

Chelators that cross cell membranes and bind metals within the cell will likely have very different effects on cellular physiology than chelators that stay within the extracellular space (8). The membrane permeance of a chelator is therefore of paramount importance in interpreting its effect on cells in vivo. It has recently been claimed that EDTA may be membrane

permeant (31). At physiological pH, the predominant species of EDTA has four negative charges and two when complexed with a metal (25). On the basis of these charges, it is unlikely that either species is membrane permeant. EDTA and EGTA have been used for more than 80 years in biology, and the preponderance of evidence suggests that they are membrane impermeant. This is not a trivial point because many experiments rely on the impermeance of EDTA. The claim that EDTA is membrane permeant requires demonstration through rigorous experiments, including those that prove that a breach in the plasma membrane—for example, by osmotic shock—does not open a passage for EDTA influx.

Modes of Zinc Release

If zinc is released during synaptic transmission, it could act at a number of potential pre- and postsynaptic sites. However, to demonstrate experimentally that it acts as a modulator, we believe it is useful to distinguish between two different modes of zinc release (Fig. 2). The first is a phasic mode, in which synaptic zinc diffuses freely into the synaptic cleft after exocytosis and then binds to post- or presynaptic receptors or channels. In this mode, free zinc appears fleetingly in the extracellular space, diffuses within the cleft, and binds to available sites. The time during which the concentration of free zinc remains elevated in the cleft is determined by extracellular molecules that bind zinc, those that pump it back into cells, and diffusion out of the synaptic cleft. Theoretical studies predict that the transient increases in extracellular free zinc concentration will last for no more than a few milliseconds (32). The second is a tonic mode, in which only a very small fraction of zinc is released into the extracellular space during an exocytotic event. After many such events, the amount of zinc that is bound to macromolecules in the extracellular space would accumulate; however, the concentration of free extracellular zinc would be negligible. How might this occur? If zinc is bound to proteins within the synaptic vesicles by two or three coordination bonds, then upon exocytosis, only a small fraction of the zinc ions would diffuse into the cleft, and most zinc ions would remain attached to proteins in the vesicle and plasma membranes. We have termed this process “externalization.” In this scenario, the vesicles could serve to supply a layer of zinc, termed the “veneer” (27), where the metal is associated with proteins on the pre- and postsynaptic membranes. It is unlikely that there is a rigid dichotomy between tonic and phasic zinc release; rather, some combination of these two modes is probable. Indeed, we suggest that neurons may be able to regulate the balance between tonic and phasic release, perhaps by varying the strength of zinc binding within vesicles.

Alternative mechanisms for zinc’s action on synaptic activity that do not depend on its release may also contribute to zinc’s action in the synapse. For example, if zinc is externalized on some vesicular protein, it may serve as a bridge between this protein and a ligand or macromolecule within the synaptic cleft (8,27,32). We postulate that the ternary complex so formed may then serve as a retrograde signaling pathway in the synaptic bouton.

Another possibility is that zinc serves a role within synaptic vesicles, perhaps changing some aspect of exocytosis. There is a growing body of evidence that the synaptic vesicles within a bouton are not homogeneous, but are segregated into separate pools that are mobilized under different conditions and may be characterized by distinct complements of proteins (33). Salazar *et al.* (34) showed that ZnT3 is specifically targeted to synaptic vesicles through the AP-3 (adaptor protein-3)–dependent recycling pathway, in which synaptic vesicles are formed from endosomes. Moreover, the coexpression of ZnT3 and the vesicular glutamate transporter, VGlut1, increases the vesicular glutamate content (35). Perhaps ZnT3 serves to increase the size of the reserve pool in synaptic boutons and alters the ability of the synapse to follow spike trains with particular tempi.

How can we demonstrate that zinc is acting as a neuromodulator? Three approaches are presently available to address this question: (i) chelate zinc within the synaptic cleft, (ii) eliminate zinc from the synaptic vesicles by knocking out the transporter, and (iii) modify zinc binding sites on pre- or postsynaptic proteins.

Zinc Chelation

Zinc may depress or augment synaptic activity depending on zinc's molecular target. Indeed, if zinc binds to a channel, then depending on the nature of the channel, this may increase or decrease its conductance. We will use the term "chelator effect" (CE) to indicate that the chelator has a measurable effect on the postsynaptic response that is attributable to the interception of zinc. The speed with which the chelator binds zinc is of paramount importance. For example, if one considers a synapse where zinc exerts a phasic action on a receptor, a fast chelator would exhibit a positive CE, whereas a slow one would have no effect. In contrast, if a receptor is subject to tonic modulation, slow and fast chelators would both show a CE.

In addition to the effects of synaptic zinc, contaminating zinc in physiological salines may also exert an influence. Typically, unless extreme precautions have been taken, the concentration of free zinc in solutions will be in the range 0.1 to 1 μM (36). How can one distinguish whether a CE results from contaminating zinc or endogenous zinc? If a zinc-free solution is used and the observed CE is abolished, then the CE can be attributed to contaminating zinc. Zinc-free solutions (<1 nM) can be produced by addition of transition metal-chelating beads (36,37) or with appropriately buffered solutions (26).

Interception of synaptic zinc by chelators was used by Vogt *et al.* (38) to investigate whether synaptically released zinc could modulate *N*-methyl-D-aspartate (NMDA) receptor-mediated events in CA3 pyramidal neurons in the hippocampus. NMDA receptors are a class of ion channels activated by glutamate that are subject to two distinct types of inhibition by zinc (19). First, a high-affinity, voltage-independent (HAVI) inhibition occurs at zinc concentrations from 0.02 to 20 μM , depending on the exact composition of the receptor. Second, a low-affinity, voltage-dependent (LAVD) inhibition arises through zinc occlusion of the permeation pathway of the channel and occurs at higher concentrations of zinc (>20 μM). Vogt *et al.* used a hippocampal slice preparation and applied 2.5 mM Ca-EDTA to chelate zinc. When the postsynaptic response to stimulation of either the mossy fiber (MF, zinc-rich) pathway or the associational commissural (AC, zinc-poor) pathway was measured in the presence of Ca-EDTA, a CE was only detected for the LAVD inhibition at the receptors activated by MF stimulation (Fig. 3). Their results are consistent with a phasic mechanism of zinc release on this neuronal pathway, because the onset of the effect of the zinc chelator and its offset as the chelator is washed off are symmetrical. If the mechanism were tonic, one would expect that restoration of the response during the wash-off period would take far longer because the veneer would take longer to reaccumulate. Similar results were obtained by Molnar and Nadler (39) in experiments that measured the effect of zinc on the recurrent MF pathway in dentate granule cells in the hippocampi of pilocarpine-treated rats, which are a rodent model of epilepsy.

Why does the application of Ca-EDTA not prevent the presumed HAVI inhibition of NMDA receptors? Vogt *et al.* postulated that the chelator is too slow to intercept zinc in the cleft to prevent HAVI inhibition but can prevent LAVD inhibition because of its lower zinc affinity. To test this hypothesis, they applied glutamate onto CA3 pyramidal neurons by iontophoresis while monitoring HAVI inhibition of the NMDA receptor responses without stimulating the synaptic pathway. In this case, the application of Ca-EDTA during iontophoresis led to an increase in the response only when applied in the stratum lucidum (zinc-rich), but not in the stratum radiatum (zinc-poor). This suggests that the high-affinity zinc-binding site on the

receptor is tonically occupied. Because the synaptically activated voltage-independent NMDA response showed no CE, this suggests that the chelator they used, Ca-EDTA, is too slow to capture synaptically released zinc.

The work of Vogt *et al.* is the clearest evidence yet for the operation of phasic zinc modulation; however, more experiments are needed to confirm the role of zinc as an endogenous neuromodulator. For example, to make the story complete, it would be necessary to show that a rapidly acting zinc chelator has a CE on the HAVI inhibition of the NM-DA response to MF stimulation.

A nice example of tonic zinc modulation of a postsynaptic response comes from the work of Ruiz *et al.* (40), who studied the GABAergic response of CA3 pyramidal neurons to MF stimulation in guinea pig hippocampus. This inhibitory response is unusual because GABA is released with glutamate from the same terminals. Application of Ca-EDTA led to an increase in the current consistent with removing zinc-dependent inhibition of the response. However, the time course of wash-in of Ca-EDTA and its wash-out was asymmetrical, with the latter taking more time. To quote the authors, “Indeed, all of the results of the present study can be explained by postulating that extracellular Zn^{2+} associated with mossy fibers exert a tonic inhibition of GABA_A receptors, which is relieved by chelation with TPEN or Ca-EDTA and that there is little dynamic modulation of this inhibition by synaptic release of Zn^{2+} with physiological patterns of stimuli.”

Kainate receptors are another abundant type of glutamate receptor (41). In experiments with extracellular zinc chelators, Mott *et al.* (42) found that synaptic zinc blocks kainate receptors at MF synapses. Moreover, this effect was abolished in mocha mice, a strain that arises from a mutation in the adaptor protein, AP-3, that reduces but does not eliminate ZnT3 or histochemically evident zinc (43).

Most thinking about how zinc might influence synaptic transmission has been largely restricted to a paradigm where zinc is released and then binds to receptors. Izumi *et al.* (44) introduced an interesting twist to this story. They found that the application of Ca-EDTA could block CA1 Schaffer collateral LTP; however, it also blocked LTP when applied before stimulation and removed prior to application of high-frequency stimulation to trigger LTP. Their interpretation, for which they provide further evidence, is that the chelator removes zinc from NMDA receptors, which then lifts the inhibition on persistently active channels and results in inhibition of LTP. This is an important finding because it shows that just because an extracellular chelator blocks LTP, this does not necessarily imply that the chelator is intercepting zinc in passage. Their procedure of applying the chelator before stimulation could also be used to distinguish between tonic and phasic zinc modulation. In the case of a phasic mechanism, the treatment should have no effect because the chelator should not alter the release of zinc at the time of the stimulus. However, in the case of a tonic mechanism, the chelator would remove zinc from the veneer, and if zinc does not accumulate between the time of chelator removal and application of the test stimulus, a positive CE will be observed.

If a CE occurs, it is important to prove that the effect is not due to a pharmacological interaction of the chelator with the channel under investigation. There are instances of chelators having effects on synaptic transmission that are not dependent on chelation (45). At least two methods are used to establish that the chelator is acting through chelation. First, a control pathway that is not zinc-rich—such as the stratum radiatum in the example from the work of Vogt. *et al.* (38)—that activates the same receptors as in the zinc-rich pathway should be unaffected by chelator application. Second, the chelator saturated with zinc should have no effect.

Knocking Out Zinc Transporters

A slew of proteins have been implicated in zinc transport; 10 Slc30 proteins may be involved in clearing zinc from the cytoplasm by transporting it out of the cell or into vesicles, and 14 Slc39 proteins may participate in zinc uptake into the cytoplasm from the extracellular space (46). The best characterized is Slc30a3 (ZnT3), which transports zinc into synaptic vesicles. Only a few members of the Slc30 and Slc39 families have been confirmed to ferry zinc, and some may indeed transport other metals.

ZnT3 is essential for the accumulation of zinc in synaptic vesicles; indeed, genetic ablation in mice entirely eliminates synaptic zinc (47–49). Mice provide a nearly ideal experimental model for assessing the role of zinc in neurotransmission. These animals showed no change in synaptic excitability in the CA3 area of the hippocampus and had normal memory functions, but showed lower threshold for kainic acid–induced seizures (5,50,51). The lack of an obvious phenotype in knockout mice is surprising. Perhaps the mice appear unimpaired either because of developmental compensation or because we have not devised realistic enough assays to coax the phenotype out into the open.

In ZnT3 knockout mice, one might expect the CE on synaptic transmission to be eliminated. However, if a tonic zinc effect is at play, there may be ways of providing zinc to the extracellular veneer other than by release from synaptic vesicles, and a CE may be evident on testing. For example, extracellular zinc may populate the veneer.

Could there be other zinc transporters that provide zinc to vesicles in other neurons? If so, one has to postulate that the zinc in these vesicles is in a different state because it is not detected by histochemical or fluorescent stains.

Modification of Zinc-Binding Sites

An alternative method to reveal the involvement of zinc is to genetically modify a zinc-binding site on a receptor believed to be involved in zinc modulation. This strategy was elegantly implemented by Hirzel *et al.* (52), who used a point mutation [Gla1(D80A)] to render glycine receptors insensitive to zinc. In wild-type receptors, zinc augments the response to glycine. The knockin mice developed a severe neuromotor phenotype that is similar to human hyperekplexia, which is characterized by an exaggerated startle response followed by a period of stiffness. Although they clearly showed that the zinc site on the glycine receptor is required for normal function, it remains an open question whether a phasic or tonic mechanism is engaged during synaptic activation (53). Unexpectedly, hyperekplexia is not observed in ZnT3-null animals, which suggests that separate zinc transporters may be involved in the glycinergic pathway or extracellular zinc from the cerebrospinal fluid (CSF) may be sufficient to populate the zinc-binding sites on the receptor.

Future Experimental Approaches

What other approaches might be used to unravel the role of zinc in the central nervous system? It would, for example, be immensely useful to have a conditional knockout of ZnT3 to avoid the developmental problems associated with a germ-line knockout. There is also an urgent need for rapid zinc chelators that are not affected by physiological concentrations of calcium and magnesium and have no pharmacological action on receptors and channels independent of the ability to chelate zinc. Tricine is an excellent candidate (19); however, there is a need for molecules that can be used at lower concentrations and measurements of how fast they can bind zinc. In addition, more probing psychological tests are needed to tease out the subtle effects that zinc might have on an animal's cognitive capabilities.

If zinc is released, does it act alone as a hydrated ion or with an accomplice like histidine, which is abundant in the CSF (54)? Xiong *et al.* (55) found that zinc:pyrithione, which forms 1:2 complexes like those formed by zinc:histidine, potentiates KCNQ potassium channels. This raises the interesting question whether similar metal:ligand complexes might serve as neuromodulators in the brain.

Perhaps the central enigma of zinc neurobiology is why some pathways are stocked with zinc, whereas others that use the same neurotransmitter are not. There may be differences in the kinetics or perhaps plasticity of the zinc-rich versus the zinc-poor pathways that only more experimental investigations will reveal. There are suggestions that ZnT3 is present in only a subset of vesicles (34); whether this is indeed true in glutamatergic terminals and, if so, how this might affect vesicle recycling are questions of great importance for the field. Zinc-rich terminals have been described throughout the vertebrate kingdom, but it is not known whether synaptic zinc is found in any invertebrate nervous systems. This may be a useful avenue to pursue given the power of model systems like *Caenorhabditis elegans*, *Drosophila melanogaster*, or *Ciona intestinalis* to shed light on biological problems.

Why animals invest energy to accumulate zinc in synaptic vesicles is likely to engage the interests of neurobiologists and chemists alike for a while. We suspect that in the pursuit of its function, as in all biological quests, our view of zinc's role will deepen and broaden as we mine the rich seams that its biology presents.

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Fig. 1. Chelators. Membrane-impermeant chelators are in black and membrane-permeant ones are in blue.

Fig. 2. Phasic (**A**) versus tonic (**B**) zinc release. Zinc ions are shown as red spheres. In the tonic mode zinc ions are bound to membrane associated macromolecules (not shown) in synaptic vesicles and on the pre- and postsynaptic membrane.

Fig. 3. Chelation removes zinc inhibition of the mossy fiber NMDA synaptic response. **(A)** CA3 pyramidal cell indicating approximate position of the stratum lucidum (sl; MF pathway) and stratum radiatum (sr; AC pathway). The CA3 neuron [from (56)] was obtained from the “Hippocampal neuronal morphology site” (www.compneuro.org/CDROM/nmorph/cellArchive.html). **(B)** (Top) Synaptic currents elicited under voltage-clamp (holding potential -40 mV) in a CA3 pyramidal cell on stimulation of the mossy fiber pathway in the presence of 0.5 mM Mg^{2+} , 12 μ M NBQX, and 100 μ M picrotoxin, in the presence and absence of Ca-EDTA. (Bottom) Response of the same cell to stimulation of the AC pathway. **(C)** Effect of the application of 2.5 mM Ca-EDTA on

the synaptic currents elicited by MF (○) or AC stimulation (●); (mean $\pm$ SEM, $n = 6$). The horizontal bar represents the duration of the Ca-EDTA application. [Modified from (38), with permission from Elsevier]