Excitotoxicity Upregulates SARM1 Protein Expression and Promotes Wallerian-Like Degeneration of Retinal Ganglion Cells and Their Axons

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PURPOSE. This study investigated the role of sterile alpha/Armadillo/Toll-Interleukin receptor homology domain 1 protein (SARM1) in Wallerian-like degeneration of retinal ganglion cells (RGCs) and their axons after inducing excitotoxicity.

METHODS. To induce excitotoxicity, kainic acid (KA) was injected into the vitreous humor of B6.Cg-Tg(Thy1-YFP)HJrs/J mice. Control mice received PBS. At 24, 48, and 72 hours after injection, degeneration of RGCs and their axons in the retina was determined by fundus imaging, and axonal degeneration in the optic nerves was determined by fluorescence microscopy. SARM1 protein levels were determined by Western blot analysis and SARM1 tissue localization was determined by immunohistochemistry. Causal role of SARM1 in KA-mediated degeneration of RGCs and their axons was determined by treating the eyes with KA along with *Sarm1* silencer siRNA.

RESULTS. Fundus imaging and microscopic analysis indicated that KA promoted Wallerian-like degeneration of RGCs and axons in KA-treated eyes, but not in PBS-treated eyes. Quantitative analysis indicated a significant increase in degeneration of RGCs and their axons in KA-treated injected eyes, but not in PBS-treated eyes. Compared with low levels of SARM1 protein in retinal protein extracts, retinal cross sections, and optic nerve from PBS-treated eyes, SARM1 protein levels were increased in KA-treated eyes. Finally, treatment of eyes with KA along with a *Sarm1* silencer siRNA attenuated KA-mediated degeneration of RGCs and their axons significantly.

CONCLUSIONS. Results presented in this study, for the first time, show that KA-mediated upregulation of SARM1 protein promotes Wallerian-like degeneration of RGCs and their axons.

Keywords: excitotoxicity, axonal degeneration, retinal degeneration

Damage to retinal ganglion cells (RGCs) and their axons are major clinical concerns in a number of blinding diseases of the retina, including glaucoma, which affects around 60 million people worldwide.^{1–3} Despite the fact that the elevated IOP has been identified as a risk factor for the degeneration of RGCs in glaucoma, the mechanisms underlying IOP-mediated retinal damage are still unclear. A number of hypotheses have been proposed, including alterations in transport of neurotrophic factors,^{4,5} retinal ischemia,^{6,7} increase in endothelial nitric oxide synthase (eNOS),^{8,9} reactive gliosis,⁹ excitotoxicity,^{10,11} and axonal self-destruction.¹² Among these hypotheses, evidence regarding the role of glutamate in RGC death has been controversial, and, to date, the role of increased levels of glutamate in RGC death in glaucomatous damage has never been substantiated.^{13,14} However, after inducing excitotoxicity by N-methyl-D-aspartic acid (NMDA) or kainic acid (KA), a number of studies on rodents and primates have shown that excitotoxicity does play a role in the death of RGCs because antagonists against NMDA and non-NMDA receptors have been shown to offer neuroprotection.^{15–18}

Although studies in animal models of excitotoxicity have shown that NMDA, and non-NMDA receptor antagonists offer neuroprotection, the underlying mechanisms are still not well understood. Previous studies have shown that excitotoxicity

causes damage not only to the cell bodies of RGCs, but also to their axons in the retina and optic nerve.^{15–17} When excitotoxicity induces apoptotic death of RGCs,¹⁸ their axons can undergo morphologic changes that lead to their fragmentation through an axonal self-destruction process known as Wallerian-degeneration, named after Augustus Waller.¹⁹ This process was traditionally viewed as a passive process due to blockade of neurotrophic factors, but a few studies on a mutant mouse strain, *Wallerian degeneration slow (Wld^s)*, have shown that this degeneration process might be driven by active molecular program similar to apoptotic cell death program.²⁰ Since *Wld^s* is not made under normal conditions, the endogenous mechanisms that promote axonal-self destruction are not well understood. Toward this goal, by employing forward genetic screen in Wallerian-degeneration model of *Drosophila melanogaster*, a recent study reported that two genes, *Drosophila* sterile alpha Armadillo motif (*dsarm*) and its mouse homolog, sterile alpha/Armadillo/Toll-Interleukin receptor homology domain 1 (*Sarm1*), plays a direct role in axonal self-destruction.²¹

Previous studies have reported that expression of *Wld^s* in glaucomatous DBA/2J mice reduces axonal loss,²² and the expression of *Wld^s* in transgenic rats delays axonal degeneration, but not the degeneration of RGC bodies.^{23,24} Another previous study has reported that NMDA-mediated excitotoxicity causes

Wallerian-like axonal degeneration in Sprague-Dawley rats.¹⁸ Studies from this laboratory have shown that excitotoxicity, induced by KA that activates non-NMDA-type receptors, causes apoptotic death of RGCs.^{25–27} Until now, except for two studies on Sprague-Dawley rats and DBA/2J mice on the role of *Wld^s* by surgically severing the axons, no studies have been conducted to investigate the role of SARM1 protein in Wallerian-like degeneration of RGCs and their axons. Since we have shown previously that excitotoxicity induced by activating non-NMDA receptors causes RGC death,²⁸ in this study, we have investigated whether KA-mediated excitotoxicity upregulates SARM1 protein in the retina, and whether upregulation of SARM1 protein promotes Wallerian-like degeneration of RGCs and their axons in a transgenic mouse line, B6.Cg-Tg(Thy1-YFPH)2Jrs/J, in which a subset of RGCs and their axons express a yellow-fluorescent protein (YFP). After inducing excitotoxicity, we have performed fundus imaging on live animals and microscopic analysis of optic nerves before and after KA or PBS treatment.

MATERIALS AND METHODS

KA and PBS were obtained from Sigma Chemical Company (St. Louis, MO). For Western blot analysis of SARM1, primary antibodies were obtained from Origene (Rockville, MD). For immunohistochemical analysis of SARM1, primary antibodies were obtained from GenWay Biotech Inc. (cat# 18-661-15183; San Diego, CA). Appropriate secondary antibodies conjugated to horse-radish peroxidase or AlexaFluor-568 were obtained from Invitrogen (Carlsbad, CA). *Sarm1* silencer predesigned siRNA (cat# AM16708) and silencer negative control siRNA (cat# AM4611) were obtained from Life Technologies (Grand Island, NY).²⁹

Animals

In this study, a mouse line B6.Cg-Tg(Thy1-YFPH)2Jrs/J in which a subset of RGCs express an YFP under the control of Thy1 promoter was used (Jackson Laboratory, Bar Harbor, ME). The advantage with these transgenic mice is that degeneration of RGCs and their axons can be assessed by live fundus imaging on the same animal before and after KA or PBS treatments.

Preparation of Transit-TKO-siRNA Complexes

Negative control siRNA and predesigned silencing siRNA against *Sarm1* gene were obtained from Life Technologies. Transit-TKO reagent was purchased from Mirus Biologicals (Madison, WI). Five nanomoles of siRNA were combined with 5 μ L Transit-TKO in 50 μ L RNase-free water and incubated for 20 minutes at room temperature.³⁰ The solution was then evaporated under vacuum and the precipitate was dissolved in 20 μ L RNase-free water for intravitreal injections.

Intravitreal Injections

All the experiments on animals were performed under general anesthesia according to Oakland University's protocol guidelines and the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research. Adult Thy1-YFP mice (8–10 weeks-old) were anesthetized by an intraperitoneal injection of Ketamine (50 mg/kg body weight) and Xylazine (8 mg/kg body weight). After instilling a drop of topical anesthetic agent, proparacaine, KA (20 nM/2 μ L volume) was injected into the vitreous humor of right eyes of each mouse by using a NanoFil syringe equipped with a 36-gauge beveled needle (World Precision Instruments, Sarasota, FL). Left eyes were injected with 2 μ L PBS (controls).

Fundus Imaging

To investigate whether KA injection causes axonal loss in the retina, fundus imaging was performed by using a recently developed Micron III camera (Phoenix Research Laboratories, Inc., Pleasanton, CA) designed for small animals. At 24, 48, and 72 hours after KA or PBS injection, mouse eyes were dilated with Atropine prior to anesthetizing the mice with an intraperitoneal (IP) injection of Ketamine (50 mg/kg body weight) and Xylazine (8 mg/kg body weight). In addition, mice were treated with *Sarm1* silencing siRNA or negative control siRNA along with KA or PBS. Mice were kept on a warm support stand and Micron III camera lens (Phoenix Research Laboratories, Inc.) was focused on the retina to capture images of YFP expressing RGCs and their axons. Fundus images were converted to gray scale images and inverted by using Adobe Photoshop Software (versions 5.5 and 7.0; Adobe Systems Inc., Mountain View, CA) to show better quality images of axonal degeneration. The number of degenerating axons in the retina was quantified, and statistical significance was determined by ANOVA, followed by a post hoc Tukey's test (GB-Stat Software; Dynamic Microsystems, Silver Spring, MD) and expressed as the mean $\pm$ SEM.

Imaging of Optic Nerves

At 24, 48, and 72 hours after KA or PBS injection, optic nerves were separated from enucleated eyes by cutting them at 1 mm behind the eye and fixed in 4% paraformaldehyde for 30 minutes at room temperature. In addition, optic nerves were isolated from mice treated with *Sarm1* silencing siRNA or negative control siRNA along with KA or PBS. After washing three times (5 minutes each) with PBS (pH 7.4), optic nerves were mounted on Superfrost plus microscope slides (Fisher Scientific, Fair Lawn, NJ) by using a cover slip and Fluoromount-G mounting solution (SouthernBiotech, Birmingham, AL). Optic nerves were fashioned in such way that the optic nerve heads oriented at the 3 o'clock position. Note that optic nerves were not cut into cryostat or paraffin sections, rather intact optic nerves were observed under a Zeiss microscope equipped with ApoTome (Zeiss Imager Z.2; Carl Zeiss Microscopy, Thornwood, NY) and a sequence of 12 to 14 optical sections (4- μ m slices) was collected for each optic nerve by using a digital camera (AxioCam; Carl Zeiss Microscopy). Finally, 12 to 14 optical sections were merged into one composite slice by using Zeiss extended focus software module (Carl Zeiss Microscopy). Images were converted to gray scale and inverted by using Adobe Photoshop Software (Adobe Systems Inc.). The number of degenerating axons in the in the optic nerves was quantified, and statistical significance was determined by ANOVA, followed by a post hoc Tukey's test and expressed as the mean $\pm$ SEM.

Protein Extraction

Following 24, 48, and 72 hours after intravitreal injection, mice were euthanized with an overdose of Ketamine plus Xylazine, and their eyes were enucleated. In addition, KA or PBS along with *Sarm1* silencer or negative control siRNA (100 pM) were injected into the vitreous humor of Thy1-YFP mice and their eyes were enucleated after 72 hours. Retinas were carefully removed and washed three times with PBS to remove any vitreous humor that may have adhered to the retina. Three to four retinas each were placed in Eppendorff tubes (Fisher Scientific, Hanover Park, IL) containing 40 μ L extraction buffer (1% nonidet-P40, 20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃VO₄, pH 7.4), and the tissues were homogenized. Tissue homogenates were centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatants were collected. Protein concentra-

FIGURE 1. Fundus imaging of RGCs and their axons in Thy1-YFP mice at 24 hours after excitotoxicity. Four representative fundus images obtained from three independent experiments indicate that RGCs and their axons started to degenerate as early as 24 hours in KA-injected eyes (boxed areas), but not in PBS-injected eyes. Note that the images obtained from the same mice before (left panels) and after (right panels) KA- or PBS- injection were converted into gray scale and inverted.

tion in supernatants was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Aliquots containing an equal amount of retinal proteins (50 μ g) extracted from KA- or PBS-injected eyes were mixed with gel loading buffer, and separated on 10% SDS-PAGE. In addition, aliquots containing equal amount retinal proteins (50 μ g) extracted from eyes treated with PBS, PBS+negative control siRNA, PBS+*Sarm1* silencer siRNA, KA, KA+negative control siRNA, KA+*Sarm1* silencer siRNA were mixed with gel loading buffer and separated on 10% SDS-PAGE. After electrophoresis, the proteins were transferred onto nylon membranes, and

FIGURE 2. Fundus imaging of RGCs and their axons in Thy1-YFP mice at 48 hours after excitotoxicity. Four representative fundus images obtained from three independent experiments indicate that when compared with the retinas of PBS-injected eyes, RGCs and their axons started to show a Wallerian-like degeneration in the retinas at 48 hours after KA-injection (arrows). Note that all the images obtained from the same animal before (left panels) and after (right panels) KA or PBS injection were converted into gray scale and inverted.

FIGURE 3. Fundus imaging of RGCs and their axons in Thy1-YFP mice at 72 hours after excitotoxicity. Four representative fundus images obtained from three independent experiments indicate that when compared with the retinas of PBS-injected eyes, RGCs and their axons show a further increase in Wallerian-like degeneration in the retinas at 72 hours after KA injection (arrows). Note that the images obtained from the same animal before (left panels) and after (right panels) KA or PBS injection were converted into gray scale and inverted.

nonspecific binding was blocked with 5% nonfat dry milk prepared in Tris-buffered saline containing 0.2% Tween-20 (TBS-T). Membranes were then probed with antibodies against SARM1 (1:1000 dilution). After incubating with primary antibodies, membranes were washed with TBS-T, and incubated with appropriate horse radish peroxidase (HRP)-conjugated secondary antibodies (1:4000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at room temperature. The proteins in the membranes were detected by using a Pierce

FIGURE 4. Quantitative analysis of degenerating axons in the retinas of Thy1-YFP mice. Average results from three independent experiments presented in the figure indicate that KA promotes increased degeneration of axons in the retinas in a time-dependent manner when compared to the axons in PBS-injected eyes. *, #, % $P < 0.05$, at 24, 48, and 72 hours, respectively.

FIGURE 5. Microscopic analysis of yellow-fluorescent protein-expressing axons in the optic nerves of Thy1-YFP mice. Representative micrographs from three independent experiments presented in the figure show Wallerian-like degeneration ([A], *arrows*) of axons in the optic nerves isolated from KA-injected eyes, but not in PBS-injected eyes (A). Note that images obtained at $\times 10$ magnification were converted into gray scale and inverted. Quantitative analysis of remaining axons indicated that a significant number of axons degenerated in KA-treated eyes in a time dependent fashion when compared with PBS-treated eyes (B). *, #, % $P < 0.05$, when compared with PBS-injected eyes at 24, 48, and 72 hours, respectively.

Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL) and by exposing the membranes to an X-ray film. Finally, X-ray films were scanned by using a densitometer (Storm 840; Amersham Pharmacia Biotech) and SARM1 protein levels from two independent experiments were normalized to beta actin and expressed as mean arbitrary densitometric units.

RESULTS

KA Induces Degeneration of RGCs and Their Axons in Thy1-YFP Mice

To determine the effect of hyperstimulation of non-NMDA receptors on RGCs and their axons in the retina, KA was injected into the vitreous humor of Thy1-YFP mice, and fundus imaging was performed at 24, 48, and 72 hours after injection ($n = 3$ mice for each treatment; three independent experiments). Results presented in Figure 1 show that KA initiates

degeneration of RGCs and their axons in the retina as early as 24 hours, when compared with PBS-injected eyes. Fundus imaging results presented in Figure 2 show increased degeneration of RGCs and fragmented appearance of axons in the retinas of KA-injected eyes after 48 hours, when compared with the retinas of PBS-injected eyes. Results presented in Figure 3 shows a further increase in degeneration of RGCs and a classic Wallerian-like degeneration of axons in the retinas of KA-injected eyes after 72 hours, when compared with PBS-injected eyes. Quantitative results indicated a significant increase in the degeneration of axons in the retinas of KA-injected eyes, but not in the retinas of PBS-injected eyes (Fig. 4).

KA Induces Degeneration of Axons in the Optic Nerves of Thy1-YFP Mice

To determine the effect of excitotoxicity on degeneration of axons in the optic nerves, KA was injected into the right eyes,

FIGURE 6. Western blot analysis of SARM1 protein in retinal protein extracts of Thy1-YFP mice. Aliquots containing an equal amount of protein (50 μ g) extracted from KA- or PBS-injected eyes were separated in 10% polyacrylamide gels, transferred to PVDF membranes, and probed with antibodies against SARM1 and beta actin. A representative Western blot from two independent experiments indicates that SARM1 protein levels were increased in KA-injected eyes when compared with PBS-injected eyes (A). Quantitative analysis (normalized to actin levels) indicate that SARM1 protein levels were increased significantly in KA-injected eyes (*, #, % $P < 0.05$), when compared with PBS-injected eyes (B).

and PBS was injected into the left eyes of Thy1-YFP mice. At 24, 48, and 72 hours after KA or PBS injection ($n = 3$ mice for each treatment; three independent experiments), optic nerves were separated by cutting them approximately 1 mm behind the globe, mounted with a coverslip, and observed under a fluorescence microscope (Zeiss Imager Z.2; Carl Zeiss Microscopy). Results presented in Figure 5A show fragmented appearance of axons as early as 24 hours in the optic nerves from KA-injected eyes. At 48 and 72 hours after KA injection a further Wallerian-like degeneration of axons was observed in the optic nerves from KA-injected eyes, but not in the optic nerves of PBS-injected eyes (Fig. 5A). Quantitative results indicate a significant increase in the degeneration of axons in the optic nerves of KA-injected eyes, but not in the retinas of PBS-injected eyes (Fig. 5B).

KA Upregulates the Expression of SARM1 Protein in the Retinas of Thy1-YFP Mice

To determine the effect of excitotoxicity on SARM1 protein expression, KA or PBS was injected into the vitreous humor of Thy1-YFP mice, and retinal proteins were extracted at 24, 48, and 72 hours after injection ($n = 3$ mice for each treatment; three independent experiments). Results presented in Figure 6A show that low levels of SARM1 protein were present in retinal proteins extracted from PBS-injected eyes. In contrast, SARM1 protein levels were increased at 24, 48, and 72 hours in retinal proteins extracted from KA-injected eyes. Densitometric analysis of SARM1 protein expression, when normalized to beta-actin levels, indicated a significant increase in SARM1 protein levels in KA-treated eyes when compared with PBS-treated eyes (Fig. 6B). Tissue localization experiments indicated that SARM1 protein levels were also increased in RGCs, but not in other cell types in retinal cross sections (data not shown) prepared from KA-injected eyes when compared with PBS-injected eyes (Fig. 7).

To determine the expression of SARM1 protein in the optic nerves, optic nerves separated by cutting them approximately 1 mm behind the globe from KA- or PBS-injected eyes were immunostained with antibodies against SARM1 protein ($n = 3$

mice for each treatment; three independent experiments). Immunoreactivity of SARM1 protein and expression of YFP in the optic nerves was assessed by collecting a sequence of 12 to 14 optical sections, by merging them into one composite slice, by using the Zeiss extended focus software module (Carl Zeiss Microscopy). Results presented in Figure 8 indicate that low levels of SARM1 protein is expressed in the optic nerves isolated from PBS-injected eyes. In contrast, results presented in Figure 9 indicate a time-dependent increase in SARM1

FIGURE 7. Immunolocalization of SARM1 protein in retinal cross sections of Thy1-YFP mice. Retinal cross sections prepared from KA- or PBS-injected eyes were immunostained with antibodies against SARM1 protein. Representative immunohistochemistry results from three independent experiments indicate that SARM1 protein expression was increased in RGCs in retinal cross sections prepared from KA-injected eyes (arrowheads), when compared with low levels of SARM1 protein in PBS-injected eyes (arrows). Images were obtained at $\times 40$ magnification.

FIGURE 8. Expression of SARM1 protein in the optic nerves of Thy1-YFP mice. At 24, 48, and 72 hours after PBS injection into the vitreous humor, optic nerves were isolated from enucleated eyes, and immunostained with antibodies against SARM1. Immunoreactivity of SARM1 protein and expression of yellow-fluorescent protein in the optic nerves was assessed by collecting a sequence of 12 to 14 optical sections by using a Zeiss microscope and merged into one composite slice by using a Zeiss extended focus software module. Results presented indicate a low level of SARM1 immunostaining in the optic nerves isolated from PBS-injected eyes at all the time points indicated in the figure. Images were obtained at $\times 10$ magnification.

protein levels in the optic nerves isolated from KA-injected eyes.

Silencing siRNA Against *Sarm1* Attenuates Wallerian-Like Degeneration of RGCs and Their Axons in the Retinas of Thy1-YFP Mice

Since increased SARM1 protein levels were correlated with degeneration of RGCs and their axons, KA (20 nM) or PBS was injected into the vitreous humor of Thy1-YFP mice along with 100 pM of control and *Sarm1* silencing siRNA. Our preliminary studies indicated 100 pM of silencing siRNA was optimum to observe a detectable protective effect against KA-induced retinal degeneration (data not shown). Therefore, we have chosen to inject 100 pM of control siRNA and *Sarm1* silencing siRNA along with KA (20 nM) or PBS into the vitreous humor of Thy1-YFP mice, and axonal degeneration in the retina and optic nerve was assessed at 72 hours after treatment ($n = 3$ mice; two independent experiments). Results presented in Figure 10A indicate that KA induces degeneration of RGCs and their axons by 72 hours (top right panel, arrow) when compared with PBS-treated eyes (Fig. 10C, top two panels). In contrast, KA-induced Wallerian-like degeneration of RGCs and their axons in the retina (Fig. 10A, center right panel; arrow) was attenuated in eyes treated with *Sarm1* silencing siRNA (Fig. 10A, bottom right panel), but not with control siRNA (Fig. 10A, center right panel). Although degeneration of RGCs and their axons was attenuated to a large extent, degeneration of a few RGCs and their axons was still apparent in the retinas of KA and *Sarm1* silencing siRNA-treated eyes at 72 hours (Fig. 10A, bottom right panel, arrowheads) after treatment. Quantitative analysis indicated a significant degeneration of axons in the retinas from KA-treated eyes (Fig. 10B). Degeneration of RGCs and their axons was not observed in the retinas from PBS, PBS+negative control siRNA, and PBS+*Sarm1* silencing RNA-treated eyes (Figs. 10C, 10D).

FIGURE 9. Expression of SARM1 protein in the optic nerves of Thy1-YFP mice. At 24, 48, and 72 hours after KA injection into the vitreous humor, optic nerves were isolated from enucleated eyes, and immunostained with antibodies against SARM1 protein. Immunoreactivity of SARM1 protein and expression of yellow-fluorescent protein in the optic nerves was assessed by collecting a sequence of 12 to 14 optical sections by using a Zeiss microscope and merged into one composite slice by using a Zeiss extended focus software module. Results presented indicate a time-dependent increase in SARM1 immunostaining in the optic nerves of KA-treated eyes, when compared with a low level of immunostaining in PBS-treated eyes.

Silencing siRNA Against *Sarm1* Attenuates Wallerian-Like Degeneration of Axons in the Optic Nerves of Thy1-YFP Mice

To determine the effect of control and silencing *Sarm1* siRNA on Wallerian-like degeneration of axons in the optic nerves, Thy1-YFP mice eyes were injected with KA or PBS along with 100 pM control and *Sarm1* silencing siRNA, and axonal degeneration in the optic nerves was assessed at 72 hours after treatment ($n = 3$ mice for each treatment; two independent experiments). Results presented in Figure 11A indicate that KA induces Wallerian-like degeneration of axons by 72 hours (bottom left panel; arrows) when compared with PBS alone treated eyes (Fig. 11C, bottom left panel). In contrast, KA-induced Wallerian-like degeneration of axons was attenuated in the optic nerves isolated from the eyes treated with KA and *Sarm1* silencing siRNA (Fig. 11A, bottom right panel). Although degeneration of axons was attenuated to a large extent, degeneration of a few axons in the optic nerves was still observed in the retinas of KA and *Sarm1* silencing siRNA-treated eyes at 72 hours after treatment (Fig. 11A, bottom right panel, arrowheads). Quantitative analysis of axons indicated a significant degeneration of axons in KA or KA+negative control siRNA-treated eyes (Fig. 11B), when compared with PBS-treated eyes at 72 hours after treatment (Fig. 11D). In contrast, when eyes were treated with KA and *Sarm1* silencing siRNA, degeneration of axons was significantly reduced at 72 hours after treatment when compared with eyes treated with KA (Fig. 11B).

Sarm1 Silencing siRNA Decreases KA-Mediated SARM1 Protein Expression in the Retina

Finally, to determine the effect of *Sarm1* silencing RNA on SARM1 protein expression, aliquots containing equal amount of total retinal proteins extracted from PBS, PBS+negative control

FIGURE 10. *Sarm1* silencing siRNA attenuates degeneration of RGCs and their axons in the retina. To determine the effect of silencing siRNA against *Sarm1* on degeneration of RGCs and their axons KA or PBS was injected into the vitreous humor of Thy1-YFP mice along with 100 pM of control and *Sarm1* silencing siRNA. Seventy-two hours after treatment, degeneration of RGCs and axons in the retinas was assessed by fundus imaging. Results presented indicate that KA induces degeneration of RGCs and their axons by 72 hours (top right panel, arrow). KA-induced degeneration of RGCs and their axons (A, center right panel, arrow) was attenuated in eyes treated with *Sarm1* silencing siRNA (A, bottom right panel), but not with control siRNA (A, center right panel). Degeneration of a few RGCs and their axons was still observed in the retinas of KA and *Sarm1* silencing siRNA-treated eyes (A, bottom right panel, arrowheads) after treatment. Quantitative analysis indicated a significant degeneration of axons in the retinas from KA-treated eyes (B); * $P < 0.05$ when compared with the axons at 0 hour; **when compared with the axons from KA-treated eyes; %when compared with the axons in KA-treated eyes). No degeneration of RGCs and their axons was observed in PBS, PBS+negative control siRNA, and PBS+*Sarm1* silencing RNA-treated eyes (C, D). NS, not significant.

siRNA, PBS+*Sarm1* silencing RNA, KA, KA+negative control siRNA, and KA+*Sarm1* silencing RNA were subjected to Western blot analysis. The results presented in Figure 12A indicate that SARM1 protein levels were upregulated in KA (* $P < 0.05$) or KA+negative control siRNA-treated retinas, when compared with PBS-treated eyes. In contrast, SARM1 protein levels were downregulated significantly in KA+*Sarm1* silencing RNA-treated retinas (Fig. 12B).

DISCUSSION

Although excitotoxicity has been implicated in the degeneration of RGCs and their axons, the mechanisms underlying excitotoxic retinal damage were still unclear. In this study, we have provided evidence that KA-mediated excitotoxicity upregulated the expression of SARM1 protein in the retinas of Thy1-YFP mice and induced Wallerian-like degeneration of

FIGURE 11. Silencing siRNA against *Sarm1* attenuates degeneration of axons in the optic nerves. To determine the effect of control and *Sarm1* silencing siRNA on Wallerian-like degeneration of axons in the optic nerves, Thy1-YFP mice eyes were injected with KA or PBS along with 100 pM of control and *Sarm1* silencing siRNA and axonal degeneration in the optic nerves was assessed at 72 hours after treatment ($n = 3$ mice for each treatment; two independent experiments). (A) Results presented indicate that KA induces degeneration of axons by 72 hours (bottom left panel; arrows) when compared with PBS-treated eyes (C, bottom left panel). In contrast, KA-induced Wallerian-like degeneration of axons was attenuated in the optic nerves of mice treated with KA and *Sarm1* silencing siRNA (A, bottom right panel). Degeneration of a few axons in the optic nerves was still observed in the retinas of KA and *Sarm1* silencing siRNA-treated eyes at 72 hours after treatment (A, bottom right panel, arrowheads). Quantitative analysis indicated a significant degeneration of axons in the optic nerves isolated from KA (* $P < 0.05$) or KA+negative control siRNA-treated eyes (B; ** $P < 0.05$), when compared with PBS-treated eyes (D; NS, not significant). In contrast, when eyes were treated with KA and *Sarm1* silencing siRNA, degeneration of axons in the optic nerves was significantly reduced (B; % $P < 0.05$).

FIGURE 12. *Sarm1* silencing siRNA decreases KA-induced expression of SARM1 protein in the retina. To determine the effect of *Sarm1* silencing RNA on SARM1 protein expression, aliquots containing equal amount of total retinal proteins extracted from PBS, PBS+negative control siRNA, PBS+*Sarm1* silencing RNA, KA, KA+negative control siRNA, and KA+*Sarm1* silencing RNA were subjected to Western blot analysis. (A) The results presented indicate that SARM1 protein levels were upregulated in the retinas from KA (* $P < 0.05$) or KA+negative control siRNA-treated eyes (** $P < 0.05$), when compared with PBS-treated eyes. (B) In contrast, SARM1 protein levels were downregulated significantly in KA+*Sarm1* silencing RNA-treated retinas (% $P < 0.05$).

RGCs and their axons in a time-dependent fashion. The findings presented in this study are significant for two reasons.

First, although a previous study showed, by evaluating optic nerves through transmission electron microscopy analysis, that NMDA-mediated excitotoxicity promotes Wallerian-like degeneration,¹⁸ to our knowledge, no other studies have investigated the role of SARM1 protein in Wallerian-like degeneration of axons in the retina and optic nerve after KA-induced excitotoxicity. Although axonal damage is delayed in *Wld^S* mutant mice, the mechanisms underlying Wallerian-degeneration are still unclear. Yet, some evidence suggested that axonal protection observed in *Wld^S* mice may be the result of chromosomal rearrangement that causes overexpression of *Wld^S*.³¹ Other studies on both fly and mouse have suggested that delayed axonal degeneration may be due to a mutation in Dual Leucine Zipper-bearing Kinase (DLK)/Wilson disease (*Wnd*)³² and suppression of ubiquitin pathway.³³ Yet, the mechanisms underlying delayed axonal degeneration in *Wld^S* mice are unclear because *Wld^S* is not expressed in normal mice. Second, we have employed a mouse line B6.Cg-Tg(Thy1-YFPH)2Jrs/J, in which a subset of RGCs express YFP and investigated the role of excitotoxicity in Wallerian-like degeneration of RGCs and their axons by performing fundus imaging on live animals, which was not attempted prior to this study. A majority of the studies to date have investigated axonal damage by axotomy or mechanical injuries, where the axons might be directly damaged at the lesion site. Therefore, in this study, we have induced damage to the RGCs and their axons by intravitreal injection of KA and investigated the effect of KA on Wallerian-like axonal damage by performing fundus imaging on live animals before and after KA injection. Our results indicate that Wallerian-like axonal damage is initiated first in the retina and then in the optic nerve. In addition, our results indicate that SARM1 protein plays a central role because silencer siRNA against *Sarm1* downregulated SARM1 protein expression and attenuated Wallerian-like degeneration of RGCs and their axons. Our fundus imaging results show that there

appear to be some axons healthy while RGC soma is shrinking. We found that this is not the case. Since the images were taken with a newly developed camera for fundus imaging of rodents, and since we cannot focus perfectly on each and every RGC and its axon, we tried to photograph as many RGCs as possible. This is one of the major reasons some somas of RGCs appeared to be shrinking.

Although the results presented in this study showed that SARM1 protein expression correlates with Wallerian-like degeneration of axons in Thy1-YFP mice, the underlying signaling mechanisms are still unclear. Some previous studies have suggested that *Sarm1* may delay axonal degeneration through Toll/interleukin-1 receptor 1 (Tir-1) because Tir-1 (a homolog of *Sarm1* in *Caenorhabditis elegans*) functions through activating Ca^{+2} -CaM kinase signaling pathway.^{34,35} Pharmacological inhibition of SARM1 may offer therapeutic potential in diseases like Parkinson's, multiple sclerosis, and glaucoma in which axonal damage is a major problem. However, no studies have investigated the role of SARM1 protein in disease models such as glaucoma in *Sarm1* knockout mice. Future studies in this direction may provide valuable information regarding the mechanisms underlying SARM1-mediated Wallerian-like degeneration of RGCs and their axons.

In conclusion, results presented in this study, for the first time, indicate that KA-mediated excitotoxicity upregulates SARM1 protein in the retina and promotes Wallerian-like degeneration of RGCs and their axons.

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