Published in final edited form as:

J Neurochem. 2014 April ; 129(2): 249–255. doi:10.1111/jnc.12627.

Cytoprotection by Endogenous Zinc in the Vertebrate Retina

Ivan Anastassov^{a,b,1}, Harris Ripps^{b,c}, and Richard L. Chappell^{a,b,d,*}

Ivan Anastassov: ivan.anastassov@bcm.edu; Harris Ripps: harrripp@uic.edu

^aDepartment of Biological Sciences, Hunter College and the Graduate Center, CUNY, 695 Park Avenue, New York, NY 10065, USA

^bMarine Biological Laboratory, 7 MBL Street, Woods Hole, MA 02543, USA

^cDepartments of Ophthalmology and Visual Sciences, Anatomy and Cell Biology, and Physiology and Biophysics, University of Illinois College of Medicine, Chicago, IL 60612, USA

^dCenter for Vision Research, Brown Institute for Brain Science, Box 1953, Providence, RI 02912, USA

Abstract

Our recent studies have shown that endogenous zinc, co-released with glutamate from the synaptic terminals of vertebrate retinal photoreceptors, provides a feedback mechanism that reduces calcium entry and the concomitant vesicular release of glutamate. We hypothesized that zinc feedback may serve to protect the retina from glutamate excitotoxicity, and conducted *in vivo* experiments on the retina of the skate (*Raja erinacea*) to determine the effects of removing endogenous zinc by chelation. These studies showed that removal of zinc by injecting the zinc chelator histidine results in inner retinal damage similar to that induced by the glutamate receptor agonist kainic acid. In contrast, when an equimolar quantity of zinc followed the injection of histidine, the retinal cells were unaffected. Our results are a good indication that zinc, co-released with glutamate by photoreceptors, provides an auto-feedback system that plays an important cytoprotective role in the retina.

Keywords

ionic zinc; chelation; glutamate excitotoxicity; retinal histology; cytoprotection

INTRODUCTION

A unique feature of the vertebrate photoreceptor is that, in darkness, a sustained inward cation current holds the cell in a depolarized state. Consequently, the photoreceptor continually releases its neurotransmitter, glutamate, into the synaptic cleft. It has long been known that excessive levels of glutamate are toxic to retinal neurons (Reif-Lehrer et al., 1975), and as shown by Rothman (1984), over-activation of glutamate receptors results in the death of neurons in culture. These findings were extended to the intact isolated retina and other nerve centers in the CNS by Olney and co-workers (cf. Olney 1982, 1994; Romano et al., 1998). In a related series of experiments we attempted to determine whether free zinc (Zn^{2+}), packaged within the synaptic vesicles of the photoreceptor terminal and co-

*Corresponding author: Dr. Richard L. Chappell, Marine Biological Laboratory, 7 MBL Street Woods Hole, MA 02543 USA, rchappell@mbi.edu.

¹Present address: Department of Biochemistry and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

The authors have no disclosures or conflicts to be reported.

released with glutamate (Redenti & Chappell, 2004, 2005; Redenti et al., 2007; Chappell et al., 2008), can serve a neuromodulatory role. This notion was based on results by Wu and co-workers (Wu et al., 1993), showing decreased calcium entry into photoreceptor terminals when *exogenous* zinc was applied to the salamander retina preparation. We have since confirmed these results, and in addition have shown that using chelators to remove *endogenous* zinc leads to a marked increase in both calcium entry (Anastassov et al., 2013) and in the photoreceptor's dark current (Chappell et al., 2008). These findings indicate that a reduced zinc concentration results in a concomitant increase in the discharge of glutamate, and led us to suggest that if endogenous zinc can suppress transmitter release, it may serve to protect the retina from the toxic effects of glutamate. In addition, we wished to determine how cell death evolves in the intact retina when challenged with kainate (cf. Olney et al., 1974) an analogue of glutamate and a potent activator of a subset of highly sensitive ionotropic glutamate receptors even at extremely low concentrations (Shen et al., 2004). To examine these issues, it was important to perform *in vivo* experiments on dark-adapted retinas in which kainate is introduced into the vitreal chamber adjacent to the retina, and also to study the pathological changes that occur when zinc is chelated and glutamate is continuously released.

MATERIALS AND METHODS

All surgical and animal handling procedures were conducted in accordance with ARRIVE guidelines.

Injections

Adult skates (*Raja erinacea*), members of the family of cartilaginous elasmobranchs that includes sharks and rays, were used in these experiments. Prior to injections, the fish were anesthetized with 0.02% tricaine-methane-sulfonate (Argent Chemical, Redmond, WA), and a local anesthetic (2% lidocaine) was applied to the cornea. Injections into the vitreal space were performed using a 33 gauge (6.35mm) Hamilton micro-syringe. Drugs were dissolved in skate Ringer solution (in mM): NaCl (250), KCl (6), CaCl₂ (4), Urea (360), D-glucose (10), NaHCO₃ (20), MgCl₂ (4), NaH₂PO₄ (0.2), HEPES (5), pH 7.6; and injected in one (test) eye. The contralateral eye was either not injected or received vehicle only. Injections were made posterior to the lens, along the horizontal midline of the eye and approximately over the tapetal area of the retina. The total volume (control or test) per injection was 10 μ L. In order to maximize vesicular release from photoreceptors, fish were kept in the dark for the entire experiment. In short: pre-injection, the animals were dark-adapted for 8–12hrs; following adaptation, an injection was performed under dim red illumination every 2hrs (total of 5 injections over 10hrs); finally, the animals were left to recover in darkness for another 12 or 24hrs post-injection. Tissue was collected after the recovery stage. Drugs and chemicals were purchased from Sigma-Aldrich, St. Louis, MO (kainate, histidine) and VWR, Radnor, PA (Ringer components).

Detection of Necrosis

Eyes were enucleated under dim red light, the cornea and lens removed, and the vitreous drained with cotton wicks. To visualize necrosis, control and drug-treated eyes from the same animal were simultaneously incubated with the nucleic acid probe ethidium homodimer III (EthD-III), which is impermeant to healthy cells with uncompromised membranes, but stains cells whose membranes have lost their integrity. To minimize the chance of false positives resulting from significant cell death due to tissue removal, the EthD-III was applied to the eye cup immediately after enucleation and the incubation was done on ice. Control and treatment eyes from the same animal were removed and incubated with the probe simultaneously. The EthD-III was dissolved in proprietary Binding Buffer, as

per the manufacturer's instructions (PromoCell, Heidelberg, Germany). After staining, the eye cup was fixed with 2% paraformaldehyde (PFA) in the dark, on ice for 1hr. Eye cups were cryoprotected by incubation in 30% sucrose in PBS at 4°C overnight, and protected from light to avoid bleaching of the fluorescent signal. Cryoprotected tissue was cut into pieces, embedded in O.C.T. (Tissue Tek, Torrence, CA) and flash frozen. Blocks of frozen tissue were stored at -80°C and sectioned at -25°C on a Leica CM 1850 cryostat in 14–18µm sections. To prevent photobleaching of the necrosis signal, we used Vectashield mounting media (Vector Labs, Burlingame, CA) that already contains DAPI at a concentration of 1.5µg/ml. As per manufacturer's instructions, 1 drop of ~25µl from the provided dispenser was used to cover each section on every slide. Slides were examined with LSM 710 and LSM 780 Zeiss confocal microscopes. Images were collected and analyzed using Zeiss ZEN imaging software.

Data analysis and statistical methods

The ratio of EthD-II and DAPI fluorescence intensity was computed from raw images of retinal sections. A transect line was drawn across each retinal layer and the fluorescence ratio for each pixel on the line was computed. The highest and lowest 5% of values were excluded because these were likely to represent 0 in either numerator or denominator, indicating non-specific or debris staining not associated with cell bodies. 2-factor ANOVA was conducted with Prism 6.0 to compare ratios between treatments and across layers. Post-hoc comparisons were made with paired, Bonferroni-corrected t-tests for comparisons across layers within treatment groups, and unpaired, corrected t-tests for comparisons of the same layers in different treatment groups. All p values reported are 2-tailed.

Histology

Eyes were enucleated under dim red light, the cornea and lens removed and the vitreous drained. For histological observation, the retina was left attached to the choroid and cartilaginous sclera to protect it from damage and aid in subsequent sectioning. The resulting eyecup was immediately fixed with 2% PFA/2% Glutaraldehyde in 0.1M Cacodylate buffer with 3% sucrose and 4.5mM CaCl₂ followed by post-fixation with 1% OsO₄. Dehydration was done with ethanol steps (30%–100%) and rotation in propylene oxide. Tissue was infiltrated with a 1:1 mixture of propylene oxide and EMBED 812 (Electron Microscopy Sciences, PA) followed by fresh EMBED 812. Polymerization was done at 60°C for 24–48hrs. Blocks of embedded tissue were trimmed by hand and subsequently sectioned (0.5–1.0µm) on a Reichart Jung Ultracut E microtome. Sections were stained with 1% Methylene Blue/1% Azure II/1% Sodium borate solution for 20sec on a gently heated hotplate. Sections were mounted and sealed and images were taken with a Zeiss AxioImager Z2 light microscope running AxioVision software.

RESULTS

In this brief report we present representative images of the results obtained from the study of eyes injected with Ringer (control), 20mM histidine, 2mM kainate, or with 10mM histidine + 10mM zinc. Eyes were prepared either for necrosis analysis (early onset cell death) or histological study (to reveal the gross cellular damage that follows).

Figure 1(A, B) shows results obtained following intraocular injections of Ringer in the eye (control) of an experimental animal. There is very limited co-staining with DAPI and EthD-III (Fig. 1A). A graphical representation of the peak DAPI (blue) and EthD-III (red) signals across a large section of the retina is shown in Fig. 1B. Note the relatively small degree of necrosis, i.e.; cells showing EthD-III signals.

In contrast, injections of 2mM kainate in the contralateral eye resulted in widespread necrosis of cells in the ganglion cell layer (GCL, Fig. 1C,D). Surprisingly, little necrosis was seen in the inner nuclear layer (INL) and outer nuclear layer (ONL). Results similar to those obtained with kainate were seen when the retina was injected with 20mM histidine. As shown in Fig. 1E,F, a significant degree of necrosis was observed after chelation of *endogenous* zinc by the histidine injection. Statistical analyses of these experimental results, illustrated by the bar graphs in Fig. 2A,B, confirm the findings that both kainate and histidine induce a significantly greater degree of necrosis (2-way ANOVA, Bonferroni posthoc test, $p < 0.0001$, $N = 9$ for Ringer/kainate, $N = 10$ for Ringer/histidine) in the GCL when compared to the Ringer injected eye. Cells in the INL and ONL showed little to no necrosis following injection of either drug.

Figure 3A shows the normal retinal structure of a Ringer-injected eye. There are no signs of damage; all nuclear and plexiform layers appear normal, and the cells are structurally intact. In contrast, Fig. 3B illustrates the abnormal cellular structure, seen throughout the inner retina, that results from exposure to 2mM kainate. Cell bodies in the INL and GCL are swollen, and processes in the inner plexiform layer (IPL) appear to have lost their protoplasmic content, giving the IPL a “sponge-like” appearance.

We have shown that the zinc released from photoreceptor terminals feeds back to reduce calcium entry, and thereby inhibits exocytosis of glutamate-containing vesicles. On this view, chelation of endogenous zinc could lead to an increased release of glutamate, and result in cytotoxic effects similar to those seen with kainate. As shown in Fig. 3C, zinc chelation by 20mM histidine (injections were done as before, i.e. 10 μ L given every 2hrs over a period of 10hrs; tissue collection after recovery period) resulted in gross morphological changes in the retina. Cells in the INL and GCL were swollen, and the IPL was severely affected. There is a significant loss of cellular material as evidenced by the sparse staining of the tissue, and the IPL has the same “sponge-like” appearance as observed with kainate. Moreover, the inner limiting membrane has been disrupted, and there is a marked loss of ganglion cells. Note that photoreceptor structure also shows signs of damage, although this was not observed in all animals.

Results illustrated in Fig. 3D show the cytoprotective effects of *exogenous* zinc, when introduced in equimolar amounts 2 to 3min following each of the 5 injections of the zinc chelator histidine (10mM each, every 2hrs). In these circumstances, no appreciable retinal damage was observed, confirming that it is the removal of *endogenous* zinc by histidine, and not histidine itself, that caused the cytotoxic effects observed in Fig. 1E and 3C.

DISCUSSION

Excitotoxicity in the CNS is a process considered to lead to neuronal cell death through the overactivation of glutamate receptors (Dong et al., 2009). It is understood that ionotropic glutamate receptors of the NMDA and non-NMDA type (AMPA and kainate) are the predominant mediators of excitotoxicity (Sattler and Tymianski, 2001), but there is some evidence to suggest that metabotropic glutamate receptors may also be involved (McDonald et al., 1993). Similarly, glutamate excitotoxicity in the vertebrate retina is a well-known phenomenon, described over the years in a number of studies (Olney, 1982, 1994; Izumi et al., 1995, 2002; Chen et al., 1998). In the retina, NMDA, AMPA and kainate have proven to be potent agents of excitotoxicity, where treatment of the isolated tissue *ex vivo* with those compounds quickly leads to damage characterized by cell swelling, pyknosis and spongy appearance of the inner plexiform layer (Olney et al., 1974; Olney, 1994).

In the experiments described here, it was necessary to modify and adapt some of the above methodologies. The goal was to chelate endogenous zinc *in vivo* and monitor the effects of zinc removal on retinas exposed to the unregulated release of glutamate from photoreceptors in darkness. To achieve this we used the skate (*Raja erinacea*), and an important consideration before starting the zinc chelation experiments was the need to confirm the viability of the skate as a model system for excitotoxicity. To that end, intraocular injections of kainate were performed and the tissue examined microscopically. The morphological changes observed following this protocol included pronounced tissue damage and cell swelling and a very characteristic spongy appearance of the inner plexiform layer. Our results confirmed that the skate retina undergoes excitotoxic events very similar to the ones observed by other groups in the retinas of chick, rat and mouse (Olney et al., 1986, Romano et al., 1998).

Severe insult to neuronal tissues usually results in necrosis and/or apoptosis, but the time course of these indices of cell death seems to vary widely depending on the type of neuronal tissue and the species of animal used. Based on the numerous studies by Olney and co-workers (cited above), it is likely that in the retina the majority of affected cells are undergoing necrosis within the first 24 hours after exposure to cytotoxic levels of glutamate. This is followed by more extensive destructive changes over a broad extent of the retina, characteristic of apoptotic cell death (Gwag et al., 1997; Joo et al., 1999; Ientile et al., 2001). Looking quantitatively at the early effects of kainate on cell death of the various cell groups within the skate retina, we found substantial evidence of necrosis in the GCL with very little necrosis observed elsewhere. It is likely that apoptosis will follow these early necrotic events, as previously reported for goldfish retina (Villani et al., 1995, 1997).

We had suggested that negative feedback control of glutamate release by zinc serves not only a neuromodulatory, but also a cytoprotective role in the vertebrate retina (Chappell et al., 2008). To test this hypothesis, we performed injections of histidine, a membrane-impermeable chelator of zinc, into the eye of the dark adapted skate. This approach allowed us to continuously chelate zinc *in vivo* in order to learn its effect while the animal was kept in the dark, when glutamate release from photoreceptors is maximal. If zinc does indeed regulate glutamate release, then over time, with chelation of zinc, we would expect to see the retina suffer from the effects of excessive glutamate exposure, i.e. excitotoxicity. Injections of histidine (20mM) did indeed result in morphological changes in the tissue that very strongly resembled what was observed with kainate injections, and in fact sometimes exceeded the effects of kainate. As was the case for kainate injections, early necrotic changes were observed almost exclusively in the GCL. At later times, tissue swelling, loss of cell material and spongy appearance of the IPL similar to kainate treatment was observed histologically.

Finally, in control experiments we found that separate injections of identical concentrations of the chelator histidine and zinc result in no significant damage to the tissue. This demonstrates the specificity of action of histidine in that it does not appear to act directly on the tissue, but rather that its effect is accomplished by the chelation of zinc and the subsequent unregulated glutamate release. Interestingly, *exogenous* zinc has been shown to have a modulatory effect on bipolar and amacrine cells (Luo et al., 2002; Zhang et al., 2002; Han & Yang, 1999). In light of our experiments, these findings do not exclude the possibility of Zn^{2+} diffusion from the outer to the inner retina. To our knowledge there is as yet no evidence for zinc and glutamate co-release from the bipolar cell terminal, perhaps due to the differences in the nature of transmitter release from photoreceptors and bipolar cells.

It should be noted that despite the millimolar concentrations listed in the figures, the actual concentration of drugs reaching the tissue was likely at least 20 to 50 times less, due to the

large volume of the skate eye, the small volume of treatment injections, and the highly viscous vitreous humor. Effectively, we believe that actual concentrations of kainate and histidine were very likely in the micromolar range. Based on histological observations in experiments like those shown in Figure 3, we were expecting a large number of cells to exhibit signs of necrosis throughout the retina. To our surprise, relatively little evidence for cell necrosis was observed in the outer retina. On the other hand, significant necrosis was observed within the GCL. This finding is consistent, however, with earlier reports that excitotoxic damage to the inner retina is generally what is first observed in other animal models of glutamate toxicity, i.e., in avian and murine retinas (Romano et al., 1998).

A study by Kikuchi et al. (1995) demonstrated the protective action of zinc against the neurotoxic effects of glutamate on retinal neurons in culture. In an alternative approach, Hyun et al. (2001) 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 UV radiation or exposure to hydrogen peroxide. Furthermore, zinc deficiency has serious consequences, and the resultant pathology has been well documented in a wide variety of tissues (reviewed in Prasad, 2013). The results reported here suggest that zinc, co-released endogenously with glutamate from vertebrate photoreceptor terminals, plays an important role in protecting the retina from the excitotoxic damage that results from unregulated tonic release of glutamate in darkness, i.e. when photoreceptors are maximally depolarized. Thus, zinc deficiency may have subtle but serious consequences for the health of the inner retina.

Acknowledgments

We thank Dr. Robyn Crook for her help with statistical analysis and with preparation of the necrosis data. We thank Marjeta Argjir for help with tissue sectioning. These studies were supported by grants from the National Science Foundation (1026531 & 1214162: RC) and NCRR/NIH (RR003037: RC).

References

- Anastassov I, Shen W, Ripps H, Chappell RL. Zinc modulation of calcium activity at the photoreceptor terminal: a calcium imaging study. *Exp Eye Res.* 2013; 112:37–44. [PubMed: 23619034]
- Chappell RL, Anastassov I, Lugo P, Ripps H. Zinc-mediated feedback at the synaptic terminals of vertebrate photoreceptors. *Exp Eye Res.* 2008; 87:394–397. [PubMed: 18638476]
- Chen Q, Olney JW, Lukasiewicz PD, Almlil T, Romano C. Ca²⁺-independent excitotoxic neurodegeneration in isolated retina, an intact neural net: a role for Cl[−] and inhibitory transmitters. *Molec Pharmacol.* 1998; 53:564–572. [PubMed: 9495825]
- Dong XX, Wang Y, Qin ZH. Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases. *Acta Pharmacol Sin.* 2009; 4:379–387. [PubMed: 19343058]
- Ganesh BS, Chintala SK. Inhibition of reactive gliosis attenuates excitotoxicity mediated death of retinal ganglion cells. *PloS One.* 2011; 6:e18305. [PubMed: 21483783]
- Gwag BJ, Koh JY, DeMaro JA, Ying HS, Jacquin M, Choi DW. Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. *Neurosci.* 1997; 77:393–401.
- Han MH, Yang XL. Zn²⁺ differentially modulates kinetics of GABA(C) vs GABA(A) receptors in carp retinal bipolar cells. *Neuroreport.* 1999; 10:2593–2597. [PubMed: 10574375]
- Hyun HJ, Sohn JH, Ha DW, Ahn YH, Koh JY, Yoon YH. Depletion of intracellular zinc and copper with TPEN results in apoptosis of cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci.* 2001; 42:460–465. [PubMed: 11157883]
- Ientile R, Macaione V, Teletta M, Pedale S, Torre V, Macaione S. Apoptosis and necrosis occurring in excitotoxic cell death in isolated chick embryo retina. *J Neurochem.* 2001; 79:71–78. [PubMed: 11595759]

- Izumi Y, Benz AM, Kurby CO, Labruyere J, Zorumski CF, Price MT, Olney JW. An ex vivo rat retinal preparation for excitotoxicity studies. *J Neurosci Meth.* 1995; 60:219–225.
- Izumi Y, Shimamoto K, Benz AM, Hammerman SB, Olney JW, Zorumski CF. Glutamate transporters and retinal excitotoxicity. *Glia.* 2002; 39:58–68. [PubMed: 12112376]
- Joo CK, Choi JS, Ko HW, Park KY, Sohn S, Chun MH, Oh YJ, Gwag BJ. Necrosis and apoptosis after retinal ischemia: involvement of NMDA-mediated excitotoxicity and p53. *Invest Ophthalmol Vis Sci.* 1999; 40:713–720. [PubMed: 10067975]
- Kikuchi M, Kashii S, Honda Y, Ujihara H, Sasa M, Tamura Y, Akaike A. Protective action of zinc against glutamate neurotoxicity in cultured retinal neurons. *Invest Ophthalmol Vis Sci.* 1995; 36:2048–2053. [PubMed: 7657543]
- Luo DG, Li GL, Yang XL. Zn⁽²⁺⁾ modulates light responses of color-opponent bipolar and amacrine cells in the carp retina. *Brain Res Bull.* 2002; 58:461–468. [PubMed: 12242098]
- McDonald J, Fix A, Tizzano J, Schoepp D. Seizures and brain injury in neonatal rats induced by 1S, 3R-ACPD, a metabotropic glutamate receptor agonist. *J Neurosci.* 1993; 13:4445–4455. [PubMed: 8410197]
- Olney JW. The toxic effects of glutamate and related compounds in the retina and the brain. *Ophthalmic Comm Soc.* 1982; 2:341–359.
- Olney JW. Excitatory transmitter neurotoxicity. *Neurobiol Aging.* 1994; 15:259–260. [PubMed: 7838306]
- Olney JW, Price MT, Samson L, Labruyere J. The role of specific ions in glutamate neurotoxicity. *Neurosci Lett.* 1986; 65:65–71. [PubMed: 2871531]
- Olney JW, Rhee V, Ho OL. Kainic acid: a powerful neurotoxic analogue of glutamate. *Brain Res.* 1974; 77:507–512. [PubMed: 4152936]
- Prasad AS. Discovery of human zinc deficiency: its impact on human health and disease. *Adv Nutr.* 2013; 4:176–90. [PubMed: 23493534]
- Redenti S, Chappell RL. Localization of zinc transporter-3 (ZnT-3) in mouse retina. *Vision Res.* 2004; 44:3317–3321. [PubMed: 15535999]
- Redenti S, Chappell RL. Neuroimaging of zinc released by depolarization of rat retinal cells. *Vision Res.* 2005; 45:3520–3525. [PubMed: 16181655]
- Redenti S, Ripps H, Chappell RL. Zinc release at the synaptic terminals of rod photoreceptors. *Exp Eye Res.* 2007; 85:580–584. [PubMed: 17825289]
- Reif-Lehrer L, Bergenthal J, Hanninen L. Effects of monosodium glutamate on chick embryo retina in culture. *Invest Ophthalmol.* 1975; 14:114–124. [PubMed: 234410]
- Romano C, Chen Q, Olney JW. The intact isolated (ex vivo) retina as a model system for the study of excitotoxicity. *Prog Ret Eye Res.* 1998; 17:465–483.
- Rothman S. Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J Neurosci.* 1984; 4:1884–1891. [PubMed: 6737044]
- Saggu SK, Chotaliya HP, Blumbergs PC, Casson RJ. Wallerian-like axonal degeneration in the optic nerve after excitotoxic retinal insult: an ultrastructural study. *BMC Neurosci.* 2010; 11:97. [PubMed: 20707883]
- Sattler R, Tymianski M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. *Mol Neurobiol.* 2001; 24:107–129. [PubMed: 11831548]
- Shen W, Finnegan SG, Slaughter MM. Glutamate receptor subtypes in human retinal horizontal cells. *Vis Neurosci.* 2004; 21:89–95. [PubMed: 15137585]
- Villani L, Carraro S, Guarnieri T. 6,7-Dinitroquinoxaline-2,3-dione but not MK-801 exerts a protective effect against kainic acid neurotoxicity in the goldfish retina. *Neurosci Lett.* 1995; 192:127–131. [PubMed: 7675320]
- Villani L, Guarnieri T, Dell'Erba G. Apoptosis is induced by excitotoxicity in the goldfish retina. *J Brain Res.* 1997; 38:481–486.
- Wu SM, Qiao X, Noebels JL, Yang XL. Localization and modulatory actions of zinc in vertebrate retina. *Vision Res.* 1993; 33:2611–2516. [PubMed: 8296456]

Zhang DQ, Ribelayga C, Mangel SC, McMahon DG. Suppression by zinc of AMPA receptor-mediated synaptic transmission in the retina. *J Neurophysiol.* 2002; 88:1245–1251. [PubMed: 12205145]

Ringer only A

2mM Kainate C

20mM Histidine E

B

D

F

Figure 1.

Kainate and histidine toxicity evidenced by necrosis in the skate retina. (A) Cross section of skate retina from a control eye receiving an injection of Ringer solution. DAPI staining of cell nuclei (blue) identifies cell bodies of the ONL, INL and GCL. Overlay of the image (red) identifying necrotic cells with ethidium homodimer III (EthD-III) shows that relatively few cells are necrotic. (B) The DAPI and EthD-III fluorescence signals measured across the entire GCL for the Ringer control condition in "A" are overlaid and displayed graphically. (C) Similar staining of a retinal cross-section from an experimental eye injected with 2mM kainate reveals pronounced EthD-III staining in the GCL. (D) The DAPI and EthD-III fluorescence signals measured across the entire GCL for tissue from the 2mM kainate treated eye; peaks in fluorescence of blue and red traces represent nuclei stained with DAPI

and EthD-III, respectively. (E) Skate retina cross-section from experimental eye which received 20mM histidine injection. EthD-III staining indicates that there is significant cell death by necrosis in the GCL. (F) The DAPI and EthD-III fluorescence signals measured across the entire GCL for tissue from the 20mM histidine treated eye. As before, peaks in fluorescence of blue and red traces in (F) represent nuclei stained with DAPI and EthD-III, respectively. Scale bars = 50 μ m.

Figure 2.

Statistical analysis of necrotic cell death in the retinal nuclear layers. The Ringer control is compared to eyes injected with kainate or histidine. (A) Kainate results based on data from Fig. 1, C and D. Cells in the GCL are significantly more necrotic after injection of 2mM kainate when compared to contralateral Ringer control (2-way ANOVA, Bonferroni posthoc test, ****, $p < 0.0001$, $N=9$; for Ringer: $\text{mean} \pm \text{SEM} = 1.541 \pm 0.237$, for kainate: $\text{mean} \pm \text{SEM} = 2.742 \pm 0.368$). (B) Cells in the GCL are significantly more necrotic as a result of 20mM histidine when compared to contralateral Ringer control (2-way ANOVA, Bonferroni posthoc test, ****, $p < 0.0001$, $N=10$; for Ringer: $\text{mean} \pm \text{SEM} = 0.299 \pm 0.035$, for histidine: $\text{mean} \pm \text{SEM} = 0.523 \pm 0.039$).

Figure 3.

Drug-induced morphological changes in the skate retina. (A) A retinal section from a control eye injected with Ringer alone. Note the absence of cell swelling or other abnormalities in the INL, IPL and GCL. (B) *In vivo* intraocular injections of the glutamate receptor agonist kainate (2mM) result in cellular changes typical of glutamate toxicity. There is pronounced swelling of cells of the INL and GCL and the structural integrity of their cellular membranes appears compromised. The inner plexiform layer exhibits the typical “sponge-like” appearance and loss of cell material associated with apoptosis. (C) Intraocular injections of 20mM histidine, a membrane-impermeable chelator of zinc, result in histological damage throughout the retina. Cells of the INL and GCL are swollen and normal morphology is lost. The IPL has the characteristic “sponge-like” appearance associated with the loss of cell cytoplasm. Note also that the photoreceptor outer segments and cell bodies are contorted and appear less dense, but this effect was not seen in every animal. (D) When both zinc (10mM) and then histidine (10mM) were injected separately into the experimental eye, the glutamate-like toxicity found following injection of histidine alone was not observed. In tissue from that eye, the nuclear layers and plexiform layers are clearly visible and appear normal. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars = 50µM.