Cell Swelling Contributes to Thickening of Low-Dose N-methyl-D-Aspartate–Induced Retinal Edema

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PURPOSE. The contribution of cell swelling versus vascular leakage in retinal edema remains largely undefined. The objective of this study was to use in vivo magnetic resonance imaging (MRI) to assess retinal cell swelling in the edematous mouse retina.

METHODS. Inner retinal edema was induced by intravitreal injection of 2.5 nmol N-methyl-D-aspartate (NMDA). To assess retinal cell swelling, diffusion MRI was performed at baseline, 3-hours, 1 day, 3 days, and 7 days ($n \geq 5$ at each time point) after NMDA injection. To detect retinal vascular leakage, gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) enhanced MRI was performed at baseline, 3 hours and 1 day ($n = 5$ for each group) after NMDA injection. Upon the completion of MRI, mouse eyes were enucleated, cryosectioned, and stained for assessing retinal layer thickness and cell death.

RESULTS. Inner retinal cell swelling was hyperintense on diffusion-weighted images at 3 hours and 1 day after NMDA injection. The thickened inner retina was also seen in anatomic MRI and histology. Quantitatively, inner retinal apparent diffusion coefficient (ADC) decreased approximately 20% at 3 hours and 1 day after NMDA injection ($P < 0.05$ compared with baseline), suggesting cell swelling. Systematic injection of paramagnetic Gd-DTPA did not alter vitreous longitudinal relaxation time (T1) at baseline or at 3 hours after NMDA injection. In contrast, vitreous T1 in mice decreased $16 \pm 6\%$ ($P < 0.05$), reflecting retinal vascular leakage at 1 day after NMDA injection.

CONCLUSIONS. Noninvasive diffusion MRI was performed to detect retinal cell swelling in vivo. Our results demonstrated that retinal cell swelling could directly lead to retinal thickening independent of vascular leakage. (*Invest Ophthalmol Vis Sci.* 2012;53:2777–2785) DOI:10.1167/iovs.11-8827

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Retinal edema impairs vision in millions. It is diagnosed as abnormal thickening of the retina that is commonly observed in ischemic and inflammatory diseases.^{1–4} Retinal edema can be classified as cytotoxic or vasogenic, which respectively reflects cell swelling induced by volumetric increase of *intracellular* water and vascular leakage induced volumetric increase of *extracellular* water.⁵ It is commonly accepted that vascular leakage is the primary pathway of retinal edema. However, in ischemic/excitotoxic diseases cell swelling may develop early or coexist with vascular leakage.^{6,7} It has also been suggested that the swelling and death of Müller cells contributes to the development of cystoid macular edema.⁴ Thus, cell swelling and vascular leakage may have different contributions to retinal edema in individual patients.

Noninvasive methods for direct assessment of retinal cell swelling are lacking. Unlike vascular leakage induced edema that can be indirectly detected using fluorescein angiography,^{2,8} retinal cell swelling can be implicated only in certain patients who develop retinal thickening without vascular leakage.⁹ Given the different mechanisms underlying cytotoxic and vasogenic edema, the capability to differentially assess retinal cell swelling and vascular leakage is therefore critical to advance the current disease diagnosis and therapy.

In addition to its detectability, the contribution of cell swelling to edematous retinal thickening remains an open question. Knowledge acquired from cerebral diseases suggested that cell swelling may not lead to increased tissue volume if the blood–tissue barrier, which limits extravasation of blood-pool water, is intact.¹⁰ Instead, cell swelling can develop through redistribution of extracellular water, accompanying the ion (primarily Na⁺) flux, into the intracellular compartment.¹⁰ Since the neural retina comprises a blood–retina barrier, if the same mechanism applies, cell swelling may not lead to retinal thickening before the onset of retinal vascular leakage.

We hypothesize that retinal cell swelling directly causes retinal thickening. The retina is a thin layer of tissue (200–300 μm thick) located adjacent to vitreous (99% of water content).¹¹ It continuously experiences a transretinal fluid flux driven by intraocular pressure gradient from the vitreous to the choroid.⁵ This unrestricted extracellular fluid supply suggests retinal cell swelling may develop without changing the extracellular water volume. A mouse model of N-methyl-D-aspartate (NMDA) excitotoxicity was used to test our hypothesis. Excessive binding of intravitreally injected NMDA to NMDA receptors causes overexcitation of neuronal cells followed by energy failure. The resulting cell swelling and edematous retinal thickening occur within several hours.¹² Due to the absence of NMDA receptors on photoreceptor cells in the outer retina, NMDA excitotoxicity primarily damages the inner retina that extends from the nerve fiber layer to the outer plexiform layer.

In this study, diffusion MRI-derived water apparent diffusion coefficient (ADC) was measured to evaluate NMDA excitotoxicity-induced cell swelling in the inner and outer

retina. Diffusion MRI is a primary clinical imaging method for assessing neural tissue injury.¹³ Despite the retinal edema-induced concomitant T1 and T2 changes during the diffusion-weighted MRI measurement, the derivation of ADC takes the ratio of image intensities with and without diffusion weighting, which essentially performed the automatic normalization of T1 and T2 effects eliminating the commonly seen “T2 shine through” effect in diffusion-weighted images.¹³ The measured ADC is by nature independent of T1 relaxation, T2 relaxation, or the magnetic field strength.^{14,15} It has long been recognized that neuronal cell swelling leads to an ADC decrease.^{16,17} When cell swelling and vasogenic edema coexist, such as that observed in ischemia/reperfusion injury,^{18,19} the relative contribution of each component at different stages of the disease confounds the interpretation of the longitudinal evolution of ADC.

Gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) enhanced MRI with T1 mapping was used in this study to evaluate retinal vascular leakage before, at 3 hours, and 1 day after NMDA treatment. Gadolinium is a paramagnetic ion that reduces ¹H T1 of surrounding water molecules. The chelated Gd-DTPA has limited permeability through intact blood-brain barrier or blood-retina barrier. In CNS diseases such as multiple sclerosis (MS), the Gd-DTPA from the blood pool into perivascular tissue through the damaged blood-brain barrier in active lesions results in signal enhancement on T1-weighted images.^{20–22} The sensitivity of this technique to MS lesions is improved by administering a higher dose of Gd-DTPA and delayed image acquisition^{23,24}; for example, it reached a 95% detection rate for MS lesions when MR images were acquired at 20 minutes after a triple dose (0.3 mmol/kg) of Gd-DTPA.²⁴ In retinal diseases such as diabetic retinopathy²⁵ and macular edema,²⁶ the leakage of Gd-DTPA into avascular posterior vitreous caused local enhancement of the T1-weighted signal. The Gd-DTPA-enhanced MRI-detected retinal vascular leakage was confirmed by histology²⁷ or electron microscopy.²⁸ Our recent work showed that retinal vascular leakage in *rd1* mice resulted in a progressive increase of vitreous signal within the first 30 minutes after injection of 1.0 mmol/kg Gd-DTPA.²⁹ Unfortunately, the exact sensitivity of this MRI technique to retinal vascular leakage, as compared with standard fluorescein angiography and histological methods, has not been defined yet. Based on the previous work of our group and others, we used a high dose of Gd-DTPA (1.0 mmol/kg, 10-folds the standard dose) and delayed image acquisition (between 30 and 45 minutes after Gd-DTPA injection) to optimize MRI sensitivity against retinal vascular leakage.

METHODS

NMDA Animal Model

All animal experiments were performed according to protocols approved by the Animal Studies Committee at Washington University and were in adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

A total of 71 3- to 4-month-old male C57BL/6 mice were used. Normal mice ($n = 5$) were used to determine the baseline parameters. Other mice were intravitreally injected with 0.5 μ L of 5 mM (equivalent to 2.5 nmol) NMDA or sham-injected with 0.5 μ L of saline. Diffusion MRI was performed at 3 hours ($n = 8$ for NMDA group vs. 5 for sham group), 1 day ($n = 8$ vs. 7), 3 days ($n = 8$ vs. 5) or 7 days ($n = 5$ vs. 5) after intravitreal injection. A separate group of mice was used to assess the development of NMDA excitotoxicity-induced retinal vascular leakage. The differences in vitreous longitudinal relaxation time (T1) after Gd-DTPA injection in normal mice ($n = 5$), and in mice at 3 hours ($n = 5$), and 1 day ($n = 5$) after NMDA injection were examined.

Intravitreal Injection Protocol

Intravitreal injection was performed according to reported procedures with some modifications.³⁰ Mice were anesthetized by inhalation of 1.5–3% isoflurane mixed with oxygen. A drop of topical antibiotics was applied to the left eye. A 34-gauge sharp needle was inserted into the posterior vitreous (1.5 mm depth) at approximately 1 mm posterior to the limbus. The other end of the needle was connected to a microinjection pump (World Precision Instruments, Sarasota, FL). A total of 0.5 μ L 5 mM (equivalent to 2.5 nmol) NMDA or saline was injected into the vitreous within 15 seconds. The needle was kept in the vitreous for 1 extra minute and then was slowly pulled out. Finally, a drop of antibiotic gel was applied to the left eye.

The injection volume was selected to avoid distortion of the globe that was frequently observed after injection of >1 μ L fluid. The 0.5 μ L fluid occupied approximately 10% of mouse vitreous volume (4.3 ± 0.6 μ L, $n = 6$) as determined by MRI (voxel size = $100 \times 100 \times 100$ μ m³ or 1 nL; Kaplan HJ, et al. *IOVS* 2010;43:ARVO E-Abstract 4414).

MRI

MRI was performed on a commercial MRI system scanner (Varian 11.74T UNITYINOVA spectrometer/scanner; Varian Associates, Palo Alto, CA) using our previously reported procedure. Mice were anesthetized by intraperitoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg) followed by constant subcutaneous infusion of ketamine (54 mg/kg/h)/xylazine (4 mg/kg/h). Animal body temperature was maintained at 37°C and its respiration was monitored using a small animal heating and monitoring system (SA Instruments, Stony Brook, NY). An active-decoupled saddle volume coil was used for radiofrequency (RF) transmission. An actively decoupled surface coil (diameter = 1 cm) was placed on top of the left eye for receiving the MR signal. All images were acquired on a nasal-temporal slice that bisects the eye through the optic nerve head.

Diffusion MRI was used to assess NMDA excitotoxicity-induced retinal cell injury. Three orthogonal diffusion gradients were applied in directions parallel to and in- and out-of-plane perpendicular to the axis of the optic nerve. In each direction, a pair of diffusion-weighted images was acquired with positive and negative diffusion gradients to minimize the background magnetic field gradient effect on the diffusion measurement. Acquisition parameters were: slice thickness 400 μ m; field-of-view 12×12 mm²; data matrix 256×256 (zero filled to 512×512); in-plane resolution 47×47 μ m²; number of averages 1; repetition time (TR) 2.0 s; echo time (TE) 34 ms; diffusion gradient on time (δ) 5 ms; duration time between two diffusion gradients (Δ) 15 ms; b-values 0 and 954 s/mm²; acquisition time 1 hour.

Gd-DTPA-enhanced MRI was used to assess the development of retinal vascular leakage in NMDA-injected mice. Two sets of T1-weighted images of mouse eye were acquired before and 30 minutes after a bolus injection of Gd-DTPA (1 mmol/kg) through an intraperitoneal catheter. Acquisition parameters were: slice thickness 400 μ m; field-of-view 6×6 mm²; data matrix 32×64 (the resolution was higher in the direction parallel to the optic nerve, zero filled to 64×64); in-plane resolution 188×94 μ m²; TR (number of averages) 0.3 s (8), 0.8 s (4), 2.0 s (2), 6.0 s (1), 16.0 s (1); TE 16 ms; acquisition time 17 minutes.

All animals underwent a single diffusion or Gd-DTPA-enhanced MRI measurement. Upon the conclusion of MRI, mice were euthanized and eyes were enucleated for histology.

MR Data Analysis

Diffusion-weighted and T1-weighted MR images were processed using previously described methods to determine ADC and T1 maps of the mouse eye.³¹

NMDA-induced cell injury in the inner and outer retina was assessed using ADC. Segmentation of the inner and outer retina was performed following a previously described procedure with minor

FIGURE 1. Representative nondiffusion-weighted (*left*) and diffusion-weighted (*right*) MR images of mouse eyes at 3 hours after saline injection (**a**), or at 3 hours (**b**) and 1 day (**c**) after NMDA injection. On nondiffusion-weighted images, the anterior chamber (AC), lens (L), vitreous (V), and sclera (S) are clearly identified. Retina/choroid layers (R/C) were bright on diffusion weighting and exhibited multiple signal intensity layers on nondiffusion-weighted images. At 3 hours and 1 day after intravitreal injection of NMDA, a hyperintense inner retina layer appeared in diffusion-weighted images, reflecting decreased diffusivity. On nondiffusion images of the same mouse eyes, more MR-detected layers in retina are visually discernible.

adjustments (see Figs. 3a and 3b in the following text).³¹ First, the retina and choroid layers were identified by the hyperintense signal on diffusion-weighted images. The retina and choroid were determined as the region with signal $> \text{mean} \pm 3\text{SD}$ of vitreous and sclera signal. Second, the choroid was manually segmented on nondiffusion-weighted images as the outermost hyperintense layer of the retina/choroid layers. The remaining tissue was considered as the MRI-detected retina. Finally, the outermost hypointense retinal layer on nondiffusion-weighted images was manually segmented and assigned as the outer retina that comprises photoreceptor cell nuclei, inner and outer segments. All other MRI-detected retinal layers were assigned to the inner retina that extends from the nerve fiber layer to the outer plexiform layer. Due to the absence of MR signal contrast at the peripheral retina, we used our previously reported protocol to define the region-of-interest (ROI) analyzing retinal ADC.³¹ Specifically, two central retinal segments, located between 250 and 750 μm away from the optic nerve head, were used to determine the mean retinal ADC.

The MRI-determined inner and outer retinal thickness was estimated as the mean thickness in the two segments.

To quantify vitreous T1 on the calculated T1-map, the ROI covering the entire vitreous space was defined. The mean T1 in this ROI was used for interanimal data comparison.

Histology

Eyes were enucleated, flash-frozen, and sectioned (8 μm thick). Tissue sections were stained with hematoxylin and eosin (H&E) for analysis of the retinal thickness and cell loss, and stained with terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) for analysis of cell apoptosis. Only those sections cut through the optic nerve head and perpendicular to the retina were selected for data analysis. The thickness of the inner and outer retina on H&E-stained slices was measured in the central retina region corresponding to that used for ADC analysis. Because sporadic tissue loss had occurred during the TUNEL staining preparation, the exactly identical ROI for

FIGURE 2. Assignment of MR-detected retinal cell layers based on the MRI and histology-measured layer thickness. The R/C layers on nondiffusion-weighted images (*left*) and the corresponding regions on H&E-stained retinal sections (*right*) in mice at 1 day after saline (**a**) and NMDA (**b**) injection are shown. The bright layer adjacent to the sclera was assigned to the choroid. The dark retinal layer adjacent to the choroid was assigned to the outer retina. The remaining retinal layers, four in mice at 3 hours and 1 day after NMDA injection and two in all other mice, were assigned to the inner retina. MRI and histology-quantified thickness of retinal layers and the choroid after saline (**c**) or NMDA (**d**) injection is shown. Each data point represents the average of mice at 3 hours and 1 day after injection. Compared with histology, MRI detected similar inner retinal thickness, underestimated the outer retinal thickness, and overestimated the choroid thickness in all mice. * $P < 0.05$ compared with histology-determined layer thickness. Data were presented as mean $\pm$ SD. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer of photoreceptor cells; IS/OS, inner and outer segments of photoreceptor cells; RPE, retinal pigment epithelium.

FIGURE 3. Histology-determined inner and outer retina thickness of normal and NMDA-injured mice. H&E-stained retinal sections of mice at 1 day after saline or NMDA injection (a) show substantially increased retina thickness in NMDA-treated mice. Arrows indicate the optic nerve head. Scale bar: 100 μ m. Quantitative measurements showed increased inner retinal thickness at 3 hours and 1 day after NMDA injection (b). The outer retinal thickness remained unchanged in all mice (c). * $P < 0.05$ compared with saline-injected mice at the same time point and normal mice at baseline. Data were presented as mean $\pm$ SD.

ADC and thickness measurement by MRI could not be obtained without encountering tissue voids by histology. Thus, four 200- μ m-wide central retinal segments from two adjacent frozen-tissue sections, a subregion representing the largest area allowing a consistent localization among tissues within the MRI analysis region, were used for counting necrotic or apoptotic cells.

Statistics

All statistical analyses were performed using commercial software (SAS software; SAS Institute, Cary, NC). Quantitative data are expressed as mean $\pm$ SD. For comparisons between two experimental groups, the significance of the difference between the means was calculated.

Two-way ANOVA was used to test the difference of: (1) the inner or outer retinal ADC for NMDA- versus saline-injected mice at different time points; (2) the inner or outer retinal thickness for NMDA- versus saline-injected mice at different time points; and (3) the number of necrotic or apoptotic cells among different retinal nuclear layers at different time points. One-way ANOVA was used to test the difference of Gd-DTPA-induced T1 shortening among normal mice, NMDA-injected mice at 3 hours, and NMDA-injected mice at 1 day. When

overall significance of $P < 0.05$ was attained by ANOVA, comparisons between the means were performed using the Freeman-Tukey test. Paired Student's *t*-test was used to test the difference of vitreous T1 before and after Gd-DTPA treatment. A statistically significant difference was defined by $P < 0.05$.

RESULTS

MRI Detected Abnormal Inner Retinal Signal at 3 Hours and 1 Day after NMDA Injection

Figure 1 shows representative diffusion- and nondiffusion-weighted MR images of mouse eyes. In nondiffusion-weighted images, three MR signal intensity defined layers were seen in the retina of normal mice (Figs. 1a, 2a). Both lens and sclera appeared dark. In contrast, the anterior chamber and vitreous were bright. In diffusion-weighted images (b -value = 954 s/mm²), the vitreous and anterior chambers were nearly invisible because of fast water diffusion in these compartments. The retina/choroid layers appeared bright because of restricted water diffusion in these tissues.

At 3 hours and 1 day after NMDA injection, diffusion-weighted images detected abnormal hyperintense signal in the inner retina, suggestive of reduced water diffusivity. Nondiffusion-weighted images showed two more MR-detected retinal layers in NMDA-treated mice than that in saline-injected mice, indicating retinal thickening (Figs. 1b, 1c, 2b).

Inner Retinal Thickness Transiently Increased at 3 Hours and 1 Day after NMDA Injection

Figures 2a and 2b show the assignment of MRI-detected retinal/choroid layers using MRI and histology-measured retinal thickness. Based on our previous reports,^{29,31,32} the outmost MR-detected retina/choroid layer, that is, the bright layer adjacent to sclera, was assigned as the choroid. The layer adjacent to the choroid was assigned as outer retinal photoreceptor cells comprising the outer nuclear layer and the inner and outer segments. The remaining MRI-detected layers were assigned as the inner retina. The assigned inner retina was confirmed by the comparable MRI and histology-determined inner retinal thickness in both saline- and NMDA-treated mice (Figs. 2c, 2d). The thickness of the MRI-detected outer retina was underestimated due to the partial volume-effect-induced overestimation of the thickness of MRI-detected choroid.³¹

Histological analysis showed NMDA injection acutely caused an approximately 50% increase of inner retinal thickness, reaching $170 \pm 17 \mu$ m at 3 hours and $163 \pm 16 \mu$ m at 1 day ($P < 0.05$ compared with saline-injected mice). From 3 days after NMDA injection, the inner retinal thickness recovered to its normal value of $115 \pm 15 \mu$ m (Figs. 3a, 3b). The outer retinal thickness remained unchanged at all time points after NMDA injection (Fig. 3c).

Inner Retinal ADC Transiently Decreased at 3 Hours and 1 Day after NMDA Injection

Figure 4 shows diffusion MRI quantified inner and outer retinal ADC. The inner retinal ADC was $0.51 \pm 0.07 \mu$ m²/ms at baseline. After NMDA injection, the inner retinal ADC decreased by approximately 20%, reducing to $0.40 \pm 0.02 \mu$ m²/ms at 3 hours and $0.39 \pm 0.05 \mu$ m²/ms at 1 day. From 3 days after NMDA injection, the inner retinal ADC recovered to baseline values. In contrast, the outer retinal ADC remained unchanged after NMDA injection. In saline-injected mice, both

FIGURE 4. Diffusion MRI determined inner and outer retinal ADC of normal and NMDA-injured mice. Representative ADC maps of mouse retina at 1 day after saline or NMDA injection were shown (a). The inner retinal ADC transiently reduced in mice at 3 hours and 1 day after NMDA injection (b); the outer retinal ADC remained unchanged after NMDA injection (c). * $P < 0.05$ compared with saline-injected mice. Data were presented as mean $\pm$ SD.

inner and outer retinal ADC remained unchanged at all time points.

Retinal Vascular Leakage Was Observed at 1 Day, but Not at 3 Hours, after NMDA Injection

Retinal vascular leakage was evaluated by Gd-DTPA-enhanced MRI (Fig. 5). Gd-DTPA treatment did not cause significant changes of the vitreous T1 in normal mice ($-0 \pm 4\%$) or mice at 3 hours after NMDA injection ($-4 \pm 3\%$). However, in mice at 1 day after NMDA injection, Gd-DTPA treatment changed the vitreous T1 by $-16 \pm 6\%$ ($P < 0.05$ compared with before Gd-DTPA treatment) due to leakage of Gd-DTPA into the avascular vitreous.²⁵

NMDA Excitotoxicity Causes Inner Retinal Cell Necrosis and Swelling

Excitotoxicity-induced inner retinal cell swelling was reflected by the ensuing retinal cell necrosis on H&E-stained slides (Fig. 6a). The number of necrotic cells peaked at 3 hours and 1 day after NMDA injection, then rapidly decreased close to zero from 3 to 7 days (Fig. 6b). No necrotic cell was observed in the

outer retina of NMDA-treated mice or in the entire retina of normal and saline-injected mice.

Retinal cell apoptosis was assessed by TUNEL staining. As early as 3 hours after NMDA injection, TUNEL-positive apoptotic cells were observed in the inner retina (Fig. 6c). The number of apoptotic cells peaked at 1 day and decreased close to zero at 3 and 7 days (Fig. 6d). Sporadic apoptotic cells were detected in the outer retinal photoreceptor cells of NMDA-treated mice, which agreed with previous reports.^{33,34} No apoptotic cells were detected in the retina of normal and saline-injected mice.

DISCUSSION

Our results demonstrated the capability of in vivo diffusion MRI to noninvasively assess retinal cell swelling in the NMDA mouse model. The cytotoxic retinal edema induced by intravitreal injection of NMDA resulted in transient inner retinal thickening and ADC decrease. Inner retinal thickening with ADC decrease was observed at 3 hours after NMDA injection, prior to retinal vascular leakage. This decreased ADC was not masked by the potential presence of vasogenic edema resulting from the onset of vascular leakage at 1 day after NMDA injection. Thus, our finding suggests that retinal cell swelling, unlike its cerebral counterpart, can directly cause retinal thickening independent of retinal vascular leakage.

The NMDA mouse model was selected in this study to take advantage of NMDA excitotoxicity-induced layer-specific injury in the inner retina.¹² Specifically, intravitreal injection of NMDA acutely causes the overexcitation of inner retinal neuronal cells due to its excessive binding to NMDA receptors. Müller cell swelling may also occur due to intracellular overloading of K^+ and glutamate that are released by overexcited neuronal cells.³⁵ However, outer retinal photoreceptor cells that do not express NMDA receptors, as well as retinal vasculature that comprises nonexcitable cells, are not directly affected by NMDA.³⁶ Thus, the NMDA mouse model is ideal to evaluate contribution of cell swelling to edematous retinal thickening.

The present study is the first in vivo investigation of layer-specific retinal cell swelling using diffusion MRI. The image resolution used ($47 \times 47 \mu\text{m}$) is among the highest resolution reported in the literature.^{29,31,37} Although five pixels across the 200- μm -thick retina could not resolve individual retinal anatomic layers (Figs. 2a, 2b), our results showed that it is sufficient to differentially detect inner retinal cell swelling from the outer retina in vivo. Using the same imaging parameters (e.g., TR and TE) as those used in our previous report,³¹ the observed inner and outer retinal ADC in normal mice were consistent between the two studies.³¹ In NMDA-injured mice, an approximately 20% decrease of inner retinal ADC was detected at 3 hours and 1 day after NMDA injection, reflecting the acute development of cell swelling in the inner retina. In agreement with MRI findings, histology analysis showed that the inner retinal cell necrosis, which typically developed after cell swelling,³⁸ also peaked at 3 hours and 1 day after NMDA injection. In the outer retina, however, no substantial change in MRI-determined ADC or histology-delineated cell viability was observed after NMDA injection. Thus, our results demonstrated that diffusion MRI may be used to detect layer-specific retinal injury in vivo.

Retinal vascular leakage was assessed by Gd-DTPA-enhanced MRI. Gadolinium ion, Gd^{3+} , is paramagnetic, which reduces the T1 of surrounding water. After intraperitoneal injection, Gd-DTPA is absorbed into the blood pool and delivered to the retina through circulation. Under normal conditions, Gd-DTPA does not penetrate the intact blood-

FIGURE 5. Gd-DTPA-enhanced MRI detected vitreous T1 in mouse eyes at baseline (a), 3 hours (b) or 1 day (c) after NMDA injection. *Top images:* representative T1 maps of each mouse eye acquired before (left) and at 30 minutes after (right) intraperitoneal injection of Gd-DTPA. *Bottom graphs:* quantified vitreous T1 before and after Gd-DTPA treatment. Each pair of open symbols, linked by a dashed line, represents the vitreous T1 of the eye measured pre- and postintravenous injection of Gd-DTPA from each individual mouse. The filled symbols represent the averaged vitreous T1 of all five mice. Statistical analysis showed the leaked Gd-DTPA resulting in a substantial decrease of vitreous T1 ($\Delta T1 = -16 \pm 6\%$) in mice at 1 day after NMDA injection (d). In all mice, the T1 of anterior chamber was reduced after Gd-DTPA treatment due to its fluid supply from the blood pool that contains Gd-DTPA. AC, anterior chamber; V, vitreous; Pre-, before Gd-DTPA treatment; Post-, 30 minutes after Gd-DTPA treatment. * $P < 0.05$ compared with vitreous T1 of the same mice before Gd-DTPA treatment. ** $P < 0.01$ compared with $\Delta T1$ of other groups. Data were presented as mean $\pm$ SD.

retinal barrier into the avascular vitreous. After blood-retinal barrier breakdown, the leakage of Gd-DTPA from the retinal vessels into the vitreous, as well as the diffusion of leaked Gd-DTPA from the choroid vascular bed to the vitreous, results in shortening of the vitreous T1 that can be quantified by MRI.^{25,29,39,40}

Our results showed that retinal thickening developed before retinal vascular leakage in NMDA-injured mice. De-

creased inner retinal ADC was observed at both 3 hours and 1 day after NMDA injection, reflecting inner retinal cell swelling. At 3 hours after NMDA injection, the vitreous T1 was not changed after systematic Gd-DTPA injection, reflecting the integrity of blood-retina barrier. Thus, the substantial thickening of the inner retina suggests a dominant contribution of cell swelling at this early time point. This is distinct from that observed in brain injury, where a volumetric increase of

FIGURE 6. Histology analysis of NMDA excitotoxicity induced cell necrosis and apoptosis in different retinal cell layers. H&E staining shows necrotic cells (arrows) that stained with eosinophilic, pink cytoplasm and pyknotic (shrunken), purple nuclei in GCL (e.g., middle insert) and INL (e.g., right insert) (a). TUNEL staining shows apoptotic cells in green color (b). Quantitative analysis showed the number of necrotic (c) and apoptotic (d) cells in GCL and INL peaked at 3 hours and 1 day. Sporadic apoptotic cells, but no necrotic cells, were observed in the ONL. * $P < 0.05$ compared with 3 and 7 days after NMDA injection. Data were presented as mean $\pm$ SD.

cerebral tissue is primarily caused by vascular leakage instead of cell swelling.¹⁰ In mice at 1 day after NMDA injection, however, Gd-DTPA treatment caused a $16 \pm 6\%$ decrease of the vitreous T1, indicating the presence of retinal vascular leakage. The coexisting vascular leakage and cell swelling at this time point may jointly contribute to the inner retinal thickening.

The different effect of cell swelling on retinal and cerebral tissue volume might be attributed to the different external fluid supply to these tissues. Excitotoxicity-induced neuron cell swelling is developed by intracellular sequestration of water with Na^+ influx.¹⁰ In the brain, the blood pool is the only source of external fluid supply. The fluid movement from the blood pool to extracellular space is tightly controlled by the blood-brain barrier, consisting of tight junctions around capillaries, largely impermeable to ions and fluids.⁴¹ If the blood-brain barrier is intact, cerebral cell swelling will develop by redistributing extracellular water into intracellular space without increasing the brain volume.¹⁰ In contrast, the retina experiences sustained transretinal fluid flux, driven by hydrostatic pressure gradient, from the vitreous to the choroid.⁵ When the blood-retinal barrier is intact, the extracellular water is likely replenished by the transretinal fluid flux and/or diffusion, resulting in an overall increase of the retinal fluid volume (i.e., retinal thickening). This mechanism of cell swelling-induced retinal thickening is consistent with previous findings of acutely developed retinal thickening, retina cell swelling, and extracellular space shrinkage in whole-mount guinea pig retina preparation exposed to 1 mM L-glutamate.⁴² The unchanged retinal thickness before L-glutamate exposure, the reduced extracellular space after L-glutamate exposure, together with the absence of blood hydrostatic pressure to drive vascular leakage suggested vasogenic edema did not contribute to the acute thickening of whole-mount retina. Thus, it is plausible that the observed cell swelling is the primary cause of L-glutamate excitotoxicity-induced retinal thickening.

The increased inner retinal thickness at 3 hours and 1 day after NMDA injection is in agreement with previous reports that excitotoxicity leads to acute thickening and chronic thinning of the inner retina.^{12,43} The acute retinal thickening was, at least partly, caused by the swelling of injured cells.^{42,44} The progressive death/loss of injured cells^{12,26,27} caused inner retinal thickness thinning has been observed at 7 days after NMDA injection.^{33,45–47} NMDA-decreased retinal thickness was shown to be dose dependent in rats.³³ Despite the NMDA dose effect on mouse retinal thickness has not been investigated in previous studies,^{30,34,43,48–51} it has been shown that at least 30 nmol NMDA is needed in mice to cause significant death of retinal ganglion cells by 7 days after NMDA injection.⁵⁰ Accordingly, the low dose of NMDA (2.5 nmol) used in this study probably did not substantially damage retinal cells to cause significant retinal thinning at 7 days after NMDA injection.

In this study, the NMDA dose was selected to induce retinal cell swelling while minimizing the extent of cell death, which could potentially cause an ADC increase, complicating data interpretation.⁵² The 0.5 μL injection volume was selected to avoid the risk of elevated intraocular pressure-induced retinal mechanical injury. Our results showed that retinal ADC was not altered after injection of 0.5 μL of saline (Fig. 4).

The current study has several limitations. First, excitotoxicity-induced cell swelling is not a major cause of retinal edema in patients. The development of cell swelling in retinal edema commonly occurred in ischemic and inflammatory diseases.^{6,53} Since diffusion MRI-derived ADC is a marker of cell swelling despite its origin, selection of the NMDA mouse model does not undermine the potential of diffusion MRI to detect cell

swelling in other retinal diseases. Second, this study did not directly evaluate glial cell swelling that has been documented in previous studies.^{35,42} Third, Gd-DTPA-enhanced MRI was performed on control and NMDA-injured mice at 3 hours and 1 day after exposure. Since our results showed retinal vascular leakage occurred at 1 day after NMDA injection, the selected study groups sufficiently support that retinal cell swelling is the dominant contributor of retinal thickening at 3 hours after NMDA injection. However, it is unclear whether vascular leakage will continue at later time points (e.g., at 3 and 7 days) when cell swelling and apoptosis reduce and ADC returns to its normal value. Further investigation is needed in the future for a better understanding of the progression of retinal edema. Finally, quantitative ADC measurement requires four images, that is, three diffusion-weighted images oriented orthogonal to each other and one nondiffusion-weighted image. For a fast screening of disease-induced cell swelling, a diffusion-weighted image along a single direction, a 75% saving in acquisition time, may be acquired to reflect the effect without quantifying ADC (Fig. 1).

In summary, we used diffusion MRI-derived ADC to detect retinal cell swelling in the edematous retina using the NMDA mouse model. Our results revealed that retinal cell swelling can directly contribute to retinal thickening, a mechanism different from that observed in cerebral diseases. Compared with existing methods to detect retinal vascular leakage and retinal thickening, diffusion MRI assessment of cell swelling provides critical and complementary information for investigating the pathomechanism of retinal edema.

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References

- Bringmann A, Reichenbach A, Wiedemann P. Pathomechanisms of cystoid macular edema. *Ophthalmic Res.* 2004;36:241–249.
- Browning DJ. Diabetic macular edema. In: Browning DJ, ed. *Diabetic Retinopathy: Evidence-Based Management*. New York: Springer-Verlag; 2010:141–202.
- Scholl S, Kirchhof J, Augustin AJ. Pathophysiology of macular edema. *Ophthalmologica.* 2010;224(suppl 1):8–15.
- Yanoff M, Fine BS, Brucker AJ, Eagle RC Jr. Pathology of human cystoid macular edema. *Surv Ophthalmol.* 1984;28(suppl):505–511.
- Marmor MF. Mechanisms of fluid accumulation in retinal edema. *Doc Ophthalmol.* 1999;97:239–249.
- Bringmann A, Uckermann O, Pannicke T, Iandiev I, Reichenbach A, Wiedemann P. Neuronal versus glial cell swelling in the ischaemic retina. *Acta Ophthalmol Scand.* 2005;83:528–538.
- Pannicke T, Uckermann O, Iandiev I, Wiedemann P, Reichenbach A, Bringmann A. Ocular inflammation alters swelling and membrane characteristics of rat Müller glial cells. *J Neuroimmunol.* 2005;161:145–154.
- Ciulla TA, Amador AG, Zinman B. Diabetic retinopathy and diabetic macular edema: pathophysiology, screening, and novel therapies. *Diabetes Care.* 2003;26:2653–2664.
- Lobo CL, Bernardes RC, Cunha-Vaz JG. Alterations of the blood-retinal barrier and retinal thickness in preclinical retinopathy in subjects with type 2 diabetes. *Arch Ophthalmol.* 2000;118:1364–1369.

10. Liang D, Bhatta S, Gerzanich V, Simard JM. Cytotoxic edema: mechanisms of pathological cell swelling. *Neurosurg Focus*. 2007;22:E2.
11. Alm A, Ehinger B. Retina. In: Kaufman P, Alm A, eds. *Adler's Physiology of the Eye*. St. Louis, MO: Mosby; 2002:319-438.
12. Sun Q, Ooi VE, Chan SO. N-Methyl-D-aspartate-induced excitotoxicity in adult rat retina is antagonized by single systemic injection of MK-801. *Exp Brain Res*. 2001;138:37-45.
13. Schaefer PW, Grant PE, Gonzalez RG. Diffusion-weighted MR imaging of the brain. *Radiology*. 2000;217:331-345.
14. Basser PJ, Jones DK. Diffusion-tensor MRI: theory, experimental design and data analysis—a technical review. *NMR Biomed*. 2002;15:456-467.
15. Hagmann P, Jonasson L, Maeder P, Thiran JP, Wedeen VJ, Meuli R. Understanding diffusion MR imaging techniques: from scalar diffusion-weighted imaging to diffusion tensor imaging and beyond. *Radiographics*. 2006;26(suppl 1):S205-S223.
16. Loubinoux I, Volk A, Borredon J, et al. Spreading of vasogenic edema and cytotoxic edema assessed by quantitative diffusion and T2 magnetic resonance imaging. *Stroke*. 1997;28:417-426.
17. Sevick RJ, Kanda F, Mintorovitch J, et al. Cytotoxic brain edema: assessment with diffusion-weighted MR imaging. *Radiology*. 1992;185:687-690.
18. Schaefer PW, Buonanno FS, Gonzalez RG, Schwamm LH. Diffusion-weighted imaging discriminates between cytotoxic and vasogenic edema in a patient with eclampsia. *Stroke*. 1997;28:1082-1085.
19. Pierpaoli C, Alger JR, Righini A, et al. High temporal resolution diffusion MRI of global cerebral ischemia and reperfusion. *J Cereb Blood Flow Metab*. 1996;16:892-905.
20. Runge VM, Clanton JA, Lukehart CM, Partain CL, James AE Jr. Paramagnetic agents for contrast-enhanced NMR imaging: a review. *AJR Am J Roentgenol*. 1983;141:1209-1215.
21. Larsson HB, Courivaud F, Rostrup E, Hansen AE. Measurement of brain perfusion, blood volume, and blood-brain barrier permeability, using dynamic contrast-enhanced T(1)-weighted MRI at 3 tesla. *Magn Reson Med*. 2009;62:1270-1281.
22. Larsson HB, Stubgaard M, Frederiksen JL, Jensen M, Henriksen O, Paulson OB. Quantitation of blood-brain barrier defect by magnetic resonance imaging and gadolinium-DTPA in patients with multiple sclerosis and brain tumors. *Magn Reson Med*. 1990;16:117-131.
23. Silver NC, Good CD, Barker GJ, et al. Sensitivity of contrast enhanced MRI in multiple sclerosis. Effects of gadolinium dose, magnetization transfer contrast and delayed imaging. *Brain*. 1997;120:1149-1161.
24. Wolansky LJ, Finden SG, Chang R, et al. Gadoteridol in multiple sclerosis patients. A comparison of single and triple dose with immediate vs. delayed imaging. *Clin Imaging*. 1998;22:385-392.
25. Berkowitz BA, Roberts R, Luan H, Peysakhov J, Mao X, Thomas KA. Dynamic contrast-enhanced MRI measurements of passive permeability through blood retinal barrier in diabetic rats. *Invest Ophthalmol Vis Sci*. 2004;45:2391-2398.
26. Trick GL, Liggett J, Levy J, et al. Dynamic contrast enhanced MRI in patients with diabetic macular edema: initial results. *Exp Eye Res*. 2005;81:97-102.
27. Sato Y, Berkowitz BA, Wilson CA, de Juan E Jr. Blood-retinal barrier breakdown caused by diode vs argon laser endophotocoagulation. *Arch Ophthalmol*. 1992;110:277-281.
28. Viores SA, Derevjanik NL, Mahlow J, Berkowitz BA, Wilson CA. Electron microscopic evidence for the mechanism of blood-retinal barrier breakdown in diabetic rabbits: comparison with magnetic resonance imaging. *Pathol Res Pract*. 1998;194:497-505.
29. Wang Q, Song SK, Zhang H, et al. Photoreceptor degeneration changes magnetic resonance imaging features in a mouse model of retinitis pigmentosa. *Magn Reson Med*. 2011;65:1793-1798.
30. Awai M, Koga T, Inomata Y, et al. NMDA-induced retinal injury is mediated by an endoplasmic reticulum stress-related protein, CHOP/GADD153. *J Neurochem*. 2006;96:43-52.
31. Chen J, Wang Q, Zhang H, et al. In vivo quantification of T1, T2, and apparent diffusion coefficient in the mouse retina at 11.74T. *Magn Reson Med*. 2008;59:731-738.
32. Chen J, Wang Q, Chen S, Wickline SA, Song SK. In vivo diffusion tensor MRI of the mouse retina: a noninvasive visualization of tissue organization. *NMR Biomed*. 2011;24:447-451.
33. Lam TT, Abler AS, Kwong JM, Tso MO. N-Methyl-D-aspartate (NMDA)-induced apoptosis in rat retina. *Invest Ophthalmol Vis Sci*. 1999;40:2391-2397.
34. Lebrun-Julien F, Duplan L, Pernet V, et al. Excitotoxic death of retinal neurons in vivo occurs via a non-cell-autonomous mechanism. *J Neurosci*. 2009;29:5536-5545.
35. Izumi Y, Kirby CO, Benz AM, Olney JW, Zorumski CF. Müller cell swelling, glutamate uptake, and excitotoxic neurodegeneration in the isolated rat retina. *Glia*. 1999;25:379-389.
36. Osborne NN, Casson RJ, Wood JP, Chidlow G, Graham M, Melenka J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res*. 2004;23:91-147.
37. Cheng H, Nair G, Walker TA, et al. Structural and functional MRI reveals multiple retinal layers. *Proc Natl Acad Sci USA*. 2006;103:17525-17530.
38. Cobb JP, Hotchkiss RS, Karl IE, Buchman TG. Mechanisms of cell injury and death. *Br J Anaesth*. 1996;77:3-10.
39. Berkowitz BA, Sato Y, Wilson CA, de Juan E. Blood-retinal barrier breakdown investigated by real-time magnetic resonance imaging after gadolinium-diethylenetriaminepentaacetic acid injection. *Invest Ophthalmol Vis Sci*. 1991;32:2854-2860.
40. Berkowitz BA, Tofts PS, Sen HA, Ando N, de Juan E Jr. Accurate and precise measurement of blood-retinal barrier breakdown using dynamic Gd-DTPA MRI. *Invest Ophthalmol Vis Sci*. 1992;33:3500-3506.
41. Rubin LL, Staddon JM. The cell biology of the blood-brain barrier. *Annu Rev Neurosci*. 1999;22:11-28.
42. Uckermann O, Vargova L, Ulbricht E, et al. Glutamate-evoked alterations of glial and neuronal cell morphology in the guinea pig retina. *J Neurosci*. 2004;24:10149-10158.
43. Ito Y, Shimazawa M, Inokuchi Y, et al. Degenerative alterations in the visual pathway after NMDA-induced retinal damage in mice. *Brain Res*. 2008;1212:89-101.
44. Hama Y, Katsuki H, Suminaka C, Kume T, Akaike A. Chloride-dependent acute excitotoxicity in adult rat retinal ganglion cells. *Neuropharmacology*. 2008;55:677-686.
45. Honjo M, Tanihara H, Kido N, Inatani M, Okazaki K, Honda Y. Expression of ciliary neurotrophic factor activated by retinal Müller cells in eyes with NMDA- and kainic acid-induced neuronal death. *Invest Ophthalmol Vis Sci*. 2000;41:552-560.
46. Luan H, Roberts R, Sniegowski M, Goebel DJ, Berkowitz BA. Retinal thickness and subnormal retinal oxygenation response in experimental diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2006;47:320-328.
47. Morizane C, Adachi K, Furutani I, et al. N(omega)-nitro-L-arginine methyl ester protects retinal neurons against N-methyl-D-aspartate-induced neurotoxicity in vivo. *Eur J Pharmacol*. 1997;328:45-49.
48. Karl MO, Hayes S, Nelson BR, Tan K, Buckingham B, Reh TA. Stimulation of neural regeneration in the mouse retina. *Proc Natl Acad Sci USA*. 2008;105:19508-19513.

49. Li Y, Schlamp CL, Poulsen GL, Jackson MW, Griep AE, Nickells RW. p53 regulates apoptotic retinal ganglion cell death induced by N-methyl-D-aspartate. *Mol Vis.* 2002;8:341-350.
50. Nakazawa T, Takahashi H, Nishijima K, et al. Pitavastatin prevents NMDA-induced retinal ganglion cell death by suppressing leukocyte recruitment. *J Neurochem.* 2007;100:1018-1031.
51. Shimazawa M, Inokuchi Y, Ito Y, et al. Involvement of ER stress in retinal cell death. *Mol Vis.* 2007;13:578-587.
52. Morse DL, Galons JP, Payne CM, et al. MRI-measured water mobility increases in response to chemotherapy via multiple cell-death mechanisms. *NMR Biomed.* 2007;20:602-614.
53. Bringmann A, Pannicke T, Grosche J, et al. Müller cells in the healthy and diseased retina. *Prog Retin Eye Res.* 2006;25:397-424.