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Chelators for Investigating Zinc Metalloneurochemistry

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Introduction

Divalent zinc (Zn^{2+}) is one of the most abundant trace elements in the human body, where it typically serves as a structural or catalytic component for numerous proteins [1]. Although the chemistry and biology of zinc metalloproteins has historically dominated the field of zinc biology, there is a growing appreciation for a role of mobile zinc (hereafter mZn) pools found in specialized secretory tissues such as the prostate, pancreas, and brain [2–4]. Investigations of the function of mZn within these tissues have revealed that the biochemical action of mZn requires careful regulation of its concentration in order to ensure proper physiological function without pathological consequences [5]. Of particular interest is the function of mZn within the central nervous system, where high concentrations of chelatable zinc occur in specific regions of the brain [6]. Elucidating the functions of mZn requires the chemists to design and implement tools to specifically intercept and report on the location and concentration of mZn at defined extra- and intracellular locales, thereby helping to elucidate function.

Among the most common tools used to investigate the role of mZn in biology are zinc-responsive fluorescent probes. Recent reviews summarize the field of fluorescent zinc sensing and detail some challenges that remain [2,7]. Far less explored are zinc-specific chelators, which serve as antagonists for mZn [8]. With appropriately designed chelators one can apply fluorescent microscopy in conjunction with electrophysiology to unravel the molecular mechanisms of mZn. Unfortunately, the lack of an adequate supply of zinc-specific chelators has resulted in confusion and controversy within the field of metalloneurochemistry [8,9].

Here, we provide a brief background on zinc metalloneurochemistry [10], direct the reader to primary literature and reviews to outline the current status and challenges in the field, and detail how judiciously designed chemical tools can address complex biological questions involving mZn.

Anatomy of mZn in the Brain

mZn is primarily restricted to the forebrain, where zinc-containing axons are particularly abundant in the hippocampus, cortex, and amygdala (Figure 1a) [11]. Within these areas, the highest levels of mZn occur in the hippocampal mossy fibers (Figure 1b). Hippocampal mossy fiber axons project from granule cells of the dentate gyrus and are composed of two types of functionally specialized terminals, small filopodial extensions and large mossy fiber

boutons [12]. Of the two, mZn is primarily localized to the mossy fiber boutons [13]. At the cellular level, mZn is loaded into presynaptic vesicles by the zinc transport protein ZnT3, which is expressed exclusively in neurological tissue and testis [14]. In mouse models, genetic deletion of ZnT3 (ZnT3 KO) abolishes vesicular zinc [15]. The glutamate transporter Vglut1 is also targeted to zinc-containing vesicles, and ZnT3 works in concert with Vglut1 to localize glutamate and zinc within the same vesicles [16].

The Role of mZn in the Hippocampus

The presynaptic location and high levels (>100 μM) of mZn within glutamatergic vesicles, in conjunction with the importance of glutamate as a neurotransmitter, led to the hypothesis that mZn may act as a neurotransmitter or neuromodulator [8]. The abundance of vesicles containing mZn within the hippocampus, the area of the brain associated with memory and learning [17], makes this idea particularly intriguing. Seminal work with ZnT3 KO mice, however, furnished enigmatic results that questioned the importance of hippocampal mZn. [18]. Studies with 6–10-week old ZnT3 KO mice revealed no change in synaptic excitability in the CA3 region of the hippocampus or impairment in spatial learning, memory, or sensorimotor function [18,19]. The only phenotypic consequences appeared to be an increased susceptibility to limbic seizures [20]. The lack of an apparent phenotype in ZnT3 KO mice was perplexing because vesicular zinc is clearly localized to discrete regions of the brain (Figure 1a). These observations raised the question as to whether zinc was a neuromodulator or even released from vesicles upon stimulation [8,21–23]. More recently, studies with older (≥ 3 months) ZnT3 KO mice revealed them to display impaired fear memory [24], accelerated age-dependent loss in cognitive ability [25], and deficiencies in social and object recognition memory [26].

Despite the emergence of these mZn-dependent neurological phenotypes, their molecular mechanisms of action are poorly understood. The lack of a clear signal transduction mechanism can be attributed to the large number of potential targets of mZn [27]. For example, mZn is a potent inhibitor of protein-tyrosine phosphatases [28]. It can also allosterically block NMDA receptors [29,30], transactivate TrK B kinase [31,32], and modulate the function of AMPA and KAR receptors [11,33]. mZn is also critical in the stabilization and formation of postsynaptic density [34,35], and dictates the calcium sensitivity of glutamatergic vesicle release from presynaptic cells [36]. In addition, exogenously applied zinc activates a postsynaptic metabotropic zinc-sensing receptor, thereby inducing intracellular release of calcium via the ErK1/2-dependent pathway [37]. A subsequent study demonstrated that endogenous mZn could trigger ErK1/2-dependent signaling [38]. In this study, a combination of in vitro and in vivo experiments revealed ZnT3 KO mice to have reduced levels of phosphorylated Erk in hippocampal mossy fiber terminals resulting from disinhibition of MAPK phosphatase [38]. As a consequence, the ZnT3 KO mice were severely impaired in forms of memory that depend on hippocampal function, such as spatial working memory and contextual discrimination [38]. One mechanism proposed by the authors entails mZn first being released into the synaptic cleft, only to reenter the presynaptic terminals where the temporary increase in cytosolic mZn concentrations function to inhibit MAPK phosphatase [38]. Apart from the many protein targets, the complexity of mZn biology is underscored by a study that revealed both pre- and postsynaptic signal transduction mechanisms, a finding that may explain some of the controversies and apparent contradictions in the literature [39]. Sorting out the activity of mZn will benefit from the answers to outstanding questions such as, how much is released upon stimulation and how long does it remain in the synaptic cleft? Moreover, although several pre- and postsynaptic targets have been identified, details regarding downstream signal transduction mechanisms need to be delineated. Addressing these questions requires chemical tools that can modulate the action of mZn on a physiological time scale.

Design Considerations for mZn Chelators

Zinc-specific chelators are important tools for studying mZn biology. By selectively sequestering endogenous sources of mZn, chelators provide controls for fluorescent microscopic, electrophysiological, and biochemical studies [38–40]. As detailed in subsequent sections, design criteria for optimizing mZn chelators for particular applications will differ, but some generalizations about ligand composition, charge, and binding kinetics can be drawn. To operate effectively, chelators must be specific for mZn over other abundant metal ions and cofactors. Specificity for mZn in the presence of other signaling ions such as calcium can be achieved by application of the hard/soft–acid/base (HSAB) theory of inorganic chemistry [41]. Soft Lewis bases will preferentially bind softer metals like zinc rather than harder alkali and alkaline-earth metals, like calcium. For intracellular applications, chelators should be neutral, or nearly so, and have sufficiently hydrophobic character so as to diffuse passively across the plasma membrane unless a specific transporter is targeted. By contrast, extracellular chelators generally carry an overall negative charge to minimize translocation across the cell membrane. For binding mZn rapidly on a physiologically time scale, the p*K*a of the donor atoms should lie below physiological pH. If the donor atoms are protonated, dissociation of these protons from the ligating atoms may become rate limiting during metal chelation [42]. Lastly, chelators need to bind with sufficient affinity to capture mZn, while minimizing non-specific binding of the zinc proteome or unintentionally altering tonic levels of the ion that may be present. The various protein targets of mZn make it difficult to predict a priori the optimal Kd value to use in a particular application. For example, NMDA receptors have multiple zinc binding sites with binding constants that can vary by three orders-of-magnitude depending on subtype [9].

Intracellular Chelators as mZn Antagonists

The most common intracellular zinc chelator used in zinc metalloneurochemistry is *N,N,N′,N′*-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN) (Figure 2). TPEN readily permeates cell membranes [43] and form a stable 6-coordinate complex with zinc ($K_{d\,\mathrm{Zn}}$ = 0.7 fM) [44]. TPEN has been used to interrogate the intracellular targets of mZn, promote zinc deficiency, and study the toxic and neuroprotective effects of zinc chelation [6]. The relatively “soft” nitrogen donor atoms on pyridines and amines make TPEN selective for zinc over other, “harder”, divalent cations such as calcium (Table 1) [43]. Although TPEN binds other transition metals such as copper with high affinity ($K_{d\,\mathrm{Cu}}$ = 17 zM at 25 °C, pH = 7, and I = 0.1) [45], copper levels are tightly regulated with negligibly small amounts of their chelatable forms available within cells [46]. Numerous studies, however, highlight the toxic effects of TPEN. TPEN can induce axon and dendrite degeneration [47], strip metalloproteins of zinc cofactors [48], and promote apoptosis [49–51]. Such toxicity reveals the need for more sophisticated intracellular chelators, particularly those having varying zinc affinity, trappability, and the ability to target distinct biological locales or proteins. Such reagents have the potential to significantly inform zinc neurobiology. There has been a recent surge in the development of advanced metal chelators proposed for the treatment of neurodegenerative disease [52]. The design and implementation of these agents provide an excellent foundation for chelator design, including prochelators that can pass the blood brain barrier [53], as well as multifunctional chelators that target specific proteins [54,55].

Extracellular mZn Antagonists

In contrast to intracellular zinc chelators like TPEN, extracellular chelators have provided more substantial insights into the functions of vesicular zinc. For a chelator to operate effectively within the synaptic cleft it must remain in the extracellular space, bind zinc tightly and rapidly (< 60 ms), and maintain calcium and magnesium levels, which

themselves regulate several important signaling pathways [12,17]. The most common extracellular zinc chelators in use today by the neuroscience community include ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), and ethylenediamine-*N*,*N*′-diacetic-*N*,*N*′-di-β-propionic (EDPA) [8,9]. Inspection of their chemical structures (Figure 2) reveals that these chelators bind metal ions primarily through "hard" anionic carboxylate ligands, which have a high affinity for "hard" calcium ions. Even though the chelators coordinate zinc tightly (Table 1), and their anionic nature provides an extracellular locale, the high affinity of these chelators for calcium and magnesium render them inappropriate for studying the physiology of zinc (Table 1).

To avoid disrupting extracellular calcium levels, the calcium salt of EDTA, $[Ca(EDTA)]^{2-}$ or (CaEDTA), is typically employed as an extracellular zinc chelator [8,9]. CaEDTA has both a substantially reduced affinity for zinc ($K_{d\ Zn}$ = 2 nM) and a slower rate of zinc binding (k = 0.0024 s^{-1}, Table 1) than EDTA, because calcium must dissociate from the chelating agent as zinc binds concomitantly. Given that mZn released from hippocampal stores has a biological lifetime of ~60 ms, the kinetics of CaEDTA chelation are too slow for physiological measurements [8,9]. For example, it has been estimated that if 100 μM zinc is released into the synaptic cleft in the presence of 1 mM CaEDTA, 94 μM free zinc will still remain after 60 ms. As an alternative, the use of tricine (*N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine, Figure 2) as a metal chelating buffer was advocated [9]. Tricine, however, has a weak affinity for zinc ($K_{d\ Zn}$ = 10 μM), thus requiring millimolar concentrations to sequester mZn released in mossy fiber synapses.

The need for better extracellular zinc chelators was first addressed by Nagano and colleagues [56]. Using TPEN as a model scaffold, this group synthesized the sodium salts of (4-{[2-(bis-pyridin-2-ylmethylamino)ethylamino]methyl}phenyl)methanesulfonic acid (DPESA) and [4-({[2-(bis-pyridin-2-ylmethylamino)ethyl]pyridin-2-ylmethylamino}-methyl)phenyl]methanesulfonic acid (TPESA) (Figure 3). Like TPEN, DPESA and TPESA use nitrogen donor atoms to achieve selectivity for zinc over calcium and magnesium. The addition of a sulfonate group on the non-coordinating phenyl ring provided a negative charge, which was sufficient to keep the chelators from crossing cell membranes. Notably, TPSEA and DPESA coordinate zinc with picomolar affinities (Table 1) and successfully sequester zinc released under conditions that mimicked ischemic insult [56]. DPESA has a rate of zinc chelation (half-life = 67 sec) that is similar to that of CaEDTA (half-life = 65 sec). In this instance, the authors defined half-life as the amount of time it takes the chelator to reduce the fluorescence intensity of the zinc-sensitive fluorophore (ZnAF2) by half [56]. The apparent sluggishness in zinc chelation for DPESA was attributed to the similarity in zinc affinity between DPESA ($K_{d\ Zn}$ = 1.6 pM) and ZnAF2 ($K_{d\ Zn}$ = 2.9 nM). Considering that DPESA binds zinc roughly three orders of magnitude more tightly then ZnAF2, another possibility might be that, owing to its pK_a value of 9.09, the most basic nitrogen atom would be protonated at physiological pH [56]. This proton would have to dissociate prior to zinc coordination, slowing the rate of zinc [9]. By comparison, the basicity and kinetics of TPESA (half-life = 18.6 sec; pK_{a3} = 7.67) are more similar to those of TPEN (half-life 12.2 sec; pK_{a4} = 7.12). Irrespective of the kinetics of DPESA binding, both chelators are superior to CaEDTA in intercepting mZn. In a proof-of-concept study, acute rat hippocampal slices were exposed to anoxic-aglycemic artificial cerebrospinal fluid for a period of 17 min. During this time, zinc levels were monitored by fluorescence microscopy. In the absence of a chelator, an increase in fluorescence was observed in the CA1 region of the hippocampus, consistent with reported increases in CA1 zinc levels upon ischemic insult [57]. Application of 100 μM DPESA or TPESA sequestered the released zinc and prevented an increase in CA1 intracellular zinc concentration, a feat that CaEDTA was not able to accomplish at the same concentration [56].

More recently, a new extracellular zinc chelator was prepared and used to assess the role of vesicular zinc in long-term potentiation (LTP) at the mossy-fiber (mf) synapse [39]. LTP is a long-lasting (hours to days) synaptic enhancement that occurs between the pre- and postsynaptic cells, the action of which it is proposed to be crucial to the formation of memories [17]. Within the CA1 region of the hippocampus, which has a low concentration of mZn, LTP is induced after a triggering of postsynaptic NMDA and AMPA receptors. NMDA and AMPA receptors are activated by stimulant responsive release of glutamate from presynaptic vesicles. Activation of glutamate receptors results in depolarization of the postsynaptic cell due to an influx of calcium, sodium, and potassium. The increase of calcium activates calmodulin-dependent protein kinase II, which goes on to trigger several signaling cascades [17].

In contrast, mossy-fiber LTP (mf-LTP) is fundamentally different from “traditional” LTP [12]. Induction of mf-LTP is independent of NMDA receptors and the origin of induction is thought to occur presynaptically [39]. The high concentration of zinc within presynaptic mossy fiber boutons, coupled with the ability of mZn to inhibit NMDA receptors at nanomolar concentrations, has implicated mZn in the molecular mechanism of mf-LTP. Despite extensive study, the role of vesicular zinc in mf-LTP remained controversial [6,8,9,11,27]. It was hypothesized that CaEDTA was a large contributing factor to conflicting observations and that a more rapid and specific mZn chelator might help resolve the issue.

The design of an improved extracellular zinc chelator began with the metal-binding site. Based on previous experience with zinc-specific fluorophores [2], a novel dipicolylamine synthon was employed as the core zinc-binding subunit [58]. Subsequent incorporation of aniline 2-sulfonic acid increased the denticity of the chelating ligand and provided an overall negative charge to favor an extracellular localization. The electron deficient aniline moiety also aided the rapidity of zinc binding by lowering the pK_a (pK_{a3} = 6.43) of the adjacent nitrogen below physiological pH. The resulting extracellular chelator, ZX1 (Figure 3), readily coordinates zinc with a $K_{d\,Zn}$ of 1 nM and a rate constant for zinc binding (k_{Zn} = 0.027 s^{-1}) that is an order-of-magnitude faster than that of CaEDTA (Table 1) [39]. Using ZX1, electrophysiology studies were conducted to probe the role of vesicular zinc in mf-LTP, with several important results. First, vesicular zinc is required for mf-LTP and the mf-LTP initiates presynaptically. Surprisingly, depletion of vesicular zinc, either by chelation with ZX1 or genetic deletion of ZnT3 (ZnT3 KO), unmasked a form of mf-LTP that originates postsynaptically. The ability of mf-LTP to be induced both pre- and postsynaptically might explain the absence of an apparent phenotype in younger ZnT3 KO mice, because, in the absence of vesicular zinc, LTP could still be induced postsynaptically as is typical in other regions of the hippocampus [17]. Such “dual control” of CA3 synapses would support physiological action, while serving to limit hyperexcitability of CA3 pyramids, which could have pathological consequences [39].

Conclusions and Outlook

Our understanding of metalloneurochemistry is expanding as chemical tools begin to unravel the interactions of inorganic agents, within the complex circuitry of the central nervous system. [2,59] From the perspective of zinc neurochemistry, we are beginning to appreciate how the brain can use a deceptively simple ion to modulate the function of numerous protein targets in a stimulus-responsive manner. The complexity of vesicular zinc is highlighted by its dual functionality, whereby it potentiates synaptic transmission but also triggers pathological events such as Alzheimer’s disease and excitotoxicity when dysregulated [6]. Comprehending the action mZn would benefit from additional chelators and fluorescent sensors that can specifically interrogate its function. A prominent

outstanding question involves the benefit of using zinc as a neuromodulator. Considering that the hippocampus can function without mZn, what is the advantage is gained from using mZn as a neuromodulator, especially considering the pathological consequences associated with its dysregulation? By creating advanced chelators and sensors, inorganic chemists working in collaboration with the neuroscience community have the opportunity to address these important fundamental problems.

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Highlights

- Background on zinc metalloneurochemistry with an emphasis on hippocampal zinc.
- Design rules for zinc chelators that can be used as antagonists for mobile zinc.
- Utility of Zn-selective chelators for understanding zinc metalloneurochemisty.

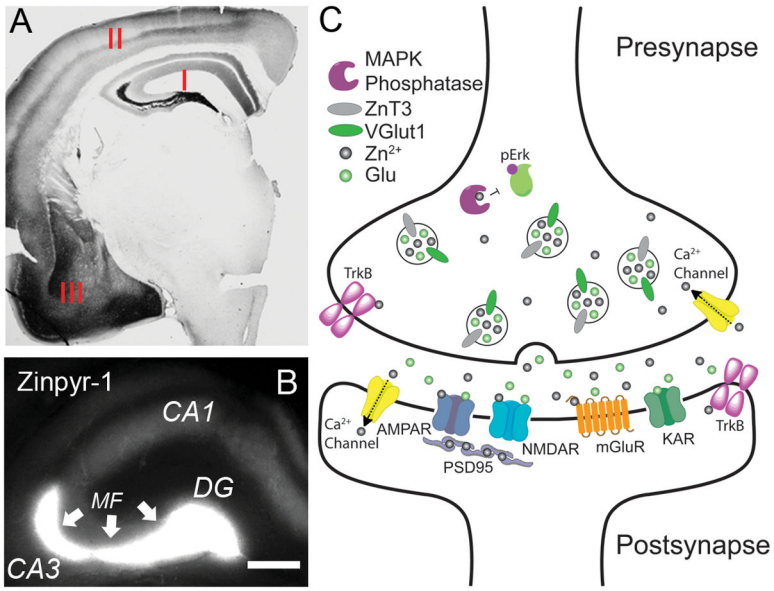

Figure 1.

(A) Timm staining of a coronal mouse brain section highlighting mobile zinc in the hippocampus (I), cortex (II), and amygdala (III). (B) The fluorescent signal from Zinpyr-1 [60] exposes the high levels of mZn held within mossy-fiber terminals. Figure adapted from reference [38]. (C) Diagram showing some pre- and postsynaptic targets of mZn.

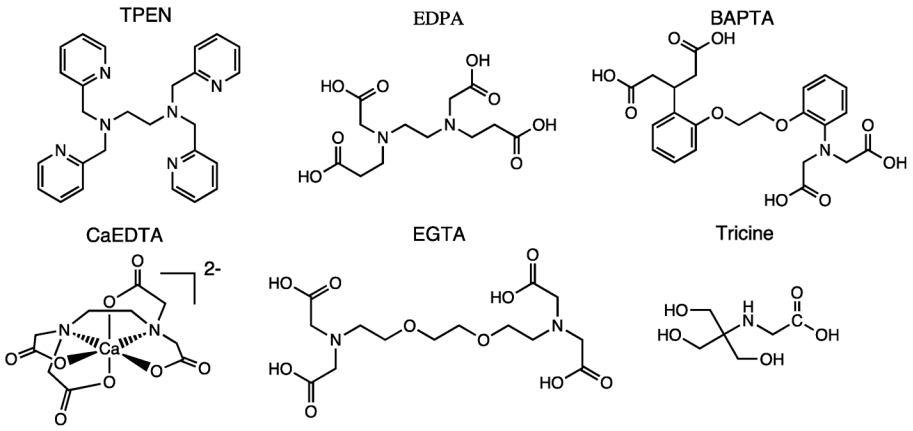

Figure 2.
Line drawings of the most common intra- and extracellular chelators used to study zinc metalloneurochemistry.

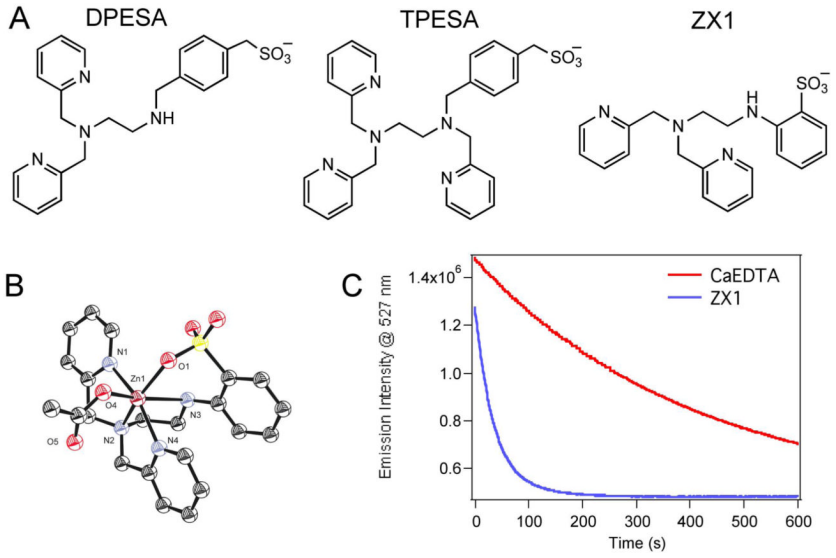

Figure 3.

(A) Line drawings of zinc-selective extracellular chelators. (B) X-ray structure of the acetate salt of the Zn:ZX1 complex. Hydrogens omitted for clarity. (C) Time dependence on the Fluorescent quenching of Zn_2:ZP1 by ZX1 and CaEDTA. (Figure 3b, c are adapted from reference [39])

Table 1

Metal dissociation ($K_{d\ M}^{2+}$, M) and pseudo-first order rate (k, s^{-1}) constants of extracellular and one intracellular (TPEN) zinc chelators.

	TPEN	EDTA	EDPA	CaEDTA	ZX1	DPESA	TPESA
$K_{d\ Zn}$	0.7 fM[a]	0.4 fM[a]	0.8 pM[a]	2.0 nM[b]	1.0 nM[b]	1.6 pM[c]	0.5 pM[c]
$K_{d\ Ca}$	68 μM[a]	19 nM[a]	0.9 μM[a]	-	n.d.	63 μM[c]	3.3 mM[c]
k_{Zn} (s^{-1})[b]	0.016	0.018	0.0034	0.0024	0.027	n.d.	n.d.
Advantages	Selective for mZn High metal affinity	Fast kinetics High metal affinity	High metal affinity	Selective for mZn Widely applied	Selective for mZn Rapid kinetics	Selective for mZn High metal affinity	Selective for mZn High metal affinity
Disadvantages	Toxic Removes metal cofactors	Non-selective Can alter Ca^{2+} levels	Non-selective Slow kinetics	Slow kinetics	Limited knowledge	Limited knowledge Slow kinetics	Limited knowledge

Adapted from references: [a][44], [b][39], and [c][56]