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Manganese-Enhanced MRI (MEMRI) via Topical Loading of Mn²⁺ Significantly Impairs Mouse Visual Acuity: A Comparison with Intravitreal Injection

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Abstract

Manganese enhanced MRI (MEMRI) with topical loading of MnCl₂ provides comparable optic nerve enhancement as seen by intravitreal injection. However, the impact of this novel and noninvasive Mn²⁺ loading method on visual function requires further assessments. The objective of this study is to determine the optimal topical Mn²⁺ loading dosage for MEMRI and to assess visual function after MnCl₂ loading. Intravitreal administration was performed to compare the two approaches of MnCl₂ loading. Twenty-four hours after topical loading of 0, 0.5, 0.75, and 1M MnCl₂, T1-weighted (T1W), T2-weighted (T2W), diffusion tensor image (DTI) and visual acuity were performed to determine the best topical loading dosage for MEMRI measurements and to assess the integrity of retina and optic nerves. Mice were perfusion fixed immediately after in vivo experiments for H&E and immunohistochemistry staining. Topical loading of 1 M MnCl₂ damaged the retinal photoreceptor layer with no detectable damage to retina ganglion cell layers or prechiasmatic optic nerves. For the topical loading, 0.75-M MnCl₂ was required to see sufficient enhancement of optic nerve. At this concentration the visual function was significantly affected, followed by a slow recovery. Intravitreal injection (0.25 μL of 0.2 M MnCl₂) slightly affected visual acuity with full recovery a day later. To conclude, intravitreal MnCl₂ injection provides more reproducible results with less adverse side effects than topical loading.

Introduction

Manganese (Mn²⁺), as a calcium (Ca²⁺) analog, enters cell through voltage-gated Ca²⁺ channels. After entry into the cell, Mn²⁺ is transported by fast axonal transport in vesicles

and released into the synaptic cleft (1). Mn^{2+} produces a concentration-dependent shortening of tissue T1 due to its paramagnetic property. Therefore, Mn^{2+} can serve as both contrast agent and axonal transport tracer. T1-weighted (T1W) imaging is usually performed to monitor real-time Mn^{2+} transport reflected by the extent of enhancement in T1W MRI (2). Therefore, manganese-enhanced MRI (MEMRI) provides a potential approach to further explore the visual pathway from retinal ganglion cells, optic nerve, to contralateral optic tract and superior colliculus (3). Indeed, MEMRI has recently been used as a biomarker and tracer to investigate optic nerve axonal integrity (4–9), retinal projections to the superior colliculus through intravitreal injection of $MnCl_2$ (10), layer-specific calcium-dependent retinal fMRI activation (11,12), or cortical response to stimulation (13). Recently, MEMRI has also been applied to assess retinal and callosal projections in early postnatal development and plasticity (14).

The cellular toxicity of Mn^{2+} has long been recognized as overexposure causes Parkinsonism (15). Therefore, it is important to determine optimal Mn^{2+} dosing, which is a tradeoff between maximizing MR detectability and minimizing toxicity (16). The toxicity of $MnCl_2$ after intravitreal injection has been examined previously by histology and visual function measurements (7,17–20). In an attempt to remedy the invasive intraocular injection of $MnCl_2$, Sun et al. proposed to deliver Mn^{2+} through topical loading (21). Topical loading is a simple, noninvasive approach for Mn^{2+} delivery, achieved by dropping $MnCl_2$ solution directly onto the eye. The effect of repetitive topical $MnCl_2$ loading was recently examined reporting RGC loss after three bi-weekly applications of 1 M $MnCl_2$ while no RGC loss was observed after three monthly applications (22). Corneal thickening was also observed after seven bi-weekly applications (22). It appeared that increasing the time interval between repetitive topical applications reduced adverse effects seen in RGC and cornea. In this study, the effect of topical and intravitreal loading of $MnCl_2$ was examined by assessing post-loading visual acuity and structural integrity of both retina (by high resolution *in vivo* T2-weighted MRI and histology) and prechiasmatic optic nerves (by *in vivo* diffusion tensor imaging, i.e., DTI). Results suggest that intravitreal injection of $MnCl_2$ is safe with limited adverse effects when administered in small volume and low doses, sufficient to monitor axonal transport.

Materials and Methods

All experimental procedures were approved by Washington University's Animal Studies Committee and conformed to the NIH Policy on Responsibility for Care and Use of Animals and the ARVO Statement for the Use Animals in Ophthalmic and Vision Research.

Topical $MnCl_2$ Loading

Thirty-five female C57BL/6 mice obtained from Jackson Laboratory (Bar Harbor, ME) at 8 weeks of age underwent topical loading of 0 (n=5), 0.5 (n=5), 0.75 (n=15), or 1 M (n=10) of $MnCl_2$ in 0.9% saline. After anesthetized using 1.5–2% isoflurane/oxygen, a 30- μ L drop of $MnCl_2$ was introduced covering the entire right eye. An additional 10 μ L of $MnCl_2$ was applied every 15 min to maintain the topical-fluid volume (total volume was $30 + 3 \times 10 =$

60 μ L), different from the original procedure reported by Sun et al.(21), assuring a consistent loading among various lab personnel.

Intravitreal MnCl₂ Injection

An additional twenty 8-week old female C57BL/6 mice were employed to compare the “safe” injection volumes of 0.25 and 2 μ L using saline (n = 5 each group), and the “safe” dose using 0.25 (n = 5) and 0.125 (n = 5) μ L of 0.2-M MnCl₂ in saline. Mice, under 1.5 – 2% isoflurane/oxygen anesthesia, were placed on a custom-made head holder for intravitreal injection at 3- μ L/min using a 34-gauge needle connected to a micro-injection pump (WPI Instrument, FL). After infusion, the needle was left in place for an extra 1 minute before withdrawal. A drop of topical antibiotic was applied after removing the injection needle (23).

Visual Acuity Assessment

Visual acuity (VA) was assessed using the OptoMotry Virtual Optometry System, (Cerebral Mechanics, Inc., Canada). Mice were placed on the platform inside the chamber with virtual rotating columns projected on four LCD monitors surrounding it. The spatial frequency in cycle/degree (c/d) starting from 0.1 c/d was incremented in steps of 0.05 c/d until the mouse stopped responding to the projected visual patterns, which was recorded as the VA (24,25). Thirty normal C57BL/6 mice were measured to establish the baseline VA for naïve animals. VA was measured before MnCl₂ loading and at days 1 through 7 after topical MnCl₂ loading, and at 4, 24, and 48 hours after intravitreal injection.

MRI: DTI and T1-weighted imaging of the visual pathway

Twenty-four hours after topical or intravitreal MnCl₂ loading, MRI measurements were performed on a 4.7T Agilent Diect Drive small-animal MRI system (Agilent Technologies, CA) equipped with a Magnex/Agilent HD gradient coil set (Magnex, Oxford, UK) with pulse gradient strength up to 58 G/cm and a gradient rise time 295 μ s. Mice were anesthetized by isoflurane/oxygen (1.5%). Respiratory rate and body temperature were monitored during experiments using a MR compatible animal monitoring system (SA Instrument, Inc., NY). The body temperature was maintained at 37°C with a regulated circulating warm water pad placed underneath mouse body along with regulated warm air blown into the magnet bore. An actively-decoupled 1.7-cm receive coil was placed on top of the mouse head. The animal holder assembly, including the receive coil was placed in an 8-cm active-decoupled volume transmit coil (26,27).

T1-weighted Imaging—A 3D gradient echo sequence was employed to image mouse brain with the following parameters: TR= 15 ms, TE= 2.63 ms, flip angle 20°, number of averages 16, FOV 15 $\times$ 15 $\times$ 22 mm³, data matrix size 128 $\times$ 128 $\times$ 64 (zero-filled to 256 $\times$ 256 $\times$ 128). Regions of interest (ROI) were manually outlined according to a previously published protocol (21) on the extracted 2D images containing retina, optic nerve, and superior colliculus using ImageJ software (NIH, <http://bigwww.epfl.ch/sage/soft/watershed/>, US). The ROI of the Mn²⁺-loaded structures along the visual pathway was outlined and mean signal intensity was obtained using ImageJ. The signal intensity of the loaded ROI was

normalized by the contralateral mean intensity to correct scan-to-scan sensitivity differences resulting from variable sample loading.

DTI of Prechiasmatic Optic Nerve—Optic nerve DTI was performed using a multi-echo spin-echo diffusion weighted imaging sequence (28) with the following parameters: TR = 1.5 s, TE = 34 ms (first echo time, including required time for diffusion-weighting gradients), inter-echo delay time 19 ms, FOV 22.5×22.5 mm², matrix size 192×192 (zero-filled to 384×384); in-plane resolution 117×117 mm² (prior to zero-filling), slice thickness 0.5 mm, two diffusion-sensitizing factors (b value) 0.0 and 1.0 ms/μm² (with δ = 5 ms and = 18 ms). The standard six-direction diffusion encoding scheme was used, with [G_x, G_y, G_z] = [1, 1, 0], [1, 0, 1], [0, 1, 1], [−1, 1, 0], [0, −1, 1], and [1, 0, −1]. A train of three echoes were co-added to generate the final MR images to increase signal-to-noise ratio. Total acquisition time was 1 hour and 40.8 minutes. We chose to image only the prechiasmatic optic nerves to minimize the potential partial volume effect. Specifically, in this region of optic nerves a straight segment of nerve is readily identifiable allowing an image plane to be as nearly perpendicular to optic nerves as possible to minimize partial volume effects. Fractional anisotropy (FA), axial (λ_{||}) and radial (λ_⊥) diffusivity maps were generated from the image data by programs written in Matlab (MathWorks, Natick, MA).

T2-weighted Imaging of Retina

For retinal imaging, 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). The infusion rate was adjusted to maintain the respiratory rate between 150 – 210 breaths/min during imaging. Retina imaging was performed on an Agilent 11.74 T DirectDrive small-animal MRI system using a spin-echo sequence with the following parameters: TR = 2 s, TE = 34 ms, FOV 12×12 mm², matrix size 256×256 (zero-filled to 512×512); in-plane resolution 47×47 μm² (prior to zero-filling), slice thickness 0.4 mm. All images were acquired on a nasal-temporal slice bisecting the eye through the retina and optic nerve head.

Histology

Optic nerve—Following MR experiments, mice were perfused with 4 % paraformaldehyde in phosphate-buffered saline (PBS). Brains were excised and post-fixed in fixative for 24 hours then transferred to 1% PBS for storage at 4°C until histological analysis. Mouse optic nerves were embedded in 2 % agar gel followed by paraffin embedding (29). Five-μm thick transverse slices were sectioned for immunohistochemical staining. All sectioned tissues were deparaffinized, rehydrated, and blocked in solution mixed with 1% bovine serum albumin (BSA) and 2% Triton-X100 for an hour at room temperature to prevent non-specific binding and to increase antibody permeability. Slides were incubated in monoclonal anti-phosphorylated neurofilament antibody (SMI-31; 1:2000, Covance) to stain non-injured axons, or in rabbit anti-myelin basic protein (MBP) antibody (1:1000 Sigma-Aldrich, MO) to stain myelin sheath at 4°C overnight. After rinsing, goat anti-mouse IgG or goat anti-rabbit IgG conjugated Alexa 488 (1:800, Invitrogen, CA) were applied to visualize immunoreactivity of phosphorylated neurofilaments and MBP. Finally, slides were covered using Vectashield Mounting Medium with 4', 6-diamidino-2-

phenylindole (DAPI) (Vector Laboratory, Inc., Burlingame, CA). Histological slides were examined with Nikon Eclipse 80i fluorescence microscope. Images were captured with a black-and-white CCD camera using MetaMorph software (Universal Imaging Corporation, Sunnyvale, CA).

Retina—Mouse eyes were flash-frozen immediately after enucleation to avoid dehydration and fixation induced tissue distortion. Frozen-cut 8- μ m-thick tissue sections were stained with hematoxylin and eosin (H&E) to assess the integrity of multiple retinal layers. Histological slices were scanned with Olympus NanoZoomer slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). For quantitative analysis of H&E staining, scanned images were exported with NDP viewer software (Hamamatsu Photonics, Hamamatsu, Japan) with 20 $\times$ objective. ROIs were selected to contain the retina segment with proximal and distal ends located at 250 and 800 μ m away from the optic nerve head on both sides of the nerve head.(30) Within the ROIs, the retinal thickness was measured from nerve fiber layer to retina pigment epithelium every 55 μ m (i.e., 10 measurements total). Ten measurements were averaged as the representative thickness for that side of retina.

Statistical analysis—Multiple comparisons for T1W enhancement in retina, optic nerve, and superior colliculus after Mn^{2+} loading were performed using a linear repeated measures model with adjustment for multiple testing using Tukey's HSD (honest significant difference). A linear repeated measures model was used to estimate the mean difference in VA between loaded and contralateral control eyes by dosages and day of experiment (from baseline to day 7). The effect of Mn^{2+} loading on the mean retinal thickness was assessed using a linear model on a log scale in each of the five study groups. Each Mn^{2+} treated group was compared with the saline control. P-values for the difference in mean retinal thickness were adjusted for multiple tests using Tukey's HSD.

Results

Topical $MnCl_2$ Loading

Manganese Enhanced T1W MRI—T1-weighted images of 1-M $MnCl_2$ loaded visual pathway including ipsilateral retina and optic nerve (ON), and contralateral lateral geniculate nucleus (LGN) and superior colliculus (SC) clearly demonstrated signal enhancement on these structures (Fig. 1A). The image of retina and optic nerve with 0.5, 0.75, and 1 M of $MnCl_2$ loading (Fig. 1B) suggested that both 1 and 0.75 M of $MnCl_2$ loading led to discernible signal enhancement in optic nerve while 0.5 M did not. Compared to the saline group, the group-averaged ROI analysis (Fig. 1C) demonstrated that 0.75 and 1 M of $MnCl_2$ loading significantly enhanced all structures along the visual pathway.

Visual Acuity—The VA of normal female C57BL/6 mice is 0.37 ± 0.03 c/d (mean $\pm$ SD; n=30). Topical loading of 1-M $MnCl_2$ caused total blindness (VA $\sim$ 0 c/d) at one day after loading without recovery up to 7 days after loading (open squares; Fig. 2). A significant VA decrease in eyes following 0.75-M $MnCl_2$ loading was also observed (open circles; Fig. 2). However, the decreased VA gradually recovered to the normal range at day 5 (VA $\sim$ 0.3 c/d). Contralateral control eyes in animals treated without 1 and 0.75-M $MnCl_2$ loading were

not affected throughout the time course (filled symbols, Fig. 2). Mice topically loaded with 0.5M MnCl₂ exhibited a slight VA decrease at one day after loading and recovered to normal at day 2; saline-loaded eyes exhibited normal VA throughout the time course (data not shown).

DTI and Immunohistochemistry of Optic Nerve—*In vivo* DTI of optic nerves was performed at days 1 and 7 after 1M MnCl₂ loading (VA = 0 c/d). Color-coded FA, maps derived from DTI analysis clearly delineated the optic nerves (Fig. 3A). There was no statistically-significant difference in group-averaged $\lambda_{||}$ and $\lambda_{\perp}$ between the loaded and control optic nerves at days 1 and 7 after MnCl₂ loading (Fig. 3B). These DTI findings suggested that there was no detectable axon or myelin injury at prechiasmatic optic nerves after topical MnCl₂ loading (31), supported by the immunohistochemical staining of phosphorylated neurofilaments (SMI-31; green dots in Fig. 3C) and myelin basic protein (MBP; red rings in Fig. 3C) from the same nerves after *in vivo* DTI.

T2W Images and H&E Staining of Retina—T2W images acquired from retinas in control and topical-loading mice at day 7 after 1M MnCl₂ loading revealed the previously reported dark-bright-dark layer contrast of the control retinas(32,33) (Fig. 4A and B). Significant thinning of the retina was seen in the 1M MnCl₂ loaded eyes (Fig. 4C and D). Retinal layers examined by H&E staining showed significant cell swelling (~26 % increased thickness) at day 1 (VA = 0 c/d; the time point of best T1W enhancement) and significant retinal thinning (~31 %) at day 7 (VA = 0 c/d) after 1M MnCl₂ loading (Fig. 4E and F). A slight swelling of the retina was seen at day 1 after 0.75M MnCl₂ loading with recovery to the normal thickness at day 5 (VA = 0.3 c/d). Retina with saline loading exhibited the same thickness as that reported in the literature for normal mice (Fig. 4E and F).(23,30)

Effect of Intravitreal Injection

Volume Effect—There was no change in VA of eyes receiving intravitreal 0.25- μ L saline injection (Fig. 5A). In contrast, VA impairment was seen in eyes at 4 and 24 hours after intravitreal 2- μ L saline injection, which recovered at 48 hours (Fig. 5A). Histological examinations revealed significant retinal swelling (~17% increase in thickness) at 24 hours after 2 μ L-saline injection while no detectable difference was seen in eyes with 0.25- μ L saline injection or topical saline loading (Fig. 5C and D).

Dose Effect—Sufficient T1W enhancement in the visual pathway was seen 24 hours after intravitreal injection of 0.25- μ L 0.2-M (50 nmol) MnCl₂ (Fig. 5B) as previously reported (17). In contrast, we failed to see a detectable T1W enhancement in the visual pathway using the other reported dosage (17), 0.125- μ L 0.2-M MnCl₂ (25 nmol; data not shown). In the 50 nmol dosage group, histological analysis of retina using H&E staining showed slight cell swelling at 4 hours, which recovered at 24 hours post-injection (Fig. 5C and D).

Discussion

This study investigated the toxicity of noninvasive topical Mn²⁺ loading using functional (VA) and structural (MR) measurements followed by histological validation. Our findings show that topical MnCl₂ loading caused photoreceptor layers degeneration without

detectable damage to the optic nerve within the 7-day time course of this investigation. In addition, we also investigated the impact of literature-reported safe dose of intravitreal MnCl_2 injection concluding that intravitreal injection causes insignificant visual impairment and is probably safer for MEMRI studies than the newly proposed topical loading.

Topical loading

The route of Mn^{2+} entry is believed to start from the conjunctiva passing through the ciliary body (34,35). Afterwards, Mn^{2+} may diffuse into the choroid vasculature passing through the blood-retinal barrier (36) to enter the retinal layers. In addition, Mn^{2+} may also pass through blood-aqueous barrier diffusing into vitreous space reaching the retina ganglion cell layer (34,37). In our hands, topical loading only resulted in anterior chamber and retina enhancement while the vitreous space was not enhanced. This is consistent with findings by Sun et al. reporting no intravitreal enhancement (21). Although a definitive conclusion may not be established from the current study, the observed lack of vitreous enhancement as well as the preferential photoreceptor layer injury after topical loading suggests that Mn^{2+} may enter the ocular space primarily through non-corneal permeation across the conjunctiva and sclera into the anterior uvea.

Visual acuity (VA) reflects the visual function and ocular integrity of retinal efferent pathways to subcortical visual structures (25). In the present study, VA was employed to assess the effect of Mn^{2+} loading since VA of normal mice has been measured consistently (24). Our VA measurements from thirty female C57BL/6 mice were in agreement with Prusky's finding with less than 10% co-variance (0.37 ± 0.03 c/d; mean $\pm$ SD; $n = 30$). The acute 73% decrease in VA after 0.75 M MnCl_2 loading followed by a slow recovery and the total loss of vision after 1 M MnCl_2 loading without recovery (throughout the course of experiment) suggests that topical loading adversely affects visual function of the loaded eye as a result of Mn^{2+} toxicity since there was no VA change after topical saline loading.

With 1M MnCl_2 topical loading, the retina thickened at 1 day followed by significant thinning at 7 days after loading [$\sim 31\%$, $p < 0.005$]; Fig. 4E and F]. Retinal thickening at 24 hours after 1M MnCl_2 loading appears to result from cell swelling (23,38), while subsequently decreased retinal thickness is likely the result of may be related to retinal degeneration and tissue loss. There was a negligible retinal thickness change by 0.75M topical loading (Fig. 4F). The increased image intensity of choroid (Fig. 4D) probably resulted from manganese accumulation in endothelial cells (39).

Photoreceptor layer degeneration was seen in H & E staining (Fig. 4E), suggesting that the entry of excess calcium analog, Mn^{2+} , could cause metabolic dysfunction and eventual photoreceptor cell loss (18). At day 1 with 1-M MnCl_2 loading, retina thickened variably (Fig. 4E) due to differential uptake of topically loaded Mn^{2+} between mice. Our T2W images exhibited strong vitreous signal intensities (Fig. 3A – D), potentially confounding layer thickness estimation although retinal swelling and thinning was clearly visible in T2W images. A balanced steady state free precession (bSSFP) MR sequence has been demonstrated to be time-efficient to detect retinal layer and layer-specific responses to hypoxia in retinal and choroidal vasculature, which are separated by an avascular region (40). This approach could be used to overcome the shortcoming of T2W measurement

employed herein to better assess retinal thickness. The optic nerve integrity was examined using in vivo DTI followed by immunohistochemistry (Fig. 3B, 3C). In order to minimize partial volume effect, DTI was performed at prechiasmatic optic nerves. Normal axial ($\lambda_{||}$) and radial ($\lambda_{\perp}$) diffusivities after MnCl_2 loading suggest axonal integrity of the loaded prechiasmatic optic nerve (31). Postmortem immunohistochemistry findings supported the conclusions drawn from in vivo DTI measurements. There was no detectable injury in either RGC layer (Fig. 4E) or prechiasmatic optic nerve (Fig. 3B and C). The visual function impairment is more pronounced with increasing retinal morphological distortions—initially retinal swelling (Fig. 2, 4E, and 4F) and subsequently photoreceptor loss and thinning of the retina. Topical saline loading did not cause retinal thickness change, or VA impairment. The results suggest that Mn^{2+} was the primary cause of the decreased VA and reduced retinal thickness.

Intravitreal injection

Intravitreal injection of MnCl_2 is a common practice for MEMRI studies. Published reports have examined toxicity of intravitreal injection of MnCl_2 in mice and rats (17–19,41). Bearer et al. used visual evoke potential (VEP) to assess visual function (17) reporting that 0.5 μL of saline injection affected VEP response. They suggested a 0.25 μL injection of 200 mM MnCl_2 (equivalent of 50 nmol) to be the safe dose for MEMRI studies in C57BL/6 mice. Haenold et al. measured VA to determine visual function suggesting that 2 μL of 0.0075 M MnCl_2 (equal to 15 nmol) as the safe dose for the same mouse strain (18). They found that 50 nmol caused photoreceptor degeneration with RGC injury observed at a dose over 50 nmol.

The volume of the vitreous space in C57BL/6 mice is approximately 5 μL . Chen et al. have previously reported that 0.5 μL of intravitreal saline injection was safe (23). Haenold et al. reported normal VA two days after 2 μL of saline injection (18). The current finding saw transient impairment of VA with 2 μL of saline injection (Fig. 5A). The recovery of VA 2 days after injecting 2 μL of saline was in agreement with that seen by Haenold et al. A significant retinal swelling was observed with 2 μL saline injection in the present study (Fig. 5C and D) while 0.25 μL saline injection did not impact VA (Fig. 5A) or retinal thickness (Fig. 5C and D) at any time point. Our findings support the conclusion of Bearer et al. suggesting the optimum dose for intravitreal injection is 0.25 μL of 200 mM MnCl_2 . We failed to see enhancement using the dose proposed by Bearer et al., 0.125 μL of 200 mM MnCl_2 (data not shown).

In conclusion, we found that both topical loading and intravitreal injection of MnCl_2 provided similar T1W enhancement in the visual system for assessing anterograde axonal transport. Despite the non-invasive nature of MnCl_2 loading, topical loading significantly impaired VA. The photoreceptor layer was significantly injured with retinal ganglion cells largely unaffected thus no detectable injury in the optic nerve. The working concentration of 0.75 M MnCl_2 offers sufficient enhancement for assessing axonal transport. However, the visual function was still affected at this dose thus cautioning functional correlation studies using topical loading. In contrast, the previously reported safe dose for intravitreal injection

proposed by Bearer et al. is ideal for axonal transport correlating with functional assessment in mouse visual system.

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Figure 1.

Representative T1W images with 1M topical MnCl₂ loading acquired from a mouse brain at 24 hours after loading (A), ipsilateral retina and optic nerve (ON), and contralateral lateral geniculate nucleus (LGN) and superior colliculus (SC) are enhanced with topical loading (arrows). The oblique images (B) bisecting retina and optic nerve after topical loading of 1, 0.75, and 0.5M MnCl₂ suggest that 0.75M MnCl₂ was sufficient to produce discernible enhancement of optic nerve. Bar graphs (C, mean $\pm$ SD) demonstrate the signal intensity ratio between labeled and control retina, ON, and SC. With 1 and 0.75M MnCl₂ loading, a significant enhancement of the structures along the visual system (retina, ON, LGN, SC) was clearly seen. In contrast, only slight retinal enhancement was seen with 0.5M MnCl₂ loading. Therefore, 0.75M was the concentration selected for topical loading in this study.

* indicates that $P < 0.005$ compared to saline group via Tukey HSD test.

Figure 2.

Visual acuity was measured daily in eyes after topical loading with 0.75 and 1M MnCl_2 (open symbols), and in contralateral, control eyes (filled symbols). Tests for difference between the dose groups indicate that there is no difference at baseline or on the first day of treatment. Between days 2 and 7, however, the difference between loaded and control eyes is not the same in the two different dosage groups (p -values < 0.0001 at days 2–7). At one day after 1M of MnCl_2 loading, the visual acuity decreased to zero (completely blind) and did not recover throughout the 7-day time course. In contrast, one day after 0.75M of MnCl_2 loading visual acuity decreased to 0.1 c/d and recovered beginning on Day 2 ($p < 0.05$, compared to 1M loading eyes) until recovering back to the normal range by Day 6.

Note 1: mean VA of normal B6 mice ($n=30$) is 0.36 ± 0.03 (c/d; mean $\pm$ SD)

Note 2: c/d denotes units of cycles per degree

Figure 3.

Brain DTI maps, containing prechiasmatic optic nerves, from mice at one and seven days after topical loading of 1M MnCl_2 were examined to assess the effect of Mn^{2+} on axonal integrity. The region-of-interest (ROI) based analysis was performed on diffusion metrics, including fractional anisotropy (FA), axial ($\lambda_{||}$), and radial ($\lambda_{\perp}$) diffusivity, from each optic nerve (A, outline in red). At one and seven days after 1M of MnCl_2 loading, there was no difference in $\lambda_{||}$ and $\lambda_{\perp}$ between the optic nerves from the control (B, black bars) and loaded (white bars) eyes suggesting no axon or myelin injury upon Mn^{2+} loading. The matched immunohistochemical staining of phosphorylated neurofilament (SMI-31) and myelin basic protein (MBP) strongly support the axonal and myelin sheath integrity seen by *in-vivo* DTI (C).

Figure 4.

In-vivo T2-weighted (T2W) imaging was performed to compare the retinal thickness between the saline sham (A) and MnCl₂-loaded (B) eyes. Zoom-in images of mouse retina (C and D) seven days after topical loading of 1M of MnCl₂ exhibit typical T2W MRI features of a mouse retina wherein sclera appears dark (white stars), choroid is bright (black arrows), and retinal layers (white double-headed arrows) exhibit intensities between sclera and choroid. Clear thinning in Mn²⁺ loaded retina was seen 7 days after topical loading in T2W MRI. The hematoxylin and eosin (H&E) staining of the frozen-embedded retina confirmed the MRI detected retinal thinning (E). At 24 hours after 1 M MnCl₂ loading, heterogeneous retinal swelling was observed. On day 7 after 1M MnCl₂ loading, photoreceptor layers degeneration was visible. 0.75 M MnCl₂ loading caused retina slightly swelling at day 1 but recovery at day 5 (E). To quantify the extent of retinal thickness change, H&E images were analyzed (F). A linear model was used to estimate mean retinal thickness on a log scale in each of the 5 study groups. Each of the MnCl₂ treated groups was compared with the saline control. P-values for the difference in mean retinal thickness are adjusted for multiple testing using Tukey's HSD test. One day after 1M MnCl₂ loading, transient significant swelling of retina ($p < 0.005$, with blindness) was seen followed by significant retinal thinning at Day 7 ($p < 0.005$, with no recovery of visual

function). At 0.75M of MnCl_2 loading, mild retinal swelling was seen at 1 day (significantly-decreased VA without blindness), which returned to normal thickness 7 days after loading (visual acuity returned to normal by 5–6 days post-loading).

Diamond symbols: mean value

Figure 5.

The effect of intravitreal Mn²⁺ injection on retinal integrity and visual function was examined on mouse eyes receiving 0.25 and 2 µL saline injection. The effect of injecting 0.25 and 2 µL saline on the visual acuity (VA) was examined where 2 µL saline injection transiently impaired visual function at 4 hours and Day 1 after injection, subsequently recovered (VA = 0.3) at Day 2 (A). The injection of 0.25 µL of saline did not impact VA (A). Representative T1W images (B) obtained at 24 hours post-injection with the working dosage (0.25 µL and 0.2M MnCl₂, 50 nmol) demonstrate the effect of Mn²⁺ induced intensity enhancement in the loaded visual pathway (left retina, left optic nerve, right LGN, and right SC). The VA was affected by the working dosage at 4 hours post-injection with nearly full recovery by 24 hours post-injection (A). Retinal thickness was examined with H&E staining of frozen-embedded mouse eyes (C and D). The previously reported safe intravitreal 2 µL saline injection caused retinal swelling at Day 2. In contrast, the other reported safe intravitreal saline injection (0.25 µL) indeed did not cause any retinal swelling of the injected eyes (C and D). MnCl₂ injection caused slight retinal swelling at 4 hours that recovered at 24 hours post-injection. Histologically-revealed retinal thickening paralleled the decreased VA caused by injection volume and Mn²⁺ (A).

Diamond symbols: mean value