Chronic Low-Dose Glutamate Is Toxic to Retinal Ganglion Cells
Toxicity Blocked by Memantine

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Purpose. It is well known that acute exposure to high concentrations of glutamate is toxic to central mammalian neurons. However, the effect of a chronic, minor elevation over endogenous glutamate levels has not been explored. The authors have suggested that such chronic exposure may play a role in glaucomatous neuronal loss. In the current study, they sought to explore whether a chronic, low-dose elevation in vitreal glutamate was toxic to retinal ganglion cells and whether this toxicity could be prevented with memantine, a glutamate antagonist.

Methods. Rats were injected serially and intravitreally with glutamate to induce chronic elevations in glutamate concentration. A second group of rats was treated with intraperitoneal memantine and glutamate. Control groups received vehicle injection with or without concurrent memantine therapy. After 3 months, the animals were killed, and ganglion cell survival was evaluated.

Results. Intravitreal injections raised the intravitreal glutamate levels from an endogenous range of 5 to 12 μM glutamate to 26 to 34 μM. This chronic glutamate elevation killed 42% of the retinal ganglion cells after 3 months. Memantine treatment alone had no effect on ganglion cell survival. However, when memantine was given concurrently with low-dose glutamate, memantine was partially protective against glutamate toxicity.

Conclusions. These data suggest that minor elevations in glutamate concentration can be toxic to ganglion cells if this elevation is maintained for 3 months. Furthermore, memantine is efficacious at protecting ganglion cells from chronic low-dose glutamate toxicity. Invest Ophthalmol Vis Sci. 1996;37:1618–1624.

Studies in the central nervous system during the past three decades have found that traumatic and ischemic neuronal injury can be mediated by excessive levels of excitatory amino acids, especially glutamate.1,2 These investigations have explored the role of glutamate in neuronal loss from stroke, trauma, epilepsy, and neurodegenerative disorders such as Huntington’s disease, amyotrophic lateral sclerosis, and acquired immune deficiency syndrome dementia.3–6

The toxic potential of an acute bolus of glutamate to mammalian retinal ganglion cells and other central neurons is well documented. In 1957, Lucas and Newhouse7 first reported the toxic effects of glutamate on the mammalian eye. Subcutaneous injection of glutamate into young mice led to severe destruction of the inner retinal layers, most notably in the retinal ganglion cell layer. Using ultrastructural techniques, Olney8 demonstrated similar glutamate-induced retinal toxicity in neonatal mice and coined the term “excitotoxicity” to describe these lesions. Sisk and Kuwabara9 injected glutamate intravitreally into adult albino rats and observed degeneration of the inner nuclear and ganglion cell layers. Azuma and coworkers10...
reported that the optic discs of neonatal rats treated with glutamate were "deeply excavated." Work in our own and other laboratories has established that the predominant form of excitotoxicity of retinal ganglion cells is mediated by overstimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor, which, in turn, leads to excessive levels of intracellular calcium. Non-NMDA receptors also may play a role in glutamate excitotoxicity.

Most efforts at exploring the toxicity of glutamate to retinal ganglion cells have focused on the effects of the administration of a single large dose of glutamate (or one of its congeners) administered either intravitreally or subcutaneously. Several investigators have described chronic glutamate toxicity in culture, but, to the best of our knowledge, there have been no studies in which chronic perturbations of glutamate levels were attempted in vivo nor have there been any efforts to explore protection against such insults.

We have shown that glaucoma in humans and monkey is associated with a significant elevation in vitreal glutamate concentration (from 23.1 ± 4.6 μM in glaucomatous vitreous compared to 10.0 ± 5.1 μM in control samples). The current study was directed at determining whether this elevation of glutamate could suffice—on its own—to cause ganglion cell damage in an animal model.

A well-characterized NMDA antagonist is dizocilpine (MK-801), which functions as an open-channel blocker of the NMDA receptor. MK-801 can protect neurons from ischemic necrosis in gerbils and rats. However, MK-801 also impairs many normal neuronal functions and can cause injury or reversible neuronal swelling at therapeutic concentrations. Memantine (1-amino-3,5-dimethyladamantane hydrochloride) is known to have anti-Parkinsonian and anti-epileptic properties and is an analog of amantadine (1-adamantanamine hydrochloride). Amantadine is a well-known antiviral agent that has been used clinically for more than 20 years in the United States. Memantine has been used with few side effects for the treatment of Parkinson’s disease in Europe for the past decade. Memantine binds to NMDA receptors and can block the effect of glutamate at NMDA receptors but have no effect on non-NMDA receptors. Memantine appears to protect against acute excitotoxic insults in vivo and in vitro. Experiments were undertaken as well to explore whether memantine could protect retinal ganglion cells against chronic minor elevations of glutamate.

METHODS

Drug Treatment

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To establish a paradigm for chronic, low-grade glutamate elevation in the vitreous, the following initial experiments were performed. Adult rats were anesthetized, and one eye of each rat was injected under direct visualization (either with loupes or with indirect ophthalmoscopy) every 5 days with 1 μl of a 2.5 mmol/l solution of glutamate prepared in 0.1 mol/l phosphate buffer, pH 7.4. The contralateral eye was used as a control and was similarly injected with 1 μl of vehicle. The pupil was dilated with phenylephrine 5% and tropicamide 0.8% before injections. Rats were killed at 1 hour, 1 day, 3 days, 5 days, 10 days, 1 month, 2 months, and 3 months. Vitreous from control and glutamate-injected eyes was aspirated and assayed in toto for amino acid concentration by cation exchange.

Histopathologic changes caused by the chronic administration of glutamate were quantified as follows. Twelve rats were subjected to injections of either glutamate or control vehicle in the right eye every 5 days for 3 months. If there was intraocular bleeding, the animal was killed, and the tissue was not evaluated. A second group of 24 rats received glutamate or control injections and intraperitoneal memantine at 1 mg/kg every 24 hours, administered as a daily single dose. This dosage was selected because it is well tolerated and neuroprotective in a murine stroke model.

Ganglion Cell Quantification

Retinal ganglion cell survival was assessed as follows. At the conclusion of the 3-month treatment period, anesthetized rats were placed in a stereotaxic frame. A portion of the skull and meninges was removed, exposing the neocortex overlying the tectum on the side contralateral to the lesioned eye. Seven intracerebral injections of 0.7 ml of a horseradish peroxidase solution (HRP; 30% (wt/vol) dissolved in 2% (vol/vol) dimethyl sulfoxide) were made, and all layers of the contralateral superior colliculus were targeted. Injections were made over a period of 2 minutes using a syringe affixed to a micromanipulator attached to the stereotaxic frame. Approximately 48 to 72 hours after the HRP injection, the animals were killed and perfused. The retina was removed in toto from the eye and rinsed, and the HRP was visualized by nickel-cobalt-enhanced diaminobenzidine (Pierce, Rockford, IL). This technique has been well validated, and it allows for a rapid assessment of ganglion cell survival, especially when it is paired with the following method.

Stereologic techniques were used for counting surviving ganglion cells. The dissector methodology, initially described by Sterio in 1984, has been applied more recently to quantitation of neurons in the central nervous system. This technique overcomes statistical biases inherent in counting objects of different
sizes and shapes using traditional two-dimensional approaches. The dissector is a three-dimensional stereologic probe that samples objects with a probability that is proportional to number rather than size or shape. Essentially, what one does is to determine whether a unique point in each object lies within the volume sampled by the dissector. This results in ascertaining volume density rather than area density. By combining this with a measurement of the volume, the total number of ganglion cells can be calculated easily. As demonstrated by West and Coggeshall, this is not only a more straightforward approach, it is also a more accurate approach for determining neuronal loss. An additional advantage of stereologic techniques has been the introduction of the quadratic approximation formula for estimating the precision of individual estimates based on systematic samples made along one axis. This statistical technique allows one to assess accurately the degree of variance caused by biologic (i.e., between-sample) differences as opposed to variance introduced by the sampling method. Finally, the stereologic literature has developed sampling strategies to provide unbiased estimates. The sampling protocol used is called "systematic random sampling." A random choice is made within the first measurement interval. Subsequently, sampling is performed at a predetermined interval thereafter throughout the entire region. This is crucial for retinal ganglion cells because their density across the retina varies according to location sampled. Therefore, one can determine easily and accurately the percentage of surviving retinal ganglion cells in the face of one or more interventions. Finally, to insure that our injections were not interfering with axoplasmic transport of HRP, six additional glutamate-treated eyes were wholemounted and stained with antibody against the ganglion cell-specific marker, Thy-1. The numbers of cells quantified by Thy-1 labeling were not statistically different from those identified by HRP labeling (data not shown). After fixation, one or more additional rat eye(s) in each group was sectioned sagitally and stained with hematoxylin and eosin.

**Statistical Analysis**

All statistical analyses of the survival rate measures between the two study groups were performed with the nonparametric Wilcoxon rank sum test. A Bonferroni procedure was used for post hoc multiple comparisons to maintain an overall 0.05 alpha level. Hence, mean comparisons were considered significant when the exact two-tailed P value for the W statistic was less than 0.05/3, or \( P < 0.012 \).

**RESULTS**

**Chronic Elevation in Vitreal Glutamate**

Our initial experiments were directed at establishing the conditions necessary to simulate the glutamate elevation we found in primate glaucoma. Table 1 lists glutamate concentrations at various time points in the eyes of animals injected every 5 days (other injection paradigms were attempted in preliminary experiments to establish the conditions necessary for a chronic elevation to approximately 30 \( \mu M \); data not shown). At all time points in this paradigm, the measured glutamate concentration was between 26 and 34 \( \mu M \) in the treated eye, compared to a range of 5 to 12 \( \mu M \) in the control eye. This experiment established that a 1 \( \mu l \) injection of a 2.5 mmol/l glutamate solution, repeated every 5 days, provided a relatively stable elevation in vitreal glutamate to approximately 30 \( \mu M \).

**Ganglion Cell Survival**

Based on these data, a second group of rats was injected in accord with this paradigm. Sections of control and glutamate-treated eyes were prepared after 10 days. No retinal pathology was observed at this time point (not shown). If the pathology were an early consequence of the limited bolus of glutamate given by injection, one might expect to see pathologic changes in the retina by this time.

Histologic cross-sections of rats treated for 3 months are illustrated in Figure 1. The normal appearance of the control retina eye after 3 months of vehicle injection indicates that the effect on the retinal ganglion cell layer is not a consequence of repeated injections per se. In contrast, the glutamate-treated eyes have pronounced loss of the retinal ganglion cell layer. Chronic administration of glutamate appears to be necessary for the observed neurotoxic effects at these concentrations. In addition, sections through the optic nerve revealed marked excavation (data not shown; as previously described in acute glutamate toxicity, reference 10). The memantine–glutamate-treated eyes appear indistinguishable from controls. Memantine treatment alone had no observable effect on the ganglion cell layer.

**TABLE 1. Intravitreal Concentration of Glutamate After Injection**

<table>
<thead>
<tr>
<th>Time</th>
<th>Glutamate-Treated Eye</th>
<th>Control Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>30 ± 1</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>1 day</td>
<td>26 ± 4</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>3 days</td>
<td>26 ± 3</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>5 days</td>
<td>29 ± 5</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>10 days</td>
<td>32 ± 3</td>
<td>12 ± 6</td>
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<tr>
<td>1 month</td>
<td>34 ± 1</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>2 months</td>
<td>30 ± 2</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>3 months</td>
<td>29 ± 2</td>
<td>11 ± 2</td>
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Values are represented as \( \mu mol/l \) and are the means of four eyes ± standard deviation.
Toxicity of Chronic Glutamate

FIGURE 1. Toxicity of chronic administration of glutamate to the retinal ganglion cell layer. Adult rats were anesthetized, and one eye of each rat was injected under direct visualization every 5 days with 1 μl of a 5-mM solution of glutamate. The contralateral eye was used as a control and was injected with 1 μl of vehicle on the same days. The glutamate-treated rat retina (right) shows a diminution of the cells in the retinal ganglion cell layer when compared to the control eye (left). Accordingly, chronic glutamate administration is toxic to cells of the retinal ganglion layer, with sparing of the other layers of the retina. GCL = ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer. Bar = 50 μm.

However, to assess accurately the effect of these interventions on ganglion cell survival, whole mounts of HRP-labeled retinas were prepared, and the surviving cells were counted. Representative sections are shown in Figure 2, and quantitative analyses are shown in Figure 3. There was a significant diminution of the number of ganglion cells after 3 months of glutamate injections, from a control value of 96,500 ± 8500 retinal ganglion cells per eye (mean, 8 eyes ± SD) to 56000 ± 9600 cells in the glutamate treated eyes (P < 0.001). Eyes from animals treated with memantine alone had 94,800 ± 8700 ganglion cells. Furthermore, when animals were treated with both glutamate and memantine, 83,000 ± 4900 ganglion cells remained after 3 months. Memantine-treated animals did not show any grossly detectable behavioral changes, but no specific behavioral testing paradigms were used.

DISCUSSION

Glutamate, or a congener, is the principal excitatory amino acid neurotransmitter in the central nervous system and specifically in the retina. Glutamate is present at high levels in neurons, but it is usually synaptically released for brief periods in localized areas; hence, it is usually not toxic. Studies in the central nervous system suggest that damage to hippocampal or cortical neurons can occur at glutamate concentrations of 2 to 5 μM. Evidence indicates that under certain conditions, these concentrations of excitotoxins can be lethal to retinal ganglion cells in vitro as well. However, factors such as extracellular Ca2+ and Mg2+, or the presence of other cell types, can be significant variables in determining the concentrations at which glutamate or its congeners are toxic to neurons in vitro. As shown in this study, a relatively minor elevation of glutamate over endogenous levels can be toxic in vivo if it is maintained for a sufficient period of time.

It is not possible—given the small size of the rodent eye—to establish the exact distance of our injection sites from the retina. It is possible that the application of glutamate provided a local, high concentration to relatively few cells rather than a diffuse elevation of the vitreal glutamate. However, this scenario may, in fact, accurately mimic the findings in glaucoma. As a retinal ganglion cell dies, it can release its intracellular concentration of glutamate—possibly as high as 10 mmol—onto surrounding cells, causing severe local toxicity. Alternatively, a diffuse chronic elevation also may be toxic. Future experiments in a larger animal model may help to separate these two possibilities and to establish the true scenario in the human glaucomatous eye.

It is perhaps surprising that high-affinity glutamate uptake systems in the retina could not clear the
FIGURE 2. Horseradish-peroxidase-stained wholemounts, illustrating the effect of a chronic elevation of glutamate on ganglion cell survival. The left panel illustrates a control rat retina, subjected to vehicle injections for 3 months. The middle panel illustrates the diminution in the number of ganglion cells after 3 months of a chronic low grade elevation of glutamate over endogenous levels. The right panel is from a rat treated with both glutamate and memantine, showing the preservation of ganglion cells. Similar retinal sections are shown in each panel. Bar = 30 μm.

FIGURE 3. Quantification of retinal ganglion cell survival. Rats were treated for 3 months with either intravitreal glutamate or control vehicle or with memantine with either glutamate or vehicle. Horseradish-peroxidase-backlabeled retinal wholemounts were prepared, and the number of surviving ganglion cells was evaluated as described in Methods. There was a significant diminution of the number ganglion cells after 3 months of glutamate injections, from a control value of 96,500 ± 8500 retinal ganglion cells per eye (mean of 8 eyes ± SD) to 56,000 ± 9600 cells in the glutamate-treated eyes (P < 0.001). Eyes from animals treated with memantine alone had 94,800 ± 8700 ganglion cells. Furthermore, when animals were treated with both glutamate and memantine, 83,000 ± 4900 ganglion cells survived after 3 months. *Statistical difference from control; P < 0.001.

Surviving Retinal Ganglion Cells

Control Glutamate Memantine Glutamate + Memantine

exogenously added glutamate from the vitreous. However, the chronicity of our elevations may perturb one or more uptake systems. Furthermore, we do not understand yet whether factors such as the internal limiting membrane or gradients in the vitreous may affect the accessibility of our injected glutamate to either ganglion cell receptors or uptake systems.

Our results also indicate that the NMDA antagonist, memantine, can protect against this chronic glutamate insult. Memantine is an uncompetitive antagonist that most likely binds to a site within the open NMDA receptor channel. In patch clamp experiments, 2 to 12 μM memantine can inhibit effectively 200 μM NMDA-activated currents at a holding potential of −60 mV. Furthermore, the degree of block is voltage dependent, manifesting considerably less inhibition at +60 mV. The dissociation time constant of memantine from NMDA receptor channels is approximately 5.2 seconds at −60 mV in the continued presence of NMDA. The real therapeutic advantage of memantine—and its potential ability to prevent neuronal damage in vivo—is from the following properties. At a given concentration of memantine, the effects of high concentrations of NMDA are blocked to a relatively greater degree than are low concentrations. This mechanism offers theoretical and practical advantages: Normal (i.e., low grade) activation of NMDA receptors will be at least partially spared (e.g., the processes involved in eliciting long-term potentiation). However, when faced with a higher, toxic concentration of glutamate, memantine can block lethal effects. In the scenario of a chronic excitotoxic insult, memantine could produce a long-lasting, as well as a
Toxicity of Chronic Glutamate

more potent, blockade. Memantine has proven effective against acute hypoxic-ischemic brain damage in vivo. The current study illustrates its efficacy at protecting against a chronic excitotoxic insult. These data indicate that memantine may merit further investigation as a protective agent in various disease states in which chronic elevations of glutamate may play a role in neuronal loss, including, perhaps, glaucoma.

Key Words
chronic, glaucoma, glutamate, memantine, N-methyl-D-aspartate, retinal ganglion cells

References
15. Hahn JS, Aizenman E, Lipton SA. Central mammalian neurons normally resistant to glutamate toxicity are made sensitive by elevated extracellular Ca2+ toxicity is blocked by the N-methyl-D-aspartate antagonist MK-801. Proc Natl Acad Sci USA. 1988;85:6556–6560.


