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Review: Zinc's functional significance in the vertebrate retina

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This review covers a broad range of topics related to the actions of zinc on the cells of the vertebrate retina. Much of this review relies on studies in which zinc was applied exogenously, and therefore the results, albeit highly suggestive, lack physiologic significance. This view stems from the fact that the concentrations of zinc used in these studies may not be encountered under the normal circumstances of life. This caveat is due to the lack of a zinc-specific probe with which to measure the concentrations of Zn^{2+} that may be released from neurons or act upon them. However, a great deal of relevant information has been garnered from studies in which Zn^{2+} was chelated, and the effects of its removal compared with findings obtained in its presence. For a more complete discussion of the consequences of depletion or excess in the body's trace elements, the reader is referred to a recent review by Ugarte et al. in which they provide a detailed account of the interactions, toxicity, and metabolic activity of the essential trace elements iron, zinc, and copper in retinal physiology and disease. In addition, Smart et al. have published a splendid review on the modulation by zinc of inhibitory and excitatory amino acid receptor ion channels.

It has long been recognized that zinc, an essential trace element, is an integral component of scores of enzymes, and thus participates in a broad range of metabolic functions [1-5]. There is mounting evidence that, owing to antioxidant properties, zinc protects cells from the damaging effects of oxidative stress, presumed to be a causative factor in the pathogenesis of various age-related retinal disorders (cf. [6-8].). In this connection, a study by Kikuchi et al. [9] demonstrated the protective action of zinc against the neurotoxic effects of glutamate on retinal neurons in culture. In an alternative approach, Hyun et al. [10] showed that zinc depletion, induced by treatment with a membrane-permeable chelator, rendered cultured human retinal pigment epithelial cells highly vulnerable to cell death from ultraviolet (UV) radiation or exposure to hydrogen peroxide. Zinc deficiency has serious consequences, and the resultant pathology has been well documented in a wide variety of tissues (cf. [11-16].). Conversely, results from an extensive study of the use of zinc supplementation for preventing and treating age-related ocular diseases (the Age-related Eye Disease Study, AREDS [17]) showed that zinc treatment significantly reduces the progression to advanced age-related macular degeneration, but other randomized controlled studies were less encouraging [18]. Nevertheless, the obvious importance of zinc has led to the development of powerful new techniques for determining zinc concentrations in plants, animals, and human donor eyes [19,20]. One such method, inductively coupled plasma mass spectrometry (ICP-MS), detects zinc isotopes as well as other transition metals in human tissues at concentrations as low as one nanogram per liter. Unlike atomic absorption spectroscopy, which can measure only a single element at a time, ICP-MS can scan for all elements simultaneously. This allows rapid sample processing and rapid recording of the entire analytical spectrum. Although impressive and accurate, the method does not distinguish between free and bound zinc, and accordingly cannot provide reliable information on the concentration of zinc ions released from neurons or present at pre-and post-synaptic sites.

Zinc in the retina: In addition to the zinc bound within the molecular structure of enzymes, there are pools of free zinc ions (Zn^{2+}) throughout the central nervous system (CNS), including the retina [21], hippocampus [22], and other regions of the cerebral cortex [23]. Based on zinc's effects on various types of neurotransmitter receptors and related signaling mechanisms, there is little doubt that Zn^{2+} has a neuromodulatory role throughout the CNS [2,24]. A comprehensive review of the many vertebrate and invertebrate sites at which zinc modulates inhibitory and excitatory amino acid ion channels was assembled by Smart et al. [25]. Interestingly, all types of retinal neurons as well as cells of the pigment epithelium contain, in varying degrees, ionic zinc [26]. This was confirmed at the ultrastructural level by Akagi et al. [27], who showed that zinc has a broad subcellular, compartmentalized localization in the rat retina, from the retinal pigment epithelium (RPE) proximally to the layer of ganglion cells. They found that zinc was associated with three compartments: In

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the outer plexiform layer (OPL) and the inner plexiform layer (IPL), zinc was associated with neural processes, whereas in the RPE, inner segments (IS), inner nuclear layer (INL), and ganglion cell layer (GCL), zinc was associated with the Golgi apparatus, presumably to catalyze metalloenzyme reactions. These loci are consistent with the immunochemical localization of the zinc transporter-3 (ZnT-3) in the rodent retina [28], i.e., in the outer limiting membrane (OLM) and the photoreceptor (PR) inner segments, and in the INL, IPL, and GCL. Using the membrane impermeable form of the Zn^{2+}-sensitive fluorescent dye Newport Green, Redenti and Chappell [29] reported increased release of Zn^{2+} in these retinal regions when the cells were depolarized with 50 mM KCl. Interestingly, a study by Tam et al. [30], using isolated rod outer segments (ROSs) and emulphogene extracts of ROSs, showed that they, too, contain appreciable amounts of Zn^{2+} that was not only stoichiometrically related to rhodopsin but was also light dependent.

Although the distribution of zinc within the retina is clearly important, the absence of a reliable (i.e., highly selective) electrochemical probe for ionic zinc has limited the measurement of the concentrations of the many histochemically detectable sources of Zn^{2+}. However, studies exploring the effects of Zn^{2+} applied exogenously at specific locations in the nervous system have provided useful information, although it is important to stress that the physiologic significance of such data is difficult to assess.

Effects on photoreceptors: Since this review focuses on the role of Zn^{2+} in the vertebrate retina, we consider some of the relevant experimental findings that have been reported in recent years on the effects of Zn^{2+} when it is applied to retinal cells. Perhaps one of the earliest observations of how zinc may influence the activity of retinal neurons was made by Wu et al. [31], who, using a modified Neo-Timm sulfide silver staining method [32,33], demonstrated the presence of a dense band of staining in the ONL of the tiger salamander retina, more specifically, in the proximity of the synaptic terminals of rod and cone PRs. Subsequent studies have reported similar findings in a wide range of vertebrate retinas [29,34,35]. Aware of the evidence that zinc is bundled in the synaptic vesicles of glutamatergic terminals and coreleased with glutamate [36,37], the researchers showed that applying zinc to the terminals of rods and cones suppressed calcium entry at these sites. This feedback mechanism would clearly regulate glutamate release, and prevent its depletion in the dark-adapted retina when PRs are maximally depolarized and actively discharging their neurotransmitter. Whole-cell recordings from cone photoreceptors in the carp retina clearly support this view. These studies revealed that the Ca^{2+} currents were suppressed by Zn^{2+} in a dose-dependent fashion [38]. In a later section of this review, we will provide experimental evidence that this autofeedback process also protects retinal neurons from glutamate-induced necrotic and apoptotic cell death.

Effects on second-order neurons: Wu and coworkers also recorded intracellularly the light responses of horizontal cells (HCs), second-order neurons that receive excitatory synaptic input from PRs and, in turn, deliver inhibitory signals to the visual cells (but see Jackman et al. [39] for evidence of a positive feedback signal to PRs). The Wu group found that Zn^{2+} selectively blocked the horizontal cells' light-induced depolarizing response to gamma-aminobutyric acid (GABA), whereas the glutamate responses of HCs were unaffected by Zn^{2+}. It appears that Zn^{2+} selectively antagonizes N-methyl D-aspartate (NMDA) receptors, and provides further evidence that the glutamate receptors on tiger salamander HCs consist of non-NMDA subtypes. The ganglion cells of this amphibian also contain NMDA receptors, and not surprisingly, the NMDA-mediated inward currents, recorded with a whole-cell voltage clamp in a retinal slice preparation, were blocked by micromolar concentrations of Zn^{2+} [40]. The neuronal receptors in rat hippocampal slices exhibit the same differential effect of zinc on NMDA and non-NMDA receptors [41].

In addition to the NMDA receptors, vertebrate horizontal cells express two distinct subtypes of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (calcium permeable and calcium impermeable), both of which have been studied regarding their responsiveness to zinc. Whole-cell voltage clamp recordings [42] revealed that zinc had dual effects on Ca^{2+}-permeable AMPA receptor-mediated currents: At low concentrations (10 μM), Zn^{2+} potentiated the current, whereas at higher concentrations (100 and 1,000 μM), Zn^{2+} reduced the current in a dose-dependent manner. However, Zn^{2+} had no effect on currents mediated by Ca^{2+}-impermeable AMPA receptors. In a follow-up to this study [43], the authors presented evidence that the Zn^{2+}-potentiating effect involves interactions with cyclothiazide (CTZ) binding sites on the AMPA receptors, while the inhibitory effect was related to the extracellular Ca^{2+} concentration, thus implicating separate mechanisms.

Although the effects of zinc on the cells of the distal retina are undoubtedly more complex than described above, vertebrate horizontal cells contain hemigap-junction channels [44,45], which, when paired with hemichannels on adjacent cells, form the gap junctions that mediate electrical coupling between adjacent cells. When individual connexins (e.g., Cx35 and Cx38) were expressed in oocytes, the results seen

with two electrode voltage-clamp recordings showed that zinc exerted a biphasic effect on the hemichannel currents [46]. Thus, a low concentration of Zn^{2+} (1 μM or 10 μM) enhanced the current response, whereas 100 μM or 1 mM Zn^{2+} greatly reduced the current. These observations suggest that zinc may interact with connexins at two external binding sites with different affinities for zinc. The high-affinity site, activated at low concentrations of zinc, gives rise to enhanced hemichannel currents, whereas the low affinity site requires high concentrations of zinc to produce an inhibitory effect. In contrast, Sun et al. [45] found that Zn^{2+} suppressed hemichannels of native bass horizontal cells at low and high micromolar concentrations. In addition, Sun et al. [45] found that zinc and calcium suppressed hemigap-junction channels in these cells, and when the two were coapplied at relatively low concentrations (20 μM Zn^{2+} + 100 μM Ca^{2+}), the total suppressive effect on the hemichannel current was approximately equal to the sum of the individual inhibitory effects. Clearly, the two divalent cations act independently at different binding sites. Another significant observation by these authors was the lack of any zinc effect on cell-to-cell coupling, a good indication that the binding sites for Zn^{2+} are located on the extracellular surfaces of uncoupled hemichannels.

One of the more unique sites at which hemichannels are expressed is on the dendritic membranes of horizontal cells that invaginate the photoreceptor terminals. At these loci, hemichannels provide an essential component of a proposed ephaptic mechanism by which horizontal cell feedback governs the center-surround receptive field organization of retinal neurons [47,48]. The effects of zinc on hemichannel currents are revealing. When zinc was applied to connexins expressed in *Xenopus* oocytes, the effects were biphasic. One to ten micrometers of zinc produced an eightfold increase in membrane current, whereas concentrations of 100 μM or greater suppressed the voltage-gated currents in a graded fashion; the effects of zinc were effectively reversed by adding 1-mM histidine [49].

The other class of second-order neurons that forms synapses with PRs is the bipolar cell, which provides parallel ON and OFF pathways to more proximal sites. These cells are activated by glutamate, and use glutamate as their neurotransmitter. Evidence that zinc neuromodulates bipolar cell activity derives from several sources in which the currents mediated by GABAa and GABAc receptors of isolated bipolar cells were shown to respond differentially to zinc. Perforated-patch-clamp recordings from isolated bipolar cells of the skate retina revealed the presence of GABAa and GABAc receptors at the dendrites and the axon terminals of these cells [50]. Whereas currents mediated by the activation of GABAc were downregulated by zinc, those mediated by GABAa receptors were biphasic: The currents were significantly enhanced by zinc concentrations in the range of 0.1–100 μM but suppressed at higher concentrations (>100 μM). The enhancing effect on the GABAa receptor had been seen earlier in the GABA-mediated responses of skate Müller cells [51]. The enhancement of GABAa receptor activity on skate bipolar cells showed little voltage dependence, suggesting that zinc acts on an extracellular domain of the GABAa receptor.

In recordings from hybrid bass bipolar cells, Qian and Dowling [52] found that zinc strongly inhibited the GABAc response of these cells, whereas the GABAa responses were not significantly affected. In addition, a study of carp retinal bipolar cells by Han and Yang [53] revealed some of the kinetics of the GABA responses. The researchers showed that zinc slowed activation and desensitization of the GABAc response, but accelerated those components of the GABAa response. Moreover, zinc accelerated deactivation of the GABAc response but had no effect on deactivation of the GABAa response. A more comprehensive study of the effects of zinc on GABAa receptors, conducted by Smart et al. [54], involved the expression of different subunits of the GABAa receptor in human embryonic kidney cell line (HEK) cells. In this system, the researchers found that zinc inhibition of GABA responses was highly dependent upon the composition of the receptor. Thus, GABAa receptors possessing the γ2 subunit were relatively insensitive to suppression by zinc, whereas those containing α1β1 subunits were inhibited by zinc.

Inner retinal neurons: Clearly, any observable effects of Zn^{2+} at second-order sites will be reflected in the behavior of amacrine cells that subserve lateral interconnections through the IPL, and in ganglion cells, the third-order retinal neurons that communicate directly with the lateral geniculate nucleus of the CNS. This is evident in the experiments of Li and Yang [55], who examined the modulatory effects of Zn^{2+} on the two major inhibitory neurotransmitters of the inner retina, namely, the GABAa receptors and strychnine-sensitive glycine receptors that coexist on the amacrine and ganglion cells of the carp retina. The results demonstrated the differential effects of zinc on these subsets of receptors. Zn^{2+} invariably suppressed the GABAa-mediated currents of these cells, but the glycine-induced currents were biphasic: Low concentrations (0.1–10 μM) of Zn^{2+} enhanced the current responses, whereas higher concentrations (>100 μM) showed a dose-dependent decrease in the responses. Similar results were obtained by Kaneda et al. [56], who demonstrated the presence of zinc in AII amacrine cells of the rat retina and,

similar to Han and Wu [57], confirmed the biphasic effects of zinc on strychnine-sensitive glycine receptors. Moreover, Han and Wu [57] showed that the different subunit composition of the 5,7-dichlorokynurenic acid (DCKA)-sensitive glycine receptors in the salamander retina made them resistant to modulation by Zn^{2+}.

In a study of the molecular basis for zinc potentiation of strychnine-sensitive glycine receptors, Miller et al. [58] identified a Zn^{2+} binding site located externally on the N-terminal domain of the α subunit of the glycine receptor that increases the efficacy of glycine receptor gating. And juxtaposed to this site, they identified another residue that acted as a key control element in the allosteric transduction pathway, enabling either potentiation or inhibition of receptor activation depending upon the zinc concentration at this location. Among the other effects of zinc, Tabata and Ishida [59] used whole-cell patch-clamp recordings from fish ganglion cells to reveal that cytoplasmic zinc (at nanomolar concentrations) facilitated the downward regulation of background chloride conductance by an endogenous protein kinase C.

Glial cells: The retinal Müller cells (radial glia) extend from the inner margin of the retina (theinner limiting membrane [ILM]) to the distal end of the ONL where they form the OLM. Here villous processes embrace the myoid region of the PRs. Along their course, Müller cells extend branches that interdigitate with every class of retinal neuron and with the blood vessels of the vascularized retina [60]. These glial cells will be exposed to zinc at every neuronal site from which it is released, resulting in a critical metabolic neuron and glial cell interaction. Since there are no extracellular enzymes to terminate glutamate's synaptic activity, the Müller cells play a major role in limiting the extracellular concentration of glutamate through the action of a high-affinity uptake mechanism [61]. Within the cell, the enzyme glutamine synthetase catalyzes the conversion of L-glutamate to L-glutamine, which is released into the extracellular space and taken up by glutamatergic neurons. The existence of glutamate transporters and the glutamate-glutamine cycle in glial cells is supported by a large body of evidence (cf. [62].). The radial glia not only take up glutamate but also release glutamate by reversing electrogenic uptake [63], and both actions are effectively blocked by zinc [64].

Zinc chelation: A zinc-specific probe that measures the concentration of free zinc within cells or in the post-synaptic region of neurons when zinc is coreleased with glutamate has yet to be developed. Nevertheless, it has been possible to circumvent this obstacle and conduct several experiments that reveal the functional and cytoprotective roles of Zn^{2+} in the vertebrate retina. In essence, the approach we took was to examine the consequences of removing zinc ions by exposing the tissue to agents that chelate zinc, and then compare neuronal activity and cellular structure before and after Zn^{2+} is removed. The experiments cited here are not the first to examine the effects of zinc chelation, or the lowering of endogenous zinc by dietary means (cf. [25,65]), but they are perhaps the most extensive studies using this procedure, and conducted on the retinas of various species of vertebrates, e.g., zebrafish (*Danio rerio*), skate (*Raja erinacea*), and tiger salamanders (*Ambystoma tigrinum*).

Modulation of electrical activity: Whole-cell voltage-clamp recordings from isolated bipolar cells of the skate retina demonstrated that 1 μM zinc blocked the glutamate-mediated current of ON bipolar cells and the kainate-induced current of OFF bipolar cells [66]. When zinc was superfused with picrotoxin to block GABAergic input, the addition of histidine resulted in a large increase in the ON and OFF components of the electroretinogram (ERG). These findings are similar to the results of ERG recordings from eyecup preparations of skate and zebrafish [67,68] in which the magnitudes of the ERG responses were significantly enhanced (approximately twofold) after the zinc chelator histidine was added. These findings are a good indication that an endogenous source of zinc suppresses the light-evoked ERG responses of the vertebrate retina, and are in line with the patch-clamp recordings of Chappell and Redenti [69] from skate horizontal cells in the retinal slice preparation, in which they showed that adding histidine increased the membrane conductance, which resulted from the chelation of endogenous zinc. This conductance increase would lead to suppression of an inhibitory feedback mechanism, thus increasing the entry of calcium into photoreceptor terminals, and to the enhancement of transmitter release. The authors speculate that "this mechanism probably represents an important component of 'neural' adaptation, comprising processes that are distinct from those governed by the bleaching and regeneration of rhodopsin."

A brief summary of retinal physiology and a key objective of this review: Here we mention a few well-established features of the vertebrate retina. (1) Zinc is readily detected in glutamatergic neurons in the retina and the CNS [26,27,31,33,35,37,70,71], and glutamate and zinc are released in the dark [29] when the photoreceptors are depolarized by a continuous dark current that carries sodium into the cell. Light blocks this current, causing the visual cells to hyperpolarize, and release is suppressed. Although unquestionably greater in darkness than in light, the release of glutamate is not in sufficiently high concentration to cause the death of retinal neurons during long hours of sleep. However, when the cells are depolarized further by the addition of K^+, the

vesicular discharge is far greater, and there follow extensive pathological changes in the morphology and physiology of the visual cells, which display a vast enlargement of the photoreceptor terminal, the depletion of vesicles within the cell and at the synaptic ribbon, and the progressive loss of the ERG light response [72].

(2) Glutamate is the amino acid that acts as the neurotransmitter by which photoreceptors and bipolar cells communicate with neurons in the inner retina. Nevertheless, a vast amount of literature demonstrates the toxic effects of glutamate when applied to the retina and brain (cf. [73] and the Olney references [74-76]).

This brings us to several questions that relate to a key thesis being developed here, namely, that free (chelatable) zinc helps protect retinal neurons from the deleterious effects of glutamate-induced toxicity during long periods of darkness. (1) Having already shown that zinc is localized within photoreceptor terminals, what is the evidence that it is coreleased with glutamate in darkness? (2) If zinc blocks voltage-gated calcium channels to suppress endogenous glutamate release, what is zinc's effect on the light-evoked responses of retinal neurons? (3) Do pathological changes result when glutamate release is enhanced by chelating zinc? In the following sections, we address each query in turn.

Dark release of zinc: Although there is abundant evidence that zinc is coreleased with glutamate [36,37,77], there has been little direct evidence of this event at glutamatergic sites in the vertebrate retina. However, Redenti et al. [78] used the extracellular form of the zinc indicator Newport Green to obtain fluorescent images of zinc in the region of the synaptic terminal of light- and dark-adapted zebrafish photoreceptors. The results provided unequivocal evidence that zinc release is reduced in light-adapted cells, but clearly reveals increased zinc release at the synaptic terminals of dark-adapted cells.

Light-evoked responses following suppression of glutamate release: As noted earlier, applying zinc to the terminals of rods and cones suppresses calcium entry at these sites, providing a feedback mechanism that regulates glutamate release and preventing its depletion in the dark-adapted retina when the visual cells actively discharge their neurotransmitter. However, as described previously, blocking glutamate release affects the response properties of second-order neurons and their subsequent transfer of signals to ganglion cells, the output neurons of the retina. To determine how removing zinc would affect the response properties of retinal neurons, Redenti and Chappell [68] showed that zinc chelation (by histidine) increased by twofold the ON (*b*-wave) and OFF (*d*-wave) components of the ERG when GABA receptor activity was blocked by 200-µM picrotoxin. In addition, whole-cell, patch-clamp recordings from horizontal cells in a skate retinal slice preparation [69] showed that zinc chelation produced an increase in the cell's membrane conductance, most likely due to an increase in the release of the neurotransmitter from photoreceptors. The authors believe this probably represents an important component of "neural" adaptation, a process that is distinct from those governed by the bleaching and regeneration of rhodopsin.

Cytoprotection: Another consequence of the zinc-mediated autofeedback mechanism is the potential protection the mechanism provides from glutamate-induced necrotic and apoptotic cell death. An earlier publication by one of us [79] reviewed the functional significance of the essential amino acid taurine. Its role in protecting cells of the distal retina from the damage associated with exposure to glutamate, a cytotoxic neurotransmitter that is abundantly released from photoreceptor terminals in darkness, was emphasized. Here we consider an analogous situation in which endogenous zinc ions (Zn^{2+}) serve a similar purpose, but at a different site and via a different mechanism. Whereas taurine was shown to protect the photoreceptors and pigment epithelium from glutamate toxicity, we review experimental evidence that zinc ions are cytoprotective for cells located more proximally in the vertebrate retina.

If zinc feedback is an important factor in providing protection from glutamate toxicity during prolonged darkness, then the ill effects of the absence of zinc should be revealed in the dark-adapted retina after chelating zinc. This is precisely the result shown by Anastassov et al. [80] when the zinc chelator histidine or the glutamate analog kainate was injected into the subretinal space of the skate eyecup in our recent studies. The chelator and the cytotoxin produced clear signs of early onset necrosis, and severe apoptosis developed later in retinal cells extending from the horizontal cells to the inner margin of the ganglion cell layer. However, the retinal cells were spared when an equimolar quantity of zinc followed the injection of histidine. Shindler et al. [81] also found that zinc-chelating agents were toxic to retinal ganglion cells through a glutamate-dependent mechanism. A clear indication of apoptosis was demonstrated by the fact that cell death could be blocked by a series of caspase inhibitors.

A final comment: We hope that this review will convince the reader of the vital functional properties of zinc, whether bound (enzymatic) or free (chelatable), in the retina and throughout the body.

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