

The Function and Regulation of Zinc in the Brain

Rebecca F. Krall,^{a,b,c,d} Thanos Tzounopoulos^{b,c,*} and Elias Aizenman^{a,c,d,*}

^a Department of Neurobiology, University of Pittsburgh School of Medicine, USA

^b Department of Otolaryngology, University of Pittsburgh School of Medicine, USA

^c Pittsburgh Hearing Research Center, University of Pittsburgh School of Medicine, USA

^d Pittsburgh Institute for Neurodegenerative Diseases, University of Pittsburgh School of Medicine, USA

Abstract—Nearly sixty years ago Fredrich Timm developed a histochemical technique that revealed a rich reserve of free zinc in distinct regions of the brain. Subsequent electron microscopy studies in Timm- stained brain tissue found that this “labile” pool of cellular zinc was highly concentrated at synaptic boutons, hinting a possible role for the metal in synaptic transmission. Although evidence for activity-dependent synaptic release of zinc would not be reported for another twenty years, these initial findings spurred decades of research into zinc’s role in neuronal function and revealed a diverse array of signaling cascades triggered or regulated by the metal. Here, we delve into our current understanding of the many roles zinc plays in the brain, from influencing neurotransmission and sensory processing, to activating both pro-survival and pro-death neuronal signaling pathways. Moreover, we detail the many mechanisms that tightly regulate cellular zinc levels, including metal binding proteins and a large array of zinc transporters. © 2021 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: zinc, zinc transporters, synapse, neurotransmitter receptors, central nervous system, neurophysiology, synaptic plasticity, sensory processing, neurodegeneration.

INTRODUCTION

Zinc is essential for survival and zinc deficiency is linked to a variety of adverse conditions including growth retardation, impaired immune function, improper skin and bone formation and repair, as well as cognitive disfunction (Prasad, 2003). Mutations in the principal zinc importer in the gut, ZIP4, lead to *acrodermatitis enteropathica*, a zinc-deficiency disease characterized by failure to thrive, severe dermatitis, hair loss, and diarrhea, which can be lethal if left untreated (Dufner-Beattie et al., 2007). These severe outcomes are largely due to the impact of the essential structural and enzymatic functions of protein-bound zinc. In fact, an estimated 10% of the human genome encodes zinc-binding proteins that serve a diverse and rich array of biological roles (Andreini et al., 2006). Zinc catalyzes reactions of all the major classes of enzymes (Laitaoja et al., 2013) and stabilizes zinc-finger proteins, whose functions include transcriptional activation, protein folding, and RNA regulation (Laity et al., 2001).

In the brain, auto-metallography, more commonly referred to as Timm staining, labels a separate reserve pool of “labile” zinc that is contained within synaptic vesicles (Timm, 1958; Haug, 1967). For the purpose of this review, labile zinc refers to zinc that is not tightly bound to proteins, but instead is buffered through loose, reversible interactions with metal-binding proteins or

*Corresponding authors. Address: Departments of Neurobiology and Otolaryngology, University of Pittsburgh School of Medicine, USA. E-mail addresses: thanos@pitt.edu (T. Tzounopoulos), redox@pitt.edu (E. Aizenman).

Abbreviations: AD, Alzheimer’s disease; A1, primary auditory cortex; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BK, large-conductance Ca^{2+} activated Slo1 potassium channel; CaEDTA, Calcium Ethylenediaminetetraacetic acid; CAMKII, Calcium/calmodulin-dependent protein kinase II; CDF, cation diffusion facilitator; CK, casein kinase; CP, calcium permeable; CREB, cAMP response element binding protein; CTZ, cyclothiazide; DCN, dorsal cochlear nucleus; DTDP, 2,2’-dithiodipyridine; Drp1, dynamin-related protein 1; EPSC, excitatory postsynaptic current; ERK, extracellular regulated kinase; GluA, glutamate AMPA receptor subunit; GluN2, glutamate NMDA receptor subunit 2; GlyR, glycine receptor; G1, Group 1; IPSC, inhibitory postsynaptic current; KA, kainate; KO, knockout; KCC2, $\text{K}^{+}/\text{Cl}^{-}$ cotransporter 2; LTCC, L-type calcium channel; LTD, long-term depression; LTP, long-term potentiation; MAPK, mitogen-activated protein kinase; mEPSC, miniature excitatory postsynaptic current; mIPSC, miniature inhibitory postsynaptic current; mRNA, messenger ribonucleic acid; mZnR, metabotropic zinc receptor; MMP, mitochondrial membrane potential; MRE, metal regulatory element; MT, metallothionein; MTF-1, MRE transcription factor 1; mGluR, metabotropic glutamate receptor; NMDA, N-methyl-D-aspartate; NO, nitric oxide; PKC, protein kinase C; P2X, ionotropic purinergic; PTP, protein tyrosine phosphatase; ROS, reactive oxygen species; RNA, ribonucleic acid; SAM, sterile alpha motif; siRNA, small interfering ribonucleic acid; shRNA, short hairpin ribonucleic acid; SNP, single nucleotide polymorphisms; TM, transmembrane domain; TRP, transient receptor potential; VGCC, voltage-gated calcium channels; ZAC, Zinc activated receptor; ZnT, zinc transporter; ZIP, Zrt, Irt-like protein; 12-LOX, 12-lipoxygenase.

sequestered into subcellular organelles (Colvin et al., 2010). The presence of zinc in synaptic vesicles suggests that the metal may have additional, unique signaling roles within the central nervous system. Importantly, the localization and content of this vesicular or synaptic zinc pool can rapidly change as a result of sensory experience (Brown and Dyck, 2002; Kalappa et al., 2015; McAllister and Dyck, 2017; Vogler et al., 2020), suggesting that neuronal zinc signaling undergoes experience-dependent plasticity. At rest, both intracellular and extracellular labile zinc levels are maintained at nanomolar or sub-nanomolar concentrations (Frederickson et al., 2006; Sensi et al., 2009; Colvin et al., 2010; Anderson et al., 2015). Following synaptic zinc release, extracellular zinc levels transiently increase to modulate neurotransmission (Frederickson et al., 2004; Sensi et al., 2009; Quinta-Ferreira et al., 2016; Wolf et al., 2018). Moreover, activity can trigger large transient increases in intracellular zinc that initiate diverse cellular signaling (Sensi et al., 2009). Under physiological conditions, these changes in zinc concentration in and out of cells are tightly regulated to prevent neuronal death that occurs with zinc dysregulation (Koh and Choi, 1994; Koh et al., 1996; Aizenman et al., 2000; Lobner et al., 2000; Koh, 2001). Tight regulation and precise spatiotemporal distribution of the metal is essential as this unconventional neurotransmitter cannot be synthesized or metabolized. As such, twenty-four distinct transporters are involved in tightly regulating the spatial and temporal distribution of zinc. In this review, we detail the current state of art in the neurobiology of zinc, from its synaptic release and known postsynaptic and signaling actions, to its regulation by a large array of zinc binding proteins and zinc transporters.

THE NEUROBIOLOGY OF SYNAPTIC ZINC

Zinc is concentrated in synaptic vesicles of large populations of glutamatergic neurons in widespread areas of the brain, primarily within the cerebral cortex, hippocampus, amygdala and the auditory brainstem (McAllister and Dyck, 2017). This pool of synaptic, labile zinc is released in an activity-dependent manner, targeting a large array of postsynaptic receptors. In this section, we summarize the diverse roles synaptic zinc plays in modulating postsynaptic function and neuronal circuits.

Evidence for the potential role for zinc as a synaptic signaling molecule arose when multiple groups observed that exogenous zinc application inhibited NMDA and GABA_A receptors (Smart and Constanti, 1982; Peters et al., 1987; Westbrook and Mayer, 1987). This suggested that zinc could act on postsynaptic targets following its release from presynaptic terminals. Within this same time period, evidence for vesicular release of zinc was observed following chemical or electrical stimulation of the mossy fiber pathway in hippocampal slices (Assaf and Chung, 1984; Howell et al., 1984). This activity-dependent increase in extracellular zinc suggested that zinc is released from presynaptic vesicles as a neuromodulator. In agreement with this, Timm-staining labeled zinc was identified in synaptic vesicles that was subsequently released by stimulation and

detected within the synaptic cleft over time (Pérez-Clausell and Danscher, 1985, 1986). The discovery of ZnT3 (Slc30a3) (Palmiter et al., 1996; Wenzel et al., 1997) as the zinc transporter sequestering the metal into synaptic vesicles, dramatically advanced the field. Indeed, the development of ZnT3 knockout (KO) mice allowed investigators to specifically and fully eliminate synaptic zinc. Namely, ZnT3 KOs showed a striking near absence of Timm staining in the brain, thereby demonstrating that most of labile zinc in the brain is contained within synaptic vesicles (Cole et al., 1999). It must be mentioned that some early studies aimed at examining the potential roles of synaptic zinc took advantage the so-called *mocha* mouse (Vogt et al., 2000). This mouse has reduced ZnT3 targeting to synaptic vesicles as a result of a deletion in the gene of the δ subunit of the adaptor protein AP-3, which interacts with the cytoplasmic tail of ZnT3 (Salazar et al., 2004). Initial characterization of the *mocha* mouse found a relative absence of zinc staining in vesicles (Kantheti et al., 1998). However, further research found that although *mocha* mice exhibit reductions in vesicular zinc compared to control animals, both Timm staining and fluorescent staining revealed residual vesicular zinc present in the hippocampus and neocortex of mutant mice (Stoltenberg et al., 2004), suggesting ZnT3 KO are the preferred tool to investigate the physiological actions of synaptic zinc.

Chelation of synaptic zinc

The ability to resolve fundamental questions about synaptic zinc was significantly advanced by the development of zinc-chelating agents optimized for interrupting localized and rapidly generated zinc signals resulting from synaptic release (Fig. 1). To be effective, chelators must target zinc with high enough affinity to outcompete binding sites on postsynaptic targets without disrupting other ions, such as calcium or magnesium. Furthermore, chelators must act rapidly in order to bind fast zinc transients produced by synaptic release (Radford and Lippard, 2013). Cell-impermeant extracellular chelators, such as CaEDTA and tricine have traditionally been preferred tools used to study synaptic zinc in order to avoid off-target effects of intracellular zinc chelation. CaEDTA, despite having high affinity for zinc, binds the ion too slowly, as zinc must displace calcium in the complex (Paoletti et al., 2009). This limitation prevents complete and effective chelation of fast zinc transients associated with synaptic release (Anderson et al., 2015). In contrast, tricine acts rapidly but its micromolar affinity for zinc is not generally sufficient to prevent zinc binding to high affinity targets such as GluN2A-containing NMDARs (Radford and Lippard, 2013; Anderson et al., 2015). A new generation chelator, ZX1 (Pan et al., 2011), has a 1 nM zinc dissociation constant and a second-order rate constant for binding zinc, which is 200-fold higher than those for tricine and CaEDTA (Anderson et al., 2015). As such, ZX1 has emerged as the most appropriate chelator for investigating the effects of fast, transient elevations of zinc on synaptic targets with nanomolar affinity. ZX1, used in conjunction with ZnT3 KO mice, has proved an invaluable asset to better

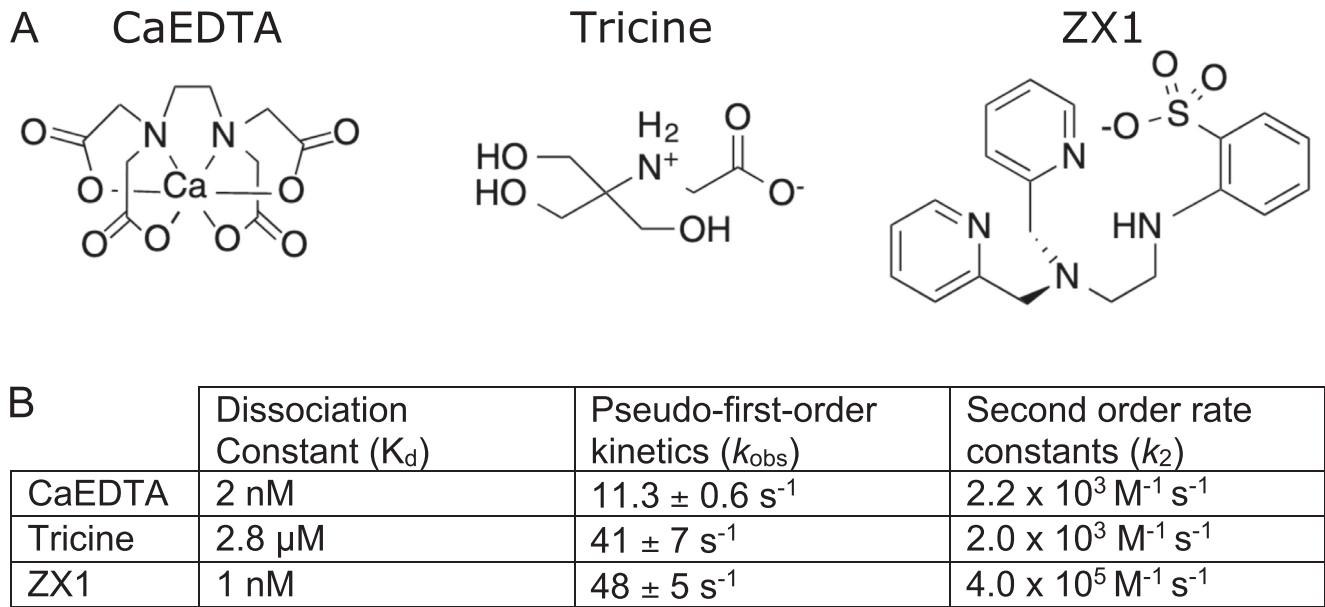


Fig. 1. Kinetics and zinc binding for the chelators ZX1, tricine, and CaEDTA. **(A)** Chemical structures of each extracellular chelator at pH 7.4 **(B)** Table showing the K_d , first- and second-order kinetics for ZX1, tricine, and CaEDTA.

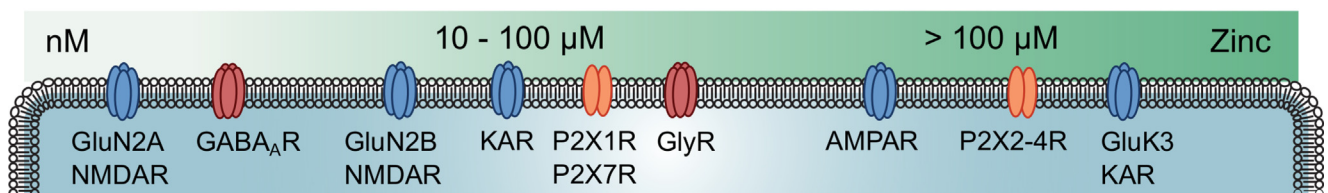
understand the physiological consequences of synaptically released zinc (Anderson et al., 2015; Kalappa et al., 2015).

Synaptic targets of vesicular zinc

NMDA receptors. The best characterized modulatory action of zinc is its inhibition of NMDARs. Initial studies found that exogenous application of micromolar concentrations of zinc inhibited NMDAR currents. These studies suggested that this inhibition had two components; a voltage-independent block at lower μM

concentrations and a voltage-dependent block at higher concentrations ($> 10 \mu\text{M}$, Fig. 2A) (Christine and Choi, 1990; Legendre and Westbrook, 1990). Buffering solutions with low affinity zinc chelators later revealed a higher-sensitivity, subunit-dependent effect of zinc. Specifically, removing low levels of contaminating zinc in the solution demonstrated that zinc inhibits GluN2A-containing NMDARs at low nanomolar concentrations (Paoletti et al., 1997). This high affinity inhibition of GluN2A occurs through binding to the N-terminal domain, allosterically reducing NMDAR channel open probability via an enhancement of proton inhibition (Low et al.,

A Inhibition



B Potentiation

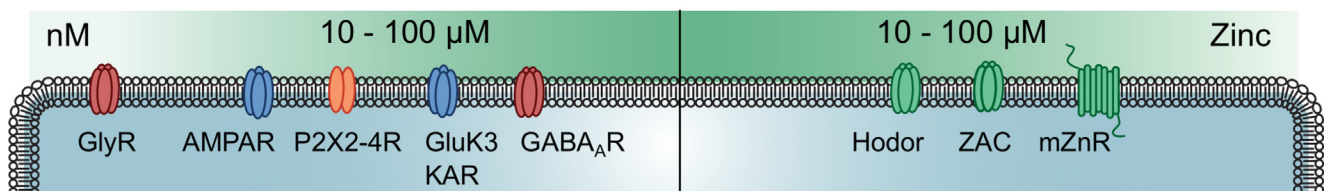


Fig. 2. Receptor targets of synaptic zinc. The green gradient represents the approximate concentration of zinc necessary for modulation each receptor **(A)** Zinc inhibits receptors with affinities ranging from nanomolar to high micromolar. **(B)** Multiple receptors that are inhibited by zinc are also potentiated by the ion at lower (GlyR, AMPAR, GluK3 KAR, and P2X2-4R) or higher (GABA_AR) concentrations. **(C)** The zinc channels ZAC, expressed in humans, and Hodor, expressed in drosophila, as well as the metabotropic zinc receptor mZnR/GPR39 are directly activated by zinc. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

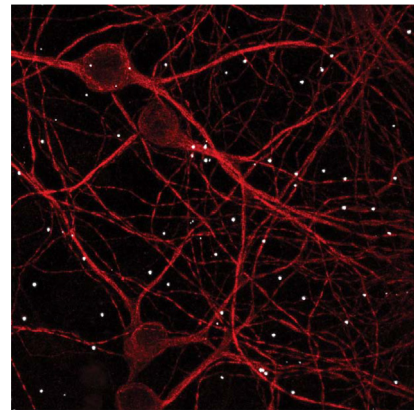
2000; Paoletti et al., 2000; Erreger and Traynelis, 2008). A comparable allosteric binding site also exists on GluN2B's N-terminal domain, albeit with micromolar affinity (Fig. 2A) (Rachline et al., 2005).

In agreement with studies that characterized zinc-mediated inhibition using exogenous zinc, endogenous zinc released from presynaptic terminals also inhibits postsynaptic NMDARs. Chelation of activity-dependent zinc signals using CaEDTA potentiated NMDAR responses evoked with the mossy fiber stimulation, suggesting zinc endogenously inhibits NMDARs at this synapse (Vogt et al., 2000). Furthermore, recordings of ZnT3-containing synapses in the hippocampus and the dorsal cochlear nucleus (DCN) showed that chelating zinc with ZX1 enhances synaptic and extrasynaptic NMDAR excitatory postsynaptic currents (EPSCs), thus revealing an effect of endogenous zinc that was not observed in ZnT3 KO animals (Pan et al., 2011; Anderson et al., 2015). Moreover, a knock-in mutation (H128S) on the N-terminal domain of GluN2A removes high affinity zinc binding from NMDARs and eliminates the effect of tricine on NMDAR EPSCs in the hippocampus (Vergnano et al., 2014). Together, these results convincingly reveal the physiological relevance of zinc modulation of NMDAR. Of interest, mutations of the GluN2A subunit that lead to altered NMDAR zinc sensitivity have been identified in patients with childhood epilepsy, suggesting that endogenous zinc inhibition may contribute to proper neurological function (Serraz et al., 2016).

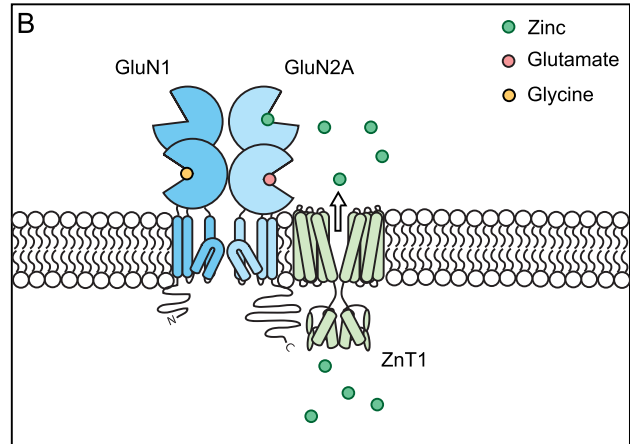
The inhibition of NMDARs by zinc has been thought to be the result of zinc diffusion across the synaptic cleft with subsequent binding to the extracellular domain of the NMDAR. However, this model does not account for the observed association of the highly zinc-sensitive NMDAR subunit GluN2A with the postsynaptic zinc transporter ZnT1 (Sindreu et al., 2014; Mellone et al., 2015), which moves intracellular zinc to the extracellular space. A recent study by our group reported that disruption of ZnT1-GluN2A association by a cell-permeant peptide strongly reduced NMDAR inhibition by synaptic zinc in mouse DCN synapses (Fig. 3) (Krall et al., 2020). Moreover, synaptic zinc inhibition of NMDARs required postsynaptic intracellular zinc (Krall et al., 2020), suggesting that cytoplasmic zinc is transported by ZnT1 to the extracellular space in close proximity to the NMDAR. These results changed a decades-old dogma on how zinc inhibits synaptic NMDARs and demonstrate that presynaptic release and a postsynaptic transporter organize zinc into distinct microdomains to modulate NMDAR neurotransmission.

AMPA receptors. In contrast to NMDARs, AMPA receptor (AMPA) modulation by zinc is less understood. Initial studies of AMPA-induced currents in oocytes expressing neuronal mRNA and cultured neurons found that exogenous zinc potentiates AMPAR responses at micromolar concentrations (30–100 μ M) but inhibits it at millimolar concentrations (Fig. 2) (Rassendren et al., 1990; Bresink et al., 1996). AMPARs composed exclusively of GluA3 display a similar biphasic response to exogenous zinc, albeit exhibiting higher affinity for zinc potentiation (4–7.5 μ M potentiation, > 100 μ M

A



B



C

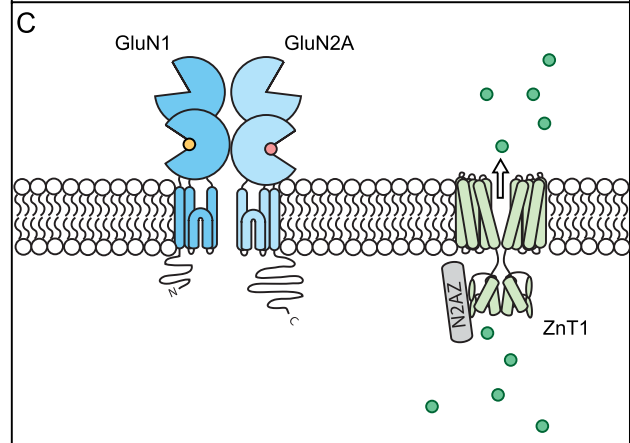


Fig. 3. ZnT1 regulation of GluN2A-containing NMDARs. Top panel shows a micrograph depicting rat cortical neurons in culture (red = microtubule associated protein) with proximity ligation assay fluorescent puncta at locations where GluN2A NMDAR subunits interact with ZnT1. Bottom panels are a model depicting how the association between ZnT1 and GluN2A localizes zinc in the proximity of the high-affinity zinc binding site on GluN2A, leading to NMDAR inhibition. When this interaction is disrupted by a cell-permeant peptide that competitively binds to ZnT1 (TAT-N2AZ), the dissociation of the two proteins removes zinc from the proximity of GluN2A and subsequently reduces inhibition of NMDARs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibition. Fig. 2). Zinc-mediated potentiation is strongly subunit dependent, as GluA1 homomers, GluA2/3 heteromers, and GluA1/2 heteromers only exhibit high-

concentration inhibition, but not potentiation (Fig. 2B) (Dreixler and Leonard, 1994). GluA2-lacking AMPARs are commonly referred to as calcium-permeable AMPARs (CP-AMPA), because GluA2 subunits prevent calcium flux. Interestingly GluA3-containing AMPARs lose their sensitivity to low concentrations of zinc when co-assembling, and thereby made calcium-impermeable, with GluA2, suggesting a possible relationship between zinc potentiation and calcium permeability (Dreixler and Leonard, 1994). In fact, studies of native AMPARs in carp retinal horizontal cells found that only CP-AMPA were sensitive to modulation by exogenously applied zinc (Sun et al., 2010a). Furthermore, recording AMPAR responses in calcium-free solution unmasks zinc potentiation of GluA1 homomers and enhances the potentiating action of zinc on GluA3 homomers, suggesting physiological calcium reduces zinc potentiation of CP-AMPA (Dreixler and Leonard, 1997). Extracellular calcium also enhances zinc-mediated inhibition of CP-AMPA in both heterologous systems and carp retinal horizontal cell preparations (Dreixler and Leonard, 1997; Sun et al., 2010b). Together these studies suggest a close relationship between calcium and zinc in the modulation of CP-AMPA.

Zinc potentiation of AMPARs is hypothesized to be the result of reduced receptor desensitization. Both the rate and degree of receptor desensitization are reduced with zinc application in parallel with its potentiation in cultured neurons (Bresink et al., 1996). Furthermore, inhibiting desensitization using high concentrations of cyclothiazide (CTZ) prevents zinc-induced potentiation of AMPARs in both olfactory bulb cultures and CA3 hippocampal neurons (Lin et al., 2001; Blakemore and Trombley, 2019). In contrast, there is enhanced exogenous zinc-mediated inhibition of AMPARs when receptor desensitization is reduced by the inclusion of the auxiliary subunits $\gamma 2$ or $\gamma 8$, or following CTZ treatment (Carrillo et al., 2020). Interestingly, at lower concentrations, CTZ has a greater affinity for the ‘flip’ splice variant of AMPARs (Johansen et al., 1995). The alternatively spliced flip/flop cassette on the ligand-binding domain of AMPARs imparts distinct receptor properties. Notably, the ‘flip’ variant is more sensitive to allosteric regulation by anions and auxiliary subunits (Dawe et al., 2019). Carp retinal horizontal preparations preferentially express the alternate ‘flop’ variant and show no basal modulation by exogenous zinc (Shen and Yang, 1999). However, low concentrations of CTZ preferentially increase ‘flip’-mediated currents, thereby unmasking zinc potentiation and inhibition, suggesting that zinc may preferentially act on these splice variants (Shen and Yang, 1999; Sun et al., 2010b). Therefore ‘flip’-splice variants, in addition to CP-AMPA, may represent a subset of zinc-sensitive AMPARs whose relative expression determine the ability of the metal to regulate neuronal function. Indeed, these difference may underlie the diversity of zinc effects observed in olfactory bulb cultures, which exhibit both zinc-sensitive and zinc-insensitive AMPAR responses (Blakemore and Trombley, 2004, 2019).

An alternative, indirect mechanism of zinc potentiation suggests that the ion modulates AMPARs through its

stabilization of the post-synaptic density scaffolding proteins Shank2 and Shank3. Zinc binds to the sterile alpha motif (SAM), a putative protein interaction domain (Thanos et al., 1999) on the C-terminal of Shank2/3, to regulate their oligomerization and localization (Arons et al., 2016). Both of these shank proteins regulate AMPAR recruitment and stabilization at the post-synaptic density. Silencing Shank3 with siRNA in hippocampal cultures blocked zinc-induced potentiation of AMPAR EPSCs in paired recordings between pyramidal neurons (Arons et al., 2016). Another study showed that shRNA against Shank2 or Shank3 prevented zinc-dependent increases in miniature excitatory postsynaptic current (mEPSC) decay time and charge transfer in hippocampal cultures. The effect was specific in younger neurons (11–14 days in vitro), which express CP-AMPA. Zinc also increased the co-localization of GluA2 at post-synaptic shank puncta and reduced the AMPAR inward rectification, a characteristic of GluA2-lacking CP-AMPA. Together, these findings led to the hypothesis that zinc enhances AMPAR EPSCs via Shank-mediated recruitment of GluA2 to the post-synaptic density (Ha et al., 2018). However, exogenous zinc-mediated potentiation has also been observed in *Xenopus* oocytes, which presumably lack the Shank-containing scaffolding complexes present in hippocampal neurons (Dreixler and Leonard, 1994, 1997). Therefore, the mechanism described here may contribute to zinc-regulation of AMPAR synaptic currents but is likely not necessary for the direct action of zinc on AMPARs.

AMPA zinc inhibition occurs following endogenous zinc release at synapses. Chelation of synaptic zinc with ZX1 in acute slices of DCN and hippocampus increased AMPAR EPSCs in cartwheel cells and CA1 cells respectively. Furthermore, this effect was absent in ZnT3 KO animals, suggesting that chelation specifically increased EPSCs through its action on synaptic zinc (Kalappa et al., 2015). Both of these synapses express GluA1–3 receptors, consistent with previous findings linking zinc’s effect to CP-AMPA (Wenthold et al., 1996; Petralia et al., 2000). A bidirectional effect of zinc was later observed during a train of high-frequency stimulation, with initial EPSCs inhibition by endogenous zinc followed by zinc-induced enhancement later in the train. However, the enhancement resulted from increased release probability and thus not via a direct action of zinc on AMPARs (Kalappa and Tzounopoulos, 2017).

Kainate receptors. Kainate receptors (KAR), much like the closely related AMPARs, are modulated by zinc in a subunit dependent manner. Studies utilizing recombinant expression of KAR subunits in *Xenopus* oocytes found that all KARs tested were inhibited by zinc but with differential affinity. Heteromers of GluK2/4 and GluK2/5 were the most sensitive, with IC_{50} values in the low micromolar range. In contrast, homomers of GluK2 or GluK1 were notably less sensitive, with GluK2/4’s IC_{50} 40-fold lower than GluK2 alone (Fig. 2). This suggests GluK4/5 subunits, whose presence in KARs impart higher glutamate affinity, also increase the zinc sensitivity. Zinc has been hypothesized to inhibit KARs via an allosteric process (Mott et al., 2008). Inter-

estingly GluK3 receptors exhibit a biphasic response to zinc, comparable to AMPARs. They are potentiated at lower concentrations, with an EC_{50} of $\sim 20 \mu M$, but are inhibited by concentrations of over $100 \mu M$ (Fig. 2). Zinc potentiation of GluK3 is mediated via a reduction in desensitization rate and increase in glutamate affinity. Mutational analysis identified two residues on GluK3 that specifically mediate zinc potentiation, aspartate 759 and histidine 763. The aspartate is unique among KAR subunits and mutation to glutamate or alanine to mimic subunits GluK1/2 or GluK4/5, respectively, abolishes zinc potentiation (Veran et al., 2012). These findings have led to the hypothesis that zinc potentiates KARs by stabilizing dimers of the ligand binding domain, thereby slowing desensitization. Interestingly GluK2/3 heteromers are also potentiated by zinc, albeit with lower affinity. The potentiation is hypothesized to depend on an aspartate residue that is conserved across KAR subunits, and which participates in the zinc-binding and subsequent dimer stabilization (Veran et al., 2012).

Although there is compelling evidence for zinc modulation of KARs, only one study has specifically examined this modulation by endogenous zinc. The synaptic contribution of KARs can be isolated using GYKI 52466, a selective AMPARs antagonist. In the presence of this drug, field EPSCs in CA3 evoked with mossy fiber stimulation are potentiated with zinc chelation. Furthermore, this effect is lost in *mocha* mutant mice, strongly suggesting that the action of chelators occurs through removal of synaptic zinc (Mott et al., 2008). Additionally, some insight into the endogenous role of zinc regulation of KARs can be inferred based on their localization. KARs are located both pre- and post-synaptically, and at the mossy fiber synapse, post-synaptic KARs are composed of GluK2, 4 and 5 subunits and presynaptic KARs are composed of GluK2 and 3 subunits (Veran et al., 2012). This suggests that synaptic zinc could simultaneously potentiate GluK3-containing KARs pre-synaptically while inhibiting post-synaptic KARs. Chelation of synaptic zinc blocks the induction of presynaptic long-term potentiation (LTP) at the mossy fiber synapse (Pan et al., 2011), which, interestingly, is a form of LTP associated with KAR activation (Schmitz et al., 2003; Bortolotto et al., 2005).

P2X receptors. P2X purinergic receptors (P2XR) are ionotropic receptors activated by extracellular ATP. The properties of these receptors have been extensively characterized using heterologous expression in xenopus oocytes. Zinc differentially modulates P2XRs depending on which of the P2X subunits are expressed. P2X1 and P2X7 are inhibited by low (μM) concentrations of extracellular zinc, whereas P2X2–4 exhibit potentiation at low (μM) concentrations and inhibition at higher concentrations (Fig. 2) (Nakazawa et al., 1997; Wildman et al., 1998, 1999a, 1999b). Interestingly, P2X2Rs have been shown to be activated by zinc alone, suggesting they may serve as both zinc- and ATP-gated receptors (Schwiebert et al., 2005). However, evidence for zinc activated P2X2R currents have not yet been observed in neurons. It is unclear if and how zinc acts on P2X5–6, although P2X4/6 heteromers exhibit similar zinc sensitiv-

ity as P2X2 homomers (Lê et al., 1998). Multiple neuronal preparations have been shown to exhibit P2X zinc modulation, including spinal cord, hypothalamus, and hippocampal cell cultures (Laube, 2002; Vorobjev et al., 2003; Lorca et al., 2011).

Zinc potentiation and inhibition of P2XRs are mediated by allosteric modulation of the receptors by the metal, although through distinct sites. Zinc binding sites were first investigated using mutational analysis of the extracellular domain, revealing two histidine residues on rat P2X2Rs, H120 and H213, critical for zinc potentiation. Mutating these residues to alanine revealed an inhibitory effect at lower concentrations that is normally masked by zinc-mediated potentiation of wild-type receptors (Clyne et al., 2002; Lorca et al., 2005). Further studies found that these residues coordinate zinc at the interface of subunits (Nagaya et al., 2005). Similarly, structural analysis of an invertebrate homolog of P2X4 identified a zinc-binding site at the trimer interface that facilitates pore opening, suggesting a conserved localization of zinc binding across species and subunits (Kasuya et al., 2016). Interestingly, human P2X2 receptors are inhibited by low concentrations of zinc, in contrast to their rat homologs. In a model of the human P2X2R, histidine residues H204 and H209 are in close proximity with H330 of the adjacent subunit, suggesting a potential zinc binding site. Indeed, mutations of these residues reduces zinc inhibition. In the rat homolog, mutation of a lysine residue to histidine mimics the human sequence and consequently reverses the zinc effect from potentiation to inhibition (Punthambaker et al., 2012). Similarly, multiple histidine residues on P2X7Rs have been identified that mediate its zinc inhibition, including H219, H267, and H62, perhaps indicative of a histidine site similar to hP2X2 (Acuña-Castillo et al., 2007; Liu et al., 2008).

Zinc may regulate neuronal function through its modulation of P2XRs. Investigations of inhibitory transmission in spinal cord cell cultures found that zinc increased the frequency of miniature inhibitory postsynaptic currents (mIPSCs) through its potentiation of presynaptic P2X2Rs and subsequent increase in presynaptic release probability (Laube, 2002). Furthermore, zinc was found to regulate synaptic plasticity in the CA1 region of the hippocampus via P2X4Rs. At relatively low concentrations ($5\text{--}50 \mu M$), application of zinc enhanced LTP evoked by theta burst stimulation of Schaffer collateral fibers. The zinc effect was lost with P2X antagonists and could be mimicked using a P2X4 positive allosteric modulator, suggesting that zinc facilitates LTP via P2X4Rs (Lorca et al., 2011).

GABA_A receptors. GABA_A receptors are allosterically inhibited by micromolar concentrations of zinc (Fig. 2) (Smart and Constanti, 1982; Westbrook and Mayer, 1987; Celentano et al., 1991; Barberis et al., 2000). Like other channels, zinc's regulation is subunit dependent. Notably, heteromers containing only α and β subunits are the most sensitive to zinc, with an IC_{50} in the range of $0.1\text{--}1 \mu M$. Inclusion of a π , δ , or ϵ subunit in the receptor moderately decreases GABA_A zinc sensitivity. Furthermore, the γ subunit causes a robust reduction of zinc inhi-

bition, increasing the IC_{50} up to 300 μ M (Nagaya and Macdonald, 2001; Hosie et al., 2003). Mutational analysis of α and β subunits identified three distinct zinc binding sites on GABA_AR. One binding site is within the ion channel on β subunit (β 3 histidine 267 and glutamate 270) and the other two occurring in the interface between the β (β 3 glutamate 182) and α subunits (α 1 glutamate 137 and histidine 141). To achieve high-affinity zinc inhibition, all three sites must be bound. Consistent with this, point mutations of the γ 2 to mimic the residues on β 3 that participate in zinc binding generates a GABA_AR that is as sensitive to zinc as $\alpha\beta$ heteromers (Hosie et al., 2003). Furthermore, α subunits exhibit different sensitivities to zinc as α 1-containing GABA_ARs are less zinc sensitive than those containing α 2-5 (White and Gurley, 1995; Fisher, 2002). In fact, α 5-containing receptors are 10 times more sensitive to zinc compared to α 1 (Fisher, 2002).

Zinc inhibition of GABA_ARs varies across developmental stage, likely reflecting the subunit composition of endogenous receptors (Smart and Constanti, 1990; Smart, 1992). For example, zinc-mediated GABA_AR inhibition of CA3 in rats is most pronounced in the first five postnatal days, decreasing as the animal matures (Martina et al., 1996). This may reflect reduced sensitivity of the α 1 subunit that begins to express in the hippocampus following the first postnatal week (Poulter et al., 1992; Fritschy et al., 1994). However, there is evidence for endogenous zinc inhibition in mature animals as well. For example, chelation of zinc disinhibits muscimol evoked GABA_AR responses, as measured by $^{36}Cl^{-}$ influx in the dentate gyrus (Gordey et al., 1995). Similarly, inhibitory postsynaptic currents (IPSCs) evoked in CA3 with mossy fiber stimulation are potentiated following zinc chelation, revealing an endogenous inhibition by zinc (Ruiz et al., 2004). Zinc was also found to selectively inhibit steroid-sensitive extrasynaptic GABA_AR currents in the dentate gyrus, thus preventing the anti-seizure effect of endogenous neurosteroids (Carver et al., 2016). Furthermore, synaptically-released zinc enables LTP in the amygdala through its reduction of feedforward GABAergic inhibition on principal neurons (Kodirov et al., 2006). It must be noted, however, zinc has also been shown to influence neuronal excitability in GABAergic interneurons, therefore zinc-dependent effects on GABA_AR currents in slice preparations may occur indirectly via changes in neurotransmitter release (Grauert et al., 2014).

Despite the documented inhibitory effect of zinc on GABA_ARs, zinc-mediated potentiation of GABA_AR currents has also been observed in bipolar retinal cells isolated from skates (Fig. 2B) (Qian et al., 1997). Moreover, recent work has suggested that endogenous synaptically-released zinc potentiates GABA_AR-mediated IPSCs at cortical synapses. In these experiments, chelation of extracellular zinc with ZX1 reduced IPSCs mediated by somatostatin, but not parvalbumin, interneurons in acute slices of mouse cortex. Furthermore, this effect was eliminated in slices from ZnT3 KO mice, suggesting that synaptic zinc endogenously potentiates GABAergic transmission (Kouvaros et al., 2020). These results are consistent with the robust expression

of ZnT3 in somatostatin but not parvalbumin neurons (Paul et al., 2017).

Glycine receptors. Glycine receptors (GlyR) are bidirectionally modulated by zinc; with potentiation at submicromolar zinc concentrations (20 nM–1 μ M) and inhibition at micromolar concentrations (20–50 μ M, Fig. 2) (Bloomenthal et al., 1994). Both actions are mediated through the α subunit via two distinct binding sites. Zinc inhibition occurs through two histidine residues (H107 and H109) on the extracellular domain, which are thought to coordinate zinc at the interface of two α subunits (Harvey et al., 1999; Nevin et al., 2003). On the other hand, potentiation is mediated through the N-terminal domain of the α subunit, which is thought to interact with the Cys-loop to influence receptor gating. (Lynch, 2004; Miller et al., 2005). Replacing a critical aspartate (D80) on the n-terminal with an alanine abrogates zinc potentiation. Mice expressing this mutation exhibit a hyperekplexic phenotype, characterized by enhanced startle response, suggesting that endogenous zinc signaling is necessary for proper neuromotor function (Hirzel et al., 2006). Furthermore, this mutation reduces ethanol-mediated enhancement of GlyRs, leading to a reduction in ethanol preference and consumption in heterozygous mutant mice (McCracken et al., 2013). Finally, zinc chelation prevents endogenous potentiation of GlyRs, resulting in increases spontaneous firing in DCN principal neurons. Interestingly, this effect is independent of ZnT3 (Perez-Rosello et al., 2015), suggesting a separate tonic source of zinc can also mediate extracellular zinc signaling. This tonic zinc pool may be generated and regulated by other zinc transporters, such as ZnT1, which, as mentioned earlier is located on the plasma membrane and can influence NMDA receptor function (Krall et al., 2020). Alternatively, tonic zinc may exist as a separate pool of extracellular metal derived from zinc-binding proteins, including matrix metalloproteinases (Yong et al., 2001).

Zinc-activated receptors

The metabotropic zinc receptor GPR39. The first evidence suggesting the existence of a metabotropic zinc receptor (mZnR) was obtained by Michal Hershfinkel and colleagues, demonstrating that the application of zinc led to IP₃-dependent increases in intracellular calcium and consequent upregulation of the Na⁺/H⁺ exchanger in the colonocyte cell line HT29 (Hershfinkel et al., 2001). These findings suggested that zinc may be a ligand of a yet unidentified G-protein coupled receptor (GPCR). Subsequently, multiple studies on the previously orphan receptor GPR39, showed that zinc, but not the peptide hormone obestatin, as had originally been suggested, activates the receptor (Fig. 2C) (Zhang et al., 2005; Lauwers et al., 2006; Holst et al., 2007). Furthermore, GPR39 mRNA expression was found in multiple zinc-rich brain regions, including the amygdala, hippocampus, and cortex, but was conspicuously absent from the hypothalamus, where obestatin was thought to act (Jackson et al., 2006). Finally, point-mutations of GPR39 identified histidine residues on the N-terminal domain critical for zinc binding (Storjohann

et al., 2008). These studies strongly suggested that GPR39 is mZnR.

GPR39 was conclusively identified as mZnR through a series of landmark studies in neuronal preparations. First, it was discovered that synaptic zinc led to a G_q , IP_3 -dependent rise in intracellular calcium, similar to what was observed in HT29 cells (Besser et al., 2009). Further studies revealed that siRNA against GPR39 as well as genetic knockdown of GPR39 prevented the intracellular calcium increase triggered by synaptic zinc release in the CA3 region of the hippocampus (Chorin et al., 2011). In addition to linking mZnR and GPR39, these studies identified downstream consequences of receptor activation such as phosphorylation of extracellular regulated kinase (ERK1/2) and CAMKII. Following mossy fiber stimulation, synaptic zinc triggers mZnR-dependent upregulation of K^+/Cl^- cotransporter 2 (KCC2) activity and subsequent hyperpolarization of GABA_AR reversal potential in hippocampal neurons (Chorin et al., 2011). This signaling pathway can act as a homeostatic protection against excitotoxic insults, including seizures. Indeed, GPR39 KO mice have dramatically increased susceptibility to kainate-induced seizures (Gilad et al., 2015), similar to what has been reported for ZnT3 null mice (Lee et al., 2000). Metabotropic zinc receptor activity is not limited to the hippocampus as synaptic zinc also triggers mZnR activation on fusiform cells of the DCN. At this synapse, mZnR activation promotes endocannabinoid synthesis, which in turn reduces presynaptic glutamate release (Perez-Rosello et al., 2013). Additionally, mZnR activation and subsequent ERK1/2 phosphorylation has also been shown to trigger Na^+/H^+ exchanger upregulation to promote recovery from intracellular acidification in cultured hippocampal neurons (Ganay et al., 2015). Finally, mZnR activity is sensitive to extracellular pH, as acidic conditions inhibit mZnR-driven Na^+/H^+ exchanger activation, suggesting mZnRs can dynamically respond to changes in pH to maintain tissue homeostasis (Cohen et al., 2012; Ganay et al., 2015).

Metabotropic zinc receptor function has been linked to a number of different nervous system disorders. As stated earlier, mZnR KO mice are more susceptible to kainate-induced seizures, suggesting that the receptor is neuroprotective against excitotoxic insults (Gilad et al., 2015). In neurons, mZnR upregulates clusterin, a stress-induced protein associated with Alzheimer's disease (AD) that is linked to pro-survival pathways (Abramovitch-Dahan et al., 2016). Interestingly, zinc binds to amyloid beta and promotes its toxic oligomerization (Bush et al., 1994), which is a hallmark of AD, and this binding reduces mZnR signaling. Additionally, mZnR signaling has been linked to depressive behaviors as KO mice exhibit depression-like phenotypes in the forced swim test and tail suspension test. Unlike wild-type mice, these behaviors are not prevented with conventional antidepressant treatment (Mlyniec et al., 2015). Furthermore, in a chronic restraint stress model, mice exhibit significant decreases in GPR39 in the hippocampus corresponding with depression-like behaviors, both of which are prevented with zinc supplementation or anti-

depressant treatment (Ding et al., 2016). Although it is unclear how mZnR contributes to depression-like behaviors, it has been shown that mZnR can form complexes with the 5HT_{1A} receptor and modulate its signaling (Tena-Campos et al., 2016). For example, zinc can prevent the formation of 5HT_{1A} – GalR1 heterodimers, suggesting it may modulate the serotonergic system, which is highly implicated in depression (Tena-Campos et al., 2015).

Ionotropic zinc receptors. A putative human zinc activated ionotropic receptor (ZAC) was identified via its homology to the Cys-loop family of receptors, which include nicotinic acetylcholine receptors, GlyR, GABA_ARs, and 5-HT₃ receptors (Davies et al., 2003). Characterization of this receptor found it to be a cation channel that is activated in response to zinc, protons, or copper (Fig. 2C). Interestingly, the gene encoding ZAC is absent from both the mouse and rat genome, which has hindered study of the receptor given the ubiquity of rodents in neuroscience research (Davies et al., 2003; Trattinig et al., 2016). As such, not much is known about ZAC's endogenous function. Finally, a different ionotropic receptor recently discovered in flies was also found to be gated by zinc. A genetic screen in *Drosophila melanogaster* identified Hodor, a chloride channel of the Cys-loop family that senses zinc in the gut and regulates food intake and insulin signaling (Fig. 2C) (Redhai et al., 2020). Although Hodor was localized to enterocytes, its discovery opens the door to the possibility of related zinc-gated chloride channels present in the nervous system.

Synaptic zinc in sensory processing and sensory-induced plasticity

Despite the evidence that zinc acts on a diverse array of postsynaptic receptors, much less is known about how this signaling influences function at the circuit and systems level. As noted above, zinc influences the induction and expression of multiple forms synaptic plasticity through its modulation of GABA_AR, GlyR, P2XR, mZnRs, and NMDARs. As synaptic plasticity is a critical process for learning and memory, zinc signaling may play a role in cognition. Notably, ZnT3 KO animals exhibit deficits in contextual discrimination, spatial working memory, and associative fear memory (Martel et al., 2011; Sindreu et al., 2011). Furthermore, synaptic zinc signaling has also been linked to cognitive decline, including in the context AD (Portbury and Adlard, 2017). As these topics extend well beyond the scope of this review, we refer the reader to earlier papers that have delved into these and other subjects (Takeda, 2014; Takeda and Tamano, 2016).

Synaptic zinc and sensory processing. A knock-in mutation (H128S) on the N-terminal domain of GluN2A removes high affinity zinc binding and zinc modulation of NMDARs. Mice with this mutation exhibit hypersensitivity to pain stimuli, suggesting endogenous zinc inhibition of the receptor may attenuate pain processing (Nozaki et al., 2011). Importantly, synaptic zinc modulates sensory processing in the auditory and

somatosensory systems. Chelation of extracellular zinc with ZX1 in the primary auditory cortex (A1) increases the responsiveness (gain) of sound-evoked responses of inhibitory interneurons, and decreases the gain of pyramidal neurons (Anderson et al., 2017). This effect of ZX1 on gain is eliminated in ZnT3 KO mice. Furthermore, synaptic zinc regulates frequency tuning in A1 in a cell-specific manner (Kumar et al., 2019). Consistent with these findings, ZnT3 KO mice exhibit reduced frequency discrimination, suggesting that zinc modulation of auditory circuits is critical for normal sensory processing (Kumar et al., 2019). Interestingly, ZnT3 KO mice also exhibit deficits in whisker texture discrimination, suggesting a similar requirement of zinc for fine-tuning of somatosensory processing (Patrick Wu and Dyck, 2018). Together, these results suggest a novel role for zinc in fine-tuning sensory processing and enhancing acuity for discrimination of different sensory stimuli.

Synaptic zinc plasticity. Sensory regions that express ZnT3 exhibit experience-dependent changes in vesicular zinc labeling, suggesting zinc signaling may contribute to plastic changes in response to sensory stimuli. For example, whisker plucking or stimulation leads to increases or decreases, respectively, in synaptic zinc staining in the barrel cortex (Brown and Dyck, 2002, 2005; Nakashima and Dyck, 2010). Similarly, zinc levels in the DCN decrease following noise exposure (Kalappa et al., 2015; Vogler et al., 2020). Recent studies in the DCN explored the signaling mechanisms underlying these activity-dependent changes in vesicular zinc. These studies showed that high-frequency stimulation of DCN parallel fiber synapses induced long-term depression (LTD) of synaptic zinc signaling (Z-LTD), evidenced by reduced zinc-mediated inhibition of EPSCs. Low-frequency stimulation induced LTP of synaptic zinc signaling (Z-LTP), evidenced by enhanced zinc-mediated inhibition of EPSCs. Pharmacological manipulations of Group 1 metabotropic glutamate receptors (G1 mGluRs) demonstrated that G1 mGluR activation is necessary and sufficient for inducing Z-LTD and Z-LTP. Pharmacological manipulations of calcium dynamics indicated that rises in postsynaptic calcium are necessary and sufficient for Z-LTD induction. Electrophysiological measurements assessing postsynaptic expression mechanisms, and imaging studies with a ratiometric extracellular zinc sensor probing zinc release, supported that Z-LTD is expressed, at least in part, via reductions in presynaptic zinc release. Finally, exposure of mice to loud sound caused G1 mGluR-dependent Z-LTD at DCN parallel fiber synapses, thus validating the *in vitro* results. Together, these results revealed a novel mechanism underlying activity- and experience-dependent plasticity of synaptic zinc signaling (Vogler et al., 2020). The mechanisms that underly changes in zinc content at the synapse remains unclear. These mechanisms could be mediated by changes in ZnT3 expression or function, resulting in alterations in presynaptic labile zinc. Indeed, ZnT3 expression has been shown to increase along with labile zinc levels in retinal ganglion following optic nerve injury, suggesting ZnT3 levels can drive changes in zinc content (Li et al., 2017). However, zinc plasticity induced by whisker pluck-

ing does not alter ZnT3 protein or mRNA levels (Liguz-Leczna et al., 2005). Alternatively, zinc content may be driven by alterations in intracellular labile zinc. For example, nitric oxide (NO), an endogenous gas that acts as a retrograde messenger, can liberate zinc from zinc-buffering metallothionein proteins (discussed below) (Lin et al., 2007). Inhibitors of NO synthase prevent the accumulation of intracellular zinc during ischemia, suggesting that NO endogenously mobilizes zinc from intracellular stores (Wei et al., 2004). As NO has been tightly linked synaptic signaling (Garthwaite, 2018), this gas may mediate activity-dependent changes in presynaptic zinc content.

ZINC IS AN INTRACELLULAR SIGNALING MOLECULE IN NEURONS

Changes in intracellular zinc can trigger a myriad of physiological and, in some, cases, pathophysiological signaling cascades (Aizenman, 2019). The various actions of zinc are, in fact, highly reminiscent of another important cation cellular messenger, calcium. Indeed, some years ago Chris Frederickson, Jae-young Koh and Ashley Bush referred to zinc as the “calcium of the XXI century” (Frederickson et al., 2005). Here, we summarize the various cellular signaling pathways known to be activated or regulated by the metal as well as its influence in neuronal circuit function.

INTRACELLULAR ZINC SIGNALING

Intracellular zinc is linked to a variety of physiological signaling pathways (Fig. 4). Notably, zinc upregulates transcription of a number of proteins involved in metal binding and regulation. Metal-regulated gene transcription was first observed with metallothionein-1 (MT-1), a metal-binding protein involved in zinc homeostasis and protection against oxidative stress. Mice injected with zinc or cadmium exhibited increased in MT-1 mRNA expression in multiple tissues (Durnam and Palmiter, 1981). Further characterization of the MT-1 gene identified a 12 base pair DNA motif in the promoter region that was necessary and sufficient for metal responsiveness, thus named metal response element (MRE) (Carter et al., 1984; Stuart et al., 1984; Searle et al., 1985). Shortly thereafter, a zinc-inducible transcription factor was found that bound MRE to induce gene transcription (Westin and Schaffner, 1988). Upon zinc binding, this metal regulatory transcription factor (MTF-1) rapidly translocates to the nucleus and increases DNA binding (Fig. 4) (Dalton et al., 1997; Smirnova et al., 2000). MTF-1 upregulates transcription of multiple gene targets including MT-2, MT-3 and ZnT1. This transcriptional pathway allows the cell to maintain homeostasis in the face of fluctuation zinc levels.

Additional there is evidence that some ZIP transporters (described in detail in the next section) are transcriptionally regulated by MTF-1. Expression of the zinc transporter ZIP10 is repressed by zinc-binding to MTF-1 through its interaction with an MRE-binding site downstream of the gene (Lichten et al., 2011). This down-regulation is unique as MTF-1 has primarily been

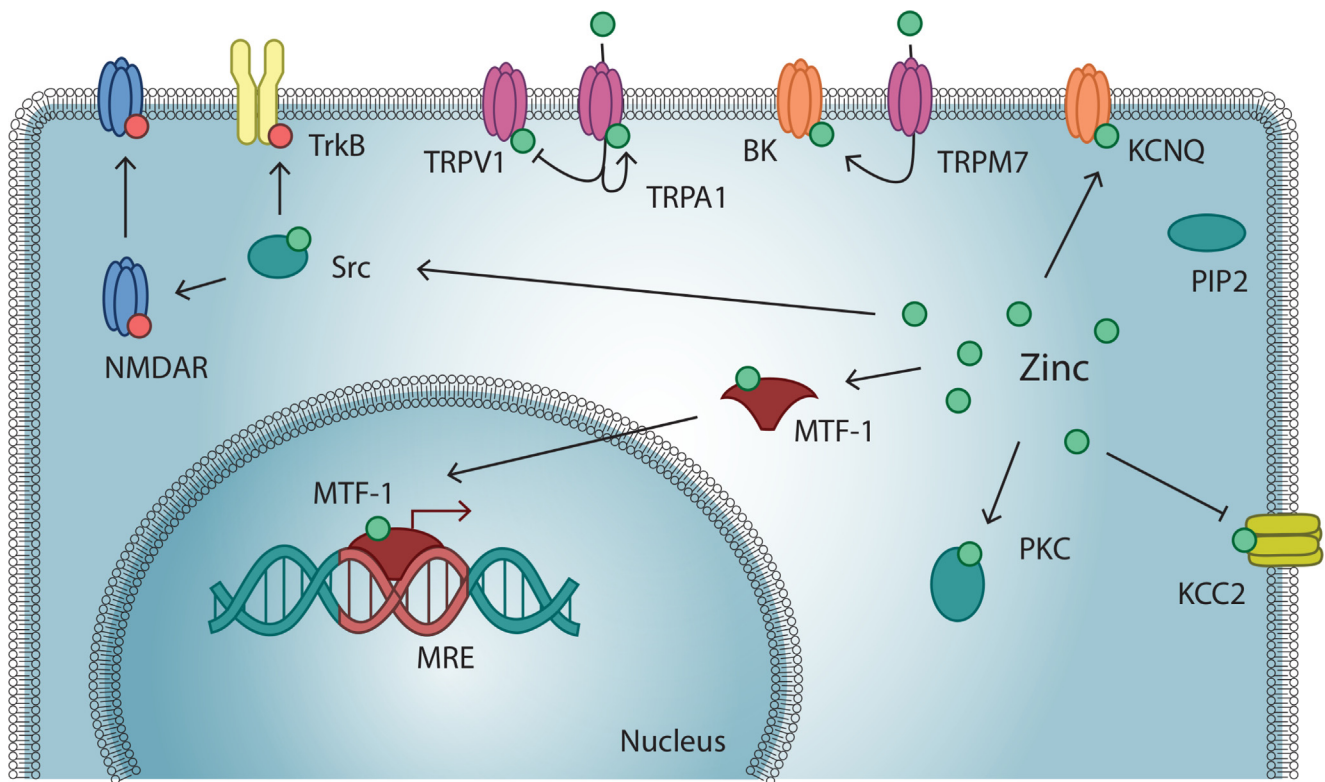


Fig. 4. Intracellular zinc signaling. Zinc acts on multiple signaling targets intracellularly. Src kinase binds zinc which leads to phosphorylation and upregulation of NMDARs as well as phosphorylation and ligand-independent activation of the TrkB receptor. Zinc influx through TRPA1 channels leads to TRPA1 activation and TRPV1 inhibition via binding to the intracellular side of the receptor. Likewise, zinc influx through TRPM7 is coupled to zinc binding and activating the BK channel. Intracellular zinc binding upregulates KCNQ activity, by reducing the channel's dependence on PIP₂. The ion also inhibits KCC2 channels intracellularly and activates PKC kinase signaling pathways. Zinc regulates transcription through MTF-1. After binding zinc, it translocates to the nucleus and binds MRE sites on zinc-responsive genes to alter their expression.

observed to increase gene expression. On the other hand, ZIP11 is upregulated by MTF-1 following zinc treatment, consistent with other MRE-regulated genes (Yu et al., 2013). Zinc-mediated upregulation appears to be unique to ZIP11 among the ZIP family of transporters.

Moderate increases in intracellular zinc modulate kinase signaling cascades. For example, zinc binds and increases the activation of PKC, a critical signaling intermediate in neurons (Murakami et al., 1987; Csermely et al., 1988; Hubbard et al., 1991). This zinc-mediated activation has been shown to constitutively downregulate a leak chloride channel in fish retinal ganglion (Tabata and Ishida, 1999). Chelation of zinc from PKC also increases the autonomous activity of the enzyme, suggesting a complex relationship between zinc binding and PKC activity (Knapp and Klann, 2000). Zinc also upregulates Src kinase activity, as measured by increased phosphorylation of PLC γ . Following activation, Src kinase signaling leads to increased phosphorylation and subsequent upregulation of NMDAR subunits GluN2A and GluN2B (Manzerra et al., 2001). This suggests intracellular zinc, when present at concentrations sufficient to induce Src activity, may be able to oppose, to a certain extent, the inhibitory effect of extracellular zinc on NMDARs. Furthermore, zinc mediated Src activation appears to directly activates the TrkB receptor via phosphorylation of tyrosines 205 and 206 (Huang and McNamara, 2010). This ligand-independent TrkB receptor

activation drives zinc-dependent long term potentiation of the mossy fiber-CA3 synapse (Huang et al., 2008). Intracellular zinc signaling also activates Ras/Raf/MEK/ERK signaling cascades. Treatments that increase intracellular zinc drive Ras-dependent increases in ERK activity in multiple cell lines, including the neuronal line HT-22 (Anson et al., 2020). Furthermore Ras/Raf/MEK/ERK signaling is regulated by the zinc transporter, ZnT1. The C-terminal domain of ZnT1 interacts directly with the N-terminal regulatory domain of Raf-1 to promote its activity. Moreover, extracellular zinc blocks the interaction of ZnT1 and Raf-1 and subsequent phosphorylation of MEK (Jirakulaporn and Muslin, 2004).

Zinc can also modulate signaling through its intracellular actions on membrane receptors. For example, the M-type potassium channels, also known as Kv7 or KCNQ, are upregulated by intracellular zinc through a reduction of KCNQ's dependence on PIP₂ (Gao et al., 2017). Similarly, intracellular zinc activates the large-conductance Ca²⁺ activated Slo1 potassium channel (BK) via a histidine residue on a cytoplasmic domain of the protein. TRP channels are also modulated by intracellular zinc, including TRPV1 inhibition and TRPA1 activation (Hu et al., 2009; Luo et al., 2018). Furthermore, TRP channels couple zinc influx to channel modulation. For instance, BK channels are activated by extracellular zinc when co-transfected in cells with TRPM7 (Hou et al., 2010). Similarly, zinc influx through

TRPA1 mediates inhibition of TRPV1 and subsequent reduction of acute nociception (Luo et al., 2018). TRPA1 itself mediates influx required for its own activation via intracellular cysteine and histidine residues (Hu et al., 2009). Intracellular zinc also inhibits KCC2, leading to a depolarizing shift in the GABA_AR reversal potential, therefore influencing inhibitory transmission (Hershinkel et al., 2009), revealing opposing functions of intracellular and extracellular zinc, as extracellular zinc increases KCC2 activity via mZnR activation (Chorin et al., 2011).

Zinc signaling in neuropathological processes

The potential pathological actions of zinc in neurons (Fig. 5) were first identified following the observation that exposure to extracellular zinc causes widespread neuronal cell death *in vitro* (Yokoyama et al., 1986; Choi et al., 1988). The extent of damage in cultured neurons varied with the concentration and duration of zinc treatment, suggesting a direct relationship between zinc and cell death (Choi et al., 1988). Furthermore, both kainate-induced seizures and ischemia were later noted to trigger

zinc translocation from presynaptic boutons to degenerating postsynaptic cell bodies (Frederickson et al., 1989; Koh et al., 1996). Chelation of zinc prior to ischemia reduced the resulting cell death, revealing a causative of the ion in degeneration (Koh et al., 1996). Zinc-induced cell death exhibits features of both necrosis and apoptosis, suggesting that zinc activates more than one signaling cascade upstream of degeneration (Kim et al., 1999; Lobner et al., 2000).

A common upstream feature of deleterious zinc signaling cascades is the generation of reactive oxygen species (ROS) (Fig. 5). Zinc activates 12-lipoxygenase (12-LOX) and NADPH oxidase to trigger ROS generation (Noh and Koh, 2000; Zhang et al., 2004). Subsequently, ROS can activate mitogen-activated protein kinase (MAPK) cascades including the Ras/Raf/MEK/ERK and p38 MAPK pathways. Zinc-induced ERK signaling causes toxicity in cortical cultures through poly(ADP-ribose) polymerase activation, DNA damage, ROS production via NADPH oxidase, and mitochondrial hyperpolarization and dysfunction (Du et al., 2002; He and Aizenman, 2010). Zinc activation of p38 MAPK leads to

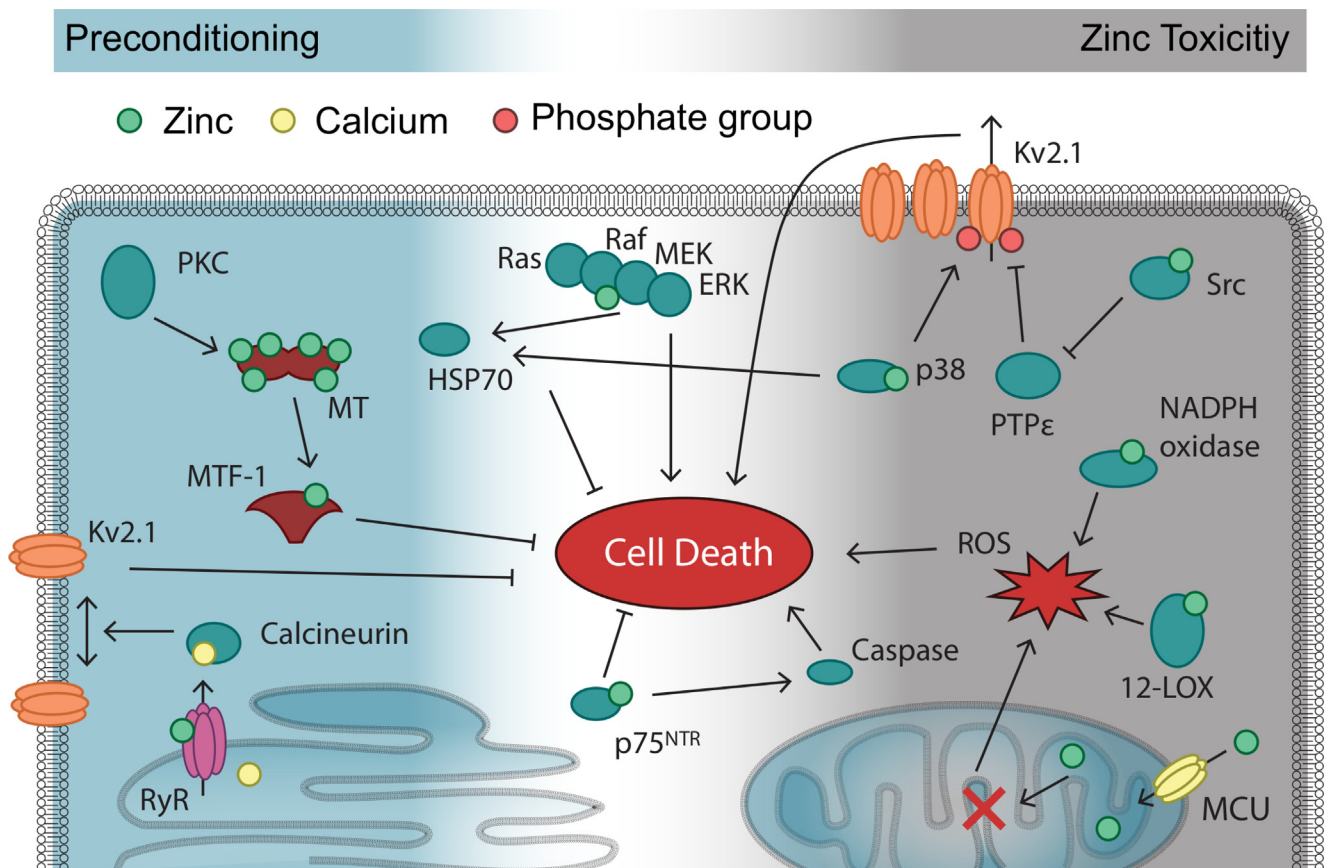


Fig. 5. Zinc signaling in preconditioning and cell death. Increases in intracellular zinc triggers both protective (light blue) and toxic (gray) signaling cascades. **(A)** Preconditioning. Sublethal increases in intracellular zinc protect against subsequent cell death. PKC driven release of zinc from metallothioneins triggers upregulation of zinc sensitive genes. Zinc binding to ryanodine receptors (RyR) triggers calcium release from ER stores and subsequent activation of calcineurin, which leads to the dispersal of Kv2.1 channels, thus preventing further, pro-apoptotic Kv2.1 insertion into the plasma membrane. ERK and p38 both activate protective HSP70 signaling. **(B)** Zinc Toxicity. Kv2.1 dual phosphorylation and insertion into the membrane is driven by p38 and Src, the latter driven by zinc inhibition of PTP ϵ , leading to apoptotic potassium efflux following SNARE-dependent Kv2.1 membrane insertion. Zinc also activates NADPH oxidase and 12-LOX which drives the generation of toxic reactive oxygen species (ROS). Similarly zinc import into the mitochondria through the mitochondrial cation uniporter (MCU) leads to dysfunction, ROS generation, and neuronal death. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phosphorylation of the c-terminal serine on the delayed rectifying voltage-gated potassium channel Kv2.1. Similarly zinc increases phosphorylation of Kv2.1 at an n-terminal tyrosine through its activation of Src kinase and concurrent inhibition of cytoplasmic protein phosphatase ϵ (McLaughlin et al., 2001; Redman et al., 2007, 2009; Huang et al., 2008). Together, this dual phosphorylation leads to the syntaxin-dependent insertion of Kv2.1 into the membrane, increased Kv2.1 activity, and, subsequent caspase activation and apoptotic cell death driven by decreasing intracellular potassium concentrations (Redman et al., 2009). Another zinc-regulated apoptotic signaling cascade is p75^{NTR} mediated cell death in which p75^{NTR} and p75^{NTR}-associated death executor induction leads to caspase activation and neuronal degeneration (Park et al., 2000).

Intracellular zinc also triggers degeneration through mitochondrial dysfunction and energy failure. Following increases in the cytosol, zinc can accumulate in the mitochondria via the mitochondrial calcium uniporter (Malaiyandi et al., 2005; Medvedeva and Weiss, 2014). Zinc accumulation is associated with a loss of mitochondrial membrane potential, subsequent mitochondrial dysfunction, and ROS production (Sensi et al., 2003; Dineley et al., 2005; Medvedeva and Weiss, 2014). Additionally, zinc-mediated signaling has been linked to opening of the mitochondrial permeability transition pore, which triggers mitochondrial failure (Jiang et al., 2001; Bonanni et al., 2006). Zinc also disrupts energy production through its inhibition of GAPDH, thus impairing glycolysis (Sheline et al., 2000).

Intracellular zinc also triggers neuroprotective signaling mechanisms at concentrations that are insufficient for toxicity (Fig. 5). This process, in which a sub-lethal insult protects cells against subsequent lethal ones, is called preconditioning. Treating neuronal cultures with metal chaperones to increase intracellular zinc protects neuron against subsequent excitotoxic and ischemic insults, suggesting zinc itself can drive preconditioning (Wang et al., 2010; Johanssen et al., 2015). In fact, studies in cortical cultures found that preconditioning with sub-lethal potassium cyanide leads to transient increases in labile zinc that are necessary and sufficient for neuroprotection against subsequent excitotoxicity. Zinc transients are triggered by protein kinase C (PKC)-facilitated release of zinc from metallothionein 1 and subsequent upregulation of gene expression (Aras et al., 2009). Similarly, ischemic preconditioning in rats leads to transient zinc increases in the cortex and striatum. Chelation of zinc is sufficient to abolish the neuroprotective effect. Furthermore, zinc induced preconditioning in cortical cultures is associated with activation of the p75^{NTR} pathway and upregulation of heat-shock protein 70, via p38 and ERK MAPK signaling (Lee et al., 2008). Sub-lethal zinc signaling also triggers ryanodine receptor mediated calcium release from the endoplasmic reticulum which drives calcineurin-dependent redistribution of Kv2.1 channels, thus preventing apoptotic insertion of additional Kv2.1 channels into the membrane (Schulien et al., 2016, 2020; Justice et al., 2017). Together these findings highlight the essential role of zinc as an intermediate in both neurotoxic and neuroprotective signaling cascades.

THE TRANSLOCATION AND REGULATION OF ZINC IN CELLS

Intracellular and extracellular labile zinc levels are normally maintained at low concentrations, despite fluctuations that result from release from synaptic terminals. This hints towards the dynamic processes that regulate zinc localization and levels. Indeed, cellular zinc is not static, but is regulated by complex mechanisms involving transporters, ion channels, and metalloproteins (Fig. 6). These systems work in tandem to spatially and temporally regulate cellular zinc signaling while protecting against the activations of injurious, zinc-activated cascades.

Translocation of synaptic zinc to postsynaptic neurons

Vesicular zinc released into the synaptic cleft can translocate into the post-synaptic cell through zinc permeable ion channels. This translocation was first hypothesized based on the observation that after seizures, ischemia, or traumatic brain injury, injured animals exhibit reduced vesicular zinc staining and increased somatic staining compared to control animals (Frederickson et al., 1989; Koh et al., 1996; Suh et al., 2000). Furthermore, extracellular chelation *in vivo* attenuates cell death and degeneration, suggesting that the extracellular movement of the vesicular pool contributes to the zinc toxicity. Moreover, stimulation of hippocampal cultures with either glutamate or high potassium leads to transient increases in post-synaptic zinc as measured by the fluorescent indicator FluoZin-3, providing evidence for zinc translocation outside of the context of neurogenerative processes (Ha et al., 2018; Sanford and Palmer, 2020).

Multiple different channels have been identified that mediate zinc translocation, including CP-AMPA, NMDARs, voltage-gated calcium channels (VGCCs), and transient receptor potential channels (TRPs) (Fig. 6). AMPARs were first linked to zinc transport with the observation that AMPAR activation increases zinc toxicity (Weiss et al., 1993). Furthermore, zinc uptake selectively labels neurons that express CP-AMPA and this subpopulation of neurons is more susceptible to zinc-toxicity (Yin and Weiss, 1995; Yin et al., 1998). Direct measurement of zinc current through CP-AMPA established that the receptor conducts zinc even in the presence of physiological calcium (Jia et al., 2002). In addition to CP-AMPA, voltage gated calcium channels also mediate zinc-toxicity and ⁶⁵zinc influx following depolarization with high potassium (Manev et al., 1997; Sheline et al., 2002). Electrophysiological recordings confirmed VGCCs conduct zinc (Kerchner et al., 2000). Furthermore, these channels are necessary for the zinc influx that occurs during spreading depression following oxygen-glucose deprivation (Dietz et al., 2008). Zinc also permeates through NMDARs, but to a lesser extent than calcium (Koh and Choi, 1994). NMDARs mediate increases in fluorescent zinc staining following treatment with low micromolar zinc concentrations, suggesting that vesicular zinc reaches concentrations sufficient to

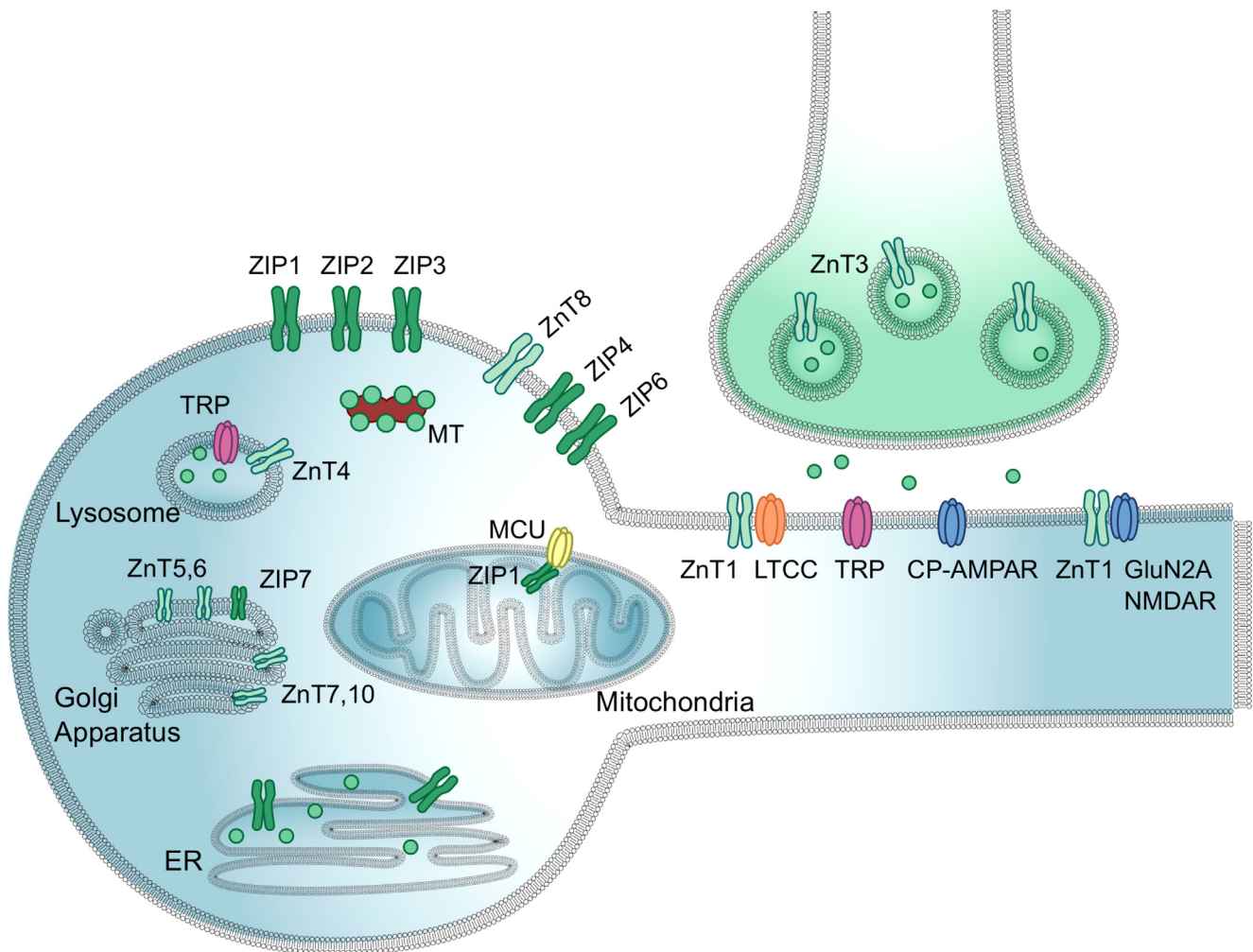


Fig. 6. Zinc transport in neurons. Illustration of the multiple transporters and ion channels that mediate movement of zinc across membranes in neurons. ZnT3 loads zinc into presynaptic vesicles where it is released into the synaptic cleft. On the plasma membrane, L-type calcium channels (LTCC), transient receptor potential channels (TRP), NMDA receptors, and calcium-permeable AMPA receptors (CP-AMPA) conduct zinc into the cell. ZIP1-4,6 and ZnT1,4,8 are localized to the plasma membrane and conduct zinc into and out of the cell, respectively. Lysosomes sequester zinc, in part through uptake via ZnT4, and can release the ion into the cytoplasm via TRPML1. ZnT5-7,10 and ZIP7 are present on the membrane of the Golgi apparatus. The mitochondrial cation uniporter (MCU) couples with ZIP1 to transport zinc into the mitochondrial matrix. ZIP7 and 8 mediate efflux of zinc from the endoplasmic reticulum (ER) into the cytoplasm.

permeate the receptor (Marin et al., 2000). Finally, TRP channels also mediate of zinc translocation in neurons. TRPA1 channels in nociceptive somatosensory neurons conduct zinc which activates the channel through interactions with cysteine and histidine residues on the intracellular side of the protein (Hu et al., 2009). TRPM7 channels contribute to zinc toxicity and oxygen-glucose deprivation cell death in cortical neurons, and silencing the channel significantly reduces zinc-induced cell death (Inoue et al., 2010). TRPM2 also mediates zinc toxicity in ischemia–reperfusion injuries, with TRPM2-KO animals protected against injury (Ye et al., 2014).

Liberation of zinc from intracellular stores

Zinc accumulation in degenerating neurons also occurs in regions of the brain that do not contain vesicular zinc, indicating intracellular zinc can increase independent of vesicular zinc translocation (Lee et al., 2000; Land and

Aizenman, 2005; Medvedeva et al., 2017). This intracellular zinc originates from metal-binding proteins and intracellular organelles that sequester zinc inside the cell. Metallothioneins buffer intracellular zinc. There are four isoforms of metallothionein, three of which (MT-1 through MT-III) are expressed in the central nervous system, with MT-III the primary form expressed in neurons (Aschner et al., 1997). These proteins contain 20 cysteine residues that can bind up to 7 zinc ions via metal-thiolate clusters (Maret and Krezel, 2007). MTs release zinc in response to oxidative stimuli (Maret, 1994, 1995). For example, the thiol oxidant 2,2'-dithiodipyridine (DTDP) causes intracellular zinc release and subsequent zinc-dependent cell death in cortical neurons *in vitro* (Aizenman et al., 2000). Nitric oxide (NO), an endogenous gas, also triggers zinc release from MTs (Lin et al., 2007), likely as a result of its interaction with superoxide and production of peroxynitrite (Zhang et al., 2004). Inhibitors of NO synthase can prevent the accumulation of intracellular zinc

following ischemia reperfusion injury, suggesting that NO endogenously mobilizes zinc from these intracellular stores (Wei et al., 2004).

Zinc can be sequestered into subcellular organelles, including the endoplasmic reticulum (ER), lysosomes, and mitochondria. The ER sequesters zinc following increases in cytosolic levels in cortical neuron cultures (Qin et al., 2011). Furthermore, zinc increases in the cytosol following inhibition of the ER calcium pump with thapsigargin or activation of the IP₃ receptor, suggesting that zinc can be released from the ER similar to calcium (Stork and Li, 2010; Qin et al., 2011). Lysosomes also can accumulate zinc. This accumulation is a prerequisite for lysosomal membrane permeabilization, and chelation can prevent permeabilization and subsequent cell death (Hwang et al., 2008; Lee and Koh, 2010). Furthermore, siRNA knockdown of TRPML1 leads to enlargement and zinc accumulation in lysosomes, suggesting that TRPML1 may endogenously release zinc from these stores (Eichelsdoerfer et al., 2010; Kukic et al., 2013). Lysosomes also protect cells against toxic increases in cytosolic zinc by sequestering the ion through the transporter ZnT4 and subsequently releasing it via lysosomal exocytosis (Kukic et al., 2013, 2014). Zinc also localizes to the mitochondria (Lu et al., 2016). Treating neurons with micromolar zinc leads to mitochondrial zinc uptake via the calcium uniporter (Malaiyandi et al., 2005). Furthermore, activation of TRPC6 channels mobilizes zinc from mitochondrial stores along with calcium (Tu et al., 2010).

The transport of zinc across biological membranes

ZIP transporters. Zrt, Irt-like proteins (ZIPs) are zinc transporters named for the first homologs of the broad family of metal transports first discovered in *Saccharomyces cerevisiae* (Zhao and Eide, 1996a, 1996b) and *Arabidopsis thaliana*, respectively (Eide et al., 1996). There are 14 ZIP transporters in mammals, encoded by the genes SLC39A1–14, which transport zinc from the extracellular space or subcellular organelles into the cytoplasm. They are organized into 4 subfamilies, ZIP subfamily I (ZIP9), ZIP subfamily II (ZIP1–3), LZT subfamily (ZIP4–8, ZIP10, ZIP12–14), and GufA subfamily (ZIP11) based on sequence similarities (Gaither and Eide, 2001a). In addition to zinc, members of the ZIP family have been shown to also transport other divalent ions, including copper, iron, manganese, and cadmium. In fact, ZIP8 seems to transport manganese better than zinc (He et al., 2006).

The structure and transport mechanism of mammalian ZIPs have not been definitively established. They are predicted to have 8 transmembrane domains with extracellular N- and C-termini, and form homo- and heterodimers in the membrane. Initial characterization of ZIPs suggested that they transport zinc in a temperature- and concentration-dependent manner (Gaither and Eide, 2000). ZIP sequences lack ATP-binding sites, which suggest that they are not active transporters, but instead act through secondary transport or facilitated diffusion (Gaither and Eide, 2001b). The observation that HCO₃[−] stimulated zinc transport in ZIP2, ZIP8,

and ZIP14 led to the hypothesis that mammalian ZIPs act as a Zn²⁺/HCO₃[−] cotransporter (Gaither and Eide, 2000; He et al., 2006). However, other studies have found that ZIPs (including ZIP2) are regulated by H⁺, not HCO₃[−], and metal transport is inhibited by depolarization of the plasma membrane (Franz et al., 2018; Hoch et al., 2020). It is possible that the discrepancies between these studies may be explained by the presence of endogenous zinc transport mechanisms in cellular systems used to identify HCO₃[−] cotransport. Specifically, HCO₃[−] stimulates zinc transport in naive oocytes, but reduces transport in ZIP2 transfected oocytes, suggesting that HCO₃[−] regulation of zinc flux is independent of ZIP expression (Franz et al., 2018).

The crystal structure of a bacterial transporter, BbZIP was recently elucidated, thus opening the door to a greater understanding of ZIP-mediated transport. The structure confirmed there are eight transmembrane domains (TM) arranged in an inward-open conformation. Furthermore, it showed a binuclear metal center localized to TM4 and TM5 coordinates zinc, conserved in human ZIP4 (Zhang et al., 2017). From the structure of BbZIP, it was hypothesized that transport was mediated via a rigid rocking mechanism that alternatively exposes the binuclear metal center to the cytoplasm and extracellular space (Zhang et al., 2017). In addition to the binuclear metal center, five other zinc coordination sites were identified that were hypothesized to facilitate transport out of transmembrane region, three of which were identified on the cytoplasmic side of the protein (Zhang et al., 2017). These sites might correspond to residues on cytoplasmic loop that lead to reduces transport when mutated in ZIP1 and ZIP4 (Milon et al., 2006; Bafaro et al., 2015). The crystal structure of BbZIP has been used to develop models of ZIP2 and ZIP4 structure. The study of ZIP2 suggest that the ZIP subfamily II lack the binuclear center found in BbZIP and ZIP4. Instead, the second binding site is occupied by a proton from a lysine residue that stabilizes the structure and is necessary for transport. Point-mutations of a histidine within the binding cavity of ZIP2 abolished its H⁺ and voltage sensitivity, suggesting that H⁺ modifies, but is not necessary for, zinc transport (Gyimesi et al., 2019). Interestingly, another group found that ZIP2, but not ZIP4, was sensitive to H⁺. However, mutating the second metal-binding site on ZIP4 led to pH-regulated transport similar to ZIP2 (Zhang et al., 2020). Together, these findings suggested that different residues alter ZIPs sensitivity to electrostatic forces, such as protons or membrane potential. However, another recent study found that ZIP4 is regulated by pH and uses the H⁺ gradient across the plasma membrane to power zinc transport (Hoch et al., 2020). Further research into the structure and transport of ZIPs is clearly required to resolve these various conflicting findings.

ZIP transporters dynamically respond to decreases in zinc to help maintain cellular homeostasis. Mice fed zinc deficient diets showed increased expression of ZIP4 (Dufner-Beattie et al., 2003) and ZIP6 (Chowanadisai et al., 2005). Multiple ZIPs increase their membrane expression and subsequent zinc influx in response to

depletion of intracellular zinc (Cao et al., 2001; Kim et al., 2004; Wang et al., 2004). Mutations to the N-terminal domain abolish ZIP4's sensitivity to intracellular zinc depletion (Wang et al., 2004). Furthermore, an N-terminal ectodomain is proteolytically processed in response to zinc deficiency which results in accumulation of ZIP4 on the membrane. In this ectodomain, there is a conserved PALV motif which resembles metalloproteinase cleavage site and is necessary for proteolysis of the ectodomain. This motif is conserved across multiple members of the LZT subfamily of transporters, indicating it may be a shared mechanism of ZIP regulation (Kambe and Andrews, 2009).

Zinc can also lead to downregulation of ZIP transport through endocytosis, ubiquitination, and protein degradation. Zinc-stimulated endocytosis has been documented in transporters of the LZT and ZIP II subfamily. ZIP1 has a dileucine sorting signal on the N-terminal domain of the protein that is necessary for zinc-induced endocytosis, trafficking to the lysosome, and subsequent protein degradation (Huang and Kirschke, 2007). In ZIP4, the N-terminal domain is also critical for regulation of endocytosis. Half of all mutations of ZIP4 associated with acrodermatitis enteropathica are localized to the extracellular N-terminal domain of the protein (Zhang et al., 2019). Studies of these mutations and a histidine-containing portion of the n-terminal domain determined that this structure is necessary for zinc-induced endocytosis (Wang et al., 2004). In addition to the extracytoplasmic N-terminal domain, a histidine-rich cytoplasmic loop between TM3 and TM4 contains two zinc binding sites. When both sites are occupied, it triggers ubiquitination and subsequent degradation of ZIP4 (Mao et al., 2007; Bafaro et al., 2015). Together these studies suggest that multiple mechanisms downregulate ZIPs to prevent overload of intracellular zinc when the ion is present.

ZnT transporters. ZnT transporters are part of the cation diffusion facilitator (CDF) family of proteins. ZnT1 was the first mammalian zinc transporter identified based on its ability to confer protection against zinc toxicity (Palmiter and Findley, 1995). Since this discovery, an additional 9 ZnTs have been identified (ZnT2–ZnT10) that transport metals out of the cytoplasm into either extracellular space or subcellular compartments such as vesicles. It should be noted that ZnT10, in contrast to the other 9 ZnTs, preferentially transports manganese. Furthermore, it was recently found that the protein TMEM163, also known as synaptic vesicle 31, effluxes zinc. Sequence alignment and phylogenetic analysis place it in the CDF family of proteins, therefore TMEM163 has been proposed to be a new member of the ZnT family, namely ZnT11 (Sanchez et al., 2019).

Multiple insights to ZnT structure and function have been gained from studies of the homolog YiiP, a member of the CDF family found in bacteria with approximately 25–30% sequence identity with ZnTs (Lu and Fu, 2007). The crystal structure of YiiP showed that the protein, with 6 transmembrane domains and cytoplasmic N- and C-termini, forms homodimers to mediate metal transport. It has four metal binding sites that coordinate either zinc or cadmium. One of these sites is located on

the extracellular side of the transmembrane domain made of three aspartate and one histidine residue (Asp^{45, 49, 157}, His¹⁵³) that are all critical for zinc transport. These residues are highly conserved in mammalian ZnTs. YiiP acts via an alternating access mechanism in which it shifts between an inward-facing and outward-facing configuration driven by the proton gradient (Lu and Fu, 2007).

Mammalian ZnTs, for the most part, share many similarities to their bacterial homolog, YiiP. Multiple members of the ZnT family form homo- and heterodimers, including ZnT1, 3, 4, and 5, which covalently dimerize via tyrosine residues on the C-terminal domain. This di-tyrosine covalent bond in ZnT3 increases zinc transport and localization to vesicles and is upregulated by oxidative stress (Salazar et al., 2009). ZnTs are thought to function as proton antiporters. In agreement with this, disruption of the vacuolar-type H⁺ ATPase blocks ZnT-mediated zinc transport into intracellular vesicles, suggesting that ZnT function requires a proton gradient. Furthermore, ZnT expression increases the rate of alkalization of intracellular vesicles, indicating that ZnTs promote efflux of protons (Ohana et al., 2009; Golan et al., 2019). Similar to YiiP, ZnTs contain four residues in the transmembrane domain essential for metal transport. However, ZnT1–8 have a histidine at the locus corresponding to Asp⁴⁵ of YiiP, which prevents cadmium transport, thus making them selective for zinc. Furthermore, mutating this histidine to aspartate on ZnT5 allows for cadmium transport, suggesting this region is essential for ion specificity (Hoch et al., 2012). Interestingly, ZnT10 primarily transports manganese, despite being classified as a zinc transporter. At the metal coordination site, ZnT10 has an asparagine residue in place of a histidine that is otherwise conserved across other ZnTs. Mutation of this asparagine to a histidine abolishes its manganese transport, further implicating the region for ion specificity. Additionally, this asparagine is essential for the unique calcium-driven antiport mechanism in ZnT10. Mutating the asparagine to a threonine decouples manganese and calcium transport, thus allowing the ions to rapidly permeate ZnT10, similar to an ion channel (Levy et al., 2019). Finally, a very recent study has provided a high-resolution structure of human ZnT8, revealing, for the first time, a plausible mechanism for the Zn²⁺/H⁺ exchange mechanism in a mammalian zinc transporter (Xue et al., 2020). ZnT8 is responsible for transferring zinc into insulin-containing granules in pancreatic β cells. The primary zinc binding site, highly conserved among the SLC30 family, is formed by the coordination of the metal by His¹⁰⁶, Asp¹¹⁰, His²²⁰ and Asp²²⁴. By resolving both the inward (cytosolic) and outward (luminal) facing states of the transporter, the results from this study suggest a simple two-state model for zinc transport whereas ZnT8, functioning as dimers, shuttle between inward and outward facing states via large structural rearrangements of the transmembrane domains, housing the zinc ion in a differential, pH-dependent manner. The lower luminal pH induces the release of zinc from the outward-facing side, while the higher pH (cytosolic) environment of outward-facing state increases the affinity of the primary binding site for the metal (Xue et al., 2020).

ZnTs expression, like other zinc proteins, is dynamically regulated. Nitric oxide-driven increases in intracellular zinc increase mRNA expression of ZnT1, 2 and 4 (Aguilar-Alonso et al., 2008). Furthermore, transient increases in zinc following KCl-mediated depolarization lead to upregulation of ZnT3 mRNA (Sanford et al., 2019). In contrast, ZnT5 and 7 are insensitive to increases in zinc, but are upregulated following chelation with TPEN (Devergnas et al., 2004). Unlike ZnT1 or ZnT2–4 which localize to the plasma membrane or intracellular vesicles respectively, ZnT5 and 7 localize to the Golgi apparatus, therefore this upregulation may reflect a mechanism to maintain Golgi zinc when cytosolic levels are depleted. On the other hand, ZnT1 transport is down-regulated via endocytosis and degradation of the transporter under zinc-deficient conditions, leading to decreased efflux from the cytoplasm. This endocytosis is regulated, in part, through N-glycosylation of asparagine 299 of the protein (Nishito and Kambe, 2019). As mentioned earlier in this review, ZnT3 is regulated by the adaptor protein AP-3 via a direct interaction on ZnT3's cytoplasmic tail. AP-3 deficiencies, as in the *mocha* mouse, reduces ZnT3 targeting to synaptic vesicles (Salazar et al., 2004). Furthermore, estrogen decreases ZnT3-dependent vesicular zinc, likely through its down-regulation of AP-3 mRNA (Lee et al., 2004). Interestingly, genetic KO of the gene *MCOLN1*, which encodes the TRPML1 receptor, reduces ZnT3 mRNA and protein levels in the brains of mice (Chacon et al., 2019). It is unclear what causes this specific downregulation of ZnT3; however, it does suggest that zinc-permeable receptors may influence synaptic zinc levels via ZnT3.

Neuronal zinc signaling associated with specific zinc transporter function

ZIP transporters. ZIP transporters in the brain have been associated with adverse conditions and disorders. Notably, ZIP1 and ZIP3 contribute to degeneration in the CA1 region of the hippocampus following kainate-induced seizures. ZIP1,3 KO animals exhibit reduced CA1 damage compared to controls resulting from decreased zinc uptake (Qian et al., 2011). On the other hand, upregulation of ZIP4 in the hippocampus following kainate injections protects neurons against toxicity. In this case, tissue plasminogen activator interacts with ZIP4 and promotes lysosomal sequestration of zinc to reduce zinc toxicity (Emmetsberger et al., 2010). Additionally, single nucleotide polymorphisms (SNPs) in ZIPs have been identified as strongly associated with psychiatric disorders. Meta-analysis of two independent gene wide association studies of bipolar disorder identified ZIP3 as a common genetic locus (Baum et al., 2008). Similarly, a gene-wide association study of schizophrenia identified a SNP in ZIP8 as significant compared to control samples (Fullard et al., 2019). Furthermore, ZIP8 gene expression was found to be significantly dysregulated in blood samples from schizophrenia patients (Hess et al., 2016). Although the mechanism linking these ZIPs to bipolar disorder and schizophrenia remain unknown, this suggests that ZIP transporters are crucial for proper functioning of the central nervous system.

ZIP transporters regulate signaling through their modulation of cytosolic zinc levels. For example, ZIP7 mediates release of zinc from the endoplasmic reticulum and Golgi apparatus into the cytosol (Fig. 6). Phosphorylation of ZIP7 by protein kinase CK2 triggers zinc release leading to downstream activation of signaling cascades, including MAPK (Taylor et al., 2012; Nimmanon et al., 2017). Interestingly, ZIP7 phosphorylation occurs following treatment of cells with 20 μ M extracellular zinc (Taylor et al., 2012). This suggests that extracellular zinc signals, such as synaptic release, could also mediate cytosolic zinc signaling via release from intracellular stores.

Neurodevelopmental signaling is also influenced by ZIP transporters. Genetic knockdown of ZIP12 in Neuro2a cells and mouse cortical neurons reduces neurite outgrowth. Similarly, this effect is mimicked by zinc chelation and rescued with zinc pyrithione, indicating that ZIP12-mediated zinc influx regulates neurite development (Chowanadisai et al., 2013). Furthermore, this effect requires phosphorylation of the cAMP response element binding protein (CREB) at serine 133, which can occur through p38 MAPK and ERK signaling pathways that are both regulated by cytosolic zinc (Xing et al., 1998; Chowanadisai et al., 2013).

ZIP transporters also influence function through their contribution to mitochondrial dynamics. ZIP1 forms a complex with the mitochondrial cation uniporter that imports zinc into the mitochondrial matrix (Fig. 6). Prior to mitochondrial fission, dynamin-related protein 1 (Drp1) is recruited to the outer membrane where it binds to ZIP1. This interaction leads to zinc influx and a focal reduction in mitochondrial membrane potential (MMP) and subsequent division. Furthermore, Drp1-ZIP1 interaction is necessary for MMP-dependent mitophagy thus long-term disruption of this interaction leads to mitochondrial dysfunction. In cortical neurons, this disruption slows neurite outgrowth, suggesting this mechanism is critical for proper neural development (Cho et al., 2019). In this case, ZIP1 transports zinc out of the cytosol, suggesting that the orientation of ZIPs and their subsequent transport may vary depending on their subcellular localization.

ZnT transporters. ZnT transporters, in addition to their role in maintaining zinc homeostasis, also influence neuronal signaling through their transport of zinc and direct interaction with other proteins. As mentioned previously, ZnT1 upregulates Ras/Raf/MEK/ERK signaling through its interaction with Raf-1. ZnT1's enhancement of Ras-ERK signaling leads to upregulation of T-type calcium channel expression on the plasma membrane to increase calcium currents (Mor et al., 2012). ERK phosphorylation is also influenced by ZnT3 in the presynaptic terminals of mossy fibers. Namely, ZnT3 KOs exhibit reduced ERK phosphorylation despite having comparable ERK expression. In KOs, protein tyrosine phosphatase (PTP) is disinhibited leading to enhanced ERK dephosphorylation, which could be slowed by the addition of 10 μ M zinc, suggesting ZnT3 zinc transport mediates its ERK effect. Furthermore, this ZnT3-regulated signaling pathway is required for

contextual discrimination behavior, as ZnT3 KO, zinc chelation, or inhibition of ERK phosphorylation all impaired the behavior (Sindreu et al., 2011).

In addition to Raf-1, ZnT1 also associates with other proteins to regulate their function. As discussed earlier, ZnT1 binds to the GluN2A subunit to regulate zinc inhibition of NMDARs. Furthermore, the C-terminal domain of ZnT1 binds to the β subunit of L-type calcium channels and subsequently reduces channel current. The reduction in LTCC current results from decreased trafficking of the α_1 subunit to the membrane (Levy et al., 2009). Furthermore, expression of the C-terminal domain of ZnT1 is sufficient to inhibit LTCC, indicating that this inhibition is independent of the zinc transport (Shusterman et al., 2017). Given that LTCC mediate zinc influx, this suggests a unique mechanism in which the same protein that removes zinc from the cytoplasm also prevents further influx.

In this review, we outlined the current state of our knowledge for diverse roles for zinc signaling in the central nervous system. The work on this metal is far from over, however, as there is still a gap in understanding how the cellular and molecular actions of zinc are functionally linked to circuit- and system-level brain processes. This is due, in part, to the limitations of the tools used to both visualize and disrupt zinc. For example, although ZnT3 KOs have been indispensable for advancing the field of zinc neurobiology, it is unclear if or how KO animals compensate for the lack of ZnT3 over the course of development. It is entirely possible that in the absence of vesicular zinc release, ZnT3 KOs upregulate other zinc-regulatory mechanisms to maintain zinc signaling. This is an important caveat to consider, particularly when interpreting studies examining the endogenous functions of synaptic zinc. Furthermore, both ZnT3 KO and zinc chelation act non-specifically on all zinc-containing neurons in a circuit. Indeed, cell- and synapse-specific actions of zinc have been observed in the cerebral cortex, hippocampus and amygdala. For instance, it has been shown that zinc chelation can have opposing effects on the *in vivo* sound-evoked activity of different cell types in the auditory cortex (Anderson et al., 2017). Therefore, the development and implementation of more precise tools, such as inducible or conditional ZnT3 KOs, will allow us to better dissect the role of zinc in neural circuits by allowing for better spatial and temporal control over ZnT3 expression. Similarly, development of a transgenic line in which genetic tools, such as Cre-recombinase, are selectively expressed in unique populations of ZnT3-containing neurons will enable for in depth characterization of the physiological consequences of zinc modulation of synaptic receptors at specific sites.

This review discussed the complex system of transporters, ion channels, and metalloproteins that work in concert to regulate the localization and concentration of zinc both in and outside of neurons. Intracellular organelles, transporters, and metal binding proteins sequester and release zinc to maintain balance between second messenger signaling, transcriptional regulation, and pathological increases in the ion. Despite this complex regulatory system, not much is

known how these systems are coordinated to drive and modulate cellular zinc signaling. We highlighted cases in which zinc transporters were directly coupled to downstream signaling cascades, such as ZnT1-mediated inhibition of NMDARs, and ZIP1-mediated decrease in MMP and subsequent mitochondrial fission. These examples suggest that transporters can generate microdomains of zinc that allow for targeted delivery of the metal to subcellular components. As these specific roles of both ZnT1 and ZIP1 were identified by using screens for protein interactions of GluN2A and Drp1, respectively, similar approaches will likely provide additional valuable insights into the complex cell biology of zinc-regulated processes. As such, the continuing study of the multiple systems regulating zinc signaling will likely open a unique window into the rich and complex nature of many roles zinc plays in regulating brain function.

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