Noninvasive Topical Loading for Manganese-Enhanced MRI of the Mouse Visual System

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Purpose. To evaluate topical loading as an alternative to intravitreal injection for Mn^{2+}-enhanced magnetic resonance imaging (MEMRI) of the visual system.

Methods. Topical administration of 0.5 to 1.5 M $MnCl_2$ and intravitreal injections with 0.5 μL 100 mM and 2 μL 1 M $MnCl_2$ for mouse MEMRI were conducted, followed by immunohistochemistry. In another mouse group, two topical administrations of 1 M Mn^{2+} were applied to the same animals 7 days apart, to evaluate the use of MEMRI in a time course study. Dynamic imaging was also conducted to reveal how Mn^{2+} travels to the retina. MEMRI with topically loaded $MnCl_2$ was also conducted in eyes with retinal ischemia, to evaluate whether the enhancements required healthy neurons.

Results. After 1 day, topical administration of 1 M and 1.5 M $MnCl_2$ rendered significant signal enhancement (up to 20%) in the superior colliculus ($P < 0.05$) that was equivalent to that of the 2-μL 1 M injection. Repeated exposure to Mn^{2+} showed reproduced enhancement. Dynamic imaging showed significant enhancement in the iris, retina, and lens boundary, but not in the vitreous space. In retinal ischemic eyes, no enhancement of MEMRI was detected in the optic nerves. The immunohistochemistry of the optic nerve (1.5 mm anterior to the chiasm) and retina showed no injury 1 week after Mn^{2+} topical administrations to each mouse.

Conclusions. The results demonstrated the feasibility of using topical administration of Mn^{2+} for MEMRI. Topically loaded Mn^{2+} did not diffuse into the vitreous space, but was it may have been absorbed into the iris to diffuse or travel via the capillary circulation to reach the retina. (*Invest Ophthalmol Vis Sci.* 2011;52:3914–3920) DOI:10.1167/iovs.10-6363

Manganese-enhanced magnetic resonance imaging (MEMRI) has been used as a noninvasive imaging modality to explore neural connectivity in the central nervous system of live animals.[1–11] Manganese (Mn^{2+}) enters cells through voltage-gated calcium channels and then moves along axons via axonal transport.[12,13] Given the paramagnetic nature of Mn^{2+}, its uptake and transport in the nervous system is detectable by MRI with T_1-weighted imaging (T1WI). MEMRI has been used to study the visual system and has been shown to be effective in characterizing ion channel regulation in rodent photoreceptors,[14,15] retinal layer-specific functionality,[9] and optic nerve axonal transport.[10,16] After an intravitreal injection of Mn^{2+}, the distribution of MRI enhancement in the superior colliculus reflects the level of optic nerve damage.[17,18] MEMRI offers a noninvasive method of performing retinotopic mapping of the superior colliculus in vivo.[18]

When MEMRI is used to investigate the visual system, an intravitreal injection is the common means of delivery of the Mn^{2+} solution into the ocular space.[2,8,9,12,17,18] The injection procedure relies on a delicate operation that requires that much care be taken, so that subconjunctival hemorrhage or trauma to the sclera does not occur. A noninvasive procedure should be used as an alternative to intravitreal injection to minimize the potential adverse effects of MEMRI in visual systems. Topical administration is currently the most common route for ocular drug delivery, but this modality has not been adequately explored in conjunction with MEMRI for studies in the visual system.

In this study, the feasibility of noninvasive topical administration of $MnCl_2$ solution for MEMRI was examined in live mice.[19] We first evaluated whether topical administration of $MnCl_2$ would lead to signal enhancement in the visual pathway. Specifically, topical administration of 5 μL of 0.5, 0.75, 1, and 1.5 M $MnCl_2$ was conducted in mouse eyes, and T1WI was performed 1 day after the $MnCl_2$ administration. Mice with 1 M $MnCl_2$ topical loading were further examined by MRI after 2 and 7 days, to evaluate the washout of Mn^{2+} from the visual system. Seven days after topical loading, immunohistochemistry of the retina and optic nerves was examined to investigate the safety of topically loaded MEMRI.

To compare the topically loaded MEMRI to images with traditional intravitreal injections, additional groups of mice were used to test MEMRI after intravitreal injection with 2 μL of 1 M and 0.5 μL of 100 mM $MnCl_2$ respectively. The volumes were chosen based on the intravitreal injections used in previous ophthalmic studies in mice, in which a fluid volume of 0.5 to 3 μL was shown to be feasible for intravitreal injections in mouse eyes.[2,20–24] The lower amount of $MnCl_2$ (0.5 μL of 100 mM $MnCl_2$) used for intravitreal injection was based on the reported literature, which supports the use of this amount to avoid an increase in intraocular volume while inducing significant enhancement of the visual system.[20] The results of topical administration were further compared with the signal enhancement produced by the traditional injection approach.

We conducted dynamic imaging of the eyes in the first 4 hours after Mn^{2+} topical loading to investigate how Mn^{2+} travels from the eye surface to the retina. To evaluate the feasibility of serial topical administration of MEMRI in the visual system, in another group of mice, we applied 1 M $MnCl_2$

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topically twice, 7 days apart. Positive outcomes of this examination would support the use of MEMRI in time course studies.

Despite enhancement of the MEMRI by topically administered Mn^{2+}, it was not clear whether Mn^{2+} may have leaked into the interaxonal space and diffused along the optic nerve to cause enhancement without the presence of live retinal ganglion cells (RGCs). In another group of mice, an MEMRI with topically administered Mn^{2+} was conducted in eyes with transient retinal ischemia. Other studies have shown that retinal ischemia can lead to severe RGC loss in mice within 1 week.[25–28] Thus, the negative result of this test would support the necessity of the presence of healthy RGCs to achieve enhancement in the visual system after topical administration of Mn^{2+}.

Materials and Methods

All animal procedures were performed in accordance with National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Institutional Animal Care and Use Committee of Loma Linda University and Washington University.

MEMRI Procedures

Female C57BL/6 mice at 8 weeks of age were anesthetized by 1.5% isoflurane/oxygen, with an isoflurane vaporizer (VetEquip, Pleasanton, CA). The body temperature was maintained with an electric heating pad. For topical administration, 5 μL $MnCl_2$ was administered to the surface of the right eye of each mouse at concentrations of 0.5 (n = 5), 0.75 (n = 7), 1.0 (n = 6), and 1.5 M (n = 7). After 1 hour, the remaining solution was carefully removed with lint-free tissue (Kimwipes; Kimberly-Clark, Irving, TX). The animals were maintained under anesthesia for 1 hour during topical Mn^{2+} administration. For the intravitreal injection (n = 5 per group), 2 μL 1 M or 0.5 μL 100 mM $MnCl_2$ was injected into the vitreous space of each mouse under anesthesia. We used a microliter syringe (2.5 μL; 62RN; Hamilton, Reno, NV) combined with a 32-gauge needle, 0.375 in. long. First, we rinsed the syringe clean with 70% alcohol five times and with sterile dH_2O five times. We drew 2.0 μL of the 1 M or 100 mM manganese-buffered solution ($MnCl_2$, dissolved in PBS) and injected either 2 or 0.5 μL into the mouse intravitreal space. The injection was slowly performed in 15 seconds, and the needle was held for another minute before it was withdrawn. The mice were then released to their original cages. Twenty-four hours after administration of $MnCl_2$, the mice were anesthetized for imaging. The core body temperature was maintained with a warm water-circulation pad. A 7-cm inner diameter linear RF coil (Bruker Biospin, Inc., Fremont, CA) was used as a transmitter, and a 2-cm surface coil was used as a receiver. T1WIs were taken with an animal scanner (4.7T; Bruker Biospin) with TR of 380 ms, TE of 8.5 ms, 32 averages, field of view of 1.5 cm, slice thickness of 0.5 mm, and data matrix of 128 × 128 (with 0-padding to 256 × 256). Nineteen contiguous transactional slices were selected to cover the visual system from the eye to the superior colliculus. The total scanning time was 28 minutes. Regions of interest (ROIs) were selected from the retina, prechiasmatic optic nerve, and superior colliculus from the left and right hemispheres. An example of the ROI is shown in Figure 1. The reference signal was selected as a 10 × 25-voxel rectangle from the septal area (Fig. 1), which was selected due to its location because of its roughly equal distance to the eye and the superior colliculus, homogeneous signal intensity, easy identification, and relatively low partial volume effects.

To evaluate whether repeated applications of Mn^{2+} for MEMRI is feasible, in another group of mice (n = 6), we conducted two applications of 1.0 M $MnCl_2$ 7 days apart and obtained images on 1, 2, and 7 days after each administration. To further explore the route by which Mn^{2+} travels from eye surface to the retina, 5 μL $MnCl_2$ was loaded on the right eye (n = 6). T1WI of a midsagittal plane of each eye was repeated every 30 minutes for 4 hours.

MEMRI and DTI on Retinal Ischemia Mice

Five C57BL/6 female mice, 8 weeks of age, underwent transient retinal ischemia of the right eye.[26–28] Briefly, under a dissecting microscope, a 30-gauge needle was inserted into the vitreous space of the right eye. The needle was connected to a saline reservoir that was hung at 1.5 m above the operating table, which produced needle water pressure equivalent to 115 mm Hg. Although we did not monitor the intraocular pressure with this procedure, based on previous studies, the severity of RGC damage

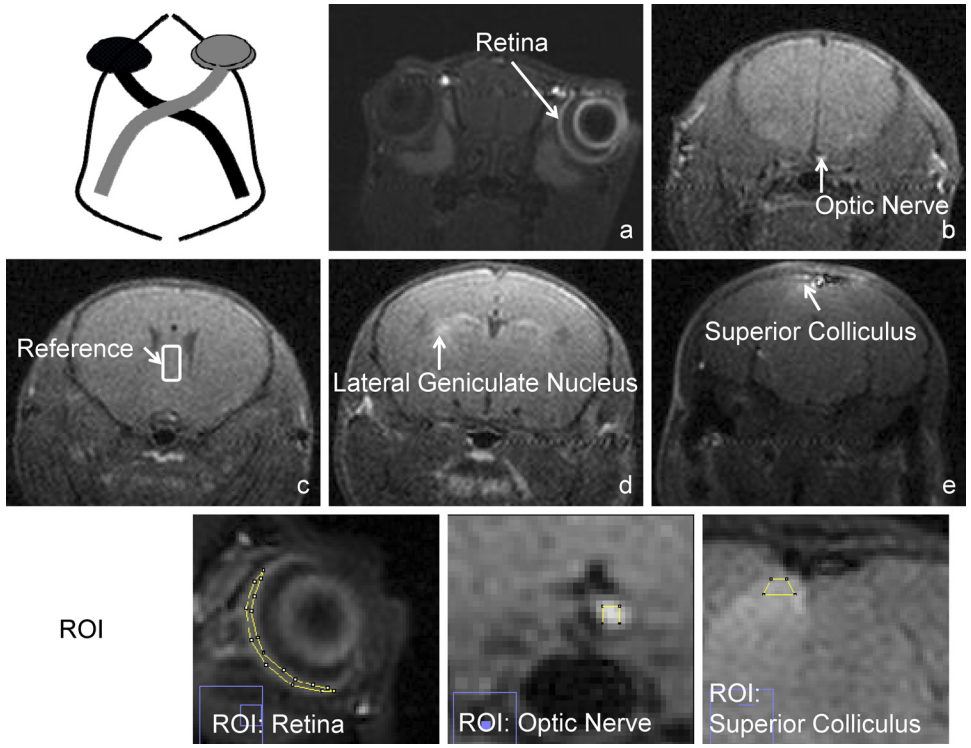

Figure 1. T1WI of a normal mouse 1 day after topical administration of 1 M Mn^{2+}. The Mn^{2+} induced signal enhancement was clearly seen in the right retina (**a**), right optic nerve (**b**), left lateral geniculate nucleus (**d**), and left superior colliculus (**e**). The ROI was selected from the retina (~80 voxels, on the slice of the middle section of an eye), optic nerve (3 × 3 voxels square, ~1.5 mm anterior to the chiasm), and the superior colliculis (~25 voxels). The internal reference was a 10 × 25 rectangle in the septal area (**c**), which was used to normalize signals measured from the visual system.

was highly consistent and reproducible.[26–28] After 1 hour, the reperfusion started immediately after removal of the cannula. An MRI was performed 2 weeks after retinal ischemia. The success of retinal ischemia was confirmed by diffusion tensor imaging (DTI). Using DTI, ~50% reduction of axial diffusivity was measured in the affected optic nerves, to confirm axonal damage.[26–28] One day after DTI, 5 μL 1.0 M $MnCl_2$ was instilled into the right eye. The next day, the mice were anesthetized for T1WI, using the parameters described in the previous section. DTI was performed with TR 3 seconds; TE 29 ms; duration between a diffusion gradient pair (Δ) 20 ms; diffusion gradient duration (δ) 3 ms; and six-direction diffusion scheme with b-values of 0 and 0.85 ms/μm^2. The geometric parameters were the same as for T1WI—that is, a spatial resolution of 117 × 117 μm^2 (or 59 × 59 μm^2 after zero-filling) and slice thickness of 0.5 mm. Using a program written in commercial software (MatLab; MathWorks, Natick, MA), the eigenvalues (λ_1, λ_2, and λ_3) derived from the diffusion tensor were used to calculate axial diffusivity ($\lambda_\parallel$), radial diffusivity ($\lambda_\perp$), relative anisotropy (RA), and the trace of the diffusion tensor (Tr), defined by the following equations:

$$\mathrm{Tr} = \lambda_1 + \lambda_2 + \lambda_3 \quad (1)$$

$$\lambda_\parallel = \lambda_1 \quad (2)$$

$$\lambda_\perp = 0.5 \times (\lambda_2 + \lambda_3) \quad (3)$$

$$\mathrm{RA} = \frac{\sqrt{(\lambda_1 - \mathrm{Tr}/3)^2 + (\lambda_2 - \mathrm{Tr}/3)^2 + (\lambda_3 - \mathrm{Tr}/3)^2}}{\sqrt{3}(\mathrm{Tr}/3)} \quad (4)$$

The ROI selections were the same as the ROIs for MEMRI analysis described in the previous section. In brief, ROIs were selected from the retina, prechiasmatic optic nerve, and superior colliculus from the left and right hemispheres (Fig. 1). Data are presented as the mean ± SD. A paired *t*-test between Mn^{2+}-affected and intact nerves was performed. *P* values < 0.05 were considered to be statistically significant.

Immunohistochemistry Examination

To investigate the toxicity of Mn^{2+} after topical administration, five mice from the 1-M and five from the 1.5-M dosage-testing experiments, were perfusion fixed. The perfusion fixing was achieved by injecting the left cardiac ventricle with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS in 1 week after Mn^{2+} treatment. A 4-mm-thick coronal section (−1 to +3 mm of bregma) was obtained from each brain and embedded in paraffin. Tissue slices with 3 μm thickness of optic nerve, ~1.5 mm anterior to the chiasm, matching the MRI (Fig. 1), were cut and deparaffinized in xylene for immunohistochemical examination. The integrity of the axons was evaluated with a primary antibody against phosphorylated neurofilament (SMI-31, 1:1000; Sternberger Monoclonals, Lutherville, MD).[27] After a 15-minute wash in PBS, the sections were incubated in a fluorescent secondary antibody (AlexaFluor 488 goat anti-mouse IgG, 1:200; Invitrogen-Molecular Probes, Carlsbad, CA) for 1 hour at room temperature. In addition, H&E staining was performed on the eye tissue of the 1.5 M $MnCl_2$–treated mice to evaluate the RGCs.

Histologic sections were examined with a confocal microscope (Fluoview; Olympus, Lake Success, NY) equipped with a 60× oil objective. Under a microscope, the images were taken from the central portions of the left (control) and right (Mn^{2+}-affected) optic nerves. The green SMI-31-positive staining, representing the normal axons, was captured, and the axons were counted through the central 100 × 100-μm^2 regions. The counts are presented as the mean ± SD. A two-tailed *t*-test was performed to compare the measurements between the control and Mn^{2+}-affected optic nerves. For RGC evaluation, the sections were examined with a 40× objective, and eight images were taken, to cover the entire RGC layer in each section. Cell density was quantified. Only the middle section was quantified to represent the RGC density in each eye. A two-tailed *t*-test was performed to compare the measurements between control and Mn^{2+}-affected eyes.

Results

Topical Administration Led to the Enhancement of the Visual Pathway

One day after topical administration of $MnCl_2$ in the right eye, the Mn^{2+} was found to be distributed along the visual pathway. Its distribution included the right retina, right optic nerve, left lateral geniculate nucleus, and left superior colliculus (Fig. 1). To quantify the enhancements, ROIs were selected in the retina, optic nerve, and superior colliculus of both hemispheres. The measured intensity of each ROI was normalized by the internal reference signal (Fig. 1).

As shown in Figure 2, the signal intensity of the control ROIs (left retinas, left optic nerve, and right superior collicu-

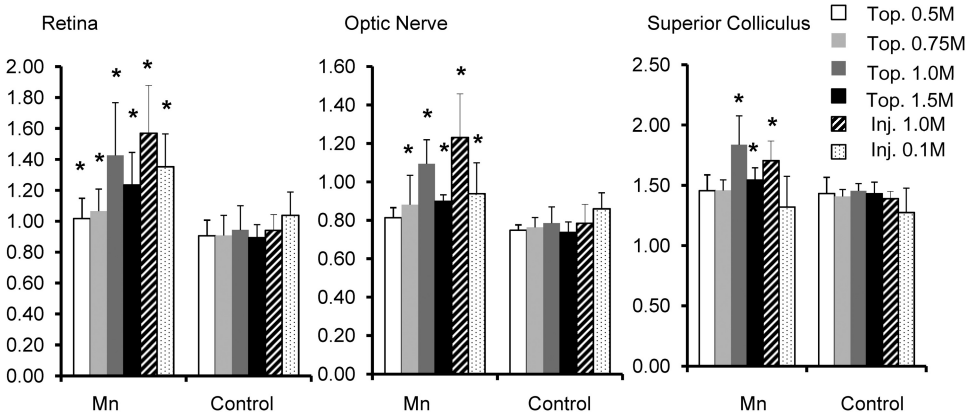

Figure 2. Signal intensity of the visual pathway 1 day after topical administration or intravitreal injection of Mn^{2+}. Signals were scaled based on the internal reference. The intensity of the control ROI was equivalent among the groups, suggestive of the consistency of the measured signals. The intensity of Mn^{2+}-affected sites increased compared with the control sites. There was a variation in the increments among the groups. For topical administration, the 1-M group provided the highest signal increments. Intravitreal injection (1 M in 2 μL) provided signal increments similar to the 1-M topical administration with no statistically significant difference. $^*P < 0.05$ compared with the control ROI.

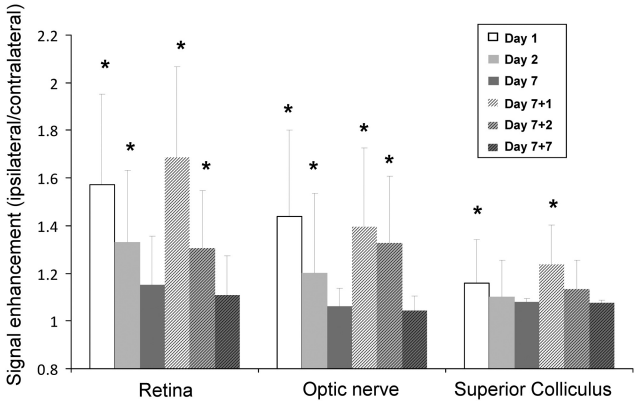

FIGURE 3. Ratio of signal increments after 1 M $MnCl_2$ topical administration. The signal reached a peak in day 1 and decayed to control levels in 7 days. Topical administration of 1 M $MnCl_2$ was conducted again on day 7. Significant enhancements were seen the following day (Day 7+1). Signals decayed in 2 days and returned to baseline in 7 days after the second Mn^{2+} administration (Days 7+2 and Days 7+7, respectively). * $P < 0.05$ comparing to control sites.

lus) was equivalent between the groups, suggesting experimental consistency. For the Mn^{2+}-affected ROIs (right retinas, right optic nerves, and left superior colliculus), the enhancement ratios varied, depending on the concentration of $MnCl_2$ provided to the eye. The higher concentration of $MnCl_2$ solution provided a higher signal increase, except the 1.5-M group, which had lower signals than did the 1-M group. Our data showed that 1 M topical administration rendered significant increments of signal enhancement of 60% in the retina, 30% in the optic nerves, and 20% in the superior colliculus ($P < 0.05$). Two groups of mice were used for intravitreal injection for MEMRI. The 2-μL 1 M intravitreal injection led to significant enhancement (70%) in the retina, 60% in the optic nerve, and 20% in the superior colliculus ($P < 0.05$). As for the 0.5-μL 0.1 M intravitreal injection group of mice, significant enhancements were measured from the retina (30% increments, $P < 0.05$) to the prechiasmic optic nerves (10% increments, $P < 0.05$), but not the superior colliculus. When compared with the intravitreal injection, the 1 M topical administration provided a degree of enhancement similar to that of the 2-μL 1 M intravitreal injection group.

Longitudinal Evolution with Serial Exposures to $MnCl_2$

After 1 M $MnCl_2$ topical administration, temporal evaluation showed that signal intensity reached the peak on day 1, decayed on day 2, and returned to baseline by day 7 (Fig. 3). To evaluate the feasibility of repeating the MEMRI with topical $MnCl_2$, 1 M $MnCl_2$ was again loaded on the same eye 7 days after the first dose. As shown in Figure 3, signal changes of the second induction were similar to the signal changes of the first route. Significant enhancements were seen on day 1 (Fig. 3, day 7+1), followed by a decay on day 2 (Fig. 3, day 7+2), and returned to baseline by day 7 (Fig 3, day 7+7).

The Uptake of Mn^{2+} Does Not Involve Vitreous Space

To further investigate how a topical drop of $MnCl_2$ travels from the cornea to the retina, we obtained repeated T1WIs of the eye, every 30 minutes within the initial 4 hours after $MnCl_2$ (1 M) loading (Fig. 4). Data collected from mice without Mn^{2+} treatments were added as a control ($n = 5$). The cornea and iris showed initial significant enhancement (four- and onefold increments, respectively) followed by gradual decreases in the cornea, but the signal was unchanged in the iris over time. The retina and lens boundary did not show significant enhancement in the beginning, but there were gradual increases that reached ~50% and ~80% increments, respectively, by the end of the test ($P < 0.05$). The vitreous space did not show significant changes in the entire temporal evaluation.

Reduced Enhancement of MEMRI on Retinal Ischemia–Injured Eyes

To evaluate whether the uptake of Mn^{2+} in the visual system is dependent on the health of the RGCs, we also performed Mn^{2+} topical-administration MEMRI on the eyes with retinal ischemia. In line with previous reports,[27] retinal ischemia caused optic nerve degeneration, which was confirmed by DTI, with a 60% decrease in RA, a 50% decrease in $\lambda_{\|}$, and a 100% increase in $\lambda_{\perp}$ (Fig. 5). Our data show that there was a significant (13%) increase in signal in the injured retina after Mn^{2+} topical loading. The increment was smaller than in the MEMRI of the healthy eyes (60% increments). No significant enhancement of MEMRI occurred in the injured optic nerve or the superior colliculus (Fig. 5).

MEMRI Safety

To evaluate the safety of eye-drop MEMRI, 1 week after topical administration of 1 M or 1.5 M $MnCl_2$, the optic nerves were examined with immunohistochemistry for axonal pathology. SMI-31 was used to stain for the phosphorylated neurofilaments, which represented the healthy axons (Fig. 6). In addition, the H&E staining of the retina showed no abnormality in

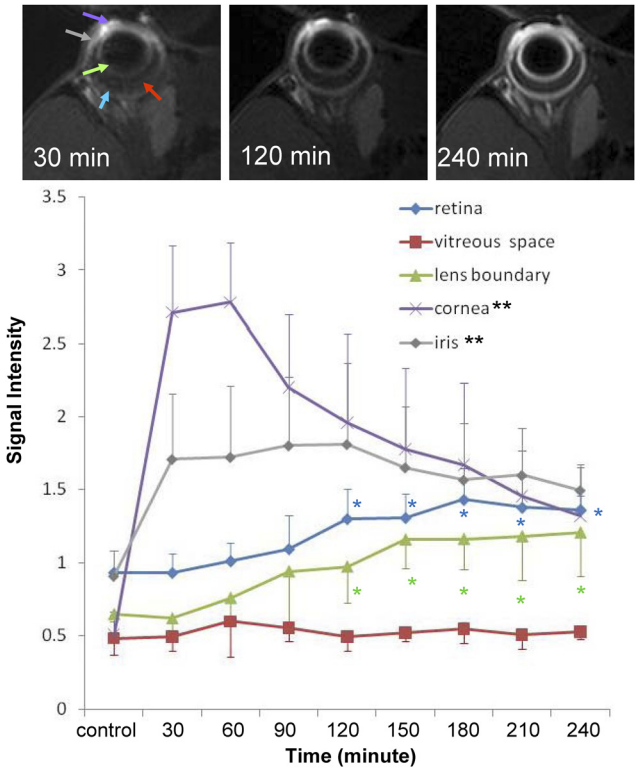

FIGURE 4. Eye T1WI after 1 M $MnCl_2$ topical administration. Corneal signals decayed over time, suggesting the release of Mn^{2+}. The signals in the vitreous space did not change. Gradually increased signals were measured in the retinal and lens boundary. *$P < 0.05$ compared with the data before Mn^{2+} treatment (control). **Cornea and iris had significant enhancement ($P < 0.05$) at all time points, compared with that of the control.

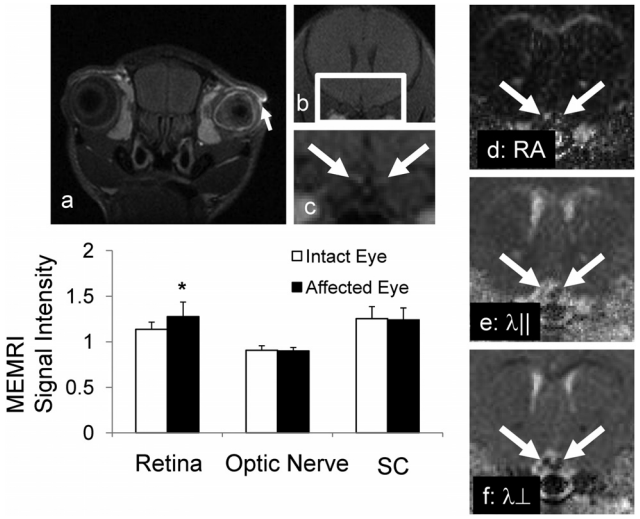

FIGURE 5. MEMRI and DTI of the eye 2 weeks after retinal ischemia. (**a**, *arrows*) The remaining Mn^{2+} in the corneal space. There was no enhancement in the optic nerve (**b**, **c**, *arrows*). (**c**) Magnified view of the rectangle in (**b**). DTI characterized injured optic nerves with decreased RA (**d**), decreased $\lambda_{||}$ (**e**), and increased $\lambda_{\perp}$ (**f**) in the affected (*right*) optic nerve, suggestive of the damage resulting from the retinal ischemia.

the Mn^{2+}-affected retina (Fig. 7). The cell density in the RGC layer showed no significant difference between Mn^{2+}-affected and intact retina (Fig. 7). Our data showed no detectable damage to the Mn^{2+}-affected nerves and retinas (Fig. 6).

DISCUSSION

The study demonstrated the possibility of using Mn^{2+} topical administration for MEMRI of the mouse visual system in vivo. Significant signal enhancement was seen in the visual system from the retina to the superior colliculus 1 day after topical administration of 1 or 1.5 M $MnCl_2$, whereas lower concentrations of $MnCl_2$ (0.5 and 0.75 M) showed significant enhancement in the retina and optic nerve but not in the superior colliculus. The enhancement peaked on day 1, decayed on day 2, and returned to baseline on day 7. Topical administration of Mn^{2+} provided MEMRI similar to the images that were obtained with intravitreal injection.[2,8,9,12,15]

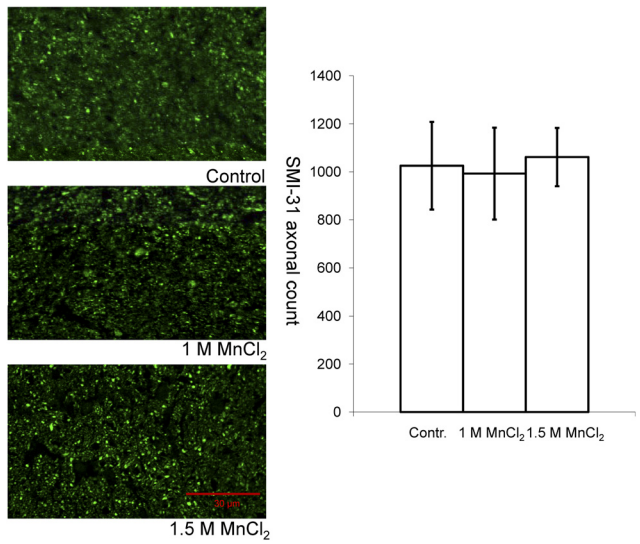

FIGURE 6. SMI-31 staining of intact axons in optic nerves at 1 week after topical administration of 1 or 1.5 M Mn^{2+}. There was no obvious axonal damage of the optic nerve after topical administration of either 1 or 1.5 M Mn^{2+}. The axonal counts were performed on the central 100×100 μm^2, and there was no significant difference in the axonal counts between the Mn^{2+}-affected and control nerves.

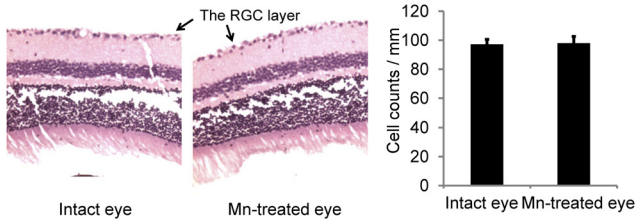

FIGURE 7. H&E staining of RGCs. There was no noticeable RGC loss in the mice after topical administration of 1.5 M Mn^{2+}.

To compare topical administration and intravitreal injection, it is critical to decide the appropriate amount of $MnCl_2$ solution for injection comparable to 1 M or 1.5 M $MnCl_2$ topical administration. The volume of fluid must be small to avoid intraocular hypertension. Learning from the published ophthalmology studies, we found that a range of fluid volume from 0.5 to 3 μL should be used for intravitreal injection in mouse eyes to provide efficient drug intervention.[2,20–24] Thus, we conducted intravitreal injection of 2 μL of 1 M $MnCl_2$ for comparison to the MEMRI with 1 M topical administration. In parallel, we also performed intravitreal injection of 0.5 μL of 100 mM $MnCl_2$ in another group of mice. We found significant increments of T1WI intensity in the retina (70%), optic nerve (60%), and superior colliculus (20%), 1 day after intravitreal injection of 2 μL of 1 M $MnCl_2$. The signal increments of T1WI intensity by the 0.5-μL 100 mM $MnCl_2$ injection were much less and showed statistical significance only in the retina and optic nerve, as compared with the control sites. It is worth noting that Lowe et al.[20] recently demonstrated that an intravitreal injection of 0.5 μL, with an $MnCl_2$ concentration smaller than 400 mM, would not disturb the focal osmotic equilibrium, thus avoiding an increase in intraorbital volume. However, although they showed that the mice with 400 mM ($n = 3$) did not have enhancement in the superior colliculus, enhancement was seen in their 800-mM group of mice, suggesting that signal enhancement can elicited by a higher concentration of $MnCl_2$, despite the possibility of intraocular hypertension. It was not clear why our 0.5-μL 100 mM–injected mice did not show significant enhancement in the superior colliculus, as presented by Lowe et al.[20] We suspect that different magnetic fields and coil setups between the two studies, as well as the use of T_1-weighted signal intensity, instead of the quantified T1 relaxation time, may have caused the discrepancy.

It is possible that the topically loaded fluid of Mn^{2+} enters the ocular space by routes different from the traditional intravitreal injection. The concentration gradients of ions may drive Mn^{2+} to diffuse across the corneal or sclera into the vitreous space, which could later reach the retina for the signal enhancement seen in the retina and optic nerve. It is also possible that the topical fluid interchanges with the anterior vascular circulation, which can also transport Mn^{2+} into the retinal layers. To further examine how Mn^{2+} travels from the cornea to the retinal tissue after Mn^{2+} topical loading, we acquired T1WIs of the eye every 30 minutes for 4 hours. As shown in Figure 4, the signal intensity of the retina gradually increased up to 50% in a period of 4 hours. Of note, the signal intensity of vitreous space did not change over time. This finding suggests that transcorneal diffusion may not be involved in the

transportation of Mn^{2+} from the corneal space to the retinal tissue.

Dynamic imaging of the eyes after Mn^{2+} topical administration showed signal changes in the cornea, iris, retina, and lens boundary, but their temporal profiles appeared to have different trends (Fig. 4). The cornea and iris initially had high signal intensities followed by a gradual decay, while the retina and lens boundary appeared normal initially, followed by a gradual increase. The synchronic enhancements of cornea and iris at the early time points suggests that Mn^{2+} applied on the cornea may be quickly absorbed and diffuse (or be transported) to the nearby iris. As the lens is attached to the ciliary body via the ciliary zonular fibers,[29] we suspect that Mn^{2+} absorbed in the ciliary–iris area diffuses into the lens boundary through the ciliary zonular fibers. Mn^{2+} may enter the retina via diffusion or transport by microvessel circulation from the iris or ciliary bodies. Worth noting, the propagation of Mn^{2+} was retrained within the recipient eye. There was no detectable signal change in the retina, optic nerve, and superior colliculus in the hemisphere opposite the hemisphere with Mn^{2+} application, regardless of the significant signal increments in the Mn^{2+}-affected visual system (Fig. 2). Although the mechanism of enhancement in the lens boundary and iris remain to be elucidated, the enhancement of these two regions has also been found in MEMRI studies using an intravitreal injection of Mn^{2+}.[17] Thuen et al.[17] used intravitreal injection of $MnCl_2$ and demonstrated significant enhancements in the lens boundary and ciliary body. The data support the relation between the lens boundary and ciliary enhancement. Cells in the ciliary body seem to attract Mn^{2+} ions despite the use of intravitreal injection or topical administration.

To further determine whether the signal enhancement was dependent on the health of RGCs, the $MnCl_2$ topical administration MEMRI was also conducted on mice with retinal ischemia. Based on our previous work,[26] the RGC layer was totally lost 7 days after transient retinal ischemia, which was in line with the findings of Zheng et al.[25] showing an 80% RGC loss 7 days after a transient retinal ischemia in mice. The subsequent optic nerve degeneration could be identified noninvasively with MR diffusion tensor imaging (DTI), which showed significant decreases in relative anisotropy (RA) on days 7 to 21.[26–28] In this study, the damage from retinal ischemia was confirmed by DTI of the optic nerve (Fig. 5). Two weeks after retinal ischemia, significant damage to the optic nerve was revealed as a 60% decrease in RA, a 50% decrease in $\lambda_{\parallel}$, and a 100% increase in $\lambda_{\perp}$ (Fig. 5), which was in line with our previous studies.[26–27] We found no detectable signal enhancement in these injured nerves with MEMRI. Although the results supported a close relation between MEMRI with topically administered $MnCl_2$ and healthy eyes, the exact mechanism that blocked Mn^{2+} uptakes is yet to be determined. The loss of RGCs can minimize the amount of Mn^{2+} that enters the cells.[25–26] The residual but impaired RGCs may have deficient axonal transport that is unable to move Mn^{2+} from the soma toward the optic nerve. Degenerated capillaries after transient retinal ischemia[25] may disturb focal capillary transport of topically loaded Mn^{2+} to the retina.

The presented study is the first to demonstrate the use of topical administration of Mn^{2+} for MEMRI in the visual system. However, this is not the first study in which a noninvasive agent-loading approach has been used as an alternative to the traditional intravitreal injection for MEMRI. Previously, transscleral or transcorneal iontophoresis[30–32] has been used and shown to be an efficient, nonintrusive delivery method of loading Mn^{2+} into vitreous space. Iontophoresis has been applied to deliver a compound across a membrane with the assistance of an electric field. In ocular iontophoresis, a donor electrode containing the drug to be delivered into the eye is placed on the eye, and the other electrode, referred to as the return electrode, is placed on another body surface, forming an electrical circuit through the body. Studies have shown the feasibility of using iontophoresis in MEMRI of the visual system. However, iontophoresis requires special equipment, which may not be practical in some MRI laboratories.

Although the signal enhancement gradually increased in parallel with the concentration of $MnCl_2$ solution, the topical administration of 1.5 M $MnCl_2$ provided a lower signal enhancement than that of the 1-M group. The reason for the lesser enhanced signal in MEMRI with 1.5 M $MnCl_2$ is unknown. Based on immunohistology, both groups of mice showed no RGC damage. However, the possibility of temporary signal blockage by higher concentrations of Mn^{2+}, causing the reduction of Mn^{2+} uptake, cannot be excluded.[33] High concentrations of Mn^{2+} also contribute to the decrease in proton T2 relaxation time,[30,34] which can compensate for the enhancement due to the short T1. Future experiments to quantify T1 and T2 of mice treated with different $MnCl_2$ concentrations will shed light on why topical administration of 1.5 M gave lower signals than 1 M.

The toxicity of Mn^{2+} in the nervous system has been a concern for adopting MEMRI in clinical practice.[35] Our immunohistochemistry showed no axonal damage in optic nerves 1 week after 1 and 1.5 M $MnCl_2$ topical loading (Fig. 6). The H&E staining on the retina showed no cell loss in RGC layers 1 week after 1.5 M $MnCl_2$ topical administration (Fig. 6). The safety of topical administration was also confirmed by repeated exposures to 1 M $MnCl_2$, as both treatments led to equivalent results (Fig. 3). However, further sophisticated investigations are still needed, to ensure the safety of MEMRI. For instance, in this study we conducted histology 1 week after Mn^{2+} treatment. It is therefore not clear whether acute or chronic damage may occur. It is also not clear how many repeated treatments are allowed before any noticeable damage occurs in animals used in a longitudinal study. One should be cautious of using this method in normal animals and those with pathologic conditions.

Our study is compromised by several limitations. The spatial resolution for dynamic imaging of eyes, $59 \times 59 \times 500$ μm, is sufficient to identify the vitreous space, lens, and retina. However, given the diameter of a mouse optic nerve (~0.2–0.3 mm, based on our MRI data), the image with slice thickness of 0.5 mm would have severe partial volume effects for measuring the optic nerve head and intraorbital optic nerve. Although we tried to capture a midsagittal image of the eye, the optic nerve head and intraorbital optic nerve were not consistently visible in our dataset. Further investigations with higher spatial resolution will be needed to resolve this issue. Furthermore, quantification of T1 will provide a better correlation to Mn^{2+} dosage than the T_1-weighted signal, as used in this study, for MEMRI. T1 maps may also enhance the image contrast to reveal Mn^{2+} uptake in mice treated with low concentration of $MnCl_2$.

In conclusion, the feasibility of topical administration of Mn^{2+} for MEMRI of the visual system was successfully demonstrated. With a drop of 1 M $MnCl_2$, significant signal increments were found within the retina, optic nerve, lateral geniculate nucleus, and superior colliculus. The signal reached a peak in 1 day and returned to baseline within 7 days. The MEMRI with topically administered $MnCl_2$ did not cause optic nerve damage, as confirmed by immunohistochemistry. Topical administration may serve as an easy and less invasive approach for loading Mn^{2+} for MEMRI analysis of the visual system.

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