Efficacy and Safety of Memantine Treatment for Reduction of Changes Associated with Experimental Glaucoma in Monkey, I: Functional Measures

William A. Hare, Elizabeth WoldeMussie, Ronald K. Lai, Hau Ton, Guadalupe Ruiz, Teresa Chun, and Larry Wheeler

PURPOSE. To determine, using electrophysiological measures of visual system function, whether oral daily dosing of memantine is both safe and effective to reduce the injury associated with experimental glaucoma in primates.

METHODS. Argon laser treatment of the anterior chamber angle was used to induce chronic ocular hypertension (COHT) in the right eye of 18 macaque monkeys. Nine animals were orally dosed daily with 4 mg/kg memantine while the other nine animals received an oral dose of vehicle only. Using both conventional and multifocal methods, recordings of the electroretinogram (ERG) were made at approximately 3, 5, and 16 months after elevation of the intraocular pressure (IOP). Recordings of the visually-evoked cortical potential (VECP) were also made at the 16-month time point.

RESULTS. Chronic ocular hypertension was associated with a reduction in the amplitude of components of the multifocal ERG response and visually-evoked cortical potential. Memantine-treated animals suffered less amplitude reduction for these measures than did vehicle-treated animals, though this treatment effect on the ERG measures was observed only at the early time points (3 and 5 months post IOP elevation). Memantine treatment was not associated with an effect on either the kinetics or amplitude of ERG or VECP response measures obtained from the normotensive eyes.

CONCLUSIONS. Systemic treatment with memantine, a compound which does not lower intraocular pressure, was both safe and effective for reduction of functional loss associated with experimental glaucoma. (Invest Ophthalmol Vis Sci. 2004;45:2625–2639) DOI:10.1167/iovs.03-0566

Glaucomatous vision loss results from injury to retinal ganglion cells (RGCs) and their axons which make up the optic nerve. Although the precise mechanism for glaucomatous insult to RGCs is unknown, RGC injury could result from a compromised vascular perfusion to the optic nerve head, retinal nerve fiber layer, or retinal ganglion cell layer; and ischemic insult to RGCs has been proposed in many models of glaucomatous pathophysiology.1–5 The extracellular concentration of glutamate, the principal excitatory neurotransmitter in the CNS, increases after ischemic insult4–7 and a significant component of ischemic injury to CNS neurons results from glutamatergic excitotoxicity. As in other regions of the CNS, glutamate is the principal excitatory neurotransmitter in the retina. Retinal ischemia is also associated with an increase in the level of extracellular glutamate8–11 and RGC sensitivity to glutamatergic excitotoxic injury has been demonstrated in a variety of both in vivo12–15 and in vitro16–18 models. RGCs express both NMDA and non-NMDA type glutamatergic receptors19–21 and, although overactivation of either receptor type can produce excitotoxic neuronal injury, excessive activation of NMDA-type receptors is particularly injurious due to their relatively high calcium permeability.22–24 NMDA-type glutamatergic excitotoxicity has been implicated as a mechanism for injury associated with ischemic and other insults to neurons in many regions of the CNS. For this reason, considerable effort has been focused on the discovery of NMDA antagonists for the treatment of both acute and chronic neurodegenerative disease.

Memantine (1-amino-3,5-dimethyladamantane) is an NMDA antagonist of the “open channel blocker” type; that is, memantine binds within the channel to a site that is not accessible unless the channel has previously been activated by glutamate binding to a different receptor site. Memantine may be distinguished from other open channel blocker type NMDA antagonists by its relatively low affinity for the channel binding site, the rapid kinetics for channel blockade, and the strong dependence of blocking kinetics on membrane voltage.25–28 It has been proposed that these properties make memantine effective for blockade of excessive (pathologic) glutamatergic activity at concentrations that have little or no effect on normal CNS function. In fact, memantine is both efficacious and well tolerated in the treatment of Parkinson’s disease29–30 and dementia.31–35 Memantine treatment is also effective in reducing injury associated with animal models for traumatic brain injury,36 cerebral ischemia,37 and retinal ischemia38–39 as well as a model for experimental glaucoma in rat.40,41

The results of memantine treatment in nine monkeys with experimental glaucoma in one eye are reported in the present article. Electrophysiological measures were used to show that memantine treatment was both safe and effective for reduction of functional changes associated with chronic ocular hypertension.

METHODS

All experimental procedures conformed to guidelines in the Statement for Use of Animals in Ophthalmic and Vision Research provided by the Association for Research in Vision and Ophthalmology and were approved by an institutional review committee. Methods for the induction of chronic ocular hypertension, histologic measures of retinal ganglion cell survival, and recording of the ERG using both conventional and multifocal techniques, are described in detail in earlier papers42,43 and are briefly described here.
Animal Subjects and Memantine Dosing

Eighteen young adult cynomolgous monkeys, Macaca fascicularis, were randomly divided into two groups and baseline measures of IOP were made. Daily oral dosing of 4 mg/kg memantine was begun for one group while the other group received oral dosing with vehicle only. This dose of memantine was previously determined to have no significant effect on IOP in normotensive or ocular hypertensive monkey eyes (data not shown). Argon laser treatment of the anterior chamber angle was performed on the right eye of all 18 animals to induce chronic ocular hypertension according to a method similar to that originally described by Gaasterland and Kupfer.44 Using an argon laser (model Novus 2000; Coherent, Inc., Palo Alto, CA), 30 to 40 spots of 50 μ diameter, 1 watt power, and 0.5 second duration were applied over the superior 180° of the internal anterior chamber angle. Two weeks later, the inferior 180° of anterior chamber angle tissue was similarly treated. IOP was measured under light ketamine sedation at regular intervals throughout the study, which lasted approximately 16 months following the laser treatment.

Measurement of IOP

IOP measurements were performed under light ketamine sedation (5 mg/kg, i.m.) by a person with >20 years experience in making tonometric measures from primates. IOP measurements from all animals were made between the hours of 8:00 AM and 12:00 noon. Previous studies in our laboratory have demonstrated that there is little diurnal variation in IOP measured over this time of day (light/dark cycle; data not shown). Light ketamine sedation is associated with little or no effect on IOP in these animals, although anesthetic doses (≥15 mg/kg, i.m.) produce a significant reduction of IOP (unpublished observations). For IOP less than or equal to 45 mm Hg, a Model RT pneumotonometer (Digilab, Norwell, MA) was used while an Alcon pneumotonometer (Alcon, Fort Worth, TX) was used for IOP greater than 45 mm Hg. In either case, stable pressure traces of approximately 4.5 seconds duration were obtained. IOP was determined as the mean value of the maxima and minima associated with cardiac pulsation, which was <2 mm Hg peak-to-peak amplitude.

Memantine Levels in Serum and Vitreous

Blood samples were obtained (2 hours after oral dosing) from each animal at 2 months after the onset of dosing and at 2-month intervals thereafter until the end of the study. A pilot study showed that memantine reached its peak plasma concentration at this time point with a half-life of approximately 6 hours (data not shown). Immediately before sacrifice, a sample of vitreous humor was also obtained from each eye. The amount of memantine in the plasma and vitreal samples was determined using an internal standard method in combination with HPLC. Forty μL memantine hydrochloride standards (0.2 M, 0.4 M, and 0.8 M) were added to 40 μL plasma or vitreous. Protein was then precipitated by adding 190 μL acetonitrile, vortexing for 2 minutes, followed by centrifugation at 500g for 4 minutes. 150 μL supernatant was then derivatized with 9-fluorenlymethyl chloroformate chloride (FMOC-Cl) by mixing with 10 μL of 15 mM FMOC-Cl in acetonitrile and 10 μL of 0.5 M pH 8.5 borate buffer. Five minutes after derivatization, 90 μL of the derivatized mixture was injected into a Gold HPLC system (Beckman Instruments, Brea, CA) coupled to a Shimadzu RF-551 fluorescence detector (Shimadzu, Sumo Sushi, Japan). Using a Beckman ODS ultrasphere C-18 column (4.6 × 150 mm), a mobile phase consisting of 60% acetonitrile and 40% 50 mM borate buffer, and a flow rate of 2 mL/min, the memantine-FMOC was eluted at approximately 40 minutes. The quantity of memantine in each plasma or vitreous sample was determined, in triplicate, from the internal standard curve generated for each individual sample.

Glutamate Level in Vitreous Samples

Samples of vitreous were obtained from each eye of all animals immediately before sacrifice (approximately 16 months after laser treatment). An 18-gauge needle was used to obtain the sample from the central vitreous. Care was taken that the vitreous sample was not contaminated with blood or any other intraocular tissue. Vitreous samples were immediately stored at −70°C and were kept for less than 3 months before glutamate assay. The concentration of glutamate in each sample was determined using HPLC and an internal standard method.
Ten μL of either a 10-, 20-, or 40-μM glutamate standard were added to 90 μL of vitreous sample. Protein was then precipitated by addition of 190 μL acetonitrile, followed by vortexing for 2 minutes and centrifugation at 500 g for 4 minutes. Twenty μL of supernatant was then mixed with 40 μL Fluoraldehyde OPA reagent (Pierce, Rockford, IL) followed, after 2 minutes, by the addition of 100 μL pH 7 phosphate buffer (PB). Sixty μL of this mixture was then injected into a Beckman Gold HPLC system (Beckman Instruments), which was coupled to a fluorescence detector (Shimadzu RF-551). A Beckman ODS Ultrasphere C-18 column was used in combination with a mobile phase gradient of

TABLE 1. IOP Peak and Mean Values for Individual Animals of Both Treatment Groups

<table>
<thead>
<tr>
<th>Monkey #</th>
<th>Baseline IOP (mm Hg)</th>
<th>Peak IOP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle-Treated Animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>79</td>
<td>18.5 (18.3)</td>
<td>34.5</td>
</tr>
<tr>
<td>105</td>
<td>19.3 (19.1)</td>
<td>46.5</td>
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<tr>
<td>106</td>
<td>18.8 (18.7)</td>
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<td>98</td>
<td>21.5 (21.7)</td>
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<tr>
<td>104</td>
<td>19.1 (19.5)</td>
<td>61.0</td>
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<tr>
<td>96</td>
<td>22.1 (21.8)</td>
<td>50.0</td>
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<td>91</td>
<td>18.5 (18.5)</td>
<td>55.0</td>
</tr>
<tr>
<td>93</td>
<td>19.0 (19.0)</td>
<td>55.9</td>
</tr>
<tr>
<td>92</td>
<td>18.7 (19.1)</td>
<td>61.5</td>
</tr>
<tr>
<td>Range</td>
<td>18.5–22.1</td>
<td>34.5–61.5</td>
</tr>
<tr>
<td>X ± SEM</td>
<td>19.5 ± 0.45</td>
<td>51.6 ± 2.8</td>
</tr>
</tbody>
</table>

Memantine-Treated Animals

| 108      | 19.3 (18.7)          | 46.5             | 25.4 (18.4)      | 24.3 (18.8)      | 23.9 (19.5) |
| 102      | 17.5 (17.0)          | 55.0             | 32.7 (18.1)      | 24.7 (18.8)      | 26.2 (20.1) |
| 99       | 21.1 (20.7)          | 55.0             | 32.7 (18.1)      | 28.47 (16.8)     | 25.6 (17.8) |
| 107      | 19.1 (19.0)          | 40.5             | 55.6 (20.5)      | 51.4 (16.0)      | 29.2 (18.6) |
| 97       | 21.1 (21.0)          | 47.0             | 43.5 (20.1)      | 38.8 (19.8)      | 29.3 (21.0) |
| 100      | 19.3 (19.5)          | 59.0             | 51.5 (21.4)      | 42.6 (20.6)      | 54.1 (20.0) |
| 94       | 18.5 (18.5)          | 53.0             | 43.6 (19.6)      | 38.9 (16.5)      | 35.3 (18.7) |
| 95       | 21.1 (14.7)          | 60.0             | 56.9 (20.4)      | 53.5 (20.6)      | 42.7 (21.1) |
| Range    | 17.5–21.1            | 31.5–61.5        | 25.2–56.9        | 24.3–58.4        | 25.9–57.0 |
| X ± SEM  | 19.8 ± 0.44          | 50.4 ± 3.3       | 41.0 ± 4.0       | 37.8 ± 4.0       | 35.7 ± 3.5 |

Values for contralateral normotensive (OS) eyes are shown in parentheses.

TABLE 2. Summary for Linear Regression Analysis of Electrophysiology Measures in Both Treatment Groups

<table>
<thead>
<tr>
<th>Measure</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
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<tr>
<td>Slope</td>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.00668</td>
<td>−0.0033</td>
<td>−0.0024</td>
</tr>
<tr>
<td>Memantine</td>
<td>−0.00183</td>
<td>0.0095</td>
<td>0.00174</td>
</tr>
<tr>
<td>P-value</td>
<td>0.40</td>
<td>0.31</td>
<td>0.973</td>
</tr>
<tr>
<td>N2-P2 vs IOP</td>
<td>−0.04054</td>
<td>−0.02898</td>
<td>−0.03155</td>
</tr>
<tr>
<td>Vehicle</td>
<td>−0.02568</td>
<td>−0.01788</td>
<td>−0.03099</td>
</tr>
<tr>
<td>Memantine</td>
<td>0.032</td>
<td>0.172</td>
<td>0.973</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2-N3 vs IOP</td>
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<td>−0.04142</td>
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<tr>
<td>Vehicle</td>
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<td>−0.00525</td>
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<tr>
<td>Memantine</td>
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<td>0.031</td>
<td>0.266</td>
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<tr>
<td>P-value</td>
<td></td>
<td></td>
<td></td>
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<td>P-N vs IOP</td>
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<td>−0.04054</td>
</tr>
<tr>
<td>Vehicle</td>
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<tr>
<td>Memantine</td>
<td>0.238</td>
<td>0.018</td>
<td>0.966</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VECP n1p1 vs IOP</td>
<td>−0.02835</td>
<td>−0.00750</td>
<td>0.040</td>
</tr>
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</table>
10% methanol/90% 50 mM PB to 75% methanol/25% PB, a flow rate of 1.5 mL/min, and a duration of 8 minutes. Elution of 1-alkylthio-2-alkylisoindole glutamate was observed at approximately 6.5 minutes. Data were collected and analyzed using PE Nelson ACCESS CHROM software (Perkin Elmer, Boston, MA). Each concentration was determined in triplicate and the quantity of glutamate in each vitreal sample was determined from the internal standard curve, which was generated for each sample. The sensitivity of this method was greater than 1 nM.

Electrophysiology
ERG recordings were made from both eyes of all animals at approximately 3 months (T1), 5 months (T2), and immediately before sacrifice at 16 months after laser treatment (T3). Baseline recordings were not made since results from initial recordings from naive monkeys suggested that variability between responses obtained from the two eyes at the same recording session was less than that obtained from the same eye at different recording sessions (unpublished observations). For this reason, each normotensive (OS) eye provided an internal control for the effects of ocular hypertension and, therefore, electrophysiological response amplitude measures from the hypertensive eyes were normalized with respect to those obtained from the contralateral eye of the same animal at the same recording session. Recordings were made under anesthesia and paralysis maintained with periodic infusion of ketamine (15 mg/kg) and constant infusion of norcuronium (Hansen Ophthalmic Laboratories, Iowa City, IA) while an s.c. needle placed at the base of the neck on the back comprised the indifferent electrode. Records of the visually-evoked cortical potential (VECP) were recorded using an active electrode located on the scalp immediately anterosuperior to the inion on the midline. The s.c. needle at the glabella comprised the indifferent electrode. Conventional ERG responses were elicited with diffuse flash stimuli of approximately 10 μscc duration, which were generated by a Grass Model P33 photostimulator (Astro Med, West Warwick, RI). The stimulus, positioned at 10 cm anterior to the cornea on the visual axis, subtended approximately 50° of visual angle centered on the fovea. Flash responses and oscillatory potentials (OPs) were elicited with flashes of 124 photopic cd s/m² intensity delivered at 10-second intervals after 5 minutes of dark adaptation at an ambient room illumination of approximately 0.05 footcandles. This initial 5-minute period of dark adaptation was chosen to ensure that the adaptational state during recording was stable and consistent from one recording session to the next. Under these conditions, the adaptational state was determined by stimulus intensity in combination with stimulus frequency. Response amplitude and kinetics stabilized rapidly after onset of a stimulus series. In fact, small adaptational effects were typically observed for the first two or three responses in a series and these initial responses were not included in response averages. For flicker responses, 30 Hz stimulus trains of 512 msec duration and flash intensity of 78 photopic cd s/m² were delivered every 1 second. Bandpass filtering from either 3–1000 Hz (flash and flicker responses) or 100–1000 Hz (OPs) was used in conjunction with 60 Hz notch filtering. Flash and OP responses obtained under these conditions likely reflect activity of both rod and cone photoreceptors with a relatively greater contribution from cone activity. Thirty Hz flicker responses reflect predominantly cone-driven activity.

For multifocal recordings, stimuli were generated on a 21-inch monitor (Radius Intelicolor; Radius Inc., San Jose, CA) using VERIS 1 software and video driver board (Electro Diagnostic Imaging, San Mateo, CA) and consisted of an array of 64 hexagonal elements of equal size. The stimulus field was positioned such that the fovea projected to the center of the central stimulus element. At the test distance of 30 cm, the stimulus field subtended approximately 50° of visual angle and thus illuminated the same retinal area which was stimulated for conventional recordings. The luminous intensity of each stimulus element was temporally modulated in a stepwise fashion at a frame rate of 67 Hz between a maximum intensity of 95 cd/m² (white) and a minimum intensity of 5 cd/m² (black) according to a binary m-sequence. An m-sequence of 15 (2¹⁰ stimulus frames) was used resulting in records of approximately 8 minutes duration. Signals were bandpass filtered from 3–300 Hz in conjunction with 60 Hz notch filtering.

Recordings of the visually-evoked cortical potential (VECP) were made using an active electrode located on the scalp immediately anterosuperior to the inion on the midline. The s.c. needle at the glabella was used as the reference and an s.c. needle placed at the base of the neck on the back comprised the indifferent electrode. Responses were elicited using the same stimuli, delivered at 2-second intervals.

Figure 2. Average IOP (± SD) for the hypertensive (OD) eyes of all 18 animals in both treatment groups over the course of the study. Note that three baseline IOP measures were obtained before laser treatment and were equal to approximately 20 mm Hg. The timing of electrophysiology measurements is indicated (T1, T2, T3). The pressure of all laser-treated eyes rose rapidly soon after laser treatment. However, the peak IOP and subsequent IOP levels varied considerably among the test eyes as indicated by the large standard deviations. Pressure histories for both eyes of the individual animals in each treatment group are summarized in Table 1.
Memantine Treatment of Glaucoma in Monkeys

TABLE 3. Serum and Vitreous Memantine Concentrations in Memantine-Treated Animals

<table>
<thead>
<tr>
<th>Monkey #</th>
<th>2 mos</th>
<th>4 mos</th>
<th>6 mos</th>
<th>8 mos</th>
<th>10 mos</th>
<th>12 mos</th>
<th>14 mos</th>
<th>15 mos</th>
<th>16 mos OD</th>
<th>16 mos OS</th>
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<td>1.7</td>
<td>1.4</td>
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<td>1.2</td>
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<td>1.5</td>
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<td>97</td>
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<td>1.5</td>
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<td>1.7</td>
<td>1.4</td>
<td>0.6</td>
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<td>108</td>
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Counts of Cells in Retinal Ganglion Cell Layer

The methods for counting RGCs are described in detail in the accompanying article and summarized briefly here. Fixed retinas were flat mounted and 3 mm x 5 mm samples obtained from eight regions including one centered on the fovea. After paraffin embedding, ten radial sections were obtained from each sample region. For the sample centered on the fovea, sections were obtained from the region of highest RGC density at 500 to 700 μm from the center of the foveal pit.

intervals, as that used for the ERG flash and OP responses. VECP signals were bandpass filtered from 3–1000 Hz in conjunction with 60 Hz notch filtering.

The same recording sequence was used for all animals at all time points: 1) multifocal ERG, OD; 2) conventional ERG (VECP), OD; 3) multifocal ERG, OS; and 4) conventional ERG (VECP), OS. During recording, the contralateral eye was always occluded. After placement of the contact lens electrode, retinoscopy determined the best spherical equivalent lens power to make the retina optically conjugate to the multifocal ERG stimulus monitor. This lens (typically 3 diopters) was then positioned at 1 cm anterior to the cornea. The stimulus monitor was then positioned at 30 cm anterior to the cornea such that the estimated visual axis projected to the center of the stimulus field. A series of multifocal recordings of approximately 2 minutes duration (m sequence = 13) was then used to adjust the monitor position such that the fovea projected to the center of the stimulus field and a clear amplitude maximum was obtained for the first-order response associated with the central stimulus element. Since a prominent central macular peak was always observed, even in eyes with severe retinal ganglion cell loss, this method provided reliable stimulus alignment in all eyes of both treatment groups. The precision of this method for stimulus alignment was verified in several eyes by optically projecting the fundus onto the stimulus monitor and noting the location of the optic nerve head and macular image. Stimulus alignment was also verified for each recording by observing the location of the optic nerve head projection (response minimum) in the first-order response trace array. After stimulus alignment, a multifocal recording of approximately 8 minutes duration (m sequence = 15) was made. Maintenance of neuromuscular block ensured that proper stimulus alignment was maintained during the entire recording session. The stimulus monitor was then covered with a light-tight shield, the corrective lens was removed, and the xenon flash stimulator was positioned. After a 5-minute period of dark adaptation, conventional recordings of the flash, OP, and flicker ERG responses were made in order. At the final time point (T3), the VECP response was recorded after the flicker ERG response.

A.  
B.  
C.  

FIGURE 3. ERG responses obtained using conventional methods from the same normotensive (OS) eye. (A) Flash response to a stimulus of 10 μsec duration delivered at 0 msec. Amplitude of the a-wave and b-wave peak voltage was measured as indicated by the arrows. Average of ten responses. (B) Oscillatory potential response to the same stimulus used to elicit the flash response in (A). Response amplitude was measured as the RMS voltage from 10 to 75 msec after the stimulus. Average of 25 responses. (C) Flicker response to a 30 Hz stimulus train of 512 msec duration beginning at 0 msec. Amplitude of the peak-to-peak voltage of the last three response cycles. Trace is average of 30 responses. See Methods for a more detailed description of the stimulus and recording parameters.
After staining with hematoxylin/eosin, the nuclei in the RGC layer were counted along the entire 3 mm length of all ten sections from each sample region. For sections from the perifoveal region, cell counts were made using a Bioquant imaging system and stereology software (R&M Biometrics, Nashville, TN). Sections from all other sample regions were counted manually. See Figure 2 in the accompanying article for the precise locations from which retinal histologic sections were obtained.

**Determination of Mean IOP**

For each animal, OD IOP was plotted as a function of time over the duration of the study. Mean IOP elevation was estimated over each time interval (T0→T1, T0→T2, or T0→T3) by first integrating the area of this plot over the limits defined by the time at which the first elevated IOP measure was obtained (soon after laser treatment; T0) and the time at which the electrophysiological measures were obtained (either T1, T2, or T3; Fig. 1). The integral was then divided by the number of days in that interval to yield the mean IOP for interval T1 (expressed in mm Hg) for each animal. This method is illustrated in Figure 1 for the OD IOP plot from one animal (M94). Values obtained from all animals for the average of the three baseline (before laser treatment) measures, the peak IOP measure, and the mean of the IOP integral for all three time intervals are summarized in Table 1, where values for the normotensive (OS) eyes are shown in parenthesis. The range and mean (± SEM) values are also shown for each treatment group. Since IOP in the normotensive eyes was similar to baseline at all time points, values for the three intervals were determined for these eyes as the simple average of measures over each interval.

**Statistical Analysis**

Linear regression analysis (see Figs. 4, 6, 7, and 9) was made using the method of least squares. To test the hypothesis that the slopes of the regressions for two data sets were the same, it was first verified that each data set could be reasonably well fit to a linear model. The probability (P) that any difference in slope could have occurred by chance was then determined by application of a general linear models
procedure for analysis of covariance. All results of ERG and VECP measures were analyzed in this manner and are summarized in Table 2.

RESULTS

Ocular Hypertension

The average IOP for all eighteen ocular hypertensive (OD) eyes of both treatment groups is summarized for the duration of the study in Figure 2. Note that laser treatment was followed by an initial decrease and then a dramatic rise in IOP. The magnitude of this initial elevation, as well as the pressure over the remainder of the study, varied considerably among eyes in both groups. This is summarized in Table 1 where mean IOP, determined for each animal by calculating the IOP integral according to Figure 1, is listed for each of the three electrophysiological time intervals (T1, T2, T3) for the hypertensive eyes of all animals in the two treatment groups. Included for each animal in Table 1 is the average of the three baseline IOP measures and the peak IOP observed soon after laser treatment. Note that at T1, approximately 3 months after IOP elevation, the range and average values are similar for the two treatment groups. The average IOP declines slightly over the following 2 months (T1 → T2) and this decline is somewhat greater in the memantine-treated animals at T2. Over the ensuing 11 months (T2 → T3), average mean pressure decreased slightly in the vehicle-treated animals while a greater decrease is seen in memantine-treated animals over this same period. The relatively greater decline, from 5 to 16 months after IOP elevation in average mean IOP for memantine-treated animals, reflects a decrease for eyes having the highest pressure in this group. IOP measures from the contralateral normotensive (OS) eyes are also included (in parenthesis) in Table 1. Since these measures showed only small variations from baseline, the mean values for the three time intervals were obtained by simply averaging the individual measures over that time interval. Note that the mean OS IOP measures are almost identical for the two treatment groups, indicating that memantine treatment likely had little or no effect on pressure in the normotensive eyes.

Serum and Vitreous Memantine Levels

Serum and vitreous memantine levels are summarized for animals in the memantine-treated group in Table 3. Note that serum levels of approximately 1 μM were obtained at all time points with the exception of the samples collected at 2 months when values ranging from 0.03 to 0.8 μM were obtained. Vitreous memantine levels obtained at the end of the study were also in the 1 μM range. Memantine was not detected in the serum or vitreous from animals in the vehicle-treated group (data not shown).

ERG Responses

Conventional ERG responses from a normotensive eye of one animal are illustrated in Figure 3. For flash responses, amplitude of the a-wave and b-wave peak voltage was measured. Amplitude of the OP was measured as the root mean squared (RMS) voltage over a window extending from 10 to 75 msec after the stimulus. Amplitude of the 30 Hz flicker response was measured as the average peak-to-peak voltage for the last three cycles of the response train. For each response measure, the amplitude of the hypertensive eye was normalized with respect to the value obtained from the normotensive eye (OD/OS). These values are plotted, for results obtained at the end of the study (T3) from each animal, in Figure 4 as a function of mean IOP elevation in the hypertensive eye.

Clearly, ocular hypertension has little or no effect on flash (Figs. 4A and 4B) or OP (Fig. 4C) responses and only a small effect on the 30 Hz flicker (Fig. 4D) response measures obtained from either treatment group. Similar results were obtained from analysis of the flash, OP, and flicker responses obtained at the earlier time points (T1, T2; data not shown). It was shown in an earlier report that severe chronic ocular hypertension (COHT)-induced loss of cells in the ganglion cell layer is associated with only a modest effect on these measures and that they have limited utility as a measure for ganglion cell injury in this model. In the same report, however, it was shown that the amplitudes of components of the multifocal ERG
response are highly correlated with the level of COHT-induced loss of cells in the retinal ganglion cell layer.

The left-hand panels of Figure 5 illustrate first order (A) and second order (C) multifocal ERG responses obtained from a normotensive eye. In each panel, responses from macular retina (approx. central 16°) are highlighted in the trace array and averaged to provide the macular responses in the right-hand panels (B, first order; D, second order). In an earlier paper it was reported that peak-to-peak measures in the first order response (N2-P2, and P2-N3) and second-order response (P-N) are highly correlated with the degree of COHT-induced loss of cells in the retinal ganglion cell layer. This is shown in Figure 6, where normalized (OD/OS) macular response amplitude measures are plotted as a function of normalized (OD/OS) perifoveal RGC counts for seven animals in the vehicle-treated group and eight animals in the memantine-treated group. Figures 6B–D show clearly that RGC loss is strongly correlated with a decreased amplitude of these response measures. For comparison, the amplitude of the first negative peak (N1) in the first order response is plotted in (A). Note that even severe RGC loss had little effect on amplitude measures of this response component. Thus, specific components of the multifocal ERG response provided a functional measure of injury to RGCs and these same measures may be used to determine the degree of functional loss associated with chronic ocular hypertension. For this reason, values for these peak-to-peak measures of the macular response from the hypertensive eye of each animal were normalized with respect to those obtained from the normotensive eye (OD/OS) and plotted as a function of mean OD IOP elevation. Results from recordings made at approximately 3 months (T1) after induction of ocular hypertension are summarized in Figure 7. Note that, for the measures...
plotted in Figures 7B–D, response amplitude in the hypertensive eye of vehicle-treated animals is inversely correlated with the mean level of IOP exposure; that is, higher IOP is associated with decreased response amplitude. However, Figure 7A shows that, for both treatment groups, IOP history has little or no effect on the amplitude of peak N1. The slope of the correlation (represented by the linear regression lines) evident in Figures 7B and 7D provides an expression for the relation between mean IOP elevation and functional measures of retinal injury changes over the three measurement time points, these results do not illustrate how these response amplitudes vary over the same time interval. For this purpose, amplitude, expressed as nV/degree², of responses from the hypertensive (OD) eyes of each treatment group were averaged and are plotted at the three measurement time points in Figure 8. Although peak N1 amplitude (Fig. 8A) changes little over time, there is a trend toward increasing amplitudes for peak N2-P2 (Fig. 8B) and peak P-N (Fig. 8D) for both treatment groups. Although measures of peak P2-N3 (Fig. 8C) show the greatest variation among animals of both groups, vehicle-treated animals show a trend for increasing response amplitude while memantine-treated animals show little change over time. Thus, measures of retinal function which are not correlated with RGC injury (N1) show little effect of IOP elevation and little or no change over time, while measures of retinal function which are highly correlated with RGC injury (N2-P2 and P-N) and severely attenuated by elevated IOP show a tendency to increasing response amplitude over time in both treatment groups. These findings are consistent with ocular hypertensive insult that is selective for injury to RGCs and is associated with functional loss that is evident by 3 months after IOP elevation.

**VECP Responses**

The VECP requires transmission of the visual signal from the retina to visual cortex. Thus, injury to retinal ganglion cells and their axons will directly (and, perhaps, indirectly) affect this response. VECP responses were recorded only at the final time (T3) immediately before sacrifice. The VECP response obtained from a normotensive (OS) eye is illustrated in Figure 9A. It was...
previously shown that VECP amplitude is highly positively correlated with the number of surviving cells in the ganglion cell layer of COHT monkey eyes. In Figure 9B, normalized n1-p1 amplitudes are plotted, for both treatment groups, as a function of mean IOP. Note that the slope for the memantine-treated group is much less than that obtained for the vehicle-treated group and this difference is significant at the $P = 0.04$ level (see Methods for statistical analysis); that is, treatment with memantine is associated with a relative preservation of the VECP response, even in animals with highly elevated IOP. A comparison of the slopes obtained from measures on the VECP responses of both groups is included in Table 2. Note also that the slopes for the ERG responses from the two groups are similar at this time point (T3).

Although the relationship between mean IOP history and VECP amplitude in Figure 9B shows that IOP elevation was associated with significantly greater VECP responses in memantine-treated animals compared with vehicle-treated animals, this comparison is limited by the fact that there are fewer memantine-treated animals with very high IOPs at T3. For example, the two highest mean IOPs in the memantine group are approximately 43 and 57 mm Hg while there are three vehicle-treated animals with mean IOP ranging from approximately 49 to 57 mm Hg. However, eyes having the highest mean IOP at T1 (see Table 1) from both groups showed histologic evidence of severe RGC loss at T3. For this reason, VECP amplitude measures are re-plotted as a function of RGC counts in Figure 10. From this figure, it is apparent that three animals in each treatment group suffered the loss of approximately 72% or more of the RGCs and that the slope for an effect of RGC loss on VECP response is much less for the memantine-treated animals than for vehicle-treated animals (though this difference was significant at only the $P = 0.06$ level). These results show that VECP responses of apparently normal amplitude were obtained from all three memantine-treated eyes which lost $>70\%$ of their RGCs and are consistent with the results which compare VECP response amplitude with mean IOP history (see Fig. 9B).

### Safety of Memantine Treatment

The same measures used for characterizing the effects of ocular hypertension on the function of the retina and central visual pathways were also used to determine whether daily oral dosing for approximately 16 months with 4 mg/kg memantine was associated with any adverse effects on visual pathway function. For this purpose, measures obtained at T3 from all normotensive (OS) eyes of the two treatment groups were compared. Results of electrophysiological recordings using both conventional and multifocal methods are summarized in Figure 11. Mean peak amplitude measures for the flash, OP, and flicker ERG responses as well as the VECP response are shown in Figure 11A where values obtained for the memantine-treated group are normalized with respect to the vehicle-treated group. In all panels of this figure, values for the averages of the normotensive eyes from vehicle-treated animals were set to equal 1.00 and included so that SE bars for this group could be shown. Figure 11C shows a similar comparison of the time-to-peak values for the same response measures represented in Figure 11A. Note that there were no significant

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**Figure 8.** Averaged response amplitudes (in nV/degree$^2$) for hypertensive (OD) eyes from vehicle- (filled squares) or memantine- (open circles) treated animals are plotted at the three measurement time points. Average values ($\pm$ SEM) for vehicle-treated eyes at T1, T2, and T3, respectively, are as follows: (A) N1: 6.4 $\pm$ 0.48, 6.9 $\pm$ 0.39, 6.9 $\pm$ 0.41; (B) N2-P2: 8.0 $\pm$ 1.30, 8.9 $\pm$ 1.5, 10.0 $\pm$ 1.40; (C) P2-N3: 1.9 $\pm$ 0.40, 2.7 $\pm$ 0.90, 2.7 $\pm$ 0.90; (D) P-N: 3.8 $\pm$ 0.62, 4.2 $\pm$ 0.75, 5.3 $\pm$ 1.03. Average values for the memantine-treated animals are: (A) N1: 6.3 $\pm$ 0.49, 6.1 $\pm$ 0.88, 6.1 $\pm$ 0.67; (B) N2-P2: 9.3 $\pm$ 2.10, 10.2 $\pm$ 3.1, 12.5 $\pm$ 2.60; (C) P2-N3: 2.7 $\pm$ 0.90, 2.9 $\pm$ 1.00, 2.6 $\pm$ 0.90; (D) P-N: 4.9 $\pm$ 0.73, 5.5 $\pm$ 1.20, 6.7 $\pm$ 1.20. There was no statistically significant difference for the average values of the two groups at any point ($P > 0.05$, student's two-tailed t-test).
differences in the amplitude or timing of any measure of conventional responses from the two treatment groups. Results for measures of amplitude and timing of peaks in the first order macular multifocal response are shown in Figures 11B and 11D, respectively. As for the conventional responses, there were no significant differences between the two treatment groups for any measure. There was also no difference for amplitude measures of the second order macular multifocal response (P-N; results not shown). Time-to-peak measures of the second order response were not made.

**Glutamate Levels in Vitreous Samples**

Results from measures of glutamate levels in vitreous samples are summarized in Figure 12. Analysis of samples obtained immediately before sacrifice from the nine memantine-treated animals showed that the glutamate levels were, on average, almost identical in the hypertensive and normotensive eyes. Vitreous samples were obtained from only six of the vehicle-treated animals since one of the animals was lost before T3 (M79) and samples from two other animals (M92 and M106) were contaminated at the time they were collected and not analyzed. Although the glutamate level in the vitreous samples from vehicle-treated animals was higher in the hypertensive eyes (1.18 μM) than that obtained from normotensive eyes (0.92 μM), this difference was not statistically significant.

**DISCUSSION**

**Ocular Hypertensive Injury**

Components of the multifocal ERG response reflect the activity of RGCs in both humans\(^{48}\) and monkeys.\(^{43-49,50}\) The amplitudes of components of the multifocal ERG are also highly correlated with the degree of histologically measured RGC loss in monkeys with experimental glaucoma.\(^{42,51}\) In the present study, the same measures (Fig. 6) indicate that eyes having the highest mean IOPs suffered severe deficits in RGC function that were apparent by 3 months after induction of ocular hypertension (Fig. 7). Evidence of injury in these same eyes was also apparent as changes in optic nerve head morphology observed in either stereo fundus photographs obtained as early as 2 months (data not shown) or from confocal tomographic scans obtained as early as 3 months after elevation of IOP (see companion article\(^{52}\)). Thus, both functional and anatomic measures indicate that, in eyes with the highest IOPs, injury progressed rapidly after induction of ocular hypertension and that considerable injury had occurred by the earliest measurement time points.

Although RGC injury was relatively rapid and severe in animals with the highest mean IOP, this insult can easily be distinguished from any contribution from an ischemic insult associated with interruption of the retinal arterial blood supply. An insult of this sort would have consequences on retinal function that are not limited to RGCs and would be evident in the flash ERG response. The observation that amplitude measures of the flash ERG a-wave, b-wave, and OP responses were unaffected at any time point by elevated IOP (Fig. 4) is not consistent with diffuse retinal ischemia, although focal ischemic insults or more subtle retinal hypoxia cannot be excluded on this basis. However, histologic examination also showed no
gross evidence for injury outside of the RGC layer and no evidence of retinal ischemia was seen in fundus photographs obtained at multiple times after IOP elevation (see companion article52).

**Efficacy of Memantine Treatment**

For eyes with the highest IOPs, the amplitudes of RGC-dependent components of the multifocal ERG response were typically larger in memantine-treated animals when compared to responses obtained from vehicle-treated animals with similar IOPs (Fig. 7, Table 2). This difference, although modest, was significant for four of six measures obtained at 3 (T1) or 5 months (T2) after IOP elevation. No difference in the two treatment groups was observed for the final measurement at 16 months (T3). It may be concluded that, in these eyes, the rate of injury to RGCs was significantly slowed by memantine treatment but that this reduction was not great enough to be evident at later time points. This apparent loss of a treatment effect may be explained by the model shown in Figure 13, where the number of surviving RGCs (RGC injury) is plotted as a function of time after induction of ocular hypertension. A linear rate of RGC loss is used here only for simplicity. According to this model, RGCs are lost at some rate represented by the slope of the plot. Treatment with memantine reduces the rate of RGC injury/loss but the magnitude of any difference in the level of injury in the two eyes will vary greatly with the measurement time point. For example, the difference will be greatest at the point when the untreated eye reaches a minimum (maximal RGC loss) and will decrease for measures made at earlier or later time points. It is also apparent from the model that the greater the rate of injury, the more critical the timing for measurement of any treatment effect. The observation of only modest levels of protection at early times, as well as the failure to observe any effect of memantine treatment to preserve ERG responses obtained at the end of the experiment, may derive, at least in part, from a relatively steep rate of injury which is suggested by the severe levels of injury observed at the early measures in animals having the highest IOP elevation.

The finding that VECP responses obtained at the termination of the study are relatively well preserved in memantine-treated animals (Figs. 9B, 10) was somewhat surprising since ERG measures of RGC injury were similar for the two treatment groups at this time point. This result may reflect the relatively greater survival in memantine-treated animals of a particular RGC subtype whose activity is not reflected in the ERG measures but makes a relatively large contribution to generation of the VECP. It seems more likely, however, that this finding may be explained by an effect of memantine treatment to enhance...
the ability of surviving RGCs to drive activity in the visual cortex; that is, treatment with memantine may be associated with plastic changes occurring at more central levels of the visual pathways. In this scheme, loss of RGC axonal inputs to the lateral geniculate nucleus and, perhaps, tectum leads to loss of innervation of neurons in these nuclei as well as the cortical neurons with which they normally make synaptic contact. Axon terminals of surviving RGCs and the brainstem neurons which they innervate may grow to form new synapses on neighboring target neurons whose inputs have been lost. Thus, a single surviving RGC axon might now drive a population of neurons in the brainstem and cortex which is greater than that obtained before ocular hypertensive injury. The consequence of this “divergence gain” for signal transfer at the brainstem and cortex would be that stimulation of the surviving RGCs with a light flash now evokes a greater level of cortical activity than would be seen if the same population of RGCs were stimulated before ocular hypertensive injury. In this sense, the effect of memantine treatment on central visual pathways may be described as one of enhancement rather than protection.

Results from in vitro26,27 and in vivo53–56 studies suggest that a plasma concentration of approximately 1 μM should be effective to reduce injury mediated by excitotoxic glutamatergic activation of NMDA-type receptors. Plasma levels on the order of 1 μM concentration or greater were obtained at all time points greater than 2 months for the memantine-treated animals of this study (Table 3). Plasma levels for samples obtained at the 2-month point ranged from 0.03 to 0.80 μM. Thus, at 2 months, plasma levels of memantine were considerably below the values obtained at later times and, in some animals, were well below the target level of 1.0 μM. Great care was exercised during oral dosing to ensure that the compound was completely ingested. For this reason, it is unlikely that the lower plasma levels resulted from problems with oral dosing. However, the plasma levels may reflect the saturation of a depot effect before the 4-month point. In any case, plasma levels of memantine were lowest during the period where the rate of injury in the eyes with the highest IOPs was greatest. It is thus likely that a greater protective effect of memantine would have been obtained if a higher plasma concentration had been achieved during this early phase of the study.

**Vitreal Glutamate Levels**

Vitreal glutamate levels are elevated in dogs57 and humans58 with primary glaucoma, and also in monkeys with experimentally induced chronic glaucoma.59 In these instances, it was presumed that elevated vitreal glutamate resulted from an accumulation of retinal extracellular glutamate with subsequent diffusion to the vitreous. More recent analyses of vitreal samples from monkeys59 or rats60 with experimental glaucoma as well as glaucoma patients undergoing vitrectomy61 failed to demonstrate any elevation of glutamate above levels found in normotensive eyes. Results from analysis of samples obtained at 16 months after IOP elevation in the present study showed no significant elevation of vitreal glutamate, when compared to the normotensive contralateral eye, in either treatment group (Fig. 12). However, the concentration of glutamate in the retinal extracellular space may not be elevated at every stage of the disease process and, furthermore, it is possible that normal levels of vitreal glutamate may be observed at times when the local retinal concentration is at pathologic levels. Thus, an observation of either normal or elevated levels of extracellular glutamate may be consistent with a contribution of glutamatergic excitotoxicity to the injury of retinal cells. It has recently been reported that experimental glaucoma results in lower levels for expression of glutamate transporters in the rat retina62 and, based on these findings, it was concluded that glaucoma may be associated with an increased potential for glutamatergic excitotoxicity to RGCs. It has also been reported that, in monkey, prior elimination of photoreceptors provides protection of the overlying RGCs from injury associated with the subsequent induction of experimental glaucoma, results that were interpreted as consistent with a contribution from glutamatergic excitotoxic insult to RGC injury in this model.63

**Safety of Memantine Treatment**

Daily oral dosing with 4 mg/kg memantine was administered to nine animals over an approximately 16-month period. Plasma levels of memantine were well below the target level of 1.0 μM. Thus, at 2 months, plasma levels of memantine were considerably below the values obtained at later times and, in some animals, were well below the target level of 1.0 μM. Great care was exercised during oral dosing to ensure that the compound was completely ingested. For this reason, it is unlikely that the lower plasma levels resulted from problems with oral dosing. However, the plasma levels may reflect the saturation of a depot effect before the 4-month point. In any case, plasma levels of memantine were lowest during the period where the rate of injury in the eyes with the highest IOPs was greatest. It is thus likely that a greater protective effect of memantine would have been obtained if a higher plasma concentration had been achieved during this early phase of the study.

**FIGURE 12.** Average glutamate levels obtained from vitreous samples from both eyes of animals in both treatment groups at approximately 16 months after elevation of IOP in the right eye. See Methods for details regarding the assay used to determine glutamate concentration. Average (± SEM) values for six vehicle-treated animals are: (OD) 1.170 ± 0.151 μM, (OS) 0.905 ± 0.055 μM. Average values for nine memantine-treated animals are: (OD) 0.898 ± 0.055 μM, (OS) 0.927 ± 0.072 μM. The difference between the measures for OD and OS from the vehicle treated animals was significant at the 0.09 level. Samples from only six vehicle-treated animals were tested, since one animal (M79) was lost before T3 and vitreous samples from two other animals (M93, M106) were contaminated at the time of collection and not measured.

**FIGURE 13.** Hypothetical model for the effect of chronic ocular hypertension on RGC injury (loss) over time. Treatment efficacy is shown as a reduction in the rate (slope) for RGC loss. Note that the magnitude of any treatment effect depends strongly on when the measure is made.
memantine concentrations greater than 1 μM were obtained for over 9 months in most memantine-treated animals of the study (Table 3). In addition, a concentration on the order of 1 μM was also found in vitreal samples from both eyes obtained at the end of the study. Results of a comparison of electrophysiological measures obtained from the normotensive (OS) eyes at the end of the study. Results of a comparison of electrophysiological measures obtained from the normotensive (OS) eyes of both treatment groups showed that these levels of memantine were not associated with a significant effect on any measure of function of the retina and central visual pathways (Fig. 11).

Thus, electrophysiological measures of function in RGCs (ERG) as well as neurons in the central visual pathways (VECP) show that memantine treatment had modest effects in reducing ocular hypertensive injury. These same measures also showed that treatment with memantine was without significant effect on the normal function of the retina and central visual pathways.

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References


