Evaluation of the retina and optic nerve in a rat model of chronic glaucoma using in vivo manganese-enhanced magnetic resonance imaging

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Glaucoma is a neurodegenerative disease of the visual system. While elevated intraocular pressure is considered to be a major risk factor, the primary cause and pathogenesis of the disease are still unclear. This study aims to employ in vivo manganese-enhanced magnetic resonance imaging (MEMRI) to evaluate dynamically the Mn2+ enhancements in the visual components following an induction of ocular hypertension in a rat model of chronic glaucoma. The episcleral and limbal veins were photocoagulated unilaterally in the right eye using an argon laser to maintain a consistent elevation of intraocular pressure by about 1.6 times above the normal level. Two and six weeks after glaucoma induction, MnCl2 solution (50 mM, 3 μL) was injected intravitreally into both eyes, and MEMRI was performed 2 to 5 h after injection. Results showed a delayed increase in T1-weighted signal intensity in the glaucomatous optic nerve at 6 weeks but not 2 weeks after glaucoma induction. In addition, there was an accumulation of Mn2+ ions in the vitreous humour of the glaucomatous eye, with a high concentration of Mn2+ ions at the optic nerve head and the retina. These MEMRI findings may help understand the disease mechanisms, monitor the effect of drug interventions in glaucoma models and complement the conventional techniques in examining the glaucomatous visual components.

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Keywords: Chronic glaucoma; Rat; Intraocular pressure; Optic nerve; Fast axonal transport; Manganese-enhanced magnetic resonance imaging

Introduction

Glaucoma is a neurodegenerative disease of the visual system characterized by retinal ganglion cell (RGC) death, optic nerve head (ONH) damage and progressive visual field loss (Thanos and Naskar, 2004). It is the second major cause of blindness in the world (Quigley and Broman, 2006). While elevated intraocular pressure (IOP) is considered to be a major risk factor, the primary cause to the disease mechanisms is still unclear (Kaufman, 1999; Morrison et al., 2005). Quantitative assessments of optic nerve (ON) axonal loss or brain changes in experimental models of glaucoma are typically performed postmortem in histological tissue sections (Lindsey et al., 2007; Morrison, 2005) or by electrophysiological techniques (Li et al., 2006a). However, these methods either fail to provide a global view of the brain or do not allow repeated measures for longitudinal studies (van der Linden et al., 2004). To understand the exact mechanisms of glaucomatous changes, there is a need to develop a novel, in vivo and three-dimensional method to investigate into the integrity of the primary visual system longitudinally.

Magnetic resonance imaging (MRI) provides a non-invasive tool to study the structural, metabolic and functional details of the inner depth of the body in vivo. Among the MRI techniques, diffusion tensor imaging has successfully detected and differentiated structurally the axon and myelin degeneration upon an evolving white matter injury in the mouse ON after retinal ischemia (Song et al., 2003; Sun et al., 2006). It has also been shown that the lamina-specific structures and functional responses in the retina can be resolved using Gd-DTPA contrast-enhanced MRI and high-resolution functional MRI (Cheng et al., 2006). Recently, manganese-enhanced magnetic resonance imaging (MEMRI) has been increasingly used to study both structural and functional changes in the central nervous system without reliance on hemodynamic response. It has been used to enhance the contrast in studies of neuroarchitecture (Aoki et al., 2004a), to trace neuronal pathways (Pautler, 2004; Pautler et al., 1998), to detect activated regions in the brain (Silva et al., 2004) and to investigate cerebral ischemic injury (Aoki et al., 2004b; Yang and Wu, 2008). Mn2+ ions are paramagnetic in nature and can shorten the T1 relaxation time of the surrounding water protons. They act as a calcium analogue and enter the
intracellular space via L-type voltage-gated calcium channels upon neuronal activation (Pautler, 2006). A fraction of the ions is then sequestered in the endoplasmic reticulum or Golgi apparatus and actively transported along the microtubules via fast axonal transport (Pautler, 2004; Van der Linden et al., 2007; Watanabe et al., 2004). On the other hand, a recent study has demonstrated the independence of electrical activity to Mn²⁺ uptake in the eye (Bearer et al., 2007), suggestive of alternative channels, e.g. divalent metal transporters (Takeda, 2003) for Mn²⁺ transport. Glial uptake and diffusion may also contribute to the cerebral pattern in Mn²⁺ enhancement (Watanabe et al., 2004). In the current study, we aimed to examine the Mn²⁺ transport in the normal and glaucomatous eyes upon intravitreal injection and to correlate the in vivo results with previous histological findings. These may help optimize the investigation into the integrity of the visual system and the possibility of ocular drug delivery into the glaucomatous eyes.

MEMRI has been used to examine the axonal transport of the central nervous system in rodents. As Mn²⁺ can access the nervous system intraxonally without the reliance on hemodynamic response, studies evaluated the axonal transport rate in Alzheimer’s disease (Smith et al., 2007) and diabetes (Serrano et al., 2007) and upon drug treatment (Smith et al., 2007). On the other hand, axon degeneration has been demonstrated by the blockade of Mn²⁺ transport at sites of radiation-induced injury (Ryu et al., 2002) and ON crush (Thuen et al., 2005) upon intravitreal injection. By applying MEMRI, it is also possible to compare the cross-sectional areas in the prechiasmatic regions of the ON induced with optic glioma (Banerjee et al., 2007). In the experimental rat model of chronic glaucoma in our laboratory, chronic IOP elevation induces a 3% RGC loss per week across the 8-week experimental period (Li et al., 2006a). Other reports indicated an early damage from an elevated IOP in the superior regions of the rat ON (Morrison, 2005, Quigley et al., 1987; Wang et al., 2004), while atrophy of large fibers was observed in areas of the ON with mild damage (Johnson et al., 2000; Quigley et al., 1987). It was also suggested that the axoplasmic flow happened to be disturbed upon chronic IOP elevation (Dandona et al., 1991; Johnson et al., 2000; Khosla et al., 1982). In experimental mouse ocular hypertension, the ON mean axon density and total number of axons in the laser-treated eyes were found to be significantly less than in the control eyes (Mabuchi et al., 2003; Mabuchi et al., 2004). Therefore, we hypothesize that there would be a reduction in Mn²⁺ transport along the glaucomatous ON, especially in the superior regions.

Further, previous studies using high-resolution MEMRI detected layer-specific retinal functional adaptation (Berkowitz et al., 2006), as well as changes in intraretinal signal intensities in rat models of ON injury and choroidal melanoma (Berkowitz et al., 2007; Braun et al., 2007). In addition to the RGC loss, it was shown that ocular hypertension appeared to accompany with ischemia, axonal swelling and mechanical alteration of the laminar layers at the ONH (Gross et al., 2003; Kaufman, 1999). To account for these, we would attempt to test if there would be signal changes in the retina and the ONH between the glaucomatous and the control eyes.

Lastly, Mn²⁺ has been applied trans-sclerally, transcorneally and intravitreally to evaluate its distribution in the eyeballs for ocular drug delivery (Li et al., 2004; Molokhia et al., 2007). The glaucoma model in the current study involves laser photoagulation of the episcleral and limbal veins, which contributes to the obstruction of venous outflow in rats (Li et al., 2006a; Morrison et al., 1995). Blockade of this drainage would induce a 1.6-fold increase in IOP (Chan et al., 2007; Fu et al., 2007; Li et al., 2006a,b). By employing dynamic MEMRI, we hypothesize that the usual pattern of Mn²⁺ clearance in the glaucomatous eyeball will be perturbed upon intravitreal injection.

To our knowledge, this is the first attempt to apply dynamic MEMRI to investigate the chronic glaucoma within the rat primary visual system in vivo. This is particularly valuable as we may be able to apply MEMRI to understand the disease mechanisms and resolve the functional loss and recovery of neuronal connectivity (van der Zijden et al., 2007) upon drug treatment (Chan et al., 2007; Fu et al., 2007).

Methods

Animal preparation

Sprague-Dawley female rats (250–280 g, 3 months old, N = 15) were reared in a temperature-controlled room subjected to a 12-h light/12-h dark cycle with standard chow and water supply ad libitum. They were divided into 3 groups (see Fig. 1). Except for the control group, the rats were induced for ocular hypertension unilaterally in the right eye by photoagulation of the episcleral and limbal veins using an argon laser. A second laser treatment in the same settings was applied 7 days later to block the neovascular flow and maintain a consistent IOP elevation by about 1.6 times above the normal level. This technique was adopted from the method by WoldeMussie et al. (2001) and has been adopted in our laboratory with track record of recent publications (Chan et al., 2007; Fu et al., 2007; Li et al., 2006a,b). The IOPs of the glaucomatous and control eyes were measured using a calibrated tonometer (Tonopen-XL, Mentor, Norwell, MA, USA) and were found to be 15.01 ± 0.50 mmHg and 14.00 ± 1.02 mmHg, respectively, before laser treatments (p = 0.41) and 24.78 ± 0.88 mmHg and 15.21 ± 0.30 mmHg, respectively, after two laser treatments (p < 0.01). After the rats were anaesthetized with an intraperitoneal injection of a mixture of ketamine (70 mg/kg) and xylazine (7 mg/kg), MnCl₂ solution (50 mM, 3 μL, diluted with Milli-Q-H₂O) was injected intravitreally into both eyes using a 10-μl Hamilton microsyringe fixed with a 26-gauge needle (Hamilton, catalog #80300). The microsyringe was held inside the eyeball for more than 1 minute before being pulled out. The rats were then returned to the cage and were laid prone under a warm lamp until their recovery. Given a recent finding of a permanent loss of 10–20% of the axons in the normal mouse ON even after a single injection of a lower amount of MnCl₂ (Bearer et al., 2007), the Mn²⁺ solution was injected into two separate groups at 2 weeks (Group 1, n = 6) and 6 weeks (Group 2, n = 6) after starting the glaucoma induction, so as to minimize Mn²⁺ toxicity to the neurons, and injuries to the eyeball from multiple injections, and to eliminate the possibility of MRI signal reduction resulting from repeated injections (Thuen et al., 2005) rather than from the disease progression in the same animal.

MRI protocols

All MRI measurements were acquired on a 7-T MRI scanner with a maximum gradient of 360 mT/m (70/16 PharmaScan, Bruker Biospin GmbH, Germany) using a 38-mm rat brain quadrature resonator for RF transmission and receiving. Rats were placed onto a head holder consisting of ear and tooth bars. Since the rate of axonal transport varies with temperature (Smith et al., 2007), under inhaled isoflurane anesthesia (2% induction and 1% maintenance), animals were kept warm on a heating pad circulated with water at 37 °C. Two syringe phantoms containing 0.05 mM of MnCl₂ solution were...
Dynamic MEMRI was performed 2 to 5 h after Mn\(^{2+}\) injection in order to capture the early arrival of Mn\(^{2+}\) ions along the ONs (see Fig. 1). Throughout the experiments, the left eye served as a control. Three-dimensional volumetric images were acquired using an isotropic T1-weighted 3D RARE sequence with fat suppression, TR/TE=300/6.6 ms, RARE factor=4, NEX=2, FOV=3.24×3.24×2.47 cm\(^3\) and voxel resolution=193×193×193 μm\(^3\).

The 3D slab was positioned to parallel the prechiasmatic ONs based on the axial, coronal and sagittal multi-slice T2-weighted RARE images acquired with TR/TE = 4200/36 ms, RARE factor = 8, NEX=1, FOV=4×4 cm\(^2\), pixel resolution=156×156 μm\(^2\), slice thickness=0.7 mm and number of slices=18. The acquisition time for each 3D T1-weighted image set was 40 minutes. Images at 4 time points (i.e. 140, 180, 220 and 260 min after Mn\(^{2+}\) injection) were acquired for each animal.

### Data analysis

All 3D images were co-registered using AIR v5.2.5 (Roger Woods, UCLA, USA). The MEMRI signal intensities in the prechiasmatic left and right ONs were measured with an identical volume-of-interest (VOI) (2×2×6 voxels) placed at about 1.5 mm anterior to the optic chiasm at all time points using BrainSuite v2.0 (LONI, UCLA, USA) to monitor the Mn\(^{2+}\) transport along the ONs (see Fig. 2). In order to evaluate the Mn\(^{2+}\) transport and to weight out the effect of possible dosage difference applied to both eyes, the values at the prechiasmatic ONs were normalized to those at the first time point (i.e. 140 min after Mn\(^{2+}\) injection). In the reconstructed 2D slice that centrally cut through the eyeball and ONH, regions of interest (ROIs) were also drawn manually on the vitreous humour and the retina, covering a distance of 2.5 mm on each side from the ONH using ImageJ v1.37 (Wayne Rasband, NIH, USA). The signal intensities at 260 min after Mn\(^{2+}\) injection were analyzed by taking their mean values. Each value was normalized to the nearby syringe phantom to avoid the effect of any system sensitivity drift. Differences between mean values of the ROIs on both sides were compared using two-tailed paired \(t\)-tests, and the mean values along the time course were compared using ANOVA. Results were considered to be significantly different when \(p<0.05\). During the analyses, the data sets were blinded by flipping the images left-right.

Fig. 1. Schedule of the 1st and 2nd laser treatments (1 week apart) for induction of chronic glaucoma, intravitreal Mn\(^{2+}\) injection and T1-weighted MEMRI. MEMRI was performed 2 weeks after the 1st laser treatment for Group 1 (n=6) and 6 weeks after 1st laser treatment for Group 2 (n=6). No laser treatment was performed in the control group. Note that Mn\(^{2+}\) was administered only once in all animals.

Fig. 2. Localization of the volume of interest (VOI, 2×2×6 voxels) onto the Mn-enhanced prechiasmatic optic nerve in the coronal (a, d), axial (b, e) and sagittal (c, f) views of the same animal. The 3D image slab was positioned to parallel with the prechiasmatic optic nerves. (a–c) T1-weighted raw images; (d–f) same images with VOI overlay.
randomly and the ROIs were drawn without realizing the actual left and right positions.

Histological analysis of the optic nerve

Given that our study was based upon the early arrival of Mn$^{2+}$ ions, to rule out the effect of Mn$^{2+}$ toxicity aforementioned to the histological results, a separate group of animals ($n=4$) were induced with chronic glaucoma and were euthanized with a lethal intravenous injection of sodium pentobarbital (1 mg/kg) 6 weeks after glaucoma induction. Segments of the prechiasmatic ON containing the VOIs analyzed in MEMRI were excised and fixed by immersing in 2.5% glutaraldehyde and 2% paraformaldehyde in PBS for at least 1 week at 4 °C after each ON was enucleated. After three washes with PBS, the ON segments were placed in 2% osmium tetroxide in saline for 2 h and washed with PBS at room temperature. Subsequently, they were dehydrated in alcohol and embedded in epoxy resin for sectioning. Cross sections of 1 μm thickness were cut on an ultra-microtome, mounted on glass slides and stained for myelin using 0.5% toluidine blue (Sasaoka et al., 2006). The axonal density was measured using a microscopic analysis system (Optiphot Fluorescence Microscope, Nikon Instruments Inc., NY) in a meshed fashion. Briefly, each ON cross section slide was divided into approximately ten 150 × 150 μm$^2$ counting frames. Axons within a 30×30 μm$^2$ sample area in each counting frame were counted at 40× magnification, representing approximately 4% of the total ON cross-sectional area. The numbers of the axons counted in the sampling areas of each ON were then averaged to compute the axonal density. Three slides were quantified per ON, whereas each estimate was based on counts of between 1500 and 2500 axons. Axonal density measurements were compared between glaucomatous and contralateral ONs using one-tailed paired $t$-tests.

Results

Mn$^{2+}$ transport in prechiasmatic optic nerve

Fig. 2 illustrates the placement of the VOI in one of the prechiasmatic ONs. As shown in Fig. 3, MEMRI signal increased significantly in both sides of the prechiasmatic optic nerves of all animals over time (ANOVA: $p < 0.05$). Reduction of Mn$^{2+}$ transport in the glaucomatous (right) optic nerve was observed in the Week 6 animals but not in the Week 2 ones by two-tailed paired $t$-tests with $^*p < 0.05$. Two-tailed unpaired $t$-tests gave no significant differences in the control optic nerves between Week 2 and Week 6 models at all time points ($p > 0.05$).

Table 1

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<th>Retina (paired $t$-test between glaucomatous and contralateral eyes)</th>
<th>Vitreous humour (paired $t$-test between glaucomatous and contralateral eyes)</th>
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<tr>
<td>Control group ($n=3$)</td>
<td>0.99±0.03 ($p=0.67$)</td>
<td>0.99±0.09 ($p=0.65$)</td>
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<tr>
<td>Group 1 ($n=6$)</td>
<td>1.22±0.11 ($^{*}p=0.006$)</td>
<td>0.96±0.05 ($p=0.09$)</td>
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<td>Group 2 ($n=6$)</td>
<td>1.09±0.05 ($^{*}p=0.005$)</td>
<td>1.24±0.16 ($^{*}p=0.02$)</td>
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<td>ANOVA among 3 groups</td>
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glaucomatous ON compared to the contralateral side in the Week 6 model in Group 1, while there was no apparent reduction in the Mn\textsuperscript{2+} transport in the Week 2 model in Group 2. Two-tailed unpaired t-tests gave no significant differences in the control ONs between Week 2 and Week 6 models at all time points (p > 0.05).

Fig. 4 shows a representative pair of glaucomatous and control ONs upon toluidine blue staining at 6 weeks after glaucoma induction. In the glaucomatous ON, degenerating axons appear swollen or dark due to collapsed myelin sheaths; whereas in the control ON, the staining looked more homogeneous. The average axonal densities in the glaucomatous and control ONs were estimated to be 0.18 ± 0.03 and 0.20 ± 0.02 axons/\(\mu m^2\), respectively, contributing to an approximation of 10% loss in the glaucomatous ON (p < 0.05).

It was suspected that many optic nerve axons in the rat are smaller than the practical limits of their resolution by light microscopy (LM) (Chauhan et al., 2006; Morrison et al., 2005). However, given that the large fibers were more vulnerable to atrophy in areas of the ON with mild glaucomatous damage (Johnson et al., 2000; Quigley et al., 1987), these fibers should practically be distinguishable by our LM analyses as shown in Fig. 4, leading to the distinct differences in our LM measurements between contralateral ONs. We have also estimated a smaller number of ON sections with the addition of a 100× oil-immersion objective, and the axonal density was found to be about 0.5 axons/\(\mu m^2\), which was similar to the literature value for the intracranial ON in the same strain and gender using electron microscopy (0.4876 ± 0.0461 axons/\(\mu m^2\)) (Lau et al., 2006). Although the use of the 100× oil-immersion objective would have allowed resolution of finer detail and application of criteria even closer to those used for counting axons with EM (Chauhan et al., 2006), we had minimized the additional work required for such method as our purpose of performing histology at the prechiasmatic optic nerve was primarily to confirm the validity and consistency of our model.

Fig. 5. Typical ROI delineations for the analysis of retina (mid row) and vitreous humour (bottom row) in the T1-weighted image of a rat from Group 1 (2 weeks after 1st laser treatment). Mean signal intensities in both retina and vitreous humour were obtained covering a distance of 2.5 mm on each side from the optic nerve head at 260 min after Mn\textsuperscript{2+} injection.

Fig. 6. Typical T1-weighted images (T1WI) of the glaucoma model (1st column) and targeted maximum intensity projection (MIP) of the optic nerves (2nd column). Accumulation of Mn\textsuperscript{2+} ions at high concentrations was clearly observed at the glaucomatous optic nerve head (ONH) (open arrow) compared to the normal ONH in Week 2 (Group 1, top 2 rows) and Week 6 (Group 2, bottom 2 rows) animals. Significantly higher signal intensity in the vitreous humour of the glaucomatous eye was also found at 6 weeks after 1st laser treatment. Note that the images shown were acquired 140 min and 260 min after Mn\textsuperscript{2+} injection.
Retina and optic nerve head

The retinal signal intensities were significantly higher in the glaucomatous eyes than in the contralateral eyes in both Week 2 and Week 6 animals as indicated in Table 1. Fig. 5 illustrates the ROI delineation for retinal and vitreous humour analysis. The typical Mn²⁺ enhancement of eye and ON structure was shown in Fig. 6. An apparent accumulation of Mn²⁺ ions at high concentrations was observed in the ONH, with more being observed in the glaucomatous eye in Group 1 (5 out 6 animals) and Group 2 (3 out of 6 animals) as indicated in the arrows in Fig. 6.

Time profile in vitreous humour

Signal intensities (SI) in the vitreous humour of both eyes decreased as time progressed for all groups after Mn²⁺ injection (data not shown). At 260 min after Mn²⁺ injection, the vitreous humour in the glaucomatous eye had a significantly higher signal intensity than that in the contralateral side in all Week 6 animals as shown in Table 1 ($p<0.05$) and illustrated in Fig. 6 and in 2 out of 6 of the Week 2 animals ($p=0.09$) but not in the control group ($p>0.05$).

Discussion and conclusion

Mn²⁺ transport along the optic nerve

Mn²⁺ ions have been verified to be uptaken by the RGCs, packed into organelles in the endoplasmic reticulum and transported along microtubules in the ONs (Pautler, 2006; Van der Linden et al., 2007). The rate of signal accumulation at a target location would be expected to depend on the uptake rate of Mn²⁺ at the injection site, the density of projections and the transport and/or diffusion away from the target location (Leergaard et al., 2003). In the current study, Mn²⁺ enhancement in ONs was seen to increase as early as at 180 min after injection. Given the length of the normal rat ON from ONH to the optic chiasm to be about 10.55±0.70 mm for the 15 samples in our study, the Mn²⁺ transport at the prechiasmatic ONs at about 1.5 mm anterior to optic chiasm should rate at approximately 3.02 mm/h, which is within the range of fast axonal transport of 2 to 16 mm/h (Elluru et al., 1995) and is consistent with reported values in previous studies along the ONs (5 mm/h, Bearer et al., 2007; 2 mm/h, Pautler et al., 1998; 2.8 mm/h, Watanabe et al., 2001).

The apparent reduction in the rate of Mn²⁺ transport (or, more precisely, the Mn²⁺ enhancement increase) in the prechiasmatic ON of the glaucomatous eye in the Week 6 model may arise from several factors that comprise its transport route. Firstly, the RGC loss in this experimental model of chronic glaucoma ranges from 22% to 25% at 4 to 8 weeks after 1st laser treatment (Li et al., 2006a). This could cause a reduction in Mn²⁺ uptake into the RGCs, and thus a reduction in the overall anterograde transport in the axons of the retinal nerve fiber layer; secondly, our current findings have also demonstrated a significant reduction in average axonal densities at the prechiasmatic ON using toluidine blue staining. This appears to correlate with the mean axonal density decrease in other chronic glaucoma studies (Mabuchi et al., 2003; Mabuchi et al., 2004; Sasaoka et al., 2006) despite their interstrain variations (Hetling et al., 2005; Lau et al., 2006) and different microscopic methods used (Chauhan et al., 2006; Morrison, et al., 2005). The decrease in ON axonal density may reduce the amount of Mn²⁺ ions transported along the ON bundles per unit time as it was observed in the current experiment. Note that the cross-sectional area in the control ON ($228221±27931 μm²$) was similar to the literature value determined by electron microscopy ($230284±41122 μm²$) (Lau et al., 2006), while the areas of the glaucomatous ONs in our glaucoma model were not statistically significant from those of the control ONs ($p=0.18$). Thirdly, there might be a blockade in axoplasmic transport along the glaucomatous ON. It has been reported that the fast axonal transport was obstructed at the glaucomatous ON by studying the movement of radioactive materials (Anderson and Hendrickson, 1974; Dandona et al., 1991; Quigley et al., 1979; Quigley and Addicks, 1980). Our histological findings have also shown swollen and enlarged ONs in agreement with a previous study of similar period of IOP elevation (39 days at 27±9 mmHg; Johnson et al., 2000). When visualized in electron microscopy, these ONs were usually characterized by accumulation of organelles, vesicles and dense bodies especially in the large diameter axons, indicative of blocked axonal transport (Grozdanic et al., 2003; Johnson et al., 2000; Morrison et al., 1997; Quigley et al., 1987). As the number of microtubules was found to increase with axonal size in the ONs of adult rats (Hernandez et al., 1989), it is likely that the delay in T₁-weighted signal increase in our findings could reflect damages to the microtubules particularly in the large fibers; In addition, abnormal processing of amyloid precursor protein (APP) has once been observed in rat glaucoma (Vickers et al., 2002). Amyloid-beta (Aβ) plaques resulting from the sequential cleavage of the APP were shown to rapidly inhibit fast axonal transport in cultured rat hippocampal neurons (Hiruma et al., 2003). Given that a previous MEMRI study has shown the decrease in fast axonal transport in Alzheimer's disease after Aβ deposition in the olfactory system (Smith et al., 2007), it is possible that the same would occur along the visual pathways of the glaucoma models as well. It is also possible that the changes in manganese concentration in the vitreous humour might affect the signal increase in the prechiasmatic optic nerve of both eyes. However, a comprehensive model that might describe the input function, the mechanisms of active Mn²⁺ transport in the visual components and the diffusion pattern along the optic nerve is still lacking, while T₁ mapping has not been carried out to quantify the Mn²⁺ concentrations in the visual components. Thus, it is inherently difficult to analyze the rate of axonal transport in the optic nerve precisely from one segment to another in the current study.

The delayed T₁-weighted signal increase was found to be significant only in the Week 6 model but not in the Week 2 one. In addition, a preliminary study on 2 Week 9 animals by the same setup has also shown an apparent reduction in Mn²⁺ signal increase as the Week 6 animals did (data not shown). These appeared to suggest the disease progression along the time course. The glaucoma model in the current study was proven to induce a chronic disease with an RGC loss of 12–14% at 2 weeks after induction (Fu et al., 2007; Li et al., 2006a), which was approximately half of that at 4 to 8 weeks after induction. Note also that histological analysis on the progression of ON damage using toluidine blue staining has been documented previously for a chronic glaucoma model, revealing a significant reduction of axonal density at Week 8 but not Week 4 or earlier after glaucoma induction (Sasaoka et al., 2006). The glaucomatous changes along the ONs in the early stage might not be detectable by MEMRI used in the current study.

Retinal enhancement and optic nerve head occlusion

Despite the proven RGC loss which might reduce the overall signal intensity of the retina, the significance of the increase in the
signal intensities of the retina measured in the glaucomatous eyes when compared to the contralateral ones remains unclear. One potential explanation could be related to the competing effect of increased reactive gliosis (WoldeMussie et al., 2001) and microglial activation (Naskar et al., 2002) on the retina, which might take up the Mn\(^{2+}\) ions concurrently (Kawai et al., 2007; Haapanen et al., 2007). Mn\(^{2+}\) might also be trapped in the retinal nerve fiber layer due to the blockade at the ONH and the ON. While some investigators have observed changes in the glaucomatous outer retina in clinical-histopathologic studies (Morrison et al., 2005), it has also been postulated that, when the intraocular pressure was increased by a tumor-induced change, outer retinal ion demand might be raised and the retinal signal intensities would increase further upon intravitreal Mn\(^{2+}\) injection (Braun et al., 2007). Note that alterations of intraretinal ion demand have previously been demonstrated in various models of ocular injury using MEMRI (Berkowitz et al., 2007).

Given that the rat ON myelination begins approximately 0.5 mm posterior to the sclera (Morrison, 2005), interestingly, we seemed to be able to delineate the unmyelinated ONH as a constriction between the sclera and the intraorbital ON in Fig. 6. The Mn\(^{2+}\) accumulation in the ONH may be due to uptake by glia and connective tissue cells via extra-axonal diffusion (Taylor and Weiss, 1965; Watanabe et al., 2004) since the rat lamina cribrosa is relatively sparse (Morrison, 2005). The apparent increase in accumulation of Mn\(^{2+}\) ions in the glaucomatous ONH might suggest the swelling of axons with a deposition of intracellular membranous organelles, whose fast axonal movement was expected to be disturbed at the pores of the ONH and the ON. While some investigators have observed changes in the glaucomatous outer retina in clinical-histopathologic studies (Morrison et al., 2005), it has also been postulated that, when the intraocular pressure was increased by a tumor-induced change, outer retinal ion demand might be raised and the retinal signal intensities would increase further upon intravitreal Mn\(^{2+}\) injection (Braun et al., 2007). Note that alterations of intraretinal ion demand have previously been demonstrated in various models of ocular injury using MEMRI (Berkowitz et al., 2007).

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**Accumulation of Mn\(^{2+}\) in vitreous humour**

Mn\(^{2+}\) might travel around in the posterior chamber by both diffusion and convection (Xu et al., 2000). Previous studies in normal primate eyes reported that intravitreal injection of similar dosages of Mn\(^{2+}\) reached the retinal boundary at 35 min after intravitreal injection, with no diffusion into the lens within the first 53 min (Li et al., 2004). The gradual reduction in signal intensities in our experiments might therefore indicate the continual clearance of Mn\(^{2+}\) away from the posterior chamber via the venous outflow, or by entering the RGCs via Ca\(^{2+}\) channels upon depolarization (Pautler, 2006) or other ion channels like divalent metal transporters (Braun, 2007; Takeda, 2003).

The higher signal intensity observed in the vitreous humour in the glaucomatous eye appeared to result from the blockade of the conventional trabecular route drained by the episcleral and limbal veins upon photoocoagulation. Further, the RGC loss in the current model, together with possible occlusion in the ONH (Thanos and Naskar, 2004), might reduce the uptake of Mn\(^{2+}\) ions into the intracellular space, causing an accumulation in the vitreous humour. It is apparent that the usual pattern of Mn\(^{2+}\) clearance in the glaucomatous eyeball was perturbed upon intravitreal injection.

By applying MEMRI, a global view for the investigation of chronic glaucoma within the rat primary visual system could be provided in vivo. This may complement other histological and MRI techniques to define the defective location specifically and conveniently for finer studies (Lin et al., 2001; Lin et al., 2003) and may help monitor the effect of drug interventions, e.g. the neuroprotective effect of *Lycium barbarum* Lynn (Chan et al., 2007) to the glaucoma models globally and longitudinally. In recent human studies, functional MRI has successfully demonstrated the relationship of the functional organization of primary visual cortex (V1) with the damage to the optic disc and the visual field loss in primary open-angle glaucoma (POAG) (Duncan et al., 2007, a,b), Given the dependency of trans-neuronal transmission of Mn\(^{2+}\) to electrical activity (Braun et al., 2007), further investigations should be performed to study the progression of trans-synaptic degeneration at the lateral geniculate nuclei (LGN) and the visual cortex (Lindsey et al., 2007; Gupta and Yucel, 2007) in the glaucoma models. We may also apply the commonly used BOLD and CBF functional MRI techniques to cross-correlate with the Mn\(^{2+}\)-based synaptic activity in the same animal model (Duong et al., 2000). This is of particular importance as lesions of the LGN and the visual cortex would induce loss of RGCs and the degenerating LGN in glaucoma may similarly disrupt trophic support to RGCs, adding further insult to their glaucomatous damage within the globe (Yucel et al., 2003). Experimentally, it is also desirable to increase the resolution of MRI acquisitions and the number of time points so as to better delineate the LGN as well as the SI profile along the entire rat ON, or specifically, in the superior regions of the ONs.

To conclude, MEMRI potentially provides an in vivo, longitudinal and three-dimensional means to investigate the abnormalities in the visual components of the rat model of chronic glaucoma. The delayed increase in T1-weighted signal intensity in the glaucomatous ON at 6 weeks but not 2 weeks after glaucoma induction might be suggestive of the disease progression related to RGC loss, axonal density decrease, and/or disturbance on fast axonal transport. The accumulation of Mn\(^{2+}\) ions in the vitreous humour of the glaucomatous eye could possibly arise from the perturbation of the usual pattern of Mn\(^{2+}\) clearance in the glaucomatous eyeball. High concentrations of Mn\(^{2+}\) ions were also observed at the ONH and the retina. The MEMRI approach may help understand the disease mechanisms, monitor the effect of drug interventions to glaucoma models and complement the conventional histological and electrophysiological techniques in examining the glaucomatous visual components.

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