

# In Vivo Evaluation of White Matter Integrity and Anterograde Transport in Visual System After Excitotoxic Retinal Injury With Multimodal MRI and OCT

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**PURPOSE.** Excitotoxicity has been linked to the pathogenesis of ocular diseases and injuries and may involve early degeneration of both anterior and posterior visual pathways. However, their spatiotemporal relationships remain unclear. We hypothesized that the effects of excitotoxic retinal injury (ERI) on the visual system can be revealed in vivo by diffusion tensor magnetic resonance imaging (DTI), manganese-enhanced magnetic resonance imaging (MRI), and optical coherence tomography (OCT).

**METHODS.** Diffusion tensor MRI was performed at 9.4 Tesla to monitor white matter integrity changes after unilateral N-methyl-D-aspartate (NMDA)-induced ERI in six Sprague-Dawley rats and six C57BL/6J mice. Additionally, four rats and four mice were intravitreally injected with saline to compare with NMDA-injected animals. Optical coherence tomography of the retina and manganese-enhanced MRI of anterograde transport were evaluated and correlated with DTI parameters.

**RESULTS.** In the rat optic nerve, the largest axial diffusivity decrease and radial diffusivity increase occurred within the first 3 and 7 days post ERI, respectively, suggestive of early axonal degeneration and delayed demyelination. The optic tract showed smaller directional diffusivity changes and weaker DTI correlations with retinal thickness compared with optic nerve, indicative of anterograde degeneration. The splenium of corpus callosum was also reorganized at 4 weeks post ERI. The DTI profiles appeared comparable between rat and mouse models. Furthermore, the NMDA-injured visual pathway showed reduced anterograde manganese transport, which correlated with diffusivity changes along but not perpendicular to optic nerve.

**CONCLUSIONS.** Diffusion tensor MRI, manganese-enhanced MRI, and OCT provided an in vivo model system for characterizing the spatiotemporal changes in white matter integrity, the eye-brain relationships and structural-physiological relationships in the visual system after ERI.

**Keywords:** glutamate excitotoxicity, optic neuropathy, corpus callosum, magnetic resonance imaging, optical coherence tomography

Excitotoxicity has been linked to the pathogenesis of several ocular diseases and injuries such as retinal ischemia,<sup>1-5</sup> traumatic injury,<sup>6,7</sup> glaucoma,<sup>8-11</sup> and diabetic retinopathy.<sup>12,13</sup> In the retina, the retinal ganglion cells preferentially express N-methyl-D-aspartate (NMDA)-type glutamate receptors and are believed to play a major role in glutamate excitotoxic retinal

injury.<sup>14,15</sup> While the cell bodies in the retina are commonly regarded as the primary site of insult, recent studies have suggested the early involvement of white matter degeneration in the posterior visual pathway after glutamate excitotoxicity in the eye.<sup>16-19</sup> Nevertheless, because of limited noninvasive techniques available for assessing the visual pathways, the

spatiotemporal patterns of neurodegenerative events in the visual system and their relationships with excitotoxic retinal injury in the eye are not fully elucidated. This in part hinders the development of effective strategies for disease monitoring and treatment.

Magnetic resonance imaging (MRI) allows noninvasive, longitudinal, and multiparametric assessments of the visual system without depth limitation.<sup>20–25</sup> Although there were existing MR reports assessing the effects of NMDA-induced excitotoxicity in developing and adult brain tissues,<sup>26,27</sup> most of the neuropathy models employed did not differentiate clearly the locations of the primary site of insult or the initial events in white matter degeneration. In addition, the early MRI studies assessing visual system injury<sup>28,29</sup> mainly used the conventional anatomical T2-weighted imaging or diffusion weighted imaging techniques at low magnetic field strengths with relatively limited sensitivity and specificity to characterize the underlying pathophysiological events. In this study, we employed the advanced MR techniques, namely diffusion tensor MRI (DTI) and manganese-enhanced MRI (MEMRI) at a high magnetic field strength, in combination with spectral-domain optical coherence tomography (OCT), with an aim to develop an in vivo model system for characterizing the spatiotemporal patterns of white matter integrity changes in the visual system and their relations to retinal integrity after glutamate excitotoxicity in the eye. Diffusion tensor MRI has been recently shown to reveal white matter integrity in normal, developing and diseased visual systems in rodent models under high magnetic field strengths.<sup>30–35</sup> In particular, the measurements of water diffusion parallel and perpendicular to the nerve fibers have been suggested to be sensitive to axonal and myelin integrity respectively.<sup>31,36</sup> Since intravitreal injection of NMDA has been commonly used as an experimental model to induce glutamate excitotoxic retinal ganglion cell death,<sup>37,38</sup> in this study, we quantified the spatiotemporal DTI profiles in the rodent visual system with a 9.4 Tesla MRI scanner after NMDA-induced excitotoxic retinal injury. In addition, DTI results were compared with OCT measurement of the thickness of the retina and MEMRI of the anterograde transport along the visual pathway, in order to determine the eye–brain relationships and to correlate between structural and physiological characteristics in the injured visual system. As the retinal ganglion cells and the sites of toxic insult after intravitreal NMDA injection in the eye are physically isolated from the axons beyond the eye in the brain's visual system, our MRI and OCT studies in this NMDA model of retinal injury are well suited for providing information about the effects of excitotoxic perikaryal injury on white matter integrity changes in both ocular diseases and other neuropathies. Our cross-sectional and longitudinal results from these in vivo multidisciplinary ophthalmic imaging techniques may allow better monitoring of the disease progression in the visual system, and provide a platform for assessing treatment effects on both the eye and the visual pathway in future studies.

## MATERIALS AND METHODS

### Animal Preparation

All animal experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and protocols reviewed and approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Ten adult Sprague-Dawley rats and 10 adult C57BL/6J mice were divided into four groups to evaluate the effects of NMDA-induced glutamate excitotoxicity in the eye on the visual system. In Group 1, six adult rats were first anesthetized by

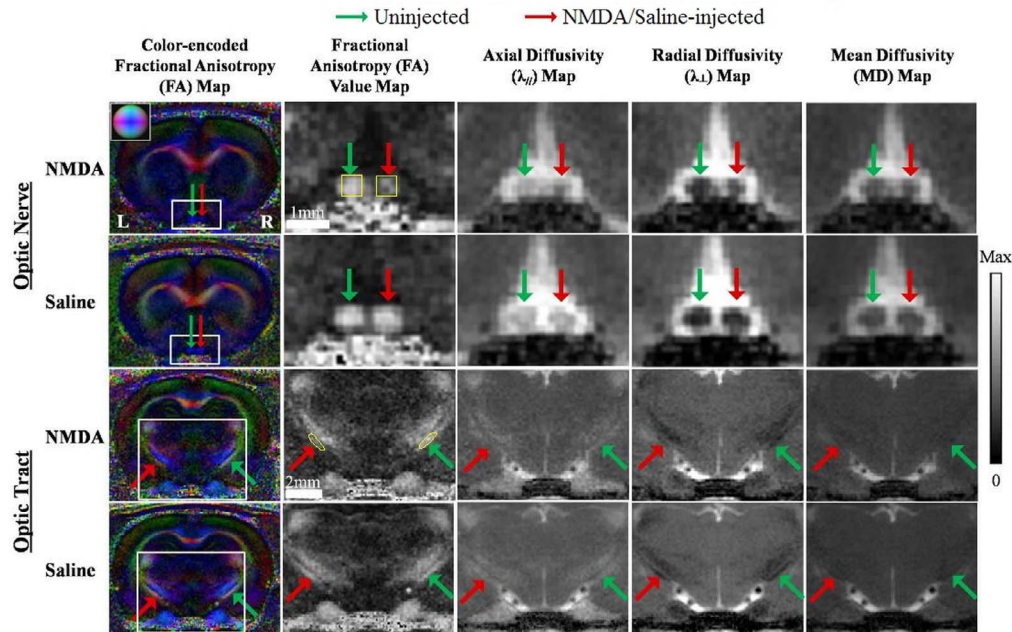
inhaling a mixture of air and isoflurane (3% for induction and 1.25% for maintenance). Proparacaine (Bausch & Lomb, Inc., Rochester, NY, USA) was topically administered to anesthetize the surface of the eye, followed by intravitreal injection of 5  $\mu$ L 40 mM NMDA (Thermo Fisher Scientific, Inc., Waltham, MA, USA) into the right eye using a 33-G microsyringe (Hamilton Co., Reno, NV, USA) to induce glutamate excitotoxicity to the retina. In Group 2, the same experimental procedures were performed to the four remaining rats, except that 5  $\mu$ L saline solution (0.9% sodium chloride; Baxter International, Inc., Deerfield, IL, USA) instead of NMDA solution was intravitreally injected to the right eye. Diffusion tensor MRI was performed to each rat at 3, 7, 14, and 28 days after NMDA or saline injection, while OCT was performed to four rats randomly selected from Group 1 and to all four rats in Group 2 after DTI at the final time point. For Group 3, 1  $\mu$ L 40 mM NMDA was intravitreally injected to the right eye of six adult mice under the same experimental settings as the rat model. For Group 4, 1  $\mu$ L saline solution was intravitreally injected to the right eye of the remaining 4 mice. Diffusion tensor MRI was performed to each mouse at 7 ( $n = 6$  for NMDA group;  $n = 4$  for saline group) and 70 days ( $n = 4$  for NMDA group;  $n = 3$  for saline group due to loss of one animal between 7 and 70 days) after NMDA or saline injection. In addition, each mouse was intravitreally injected with 0.5  $\mu$ L 100 mM manganese chloride (MnCl<sub>2</sub>) solution into both eyes after DTI at 7 days after NMDA or saline injection, and T1-weighted MEMRI was performed before and at 8 hours after Mn injection. A saline syringe phantom was placed next to the mouse head for T1-weighted MR signal normalization to counter for system instability between imaging sessions.

The current NMDA doses were chosen with reference to recent studies investigating NMDA-induced injury in rodent retina.<sup>37,38</sup> Since the main purpose of this study was to examine the effects of NMDA-induced glutamate excitotoxicity in the eye on the visual pathway via in vivo imaging, whereas intravitreal NMDA injection led to excitotoxic retinal injury in a dose-dependent manner,<sup>37,39,40</sup> we induced glutamate excitotoxicity at a relatively high NMDA dose to maximize retinal damage while minimizing the number of animals required to first prove the concept that DTI, MEMRI, and OCT could detect the spatiotemporal changes in the white matter integrity and their relations to retinal integrity after NMDA-induced excitotoxicity in the eye.

### DTI and MEMRI

All MRI experiments were performed using a 9.4-Tesla/31-cm Varian/Agilent horizontal bore scanner (Santa Clara, CA, USA) with a volume transmit coil and a surface receive coil for rats and a volume transmit and receive coil for mice. Animals were anesthetized with a mixture of air and isoflurane (3% for induction and 1.5% for maintenance) and were kept warm under circulating water during MRI experiments. To ensure reproducible slice orientation and positioning, scout T2-weighted images were first acquired in the coronal, transverse, and sagittal planes with a spin-echo pulse sequence.<sup>23</sup> Diffusion tensor MRI slices were oriented orthogonal to the prechiasmatic optic nerves and were acquired using a fast spin-echo sequence, with 12 diffusion gradient directions at  $b = 1.0$  ms/ $\mu$ m<sup>2</sup> and two  $b = 0$  ms/ $\mu$ m<sup>2</sup> ( $b_0$ ). Other imaging parameters included: repetition time/echo time = 2300/27.8 ms; echo train length = 8; duration of diffusion gradient pulses ( $\delta$ )/time between diffusion gradient pulses ( $\Delta$ ) = 5/17 ms; number of averages = 4; field of view = 26  $\times$  26 (rat) and 20  $\times$  20 mm<sup>2</sup> (mouse); in-plane resolution = 102  $\times$  102 (rat) and 78  $\times$  78  $\mu$ m<sup>2</sup> (mouse); slice thickness = 1 (rat) and 0.5 mm (mouse). Manganese-enhanced MRI was acquired using a T1-weighted fast spin-echo sequence, with the same geometric parameters,

## Diffusion Tensor MRI of Rat Visual Pathway



**FIGURE 1.** Representative DTI parametric maps of the rat brain at the levels of the prechiasmatic optic nerve (*top 2 rows*) and optic tract (*bottom 2 rows*) at 4 weeks after NMDA (*first and third rows*) or saline injection (*second and fourth rows*) to the right eye. The *leftmost column* shows the whole-brain color-encoded FA directionality maps, whereas the FA value maps, and axial diffusivity ( $\lambda_{//}$ ), radial diffusivity ( $\lambda_{\perp}$ ), and mean diffusivity (MD) maps on the *right columns* are enlarged from the *white boxes* in the whole-brain color-encoded FA maps. *Red arrows* indicate the visual pathway projected from the injected right eye. *Green arrows* indicate the visual pathway projected from the uninjected left eye. The right optic nerve and left optic tract projected from the NMDA-injected right eye showed lower FA and  $\lambda_{//}$ , and higher  $\lambda_{\perp}$  when compared with the contralateral visual pathway projected from the uninjected left eye. No apparent change in any DTI parameters was observed between the left and right visual pathways after saline injection to the right eye. The regions of interest in the optic nerves and optic tracts for DTI quantitation are outlined in *yellow* (color representations for the principal diffusion directions in the color-encoded FA maps: *Blue*, caudal-rostral; *red*, left-right; *green*, dorsal-ventral; *grayscale bar* indicates the range of values displayed in the DTI parametric maps. The maximum values for FA,  $\lambda_{//}$ ,  $\lambda_{\perp}$  and MD maps are 1.0, 3.0, 2.0, and  $3.0 \mu\text{m}^2/\text{ms}$ , respectively).

repetition time/echo time = 1060/9.35 ms and echo train length = 4.

### Optical Coherence Tomography

Prior to OCT imaging, the rats were anesthetized with an intraperitoneal injection of ketamine (Butler Schein, Dublin, OH, USA) and xylazine (Lloyd Laboratories, Shenandoah, IA, USA). A thin glass coverslip and hydroxypropyl methylcellulose (HUB Pharmaceuticals, Rancho Cucamonga, CA, USA) was applied to neutralize the corneal curvature and keep the cornea hydrated. The scans were acquired within 15 minutes of anesthesia to limit the effects of lenticular opacities. The spectral-domain OCT device (Bioptigen, Inc., Research Triangle Park, NC, USA) is equipped with a wide-bandwidth light source centered on 870 nm (Superlum Ltd., Dublin, Ireland), with a theoretical axial resolution of  $1.3 \mu\text{m}$  in tissue. A  $2.5 \times 2.5 \times 2 \text{ mm}^3$  ( $512 \times 512 \times 1024$ ) volume centered on the optic nerve head was taken on both eyes.

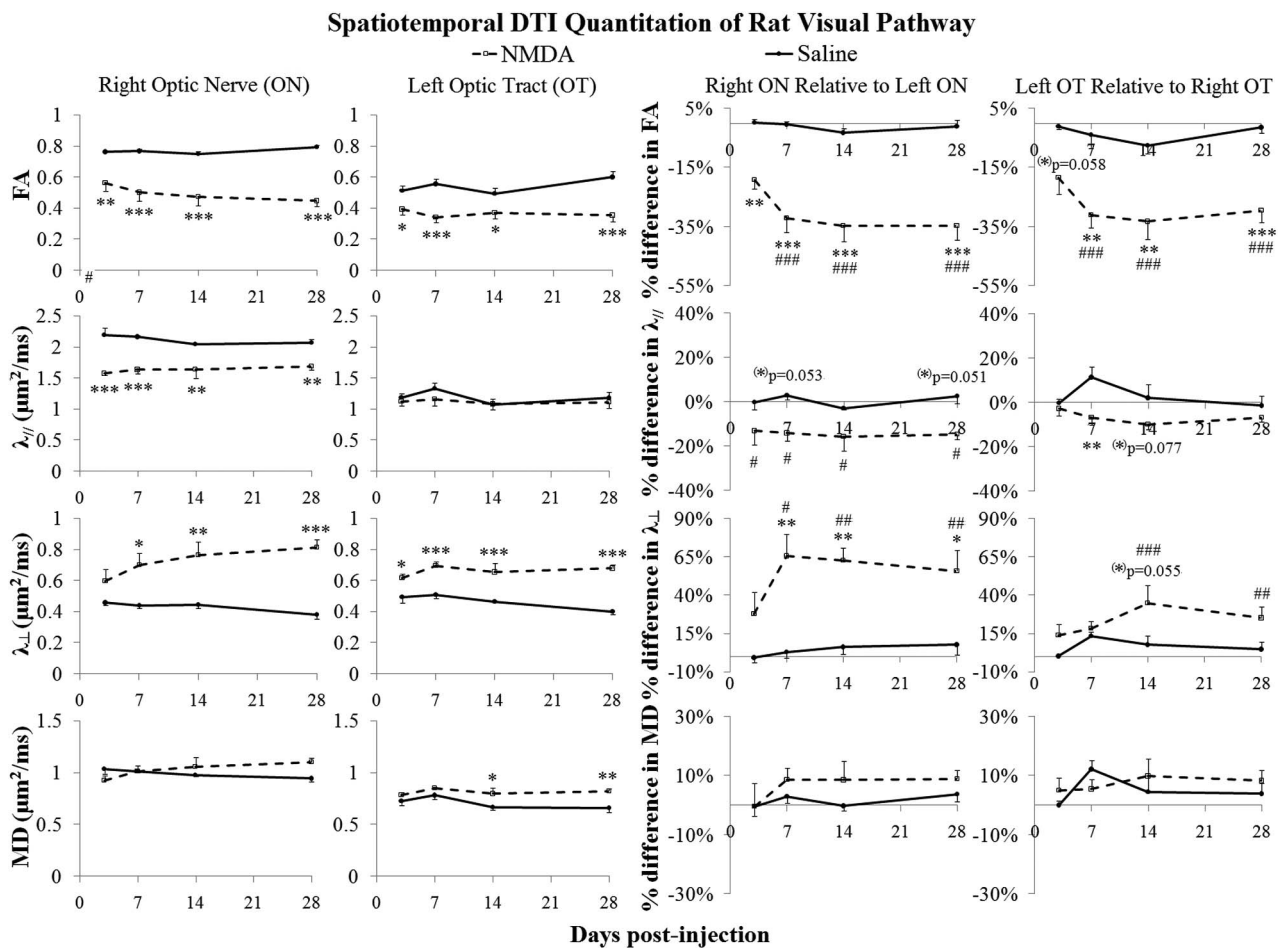
### Data Analysis

For DTI, coregistration between nondiffusion-weighted  $b_0$  images and diffusion-weighted images was performed using SPM8 (Wellcome Department of Imaging Neuroscience, University College, London, UK). Using DTIStudio v3.02 (Johns Hopkins University, Baltimore, MD, USA),  $3 \times 3$  diffusion tensors were fitted on a pixel-by-pixel basis from the nondiffusion-weighted  $b_0$  images and the diffusion-weighted images. The eigenvectors and eigenvalues of the

diffusion tensors were derived to compute the DTI parametric maps including fractional anisotropy (FA) directionality color map, FA value map, and axial diffusivity ( $\lambda_{//}$ ), radial diffusivity ( $\lambda_{\perp}$ ), and mean diffusivity (MD) maps. Regions of interest were drawn manually using ImageJ v1.47 software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) on the prechiasmatic optic nerves at Bregma 1.5 mm, the optic tracts at Bregma  $-3.5$  mm, and the splenium of corpus callosum at Bregma  $-5.04$  mm for rats based on the FA directionality map, FA value map,  $\lambda_{//}$  and  $\lambda_{\perp}$  maps, and the rat brain atlas.<sup>41</sup> Regions of interest were also drawn on the mouse prechiasmatic optic nerves at Bregma 1.0 mm and the optic tracts at Bregma  $-1.8$  mm based on the FA directionality map, FA value map,  $\lambda_{//}$  and  $\lambda_{\perp}$  maps and the mouse brain atlas.<sup>42</sup>

For OCT, the volumes centered on the optic nerve head on both eyes were automatically segmented using a custom software<sup>43</sup> to determine the internal limiting membrane (ILM) and retinal pigment epithelium (RPE). The center of the optic nerve head was manually delineated. Total retinal thickness, or the distance between the ILM and RPE, was determined along a sampling band with radius 0.39 to 0.49 mm centered on the optic nerve head.

For MEMRI, regions of interest were drawn manually using ImageJ on the optic nerves at Bregma 1.0 mm, the lateral geniculate nuclei at Bregma  $-3.0$  mm and the superior colliculi at Bregma  $-4.0$  mm based on T1-weighted anatomical images and the mouse brain atlas.<sup>42</sup> T1-weighted signal intensities pre- and post Mn injection were extracted and normalized to the signal intensity of the nearby saline syringe phantom.



**FIGURE 2.** Spatiotemporal DTI quantitation (mean  $\pm$  SEM) in the right optic nerve (*first column*) and left optic tract (*second column*) and the percentage DTI difference (mean  $\pm$  SEM) in the right optic nerve (*third column*) or left optic tract (*fourth column*) relative to the contralateral visual pathway at 3 to 28 days after NMDA or saline injection to the right eye in rats (Bonferroni's multiple comparisons tests between NMDA and saline groups, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; between left and right visual pathways in the NMDA group, # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ ). The right optic nerve projected from the NMDA-injected eye showed significantly lower FA and axial diffusivity ( $\lambda_{//}$ ), and higher radial diffusivity ( $\lambda_{\perp}$ ) compared with the saline group. The left optic tract projected from the NMDA-injected eye generally showed smaller DTI changes than the right optic nerve of the same group, or the left optic tract from the saline group. No apparent difference was observed between the left and right visual pathways in the saline group.

### Statistical Analysis

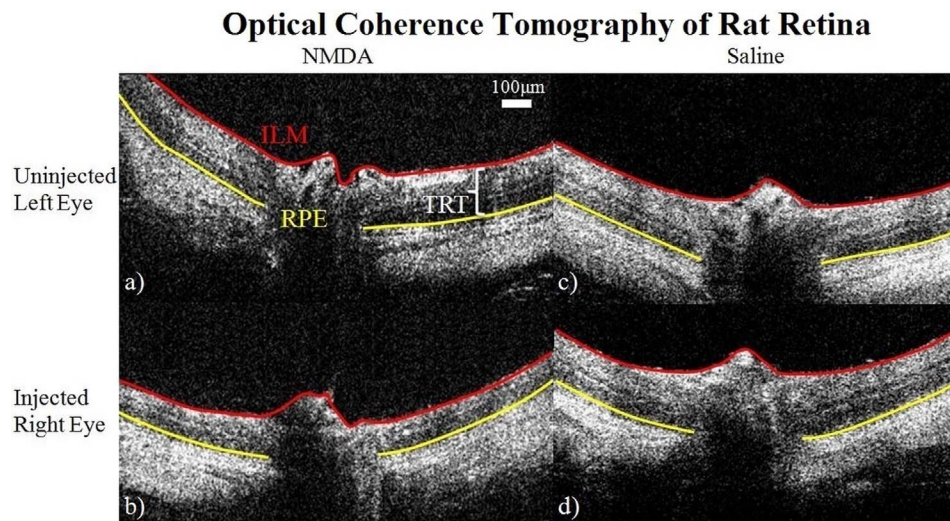
As previous studies suggested that unilateral injury to one eye may affect the contralateral eye,<sup>44-47</sup> whereas in adult rodents, more than 90% of the optic nerve fibers decussate at the optic chiasm to the contralateral optic tract,<sup>48</sup> in this study, DTI, MEMRI, and OCT measurements were first compared between the visual pathways projected from the NMDA- and saline-injected eyes (i.e., right retina, right optic nerve, left optic tract, left lateral geniculate nucleus, and left superior colliculus) and in the splenium of corpus callosum using ANOVA followed by post hoc Bonferroni's multiple comparisons tests by GraphPad Prism v5.00 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between left and right visual pathways were also evaluated and compared between NMDA and saline groups using ANOVA and post hoc Bonferroni's tests. In addition, random intercept mixed-effects models were included to determine the relationships between DTI and OCT data, and between DTI and MEMRI data. For the analyses of DTI and OCT data, the random intercept mixed-effects model was structured to assess the effect of total retinal thickness in both eyes and NMDA-induced injury on each DTI parameter

(FA,  $\lambda_{//}$ ,  $\lambda_{\perp}$ , and MD), with rat as the random effect. For the analyses of DTI and Mn data, the random intercept mixed-effects model was structured to assess the effect of each DTI parameter (FA,  $\lambda_{//}$ ,  $\lambda_{\perp}$ , and MD) in the optic nerve and NMDA-induced injury on MEMRI of both left and right visual pathways, with mouse as the random effect. The inter-eye dependency was accounted for when evaluating the models. The slopes among DTI, MEMRI, and OCT parameters in the models were also estimated. Data were presented as mean  $\pm$  SEM unless otherwise specified. Results were considered statistically significant when  $P$  was less than 0.05.

## RESULTS

### Spatiotemporal DTI Profiles of Rat Optic Nerve and Optic Tract Integrity After Unilateral NMDA-Induced Retinal Injury or Intravitreal Saline Injection

Figure 1 shows the qualitative comparisons of white matter integrity along the rat visual pathways at 1 month after



**FIGURE 3.** Cross-sectional images from OCT of the rat retina in the uninjected left eye (a, c) and injected right eye (b, d) at 1 month after intravitreal NMDA (left column) or saline injection (right column). The internal limiting membrane (ILM; white) and retinal pigment epithelium (RPE; red) were segmented in order to determine the total retinal thickness (TRT). Retinal thinning was observed in the NMDA-injected right eye (b) compared with the saline-injected right eye (d) or the uninjected left eye of either NMDA (a) or saline group (c).

NMDA-induced glutamate excitotoxicity or saline injection in the right eye in Groups 1 and 2. As shown in the DTI parametric maps, the right optic nerve and left optic tract projected from the NMDA-injected right eye showed lower FA, lower  $\lambda_{//}$ , and higher  $\lambda_{\perp}$  compared with the left optic nerve and right optic tract. No apparent difference in any DTI parameters was observed between left and right visual pathways after saline injection to the right eye. Diffusion tensor MRI quantitation in Figure 2 reveals the progressive changes in optic nerve and optic tract integrity from 3 to 28 days after NMDA-induced retinal injury or saline injection. When comparing the right optic nerve between NMDA and saline groups, significantly lower FA and  $\lambda_{//}$  were detected in the NMDA group as early as 3 days after NMDA injection, whereas significantly higher  $\lambda_{\perp}$  occurred later at 7 days and onward after NMDA injection. The left optic tract projected from the injected eye showed generally smaller DTI differences between NMDA and saline groups when compared with the right optic nerve. The percentage DTI differences between visual pathways projected from the injected and uninjected eyes of the same groups were also quantified in Figure 2. The spatiotemporal patterns of the DTI percentage differences between left and right visual pathways in the NMDA group appeared comparable to the DTI differences in the right optic nerve or left optic tract between NMDA and saline groups. In addition, continuously increasing FA decrease and  $\lambda_{\perp}$  increase were apparent in the NMDA group within the first week post ERI, whereas  $\lambda_{//}$  decrease in NMDA group remained relatively constant throughout the experimental period from 3 to 28 days post ERI. No significant DTI difference was observed between visual pathways projected from the injected and uninjected eyes of the saline group throughout the experimental period.

#### OCT of Retinal Thickness and Correlations With DTI of Visual Pathway Integrity

Figure 3 shows the representative OCT images of the rat retina in both eyes at approximately 1 month after NMDA or saline injection to the right eye. Significant retinal thinning was observed in the NMDA-injected right eye compared with the saline-injected right eye or the uninjected left eye of either

NMDA or saline group. Quantitatively, the mean total retinal thickness in the injected and uninjected eyes were comparable at  $237.8 \pm 27$  and  $231.6 \pm 26$   $\mu\text{m}$  respectively in the saline group (Bonferroni's post hoc test,  $P > 0.05$ ), and  $174 \pm 13$  and  $223 \pm 16$   $\mu\text{m}$ , respectively, in the NMDA group (Bonferroni's post hoc test,  $P < 0.001$ ), indicative of a significant reduction in total retinal thickness by 22% in the injected right eye relative to the uninjected left eye in the NMDA group. The total retinal thickness measured from OCT was compared with the DTI measurements in the optic nerve and optic tract with the random intercept mixed-effects model. As shown in Figure 4, correlation plots between retinal thickness and DTI parameters generally showed steeper slopes for the optic nerve than the optic tract. The total retinal thickness was found to be significantly and positively correlated with FA in both optic nerve and optic tract. It was also positively correlated with  $\lambda_{//}$  in the optic tract and negatively correlated with  $\lambda_{\perp}$  in the optic nerve with statistical significance. The effects of NMDA-induced injury on total retinal thickness were also significantly correlated with the random intercept mixed-effects model.

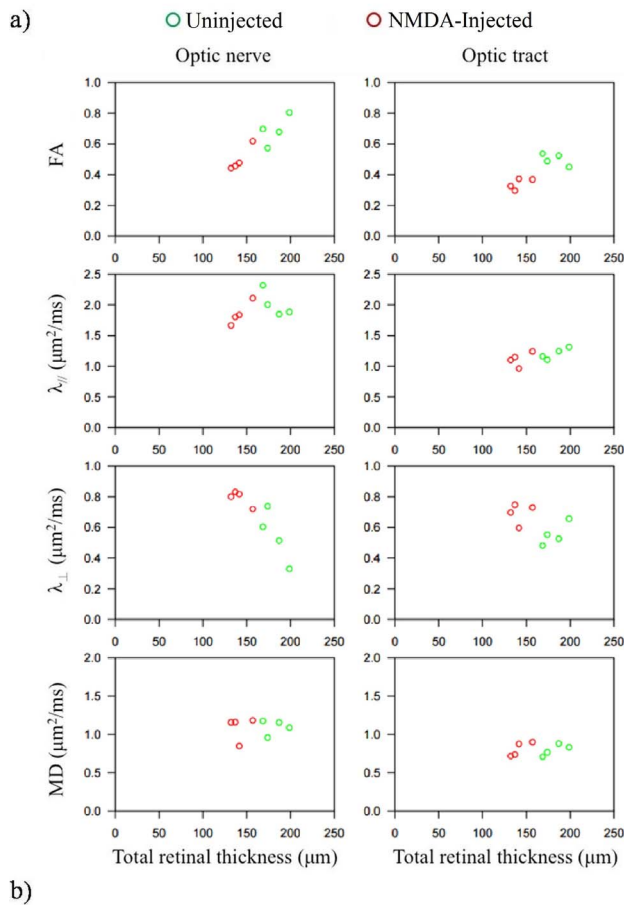
#### DTI of White Matter Integrity in Splenium of Corpus Callosum

The splenium of corpus callosum in the NMDA group showed a significant increase in FA and  $\lambda_{//}$  by 17.1% and 13.1%, respectively, at 4 weeks relative to 3 days after NMDA injection to the right eye (Fig. 5). None of the DTI parametric measurements in the saline group showed significant changes over the experimental period.

#### DTI of Visual Pathway Integrity and Correlations With MEMRI of Anterograde Transport in Mice

As shown in Figure 6, the NMDA mouse model in Group 3 showed comparable spatiotemporal DTI profiles to the rat model in Group 1. Specifically, significantly lower FA and  $\lambda_{//}$  were observed in the right optic nerve and left optic tract projected from the NMDA-injected eye relative to the visual pathway projected from the saline-injected eye in Group 4 at 7 to 70 days post injection. No significant difference in any DTI parametric measurements was observed between visual

## Relationships between OCT of Retina and DTI of Optic Nerve and Optic Tract



**FIGURE 4.** (a) Relationships between total retinal thickness in OCT (*x*-axis) and DTI parameters (*y*-axis) in the rat optic nerve (*left column*) and optic tract (*right column*) of both left and right visual pathways at 1 month after NMDA-induced glutamate excitotoxicity in the right eye. The data points for the visual pathways projected from the uninjected eye and the NMDA-injected eye are labeled in *green* and *red*, respectively. (b) The estimated slopes of DTI parameters in the optic nerve or optic tract against total retinal thickness ( $P < 0.05$ : estimated slopes are significantly different from 0). Fractional anisotropy and  $\lambda_{||}$  generally showed positive correlations with total retinal thickness, whereas  $\lambda_{\perp}$  and MD generally showed negative correlations with TRT. The magnitudes of the slopes between DTI parameters and retinal thickness were larger in the optic nerve than optic tract.

pathways projected from the injected and uninjected eyes in the saline group throughout the experimental period.

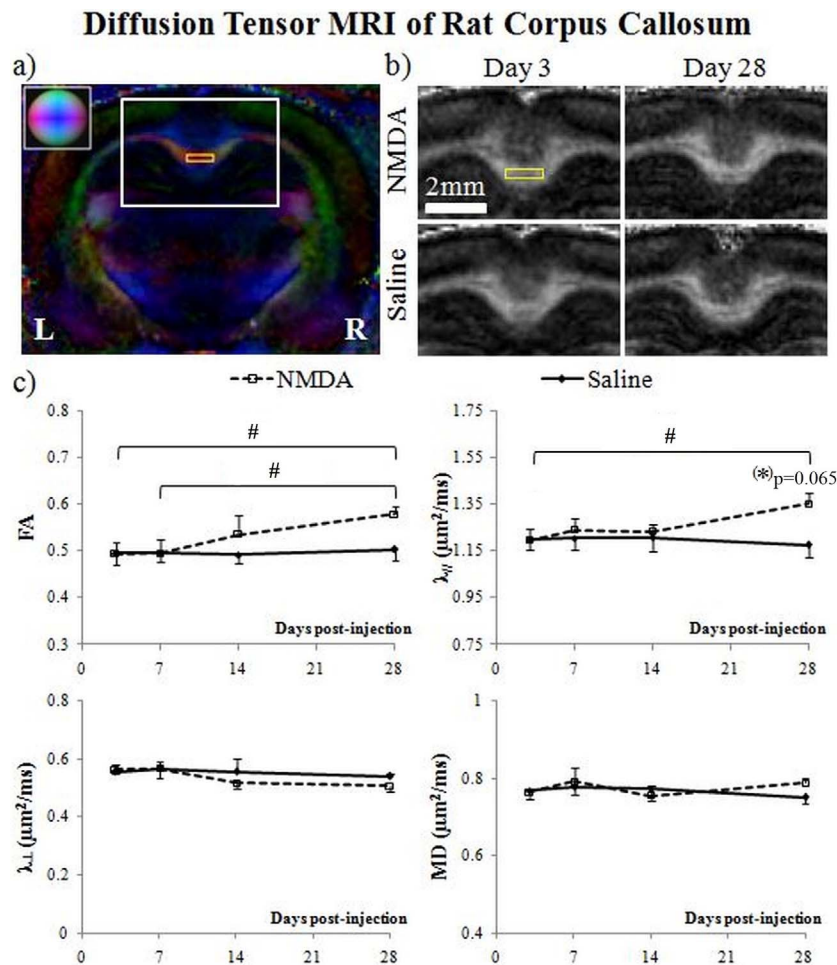
In the T1-weighted MR images at 1 week after NMDA or saline injection to the right eye and 8 hours after binocular Mn injection (Fig. 7). The Mn enhancement along the visual pathway projected from the NMDA-injected eye was significantly weaker than that projected from the uninjected eye of the same group, or from the saline-injected eye. Using the random intercept mixed-effects model (Fig. 8), FA and  $\lambda_{||}$  in the

optic nerve were significantly and positively correlated with Mn signal enhancement in the optic nerve, lateral geniculate nucleus and superior colliculus. No significant correlation was observed between  $\lambda_{\perp}$  in the optic nerve and Mn signal enhancement along the visual pathway.

## DISCUSSION

In this study, we characterized quantitatively by *in vivo* DTI the effects of NMDA-induced glutamate excitotoxicity in the eye on the spatiotemporal profiles of white matter integrity in the brain's visual system. Our DTI findings of the early  $\lambda_{||}$  decrease and delayed  $\lambda_{\perp}$  increase in the optic nerve within the experimental period may reflect different progression rates for several pathophysiological events known to occur in distal neural pathways such as early axonal injury and delayed demyelination. The stronger correlations between OCT of retinal loss and DTI of white matter integrity in the optic nerve than optic tract, and the late reorganization of the splenium of corpus callosum appeared consistent with the patterns of anterograde degeneration in the visual system after NMDA-induced excitotoxic retinal injury. In the NMDA mouse model, reduction of physiological anterograde transport of the active Mn tracer was observed along the injured visual pathway projected from the NMDA-injected eye. In addition, the strong preferential correlations of Mn enhancement with  $\lambda_{||}$  and FA but not  $\lambda_{\perp}$  in the optic nerve suggested the potential linkage between anterograde Mn transport disruption and the compromise of microstructures that predominated water diffusion changes parallel to the optic nerve such as microtubule and neurofilament injuries. Taken together, our results demonstrated the capability of *in vivo* imaging markers to reveal the pathological events, the eye-brain relationships and structural-functional relationships in the brain's visual system after eye injury. These results are potentially important in offering a model system to assess treatment strategies to both the eye and the brain in future studies.

Previous histologic studies have suggested the spread of neuronal cell degeneration to the posterior visual pathway early after excitotoxic retinal injury.<sup>16,19,30</sup> Our DTI findings indicated that these degenerative processes could be detected *in vivo* though indirectly by probing water diffusion properties noninvasively. Fractional anisotropy in DTI may reflect the directionality and overall integrity of the white matter. In the optic nerve, the continual FA decrease within the first 7 days after excitotoxic retinal injury appeared to be concurrent with ultrastructural findings revealed by *ex vivo* transmission electron tomography, whereby only parts of the neural components were injured at 3 days after injury, but almost all the optic nerve fibers were altered at 7 days after intravitreal NMDA injection at similar doses.<sup>16</sup> On the other hand, the directional diffusivities  $\lambda_{||}$  and  $\lambda_{\perp}$  have been reported to be sensitive to axonal and myelin integrity changes, respectively, in the white matter.<sup>30,31,49-52</sup> Upon excitotoxic retinal injury at similar NMDA doses, axonal swelling, and loss of microtubules had been found in the distal optic nerve within 3 days after NMDA injection,<sup>16</sup> with relative preservation of neurofilaments and myelin sheath. These phenomena appeared to restrict water diffusion preferentially along the optic nerve leading to the observed  $\lambda_{||}$  decrease. As the degeneration progressed, almost all axonal microstructures were collapsed and demyelination was frequently seen in the optic nerve by 7 days after NMDA injection.<sup>16</sup> Such patterns of disease progression may explain the delayed  $\lambda_{\perp}$  increase in the optic nerve at 7 days relative to early  $\lambda_{||}$  decrease observed at 3 days after NMDA injection. Fractional anisotropy,  $\lambda_{||}$ , and  $\lambda_{\perp}$  may offer sensitive *in vivo* imaging markers to help monitor the progression rates



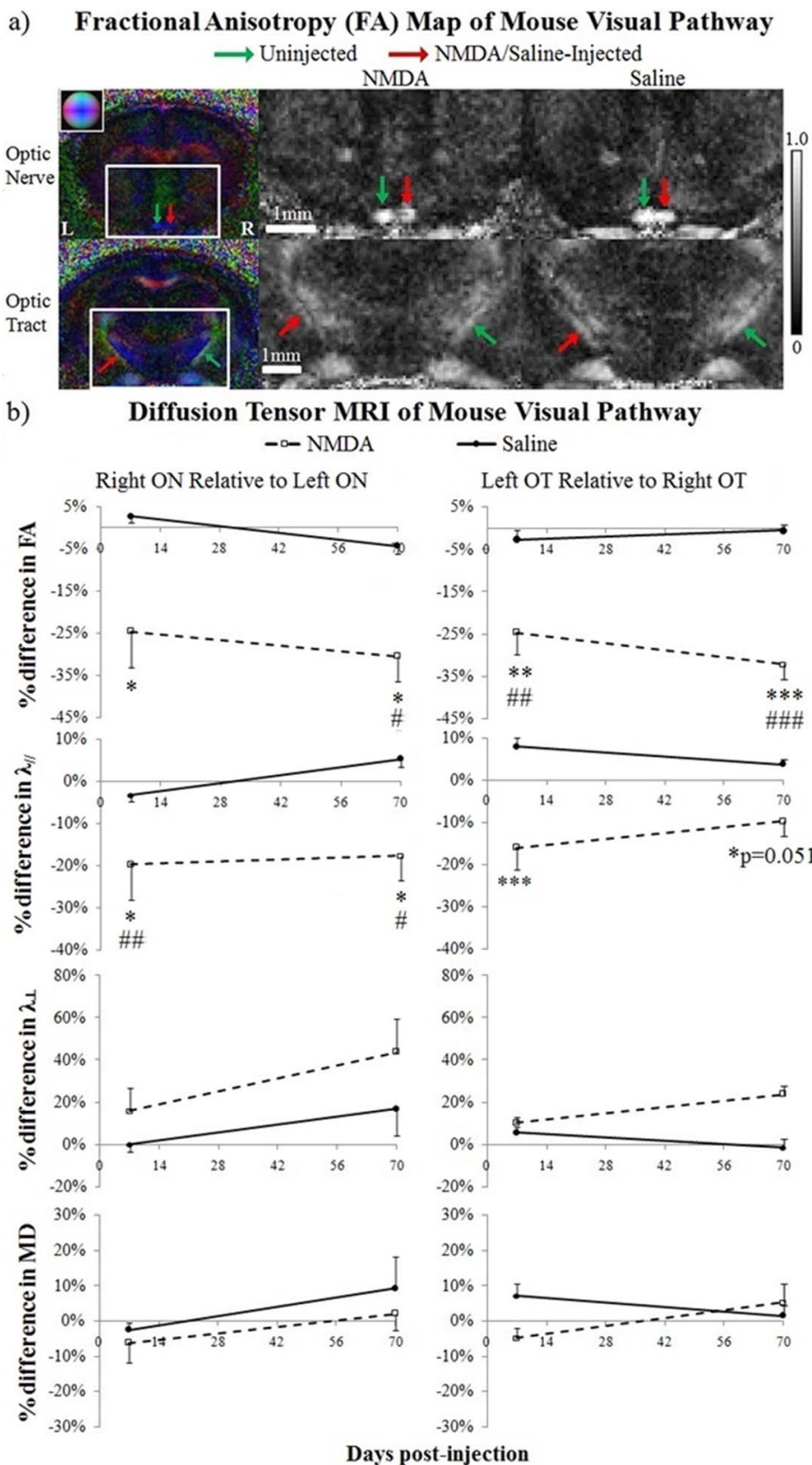
**FIGURE 5.** (a) Representative color-encoded FA maps of the rat brain at the level of the splenium of corpus callosum. (b) Representative FA value maps enlarged from the white box in (a) showing the splenium of corpus callosum at 3 and 28 days after NMDA or saline injection to the right eye. The region of interest for DTI quantitation of the splenium of corpus callosum is outlined in yellow. (c) Diffusion tensor MRI quantitation (mean  $\pm$  SEM) of the splenium of corpus callosum throughout the experimental period. Significant increases in FA and  $\lambda_{//}$  were observed in the NMDA group at day 28 relative to day 3 post injection (Bonferroni's multiple comparisons tests across time, # $P < 0.05$ ; between NMDA and saline groups, \* $P < 0.05$ ; color representations for the principal diffusion directions in the color-encoded FA maps: blue, caudal-rostral; red, left-right; green, dorsal-ventral).

for different pathological events in the optic nerve after glutamate excitotoxicity in the eye.

By comparing the DTI parametric changes between the optic nerve and optic tract, the directional diffusivities  $\lambda_{//}$  and  $\lambda_{\perp}$  appeared to be more sensitive markers than FA and MD in differentiating the spatial relationships of neurodegeneration in the brain's visual system after excitotoxic retinal injury. The relatively larger  $\lambda_{//}$  and  $\lambda_{\perp}$  changes in the optic nerve than the optic tract indicated that the compromise in white matter integrity was more pronounced in the proximal than distal visual pathway, likely as a result of the involvement of Wallerian-like anterograde degeneration in the brain's visual system.<sup>16</sup> Such patterns of degenerative processes were further supported by the stronger eye-brain relationships between OCT's retinal thickness and DTI parameters in the optic nerve compared with the optic tract. The retinal thinning detected in this study apparently reflected the NMDA-induced damages to the ganglion cell and inner plexiform layers in the ganglion cell complex.<sup>40</sup> The NMDA-injected eyes in our OCT images also experienced thinning of the inner hyperreflective region and the outer retinal regions, which likely represented some loss of the retinal nerve fiber layer and the outer retinal layers.

However, these individual layers were not segmented in the current study because image quality was not sufficient in all scans to consistently and reliably delineate these regions.

In the occipital brain, axonal projections from the visual cortex of both hemispheres interconnect through the splenium of corpus callosum for interhemispheric transfer of visual information.<sup>53-57</sup> In this study, significant FA and  $\lambda_{//}$  increases in the splenium of corpus callosum occurred later than DTI changes in the optic nerve and optic tract at 4 weeks after glutamate excitotoxicity in the eye. While optic nerve injury may lead to anterograde degeneration in the visual cortex and subcortical visual nuclei at similar experimental time points,<sup>17,19</sup> it remains to be elucidated whether the integrity of the splenium of corpus callosum was altered as a result of the neurodegeneration in the visual cortex after NMDA-induced injury in the visual pathway. Fractional anisotropy and/or  $\lambda_{//}$  increases have been suggested to reflect white matter remodeling during neuroplasticity.<sup>32,58-61</sup> There was also experimental evidence showing structural or functional reorganization mediated by the splenium of corpus callosum<sup>62-64</sup> and cross-modal plasticity in the visual cortex<sup>65-67</sup> after unilateral loss of visual input in adults. Future studies will

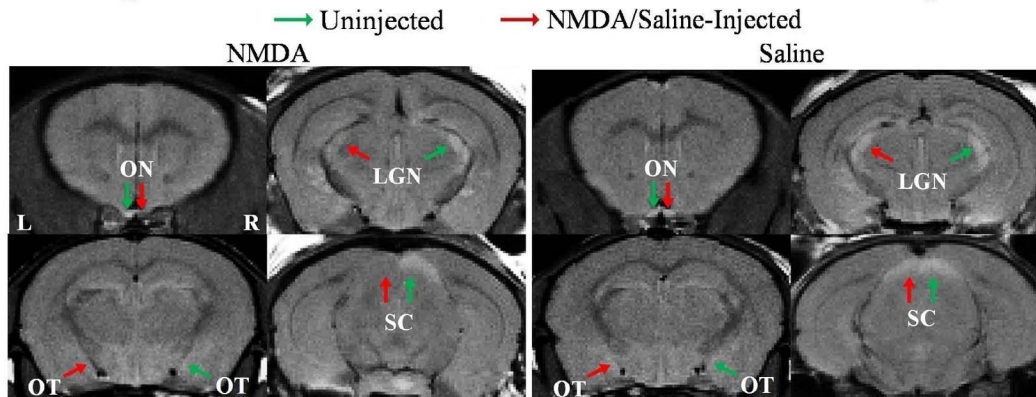


**FIGURE 6.** (a) *Left column:* Representative color-encoded FA directionality maps of the mouse brain at the levels of the prechiasmatic optic nerve (top) and optic tract (bottom) at 7 days post NMDA injection. *Middle and right columns:* Representative FA value maps enlarged from the white boxes in the color-encoded FA maps at 7 days after NMDA (middle column) or saline injection (right column) to the right eye. Red arrows indicate the visual pathway projected from the injected right eye. Green arrows indicate the visual pathway projected from the uninjected left eye. The grayscale bar indicates the range of FA values from 0 to 1.0. (b) Spatiotemporal DTI quantitation of percentage difference (mean  $\pm$  SEM) in the right optic nerve or left optic tract relative to the left optic nerve or right optic tract at 7 and 70 days after NMDA or saline injection to the right eye



(Bonferroni's multiple comparisons tests between NMDA and saline groups, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; between left and right visual pathways in the NMDA group, # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$ ). Significantly larger FA and  $\lambda_{//}$  decreases were observed in the optic nerve and optic tract of the NMDA group throughout the experimental period. No apparent difference was observed between left and right visual pathways in the saline group (color representations for the principal diffusion directions in the color-encoded FA maps: blue, caudal-rostral; red, left-right; green, dorsal-ventral).

a) T1-weighted MRI at 8 Hours after Binocular Intravitreal Mn Injection



b) Manganese-enhanced MRI Quantitation of Mouse Visual Pathway

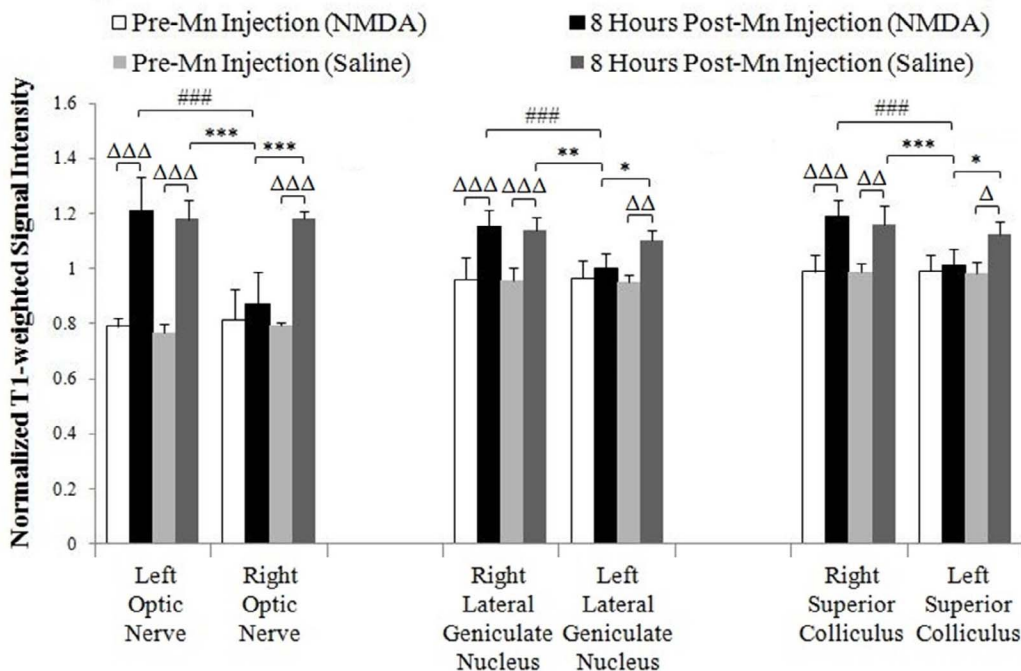
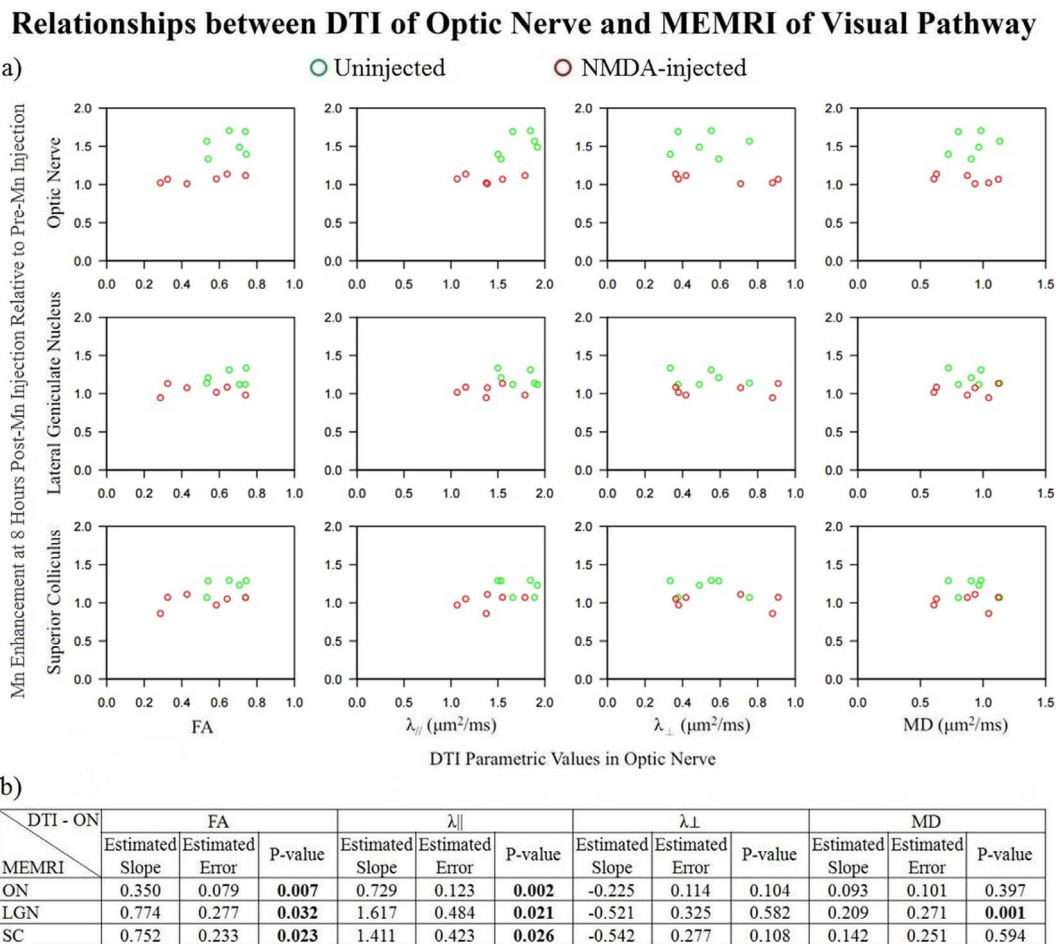


FIGURE 7. (a) T1-weighted MRIs of the mouse optic nerve (ON), optic tract (OT), lateral geniculate nucleus (LGN), and superior colliculus (SC) at 1 week after NMDA (first two columns) or saline injection (last two columns) to the right eye and 8 hours after intravitreal Mn injection to both eyes. Red arrows indicate the visual pathway projected from the NMDA- or saline-injected right eye. Green arrows indicate the visual pathway projected from the left eye without NMDA or saline injection. Manganese enhancement was observed as T1-weighted hyperintensity along the bilateral visual pathways in the saline group and in the visual pathway projected from the uninjected left eye in the NMDA group. Less apparent Mn enhancement was found along the visual pathway projected from the NMDA-injected right eye. (b) Quantitative comparisons of T1-weighted signal intensities (mean  $\pm$  SEM) of the mouse ON, LGN, and SC before (Pre-Mn) and at 8 hours after intravitreal Mn injection (post-Mn) to both eyes in the NMDA or saline group. Signal intensities were normalized to the nearby saline phantom to counter for system instability between imaging sessions. Before Mn injection, no significant difference in T1-weighted signals was observed between left and right visual pathways in either NMDA or saline group. At 8 hours post Mn injection, significant Mn enhancement was detected in the ON, LGN, and SC bilaterally in the saline group compared to pre-Mn injection. Significant Mn enhancement was also detected unilaterally along the visual pathway projected from the uninjected left eye in the NMDA group. No apparent Mn enhancement was observed along the visual pathway projected from the NMDA-injected right eye (Bonferroni's multiple comparisons tests between pre-Mn injection and 8 hours post Mn injection in the same visual pathway,  $\Delta P < 0.05$ ,  $\Delta\Delta P < 0.01$ ,  $\Delta\Delta\Delta P < 0.001$ ; between NMDA and saline groups at 8 hours post Mn injection, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; between left and right visual pathways in the NMDA group at 8 hours post Mn injection, ### $P < 0.001$ ).



**FIGURE 8.** (a) Relationships between Mn enhancement ( $y$ -axis) and DTI parameters ( $x$ -axis) along bilateral visual pathways at 1 week after NMDA injection to the right mouse eye. Manganese enhancement was derived from T1-weighted signal intensities in the ON, LGN, or SC at 8 hours after binocular Mn injection relative to pre-Mn injection. Diffusion tensor MRI parameters include FA,  $\lambda_{||}$ ,  $\lambda_{\perp}$ , and MD in the ON. (b) The estimated slopes of Mn signal enhancement in the ON, LGN, or SC against each DTI parameter in ON of both hemispheres at 1 week after NMDA injection to the right eye ( $P < 0.05$ : estimated slopes are significantly different from 0). Fractional anisotropy and  $\lambda_{||}$  in the ON showed significant positive correlations with Mn enhancement in the ON, LGN, and SC.

examine how activity-induced plasticity or other neurophysiological events might be involved in the splenium of corpus callosum leading to the observed late DTI changes after excitotoxic retinal injury.

Upon intravitreal Mn injection, the exogenous Mn ions could be taken up by the retinal ganglion cells and transported anterogradely along the visual pathway to the superior colliculus and lateral geniculate nucleus.<sup>68,69</sup> Since Mn is paramagnetic and can lead to hyperintensity in T1-weighted images, MEMRI is a robust technique for neuronal tract tracing and for examining anterograde transport in healthy and injured brains.<sup>68,70-75</sup> Nevertheless, the relative contributions of visual pathway integrity to the Mn transport and signal enhancement along the neuronal tract have not been fully understood.<sup>76-80</sup> In the NMDA mouse model, reduced Mn transport was observed along the NMDA-injured visual pathway after intravitreal Mn injection. However, only  $\lambda_{||}$  and FA but not  $\lambda_{\perp}$  in the optic nerve were significantly correlated with Mn enhancement in the optic nerve, lateral geniculate nucleus, and superior colliculus. Although both axonal degeneration and demyelination had been reported in the visual pathway in the same NMDA model at similar doses and experimental time points,<sup>11,57</sup> the Mn transport appeared to be preferentially susceptible to white matter integrity changes that primarily

altered water diffusion along the optic nerve in events such as microtubule and neurofilament damages as compared with the perpendicular direction in events such as myelin disruption. Because DTI may not discriminate axonal and myelin integrity specifically in the presence of other concurrent factors that may affect water diffusion such as inflammation, future studies may use more advanced diffusion MRI acquisition and modeling<sup>81-83</sup> to probe more specifically the structural-physiological relationships of visual pathway integrity, and the microstructural basis of Mn transport mechanisms. Future studies may also employ larger samples to investigate in more detail the relationships between MEMRI and DTI in separate injured and uninjured visual pathways. Although measuring Mn transport in the visual system using MEMRI would not be a viable option in humans at present due to the potential toxicity of  $\text{MnCl}_2$ ,<sup>84-86</sup> by understanding the microstructural basis of Mn transport via relating Mn enhancement to diffusion properties in the neural pathway, it may be possible to use noninvasive diffusion MR parameters such as axial diffusivity change as outcome measures to assess neuroprotective approaches targeting at axonal transport in the future. There were recent initial MEMRI studies using the relatively less toxic and Food and Drug Administration-approved Mn-chelates (e.g., Mn-DPDP) to perform neuronal tract tracing in normal rodent

optic nerves.<sup>87</sup> Future studies may also evaluate the potentials and safety of such Mn-chelates for assessing transport obstruction and repair in visual pathway injury compared with MnCl<sub>2</sub>.

Intravitreal injection of NMDA can induce excitotoxic retinal injury and optic neuropathy in a dose-dependent manner.<sup>37,39,40</sup> The effects of glutamate excitotoxicity on necrotic and apoptotic cell death in the retina and the brain are also dependent on the types and amount of glutamate receptors involved at the site of insult.<sup>16,39</sup> As abnormalities in glutamate metabolism were suggested to play a vital role in human diseases such as retinal ischemia,<sup>5</sup> glaucoma,<sup>11</sup> and diabetic retinopathy,<sup>12,13</sup> the current model may hint on the mechanisms of visual pathway degeneration associated with these diseases. Future studies may refine and combine the established MRI and OCT techniques with visual psychophysical assessments<sup>88</sup> to evaluate the eye-brain-behavior relationships upon altering the types and degrees of excitotoxic insults mimicking different pathological conditions of human ocular diseases and injuries. It has been suggested that treatment to both the eye and the brain upon optic nerve injury may provide better outcomes than treating the eye alone.<sup>89</sup> Our MRI and OCT examinations of retinal loss, optic neuropathy, and corpus callosum reorganization after excitotoxic retinal injury may also provide an in vivo model system to assess the beneficial effects of different neuroprotective strategies on both the eye and the brain in future studies. Future studies may also use more advanced diffusion MRI methodology (e.g., diffusion kurtosis imaging and diffusion based spectrum imaging) to better characterize the pathophysiological events in the visual system upon ocular diseases and injuries.

## CONCLUSIONS

Optical coherence tomography, DTI, and MEMRI provided an in vivo model system to monitor the spatiotemporal patterns of white matter integrity changes, the eye-brain relationships and the structural-physiological relationships in the visual system after NMDA-induced glutamate excitotoxic injury to the retina. The current results may offer in vivo imaging markers to assess the beneficial effects of neuroprotective strategies on both the anterior and posterior visual pathways upon ocular diseases and injuries in future studies. The preferential influence of reduced anterograde Mn transport by  $\lambda_{//}$ , but not  $\lambda_{\perp}$  in the neural pathway may also shed light on the microstructural basis of Mn transport mechanisms.

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