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Mobile zinc increases rapidly in the retina after optic nerve injury and regulates ganglion cell survival and optic nerve regeneration

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Retinal ganglion cells (RGCs), the projection neurons of the eye, cannot regenerate their axons once the optic nerve has been injured and soon begin to die. Whereas RGC death and regenerative failure are widely viewed as being cell-autonomous or influenced by various types of glia, we report here that the dysregulation of mobile zinc (Zn$$^{2+}$$) in retinal interneurons is a primary factor. Within an hour after the optic nerve is injured, Zn$$^{2+}$$ increases several-fold in retinal amacrine cell processes and continues to rise over the first day, then transfers slowly to RGCs via vesicular release. Zn$$^{2+}$$ accumulation in amacrine cell processes involves the Zn$$^{2+}$$ transporter protein Znt-3, and deletion of slc30a3, the gene encoding Znt-3, promotes RGC survival and axon regeneration. Intravitreal injection of Zn$$^{2+}$$ chelators enables many RGCs to survive for months after nerve injury and regenerate axons, and enhances the prosurvival and regenerative effects of deleting the gene for phosphatase and tensin homolog (pten). Importantly, the therapeutic window for Zn$$^{2+}$$ chelation extends for several days after nerve injury. These results show that retinal Zn$$^{2+}$$ dysregulation is a major factor limiting the survival and regenerative capacity of injured RGCs, and point to Zn$$^{2+}$$ chelation as a strategy to promote long-term RGC protection and enhance axon regeneration.

Significance

The inability of CNS pathways to regenerate after injury can lead to devastating, life-long losses in sensory, motor, and other functions. We report that after injury to the optic nerve, a widely studied CNS pathway that normally cannot regenerate, mobile zinc (Zn$$^{2+}$$) increases rapidly in the processes of retinal interneurons (amacrine cells) and then transfers via vesicular release to retinal ganglion cells (RGCs), the injured projection neurons. Eliminating Zn$$^{2+}$$ leads to both persistent RGC survival and substantial axon regeneration with a broad therapeutic window. These findings show that signaling between interneurons and RGCs contributes to regulating the fate of RGCs after optic nerve injury, and that Zn$$^{2+}$$ chelation may provide a potent therapeutic approach.


The authors declare no conflict of interest.

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significant human disability (43–46). Zinc also contributes to neuronal death in hypoxic-ischemic injury (34, 35, 47–49) by interfering with mitochondrial and metabolic functions (50, 51), altering ion fluxes (52), and other mechanisms (53). Abnormalities in zinc homeostasis may be important in chronic neurodegenerative diseases, such as Alzheimer’s (54) and amyotrophic lateral sclerosis (55–57), and have been shown to play a key role in oxidative stress-induced death in neurons and oligodendrocytes (58, 59). Given the abundant evidence for the importance of zinc in acute and chronic neurodegeneration, we have investigated its involvement in the death of RGCs following optic nerve injury. We show here that Zn\(^{2+}\) accumulates in amacrine cell processes shortly after optic nerve injury before being transferred to RGCs, and that Zn\(^{2+}\) chelation allows many injured RGCs to survive for months. Remarkably, Zn\(^{2+}\) chelation also provides a strong stimulus for axon regeneration.

Results

\textbf{Zn}\(^{2+}\) Increases in the Inner Retina Shortly After Optic Nerve Crush.}

Most of our studies used autometallography (AMG) (60) to visualize Zn\(^{2+}\) in the retina because of the selectivity of this method for Zn\(^{2+}\) per se, its utility for semiquantitative studies, the stability of the reaction product, and high level of reproducibility (61, 62). Whereas AMG revealed only modest levels of Zn\(^{2+}\) in the normal retina, by 6 h after optic nerve crush (NC), the earliest time point we examined using AMG (Methods), the Zn\(^{2+}\) signal in the inner plexiform layer (IPL) was 4.4 ± 0.3-fold above baseline (mean ± SEM) (Fig. 1 A, B, and D) and continued to rise over the next 18 h (Fig. 1 B and D). Staining was particularly prominent in the inner- and outermost sublaminae of the IPL. Although our standard staining protocol showed little signal in the normal retina, extending the development time revealed definite Zn\(^{2+}\) AMG staining (Fig. S1 A and B), in agreement with earlier studies (41, 63). Quantitatively, the staining intensity in the IPL after NC relative to that of the intact retina was largely insensitive to the reaction time over a range of 0.5–3 h (Fig. S1 A and B). The IPL in the retina contralateral to the injured optic nerve showed a small, transient elevation of Zn\(^{2+}\) (Fig. S2 A–C), reminiscent of other contralateral effects reported after unilateral optic nerve injury (64). Because the contralateral signal changes over time, we used the normal, intact retina as a reference throughout the study, always staining control and experimental retinas together.

After 2–3 d, whereas the Zn\(^{2+}\) signal in the IPL had declined, cells within the ganglion cell layer (GCL) showed strong staining (Fig. 1 B and E and Fig. S1 C and D). To investigate whether the AMG signal accurately reflected Zn\(^{2+}\), we tested whether it could be eliminated by the high-affinity, membrane-permeable Zn\(^{2+}\) chelator TPEN [N,N,N’-tetrakis[2-pyridyl methyl] ethylenediamine] (65) or the recently developed, highly selective, membrane-impermeable Zn\(^{2+}\) chelator ZX1 (38). A single intracocular injection of either TPEN or ZX1 immediately after NC eliminated the AMG signal in the IPL when examined at 6 h and 1 d (Fig. 1 B and D and Fig. S1E) and strongly suppressed, but did not fully eliminate, the IPL signal and the number of positively stained cells in the GCL at 2–3 d (Fig. S1F).

We extended these results by using the membrane-permeable, highly selective fluorescent Zn\(^{2+}\) sensor Zinpyr-1 (ZP-1) (38, 66), which affords higher spatial and temporal resolution than AMG. By 1 h after NC, the ZP-1 signal in the IPL was 2.8 ± 0.3-fold above baseline (mean ± SEM; Bonferroni-corrected \(P = 0.036\)) and continued to rise over the first 24 h (Fig. 1 C and F). In agreement with the AMG results, the ZP-1 signal in the IPL declined by 3 d while becoming strongly elevated in cells of the GCL (Fig. 1 C). ZX1 and TPEN both eliminated the ZP-1 signal at 6 h, verifying its association with Zn\(^{2+}\) per se (Fig. 1F) (all Bonferroni-corrected \(P < 0.0001\)). The nearly identical changes in Zn\(^{2+}\) levels observed in the retina over time using two completely different methods further validates the use of AMG for semiquantitative studies. Intracocular injection of inorganic Zn\(^{2+}\) (ZnCl\(_2\), 100 μM, 1 mM) failed to elevate the Zn\(^{2+}\)-AMG signal in the retina (Fig. S2 D–F) and did not impair RGC survival (Fig. S2 G and H), although we do not know how well exogenous Zn\(^{2+}\) reaches the neural retina nor whether it enters cells in the absence of nerve injury or manipulations that open zinc permeation pathways.

\textbf{Zn\(^{2+}\) Accumulation Requires the Zinc Transporter Protein ZnT-3.}

ZnT-3 loads Zn\(^{2+}\) into synaptic vesicles of glutamatergic, monoaminergic, and GABAergic neurons (67–69). Immunohistochemistry revealed a faint ZnT-3 signal in the IPL of intact mice. In agreement with the AMG results, the ZP-1 signal in the IPL of NC mice was 2.8 ± 0.3-fold above baseline (mean ± SEM; Bonferroni-corrected \(P = 0.036\)) and continued to rise over the first 24 h (Fig. 1 C and F). In agreement with the AMG results, the ZP-1 signal in the IPL declined by 3 d while becoming strongly elevated in cells of the GCL (Fig. 1 C). The nearly identical changes in Zn\(^{2+}\) levels observed in the retina over time using two completely different methods further validates the use of AMG for semiquantitative studies. Intracocular injection of inorganic Zn\(^{2+}\) (ZnCl\(_2\), 100 μM, 1 mM) failed to elevate the Zn\(^{2+}\)-AMG signal in the retina (Fig. S2 D–F) and did not impair RGC survival (Fig. S2 G and H), although we do not know how well exogenous Zn\(^{2+}\) reaches the neural retina nor whether it enters cells in the absence of nerve injury or manipulations that open zinc permeation pathways.
ZnT-3 expression was elevated 1.8-fold in cells of the GCL, most of which are RGCs (Fig. S3 C and D). Introducing TPEN immediately after nerve injury suppressed the elevation of ZnT-3 (Bonferroni-corrected P < 0.0001) (Fig. 2 A and B), indicating that expression of the transporter protein is regulated by Zn²⁺ concentration. ZnT-3 expression was not elevated in the eye contralateral to the NC (Fig. S3 A), nor was it elevated in either eye by intravitreal injection of exogenous ZnCl₂ (100 μM, 1 mM) (Fig. S3 B). To investigate the role of ZnT-3 in Zn²⁺ accumulation, we used mice carrying a deletion of slc30a3, the gene encoding ZnT-3, along with wild-type littermates. slc30a3 deletion eliminated the ZnT-3 signal in the retina (P < 0.0001, t test) (Fig. 2 A and C), verifying the validity of the immunostaining. Importantly, slc30a3 deletion eliminated the ZnT-3 signal in the IPL 1 d after NC (P = 0.0008, t test) (Fig. 2 D and E) and eliminated ~two-thirds of the cellular Zn²⁺ staining in the GCL at 3 d (P < 0.0001, t test) (Fig. 2 F). Thus, ZnT-3 increases in response to elevated Zn²⁺, enables Zn²⁺ to be sequestered in synaptic vesicles of the IPL, and is required for most, although not all, of the delayed Zn²⁺ accumulation in cells of the GCL (shown below to be RGCs).

The loss of Zn²⁺ accumulation in cells of the IPL following slc30a3 deletion suggests that cellular accumulation may result from vesicular release of Zn²⁺ from the processes of interneurons in the IPL. To test this idea, we injected tetanus neurotoxin (TeNT; 20 nM), an inhibitor of vesicular transmitter release, into the eye immediately after NC. TeNT blocked the decline in Zn²⁺ that normally occurs in the IPL 3 d after NC, causing Zn²⁺ levels in the IPL to increase 6.6 ± 0.4-fold (P < 0.0001) over those seen in PBS-treated retinas after NC (16.3 ± 1.0-fold increase compared with normal retina) (Fig. 2 G and J). Concurrently, TeNT caused the number of AMG⁺ cells in the GCL to decline by ~two-thirds relative to the number seen in PBS-injected controls (Fig. 2 G and H). Deletion of slc30a3 suppressed the effects of TeNT on Zn²⁺ accumulation in the IPL, confirming that Zn²⁺ accumulation reflects presynaptic vesicular Zn²⁺ (Fig. 2 G and I). Even in the absence of NC, TeNT caused an 11.4 ± 0.8-fold increase in Zn²⁺ accumulation in the IPL compared with the normal retina (P < 0.0001). This finding suggests that vesicular release of Zn²⁺ may occur in the normal IPL.

Cellular Localization of ZnT-3 and Zn⁺. The stratified, punctate distribution of ZnT-3 and Zn⁺ in the IPL resembles the laminar distribution of synapses that arise from particular classes of interneurons (70), raising the question of cell-type specificity. Double-immunostaining and confocal microscopy revealed a strong overlap between ZnT-3 and two markers for amacrine cell synapses, the vesicular GABA transporter VGAT (85 ± 2.3% overlap, mean ± SEM) and glutamic acid decarboxylase (GAD)-65/67 (73.3 ± 4.0% overlap) (Fig. 3 A and D and Fig. S3 E and F). Conversely, ZnT-3 showed only a small overlap with two markers for bipolar cell synapses, vesicular glutamate transporter-1 (VGLUT1, 7.1% overlap, Bonferroni-corrected P < 0.0001) (Fig. 3 B and D and Fig. S3 G and H). ZnT-3 is also reported to be expressed in Müller cells (71, 72), and we therefore carried out colocalization studies for ZnT-3 and the Müller cell marker, cellular retinaldehyde binding protein (CRALBP). The overlap was found to be 2.8% (Fig. 3 C and D and Fig. S3 G). We were unable to localize the Zn⁺ signal itself by double-labeling because of the incompatibility of

![Image](https://i.imgur.com/38x612.png)

**Fig. 2.** Zn⁺ accumulation in the IPL and subsequent transfer to cells of the GCL: role of ZnT-3. (A) ZnT-3 immunostaining in retinas of slc30a3/+ and slc30a3−/− littermates. (Scale bar, 25 μm.) (B) Quantitation of ZnT-3 immunostaining in the IPL before and after NC in wild-type mice with and without TPEN treatment (normalized to normal control; n = 10, 8, 6, 6). One-way ANOVA, **P < 0.01, ***P < 0.001 compared with uncrushed controls; ††P < 0.001 compared with 1d pNC. (C) Quantitation of ZnT-3 expression in IPL of slc30a3−/− retinas and slc30a3+/+ littermates (normalized; n = 10, 8, 6 retinas per group). Unpaired t test, **P < 0.001 compared with slc30a3−/− littermate controls. (D) Images (D) and quantitation of AMG staining in IPL (D, n = 6 retinas per group) and GCL (F, cells per 14 μm section; n = 6 retinas per group) of slc30a3−/− and slc30a3+/+ littermates. Unpaired t test. **P < 0.01, ***P < 0.001 compared with slc30a3+/+ littermate controls. (Scale bar, 25 μm.) (G)−(I) TeNT blocks vesicular release of Zn⁺⁺, causing continued Zn⁺⁺ build-up in the IPL and diminished accumulation in cells of the GCL. (G) Images show AMG staining 3 d after intraocular injection of TeNT (20 nM). Note reduced number of Zn⁺⁺-positive cells in the GCL. Deletion of slc30a3, the gene encoding ZnT-3, eliminates Zn⁺⁺ accumulation in IPL. (Scale bar, 50 μm.) (H) and (I) Quantitation of Zn⁺⁺-positive cells in the GCL (H, cells per 14 μm section and intensity in the IPL (I; normalized). n = 6, 7 in H and n = 12, 4, 6, 7, 6 in I. Unpaired t test, **P < 0.001, comparison between indicated groups. All data represent mean ± SEM.
immunostaining with either AMG (which forms an electron-dense reaction product and requires glutaraldehyde fixation, thereby suppressing antigenicity and causing high autofluorescence) or with ZP-1, because of a rapid loss of the fluorescent signal during the course of immunostaining. We therefore used adenov-associated viruses (AAVs) that preferentially infect different cell types to express the fluorescent protein mCherry primarily in either RGCs (AAV2) or interneurons (AAV6) (73, 74). As expected from the double-immunostaining results for ZnT-3, at 24 h after NC the ZP-1 signal partially overlapped with mCherry encoded by AAV6 (Fig. S4A), which in turn overlapped with the amacrine cell markers VGAT (Fig. S4B) and GAD-65/67 (Fig. S4C). The ZP-1 signal at 24 h after NC did not overlap with the bipolar cell markers VGLUT1 or PKCs (Fig. S4 E and F) nor with βIII-tubulin, which in the retina is only expressed in RGCs (75) (Fig. S4D). At 3 d, however, the ZP-1 signal overlapped with mCherry expressed by AAV2 (Fig. S4G), which in turn colocalized with the RGC marker βIII-tubulin (Fig. S4F). These findings indicate that Zn²⁺ first accumulates primarily in ZnT-3-containing vesicles in amacrine cells and subsequently appears within RGCs. AMG staining revealed the presence of some Zn²⁺ within the optic nerve, but this signal was only mildly altered by NC and was unaffected by intraocular injection of chelators (Figs. S5 A and B). Mice lacking ZnT-3 had somewhat lower Zn²⁺ levels in the optic nerve than slc30a3-/- littersmates (Fig. S5 C and D).

Elimination of Vesicular Zn²⁺ Promotes RGC Survival and Axon Regeneration. To determine the consequences of Zn²⁺ elevation in amacrine cell processes, we examined whether elimination of ZnT-3 would alter RGC survival and axon regeneration after NC. Mice lacking slc30a3 showed twice the number of RGCs surviving 2 wk after NC as slc30a3+/+ littersmates (unpaired t test, P < 0.0001) (Fig. 4 A and B) and nearly 10 times the level of axon regeneration (unpaired t test, P < 0.0001) (Fig. 4 C and D). It is important to note that, because slc30a3 deletion does not fully eliminate Zn²⁺ accumulation in RGCs (Fig. 2F), the effects of gene deletion on cell survival and axon regeneration may not fully represent the effects of removing Zn²⁺ from the system. Two weeks after NC, the survival of RGCs in wild-type slc30a3+/+ littersmates of slc30a3-/- mice (23.8%) was somewhat greater than that of C57 mice (16.3%) (Fig. 5G). The likely basis for this difference is that the slc30a3-/- mice were generated on a mixed 129S/C57 background, and pure 129S mice have a 2-wk survival rate of ~26.1% (Fig. S6D). Finally, as an additional test of whether vesicular Zn²⁺ is the pool responsible for RGC death, we investigated RGC survival 2 wk after intraocular injection of TeNT. TeNT increased RGC survival more than twofold (39.1%) (Fig. S6 G and H), similar to the effect seen after slc30a3 deletion (Fig. 4B).

Intraocular Zn²⁺ Chelation Attenuates RGC Death. To investigate whether the effects of suppressing Zn²⁺ elevation in the retina can be achieved pharmacologically, we examined whether Zn²⁺ chelators could affect RGC survival when administered after NC. Intraocular injection of TPEN or ZX1 (100 μM) immediately after NC and 4 d later nearly doubled overall RGC survival compared with controls receiving PBS injections (both Bonferroni-corrected P < 0.0001) (Fig. 5 A–D and G; dose–response data in Fig. S6 A and B). The effects of the chelators on cell survival were independent of the distance of RGCs from the center of the retina (Fig. S6C). The survival effects of the chelators were suppressed when saturated with equimolar Zn²⁺ (Bonferroni-corrected P = 0.011) (Fig. 5 E–G). Because Zn²⁺ increases so rapidly after NC, we tested whether having chelators present at the time of NC would have an even stronger benefit. However, an additional injection of either chelator 1 d before NC did not increase RGC survival above the level achieved by postinjury treatment (Fig. 5G and Fig. S6E). We also investigated whether Zn²⁺ elevation lies upstream of other changes known to occur in RGCs after NC. TPEN and ZX1 suppressed the injury-induced elevation of active caspase-3 and the loss of Bcl-xL that occur after NC (76, 77) (all Bonferroni-corrected P < 0.01) (Fig. 5 I and J and Fig. S6 I and J). TPEN also suppressed the elevation of CCAAT-enhancer binding protein homologous protein (CHOP) (4) (Bonferroni-corrected P = 0.0029) (Fig. S7), whereas ZX1 had a somewhat lesser effect, although in the same direction (not significant). Combining slc30a3 deletion with TPEN did not augment 2-wk survival beyond the level achieved with either one alone (Fig. 5H) (Bonferroni-corrected P > 0.999).

Combinatorial Therapy and Enduring RGC Survival. In the absence of any interventions, RGCs continue to die after NC and only ~2%
remain alive after 12 wk (two-way ANOVA, Bonferroni-corrected \( P = 0.0006 \)) (Fig. 6). In marked contrast, the effects of Zn\(^{2+}\) chelation on RGC survival endured, with the number of viable RGCs remaining nearly constant for 12 wk (two-way ANOVA, Bonferroni-corrected \( P > 0.99 \), comparing survival at 12 wk vs. 2 wk). Even more striking effects were seen when TPEN was combined with deletion of phosphatase and tensin homolog (\( pten \)), a suppressor of the PI3 kinase-Akt pathway. Although \( pten \) deletion has been shown to promote RGC survival and axon regeneration after optic nerve injury (13) (Fig. 6), this effect declined sharply over time, with only \( \sim 12\% \) of RGCs remaining alive at 12 wk (two-way ANOVA, Bonferroni-corrected \( P > 0.99 \), survival at 12 wk vs. 2 wk) (Fig. 6). Combining TPEN and deletion of \( pten \) (via AAV2-Cre-induced \( pten \) deletion in \( pten^{flx/flx} \) mice) stabilized the high level of neuroprotection afforded by \( pten \) deletion, enabling nearly half of all RGCs to survive to at least 3 mo after NC (two-way ANOVA, Bonferroni-corrected \( P = 0.92 \) comparing survival at 12 wk vs. 2 wk) (Fig. 6). Thus, early blockade of Zn\(^{2+}\) elevation provides long-term protection for many RGCs and stabilizes the high but otherwise transient neuroprotective effect of \( pten \) deletion. \( slc30a3 \) deletion also maintained high levels of RGC survival over a period of months, although to a somewhat lesser extent than TPEN (Fig. S6L and M).

**Zn\(^{2+}\) Chelation Promotes Axon Regeneration.** We next investigated whether Zn\(^{2+}\) influences RGCs’ ability to regenerate axons. For this, we used GAP-43 immunostaining to quantify the number of axons extending selected distances beyond the injury site 2 wk after NC in the presence or absence of chelators (78). Whereas untreated controls showed only a handful of axons beyond the injury site 2 wk after NC and PBS injection (7.0 ± 2.5; mean ± SEM) (Fig. 7A), mice receiving intraocular TPEN or ZX1 (100 \( \mu M \)) immediately after NC and 4 d later showed a 25-fold increase in regeneration (180.7 ± 30.2 axons for TPEN, 164.4 ± 31.3 axons for ZX1; Bonferroni-corrected \( P_{\text{TPEN}} = 0.0002 \), \( P_{\text{ZX1}} = 0.0029 \)) (Fig. 7B and Fig. S8 A–C). To verify that the observed differences in axons counted in the nerve were not a result of changes in GAP-43 expression or to a differential survival of axons after injury, we generated a separate cohort of mice to compare the numbers of axons distal to the injury site by GAP-43 immunostaining vs. anterograde tracing with cholera toxin B fragment (CTB, injected intraocularly 4 d before tissue harvest). The two methods gave nearly identical results (Fig. S9C) when analyzed by either anatomical colocalization (Fig. S9 A and B) or by quantitation of each label separately (Fig. S9D). The effects of TPEN and ZX1 were nearly eliminated by presaturating chelators with equimolar Zn\(^{2+}\) (Bonferroni-corrected \( P_{\text{TPEN}} = 0.0033 \), \( P_{\text{ZX1}} = 0.013 \)) (Fig. 7B and Fig. S8 A–C), demonstrating the specificity of these effects to Zn\(^{2+}\). As with cell survival, we also examined the effect of introducing chelators before the time of NC. Whereas pretreatment did not augment RGC survival, introducing an additional injection of TPEN or ZX1 before nerve injury doubled the amount of regeneration compared with that seen when chelators were only introduced afterward (Bonferroni-corrected \( P_{\text{TPEN}} = 0.0003 \), \( P_{\text{ZX1}} = 0.0013 \)) (Fig. 7D) and Fig. S8 B and C). Combining Zn\(^{2+}\)-3 deletion with TPEN-mediated Zn\(^{2+}\) chelation immediately after NC and 4-d later resulted in a level of regeneration similar to that of either treatment alone (Bonferroni-corrected \( P > 0.99 \)) (Fig. 7E; compare with Fig. 7B).

**Combinatorial Therapy.** The level of regeneration seen after introducing chelators before and after NC is comparable to that obtained by preinjury deletion of \( pten \) or by intraocular injections of Zymosan with a cAMP analog, two of the strongest treatments reported to date (13, 19). As noted above, \( pten \) deletion in RGCs was achieved by intraocular injection of an AAV-expressing Cre recombinase (AAV2-Cre) in \( pten^{flx/flx} \) mice 2 wk before NC. Combining Zn\(^{2+}\) chelation with \( pten \) deletion had a much greater effect than either treatment alone, and enabled some axons to extend to the far end of the optic nerve in just 2 wk (all Bonferroni-corrected \( P < 0.05 \)) (Fig. 7C and D, and Fig. S8D). By 12 wk, the number of axons growing the full length of the nerve continued to increase and many crossed the optic chiasm (all Bonferroni-corrected \( P < 0.05 \)) (Fig. 7 F–H). TPEN did not, however, augment regeneration induced by Zymosan/CPT-cAMP (Fig. 7D).

To examine the effects of Zn\(^{2+}\) chelation on gene transcription, we used a panel of probes similar to those recently used to investigate the role of dual-leucine kinase (DLK) following NC (2). TPEN increased the expression of multiple genes associated with axon regeneration, including gap43, sprouty1, fn14, atf3, and Klf6 (all Bonferroni-corrected \( P < 0.05 \)) (Fig. S7). ZX1 had
qualitatively similar effects, although in some cases these did not achieve statistical significance (Fig. S7).

**Therapeutic Window.** Because treatment in a clinical setting might be delayed for hours or days, we investigated the efficacy of beginning Zn\(^{2+}\) chelation well after injury had occurred. TPEN had equally strong effects on RGC survival whether administered immediately after injury or 1 d later (Fig. 8A) (both Bonferroni-corrected \(P < 0.0001\) compared with untreated control) and was only slightly less effective when applied after a 5-d delay (Fig. 8A) (Bonferroni-corrected \(P < 0.0001\) compared with untreated control, \(P = 0.2762\) compared with D0). Our standard treatment regimen, injecting TPEN immediately after injury and again 4 d later, was more effective than a single early injection (Fig. 8A) (unpaired \(t\) test, \(P = 0.02\) compared with D0 only), and the addition of a third injection on day 7 increased RGC survival to nearly 40% (Fig. 8B and Fig. S6F) (unpaired \(t\) test, \(P = 0.0052\) compared with two treatments at D0 and D4).

The benefit of Zn\(^{2+}\) chelation on axon regeneration followed a different pattern from that seen for cell survival. A single injection of TPEN (100 \(\mu\)M) stimulated regeneration even if delayed for 5 d (Fig. 8C) (all Bonferroni-corrected \(P < 0.05\)). However, treatment at 5 d resulted in approximately half the level of regeneration observed when TPEN was introduced immediately after nerve injury. The standard treatment regimen used in our study, one injection shortly after injury and a second one 4-d later, was about twice as effective as a single early postinjury injection (unpaired \(t\) test, \(P = 0.0033\)) (Fig. 8C and D), although the inclusion of a third injection on day 7 had no further benefit. The relative benefits of early treatment are consistent with the dramatic regeneration seen when chelators were present before optic nerve injury (Fig. 7B).

Taken together, these results indicate that the Zn\(^{2+}\)-dependent processes that suppress RGC survival and axon regeneration persist for several days after optic nerve injury, affording an appreciable therapeutic window, and that repeated treatments further augment survival and regeneration.

**Discussion**

Recovery after CNS injury is restricted in part by the inability of mature neurons to regenerate axons over long distances, the limited capacity of neurons to form compensatory circuits, and in
the case of optic nerve damage, the death of RGCs. Although RGC death and regenerative failure have been widely attributed to cell-intrinsic processes, the glial environment, and an insufficiency of trophic agents, addressing these factors has thus far resulted in transient RGC survival, limited regeneration, or persistent RGC survival in a compromised state (79–82). Our results show that both RGC death and regenerative failure are caused in part by Zn2+ dyshomeostasis, and that chelating Zn2+ enables many injured RGCs to survive for months and regenerate axons, with a therapeutic window that persists for several days after injury.

The vast majority (~90%) of total zinc in neurons is tightly bound in metalloenzymes, transcription factors, and other zinc-containing proteins, in which zinc serves as a cofactor for enzymatic activity or maintaining the three-dimensional structure of proteins (83, 84). The remaining 10% of total zinc is referred to as chelatable, free, or mobile zinc, and is present as hydrated ions or is loosely bound to protein. These chelatable Zn2+ ions, which in neurons are localized primarily in synaptic vesicles of so-called zinc-enriched neurons, can be detected by either fluorescence or AMG techniques. Although AMG detects copper (85) and iron (86), as well as zinc (62, 87, 88), ZP1 is highly selective for Zn2+ and its intrinsic fluorescence is quenched by copper, iron, manganese, and cobalt ions (38, 66, 89). Thus, the fact that we observed similar changes in Zn2+ with AMG and ZP1 reinforces the conclusion that elevation of Zn2+ itself is occurring after NC. However, the question remains as to whether the functional effects of TPEN and ZnT1 are a result of chelating Zn2+ rather than another divalent cation. Ca2+ can be ruled out because it does not interact with ZX1 (38, 66), which has the same effects as TPEN on survival and regeneration following optic nerve injury. The observation that the functional effects of TPEN are abrogated when it is presaturated with Zn2+ argues against involvement of biologically relevant divalent cations that bind the chelators with a higher affinity than Zn2+ (Kd = 2.6 × 10⁻⁷ M), including Cu2+ (Kd = 3 × 10⁻⁰ M), although not, strictly speaking, of cations that bind TPEN with a lower affinity than Zn2+ (e.g., Fe2+, Kd = 2.4 × 10⁻³ M; Mn2+, Kd = 5.4 × 10⁻¹ M; Ca2+, Kd = 4 × 10⁻⁵ M; Mg2+, Kd = 2 × 10⁻² M) (90–92). However, several other lines of evidence point to the relevant ion being Zn2+. First, the effects of the chelators in promoting RGC survival and axon regeneration closely match their ability to remove Zn2+ per se, as visualized by the staining. Second, deletion of the Zn2+ transporter ZnT3 (68, 93, 94) similarly renders Zn2+ undetectable in the IPL (Fig. 2 D and E) and has the same functional effects as the chelators (Fig. 4). Similarly, blocking vesicular release with TeNT suppresses the transfer of Zn2+ to RGCs and attenuates RGC death.

The IPL, the initial site of Zn2+ accumulation, contains distinct sublayers in which diverse types of interneurons synapse onto the dendrites of particular RGC subtypes or other interneurons in a stereotyped manner (70). RGCs receive excitatory inputs from bipolar cells that use glutamate as a transmitter and inhibitory inputs from amacrine cells that use either glycine or GABA as a transmitter. Many interneurons also contain peptides or monoamine neurotransmitters (95, 96). Like Zn2+ localization in other parts of the CNS (93), Zn2+ accumulation in the IPL was found to require ZnT3, as evidenced by the loss of detectable Zn2+ when slc30a3 was deleted. Reciprocally, increased ZnT3 expression required elevation of free Zn2+, implying that ZnT3 expression depends on Zn2+ concentration and that Zn2+ sequestration in turn relies on ZnT3 levels. The loss of detectable Zn2+ after NC when ZnT3 was deleted is consistent with prior work showing that AMG preferentially detects Zn2+ that is present at high concentrations within vesicles (93). However, the absence of an AMG signal does not imply that Zn2+ is not being liberated by upstream signals (see below) or that it might not still be present in other cellular compartments at lower concentrations. It is also possible that the failure to detect Zn2+ in the absence of ZnT3 is because vesicular sequestration acts as a sink for Zn2+ (97), and other transporters and homeostatic mechanisms prevent the accumulation of high levels of cytoplasmic Zn2+. Within the first day after NC, Zn2+ and ZnT3 were localized primarily in amacrine cell processes, whereas at 3 d, Zn2+ was found mostly in RGCs. Elsewhere in the CNS, although most Zn2+-containing synapses are glutamatergic (47, 97), prior studies have reported the presence of Zn2+ in amacrine cells (42) and in inhibitory synapses of the brainstem (98).

Along with suppressing vesicular Zn2+ accumulation in the IPL, deletion of slc30a3 suppressed the subsequent signal in RGCs, as did the chelators. These observations, together with our finding that TeNT causes Zn2+ to continue building up in the IPL while decreasing Zn2+ accumulation in RGCs, point to the transfer of Zn2+ from synaptic vesicles in amacrine cell processes as the primary source of Zn2+ elevation in RGCs. Importantly, the functional consequences of slc30a3 deletion and Zn2+ chelation were non-additive, implying that the negative effects of Zn2+ on both RGC survival and axon regeneration are associated primarily with its intra- and extracellular sequestration in amacrine cells. It is important to note, however, that even with slc30a3 deletion, Zn2+ chelation, or intraocular TeNT, many RGCs go on to die within the first 2 wk. This result may be a consequence of the continued low-level accumulation of Zn2+ in RGCs that we observed after early chelator application (Fig. 5 and Fig. S1 F) and even after preventing vesicular sequestration by slc30a3 deletion (Figs. 2 F and 4) or after blocking vesicular release (with TeNT) (Fig. 2 G–I and Fig. S6 G and H). Continued low-level release of zinc has also been demonstrated after ZnT3 deletion in the auditory brainstem (40), suggesting the presence of nonvesicular Zn2+ release. In addition, it is possible that RGCs possess both Zn2+-dependent and Zn2+-independent pathways for cell death, or that some Zn2+ accumulation may occur in RGCs via cell-autonomous mechanisms (58, 59). Finally, because Zn2+ has beneficial effects, such as modulating synaptic transmission and BDNF synthesis, removing Zn2+ could potentially have mixed positive and negative effects on RGCs (38, 40, 99, 100).

The rapid elevation of Zn2+ in the IPL following optic nerve injury is concurrent with other early changes that occur in this system: that is, Ca2+ elevation and activation of MAP kinase cascades that include DLK, c-jun N-terminal kinases (Jnk2 and -3), ASK-1 and P38, and c-Jun phosphorylation (2, 3, 7, 9, 10, 101). Blocking these latter changes results in substantial cell survival that diminishes over time and modest or no regeneration (2, 3, 7, 9, 10). Subsequent changes include down-regulation of IGF1 and phospho-Akt, increases in reactive oxygen species, the unfolded protein response and endoplasmic reticulum stress,
argue for chelation as a therapeutic strategy. Moreover, even if Zn\(^{2+}\) chelation alone is insufficient to restore central connections, long-term preservation of RGCs could have considerable value in enabling a prosthetic device to read out the activity of RGCs transduced to express a fluorescent reporter of activity and represent the code the eye sends to the brain more accurately than reading out the activity of photoreceptors (105). Zn\(^{2+}\) chelators are already in clinical use for other purposes (106), and it will be important to determine whether these agents can augment axon regeneration after other types of CNS injury or protect RGCs in neurodegenerative diseases such as glaucoma.

### Methods

#### Animal Use, Surgeries, and Intracocular Injections

Animal studies were performed at Boston Children’s Hospital with approval of the Institutional Animal Care and Use Committee. Housing conditions, strains of mice used, and surgical procedures are described in SI Methods. Animals were assigned to different treatment groups and, in any given experiment, surgeries were done for several groups at a time. Subsequent processing was performed blinded to treatment. Reagents that were injected intracocularly included the specific Zn\(^{2+}\) chelators TPEN [20–500 μM; Calbiochem; K\(_{f}\) for Zn\(^{2+}\) = 2.6 × 10\(^{-10}\) M, K\(_{f}\) for Ca\(^{2+}\) = 4 × 10\(^{-10}\) M (92)] and ZN1 [10–1,000 μM, K\(_{f}\) for Zn\(^{2+}\) = 1.0 nM; no detectable interaction with Ca\(^{2+}\) (38); Zymosan (12.5 μg/mL; Sigma, sterilized); 8-(4-chlorophenylthio) (CPT)-cAMP (50 μM; Sigma), a membrane permeable, nonhydrolyzable cAMP analog, and TeNT (20 nM; List Biological Laboratories), which cleaves the synaptic vesicle protein synaptobrevin and prevents transmitter release (107). In most cases, the Zn\(^{2+}\) chelators TPEN or ZN1 were injected right after NC and 4 d later. In the time-course study, however, we administered single injections at 0, 1, 3, or 5 d after NC. In some groups, to chelate the earliest wave of Zn\(^{2+}\) elevation, an additional injection of either chelator was given 1 d before NC (pre-treatment). Vehicle-treated controls received PBS alone. To delete the pten gene in RGCs, AAV2-Cre (5 × 10\(^{12}\) GCU/mL; Vector Laboratories) was injected intracocularly (3 μL in pten\(^{flx/flx}\) mice, 6–8 wk of age, avoiding injury to the lens. In some studies, intracocularly injected AAV2 expressing an mCherry reporter (AAV2-mCherry, 2 × 10\(^{12}\) GCU/mL; gift from Luk Vandenberghe, Massachusetts Eye and Ear Infirmary and Harvard Medical School, Boston, MA) was used to label RGCs, whereas AAV6-mCherry (5 × 10\(^{12}\) GCU/mL; Virovec) was used to label retinal interneurons and their processes in the IPL. Viruses (3 μL per eye) were injected 2 wk before optic nerve surgery to allow for sufficient labeling at the time of NC.

#### Zinc\(^{2+}\) AMG and Quantitation

The selectivity of AMG for mobile Zn\(^{2+}\) is described in Discussion and SI Methods. Using a modification of a published AMG procedure to visualize mobile Zn\(^{2+}\) (60), mice were injected intraperitoneally with sodium selenite (Na\(_{2}\)SeO\(_{3}\), 1.5 mg/mL in distilled H\(_{2}\)O; Sigma, 15 mg/kg) and were kept alive for 4 h to allow for optimal zinc-selenite precipitation in vivo, measured empirically. Our pilot studies showed that we needed to wait at least 2 h after surgery for animals to recover before administering Na\(_{2}\)SeO\(_{3}\), and that we obtained optimal staining in the retina ∼4 h after Na\(_{2}\)SeO\(_{3}\) injections. Thus, the minimum survival time after surgery that we could examine with this technique was 6 h. An overdose of anesthesia was then given followed by transcardial perfusion with isotonic saline (Sigma) and 2.5% (vol/vol) glutaraldehyde (GA; Ted Pella) in 0.1 M phosphate buffer (PB; pH 7.4). Details of tissue processing are given in SI Methods. To verify the specificity of the AMG signal for zinc, TPEN or ZN1 was injected intraocularly (100 μM, 3 μL per eye) following NC, or PBS was injected as a vehicle control. Five images from different areas of each retinal section were captured under bright-field illumination (600x; E800; Nikon). The intensity of the Zn\(^{2+}\)-AMG signal in the IPL was analyzed using ImageJ software. Zn\(^{2+}\)-positive cell numbers in the GCL of each retinal section were determined under light microscopy. AMG staining and imaging were done simultaneously for all samples to be compared with each other. To obtain a representative sampling, we analyzed five areas in each of three to five sections per case with −150-μm intervals between sections. Average signal intensity as well as Zn\(^{2+}\)-positive cell numbers were calculated from 6 to 14 individual cases per condition. All images are representative of multiple samples. The small SEMs in Zn\(^{2+}\) intensity and cell numbers throughout the study point to a relatively small sampling error.

#### Zinc\(^{2+}\) Fluorescence in Retina and Quantitation of Intensity

Zn\(^{2+}\)\(100\) (108) is a highly selective, membrane-permeable, fluorescent probe for Zn\(^{2+}\) (K\(_{f}\) for Zn\(^{2+}\) = 0.7 ± 0.1 nM with no detectable interaction with Ca\(^{2+}\) (32)). ZP-1 gave a strong, reproducible signal when injected intraocularly and had no apparent
systemic effects. We determined empirically that optimal results were obtained after a 24-h labeling period. Thus, in all cases we injected ZP-1 intraocularly (500 μg, 3 μl per eye) 24 h before killing (e.g., 18 h before surgery to examine Zn²⁺ accumulation 6 h after surgery). Details of tissue processing are in SI Methods. Fluorescent images were captured and relative ZP-1 intensity in the IPL of the retina was analyzed using ImageJ software. Average intensities were calculated from five to seven individual cases per condition and images are representative of these results.

Colocalization with Synaptic Markers. Standard histological procedures and antibody information are described in SI Methods. All tissues to be compared were stained at the same time and all settings were kept constant for imaging. Colocalization analysis was done by ImageJ software using 10 cases from each group. Mander’s value (TM) and Pearson’s r value (r) were used to represent the extent of colocalization. All images are representative of signals obtained from multiple cases across two or three independent experiments.

Quantitation of RGC Survival and Axon Regeneration. Details are described in SI Methods.

Quantitative Reverse-Transcription PCR. To investigate molecular events associated with Zn²⁺ elevation, we examined changes in the expression of multigene families by quantitative PCR (qPCR). Gene names and sequences of primers used for qPCR are shown in Fig. S7A. Methods are described in SI Methods.

Immunostaining and Quantitation of Cell Death Markers. We visualized changes in the expression of representative pro- and antiapoptotic cell death markers with and without Zn²⁺ chelation 5 d after NC. Further details are provided in SI Methods.

Statistical Analyses. All tissue processing, quantification, and data analysis were done blindly throughout the study. Sample sizes were based on accepted standards in the literature and prior experience from our laboratory. Sample size (n) represents total number of biological replicates in each condition across all experiments. Statistical experiments performed (n ≥ 2) experiments. All experiments contained positive and negative controls and multiple experimental conditions. After establishing the methodology for any type of study, no cases were excluded in our data analysis. Parametric tests (ANOVA with Bonferroni post hoc tests or unpaired Student’s t tests, two-tailed) were used after determining normality of distribution of data (SPSS v18.0 and Graphpad v6.0). Data are presented as Means ± SEM. All differences were considered significant with P < 0.05.

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