Spermidine Ameliorates Neurodegeneration in a Mouse Model of Normal Tension Glaucoma

Takahiko Noro,1,2 Kazuhiko Namekata,1 Yuriko Azuchi,1 Atsuko Kimura,1 Xiaoli Guo,1 Chikako Harada,1 Tadashi Nakano,2 Hiroshi Tsuneoka,2 and Takayuki Harada1

1Visual Research Project, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan
2Department of Ophthalmology, The Jikei University School of Medicine, Tokyo, Japan

Correspondence: Takayuki Harada, Visual Research Project, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan; harada-tk@igakuen.or.jp.
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PURPOSE. To assess the therapeutic potential of spermidine in mice with excitatory amino acid carrier 1 (EAAC1) deletion (EAAC1 knockout [KO] mice), a mouse model of normal tension glaucoma.

METHODS. Spermidine, at 30 mM in drinking water, was administered to EAAC1 KO mice from 5 to 12 weeks old. Optical coherence tomography, multifocal electroretinograms, and the measurement of intraocular pressure (IOP) were performed at 5, 8, and 12 weeks old. Histopathology analyses were carried out at 8 and 12 weeks old, and immunoblot and immunohistochemical analyses of 4-hydroxy-2-nonenal (4-HNE) in the retina were performed at 8 weeks old.

RESULTS. Spermidine ameliorated retinal degeneration and improved visual function in EAAC1 KO mice at both 8 and 12 weeks old, without affecting IOP. A significant increase of 4-HNE was observed in vehicle-treated EAAC1 KO mice, but spermidine treatment reduced this increase, suggesting that spermidine alleviated the severity of the glaucoma-like phenotype by acting as an antioxidant.

CONCLUSIONS. The results from this study suggest that oral spermidine administration could be a useful treatment for retinal degenerative disorders including glaucoma.

Keywords: spermidine, oxidative stress, neuroprotection, glaucoma, animal model
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Spermidine alleviated the severities of retinal degeneration in EAAC1 KO mice, at least partly by acting as an antioxidant.

MATERIALS AND METHODS

Mice

Experiments were performed using EAAC1 KO mice (Milenyi Biotec GmbH, Bergisch Gladbach, Germany). On a C57BL6 background in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drug Administration

Spermidine (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to drinking water at 30 mM for the treatment groups from 5 to 12 weeks old. The control group received normal drinking water. Drinking water was replaced every 2 to 3 days, and spermidine was freshly added from 1 M aqueous stock (spermidine/HCl, pH 7.4), which was kept at −20°C for no longer than 1 month.

Histological and Morphometric Studies

Mice were perfused with Zamboni’s Fixative (2% paraformaldehyde and 15% picric acid in 0.1 M phosphate buffer) at 8 and 12 weeks old. Eyes were enucleated and postfixed in 3% glutaraldehyde solution (5% glutaraldehyde, 9% formaldehyde, 37.5% ethanol, and 12.5% acetic acid in distilled water) for 2 hours. Paraffin embedded retinal sections of 7-μm thickness were cut through the optic nerve and stained with hematoxylin and eosin (H&E). A microscopic image of each section within 0.5 to 1 mm from the optic disc was scanned. The extent of retinal degeneration was quantified in two ways. 27 First, the number of neurons in the ganglion cell layer (GCL) was counted from one ora serrata through the optic nerve to the other ora serrata. Second, in the same sections, the thickness of the inner retinal layer (IRL; between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer) was analyzed.

Imaging Acquisition of Spectral-Domain OCT (SD-OCT)

Spectral-domain OCT (RS-3000, Nidek, Aichi, Japan) examinations were performed at 5, 8, and 12 weeks old. For fundus imaging, polymethyl methacrylate contact lenses optimal for mice (UNICON, Osaka, Japan) were placed on the corneas. Use of the contact lenses prevents anesthesia-induced cataract formation. A 60-D adaptor lens was placed on the objective lens of the Multiline OCT to focus on the mouse retina. For imaging of the IRLs on SD-OCT using the speckle noise-reduction method, line scans and a circular scan around the optic disc were performed. All the line scan images were location matched, scanning vertically through the center of the optic nerve head at three disc diameter lengths above the optic nerve head. All the circular scan images were obtained by scanning a circle centering around the optic nerve disc. The average thickness of the ganglion cell complex (GCC, between the internal limiting membrane and the interface of the inner plexiform layer and the inner nuclear layer) was measured. In this study, the maximum number of B-scans set by the manufacturer (50 for line scans and 10 for circular scans) was used for averaging.

Multifocal Electroretinogram

Mice were anesthetized by intraperitoneal injection of 87.5 mg/kg sodium pentobarbital. The pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. miERGs were recorded using a VERIS 6.0 system (Electro-Diagnostic Imaging, Redwood City, CA, USA). The visual stimulus consisted of seven hexagonal areas scaled with eccentricity. The stimulus array was displayed on a high-resolution black and white monitor driven at a frame rate of 100 Hz. The second-order kernel (2K), which is impaired in patients with glaucoma, was analyzed as previously reported. 28–30

IOP Measurement

Intraocular pressure was measured by a commercial rebound tonometer (TonoLab; Colonial Medical Supply, Franconia, NH, USA) in anesthetized mice as reported previously. 12–14 To minimize variation, the data were collected during a time window of 4 to 6 minutes after injection of the anesthetic, during which IOP plateaus. Intraocular pressure was measured at 5, 8, and 12 weeks old. Since the 24-hour IOP pattern in mouse eyes is biphasic, with IOP being highest at around 9 PM, 28 we examined IOP between 8 PM and 11 PM.

Immunohistochemistry

Mice were perfused with Zamboni’s Fixative at 8 weeks old. Eyes were enucleated, postfixed in Zamboni’s Fixative for 1 hour and then transferred into a sucrose buffer (30% sucrose in a 0.1-M phosphate buffer) for cryoprotection. Retinal cryostat sections of 10-μm thickness were prepared and examined by immunostaining using a 4-hydroxy-2-nonenal (4-HNE) mouse monoclonal antibody (0.2 μg/mL; Japan Institute for the Control of Aging, Shizuoka, Japan). The intensity of 4-HNE at the GCL was analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Immunoblot Analyses

Immunoblotting was performed as previously reported. 25 Membranes were incubated with an antibody against 4-HNE (0.1 μg/mL; Japan Institute for the Control of Aging). The density of 4-HNE was analyzed using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Statistics

For statistical comparison of two samples, we used a two-tailed Student’s t-test. Data are presented as means ± SEM. P < 0.05 was regarded as statistically significant.

RESULTS

Spermidine Suppresses Retinal Degeneration in EAAC1 KO Mice

The retinas of EAAC1 KO mice show normal organization at 5 weeks old and retinal degeneration starts thereafter. 6,13,14 The cell number in the GCL and the thickness of the IRL were significantly decreased in EAAC1 KO mice compared with wild type (WT) mice at 8 and 12 weeks old (Figs. 1B–D). To investigate whether spermidine is capable of preventing the NTG-like phenotypes in EAAC1 KO mice, spermidine (30 mM) was given in their drinking water from 5 to 12 weeks old (Fig. 1A). Histopathological analysis revealed that the number of cells in the GCL in spermidine-treated mice was significantly higher than that in vehicle-treated (control) mice (Figs. 1B, 1C). In addition, spermidine treatment prevented the thinning of the IRL (Figs. 1B, 1D).

We also visualized retinal layers in living mice using SD-OCT, a noninvasive imaging technique that can be used to acquire cross-sectional tomographic images of the retina in vivo. 15,14,25–26 The average thickness of the GCC, which includes the nerve fiber layer, GCL, and inner plexiform layer, was decreased in control eyes, but it was unchanged in spermidine-treated eyes (Fig. 2A). For quantitative analysis,
GCC was measured by scanning the retina in a circle centering around the optic nerve disc (Fig. 2B), and the average GCC thickness was determined from acquired images (Fig. 2C). The GCC thickness at 8 and 12 weeks old was significantly reduced in control mice, but it was unchanged in spermidine-treated mice (Fig. 2D), indicating that spermidine suppresses the retinal degeneration in EAAC1 KO mice.

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In order to determine if the histological observation of spermidine-mediated neuroprotection in EAAC1 KO mice reflects functional aspects, we examined visual function using mfERG. We analyzed the 2K component, which appears to be a
sensitive indicator of inner retinal dysfunction and is impaired in glaucoma patients. The response topography demonstrating the 2K component revealed that the average visual responses were impaired in all visual fields in EAAC1 KO mice, but spermidine treatment ameliorated the deterioration in visual function (Figs. 3A, 3B). These results verify that the neuroprotective effects of spermidine on glaucomatous retinal degeneration in EAAC1 KO mice are functionally significant.

We next examined the effects of spermidine on IOP. The previous study showed that IOP in EAAC1 KO mice is similar to that in WT mice. The intraocular pressure values of spermidine-treated EAAC1 KO mice were not significantly altered compared to those of control mice (Fig. 4). These results suggest that spermidine prevents NTG-like pathology in EAAC1 KO mice and this neuroprotective effect is IOP-independent.

Spermidine Reduces the Oxidative Stress Level in the EAAC1 KO Mouse Retina

We next investigated potential mechanisms underlying spermidine-mediated neuroprotection. One of the major causes that is associated with glaucomatous retinal degeneration in EAAC1 KO mice is increased oxidative stress levels. Therefore, we examined if spermidine treatment suppresses induction of oxidative stress in EAAC1 KO mice. For this purpose, we utilized 4-HNE, which provides a reliable measure of oxidative stress. 4-HNE was mainly observed in the GCL of EAAC1 KO mice, but it was hardly detected in WT mice or spermidine-treated EAAC1 KO mice (Fig. 5A). Quantitative analyses confirmed that the oxidative stress level in the GCL is significantly suppressed with spermidine treatment in EAAC1 KO mice (Fig. 5B).

We also carried out immunoblot analyses of 4-HNE in the retinas of WT, EAAC1 KO mice, and spermidine-treated EAAC1 KO mice at 8 weeks old. A significant increase of 4-HNE intensity was observed in EAAC1 KO mice, but spermidine treatment suppressed this increase (Figs. 5C, 5D). These results suggest that spermidine prevents retinal degeneration in EAAC1 KO mice by suppressing the induction of oxidative stress in the retina.

DISCUSSION

In this study, we show that oral administration of spermidine prevents glaucomatous retinal degeneration in EAAC1 KO mice. Spermidine suppressed cell loss in the GCL and thinning of the IRL without affecting IOP. To demonstrate these findings in the same animal over a period of time, we utilized OCT and substantiated these histological observations with physiological significance by employing mfERG, an effective noninvasive method to measure visual function in living animals. It may seem that the thinning of the GCC thickness plateaus at 8
weeks old (Fig. 2D), while IRL thinning was still progressive between 8 and 12 weeks old in EAAC1 KO mice (Figs. 1C, 1D). This difference may be due to the different resolution between OCT and pathological analyses. However, both methods successfully demonstrated the quick inner retinal degeneration and reliably showed the neuroprotective effect of spermidine in NTG model mice.

Oxidative stress is an important risk factor in human glaucoma,7,9,10 and suppression of oxidative stress in RGCs is a potential treatment strategy for glaucoma.31,32 In this study, we showed that spermidine reduces 4-HNE, which represent oxidative stress levels, in the GCL of EAAC1 KO mice. Consistently, we previously showed that spermidine exerts neuroprotective effects by reducing oxidative stress levels in the RGCs of an animal model of ONI.25 In addition, other studies have shown anti-oxidant properties of spermidine in the brain.33,34 These findings support our conclusion that one of the spermidine-mediated neuroprotective mechanisms in the EAAC1 KO mouse retina is suppression of oxidative stress.

We previously reported that spermidine suppresses ONI-induced activation of the apoptosis signal-regulating kinase 1 (ASK1) and protects RGCs, indicating spermidine’s therapeutic potential in traumatic optic neuropathy.25 ASK1 is an evolutionarily conserved mitogen-activated protein kinase.
ASK1 is activated in response to stress stimuli and induces neural cell death in a mouse model of ischemic injury and optic neuritis. Interestingly, glaucoma-like phenotypes in GLAST KO mice were partially suppressed in GLAST/ASK1 double KO mice. These findings suggest that another mechanism associated with spermidine-mediated neuroprotection may be suppression of the ASK1 activity in EAAC1 KO mice. We are planning to examine the phenotypes of EAAC1/ASK1 double KO mice in future.

Spermidine and spermine are naturally occurring polyamines and are almost exclusively accumulated in glial cells but not in neurons in the brain and in the retina. Therefore, specifically in the retina, spermidine may be stored in Müller glial cells in many species including man. Thus, it is speculated that endogenous spermidine accumulated in retinal glia may be an initial resource for RGC survival following insult and if such resources are depleted due to various conditions including aging, disease, and trauma, then exogenous spermidine may be particularly useful for protection of RGCs. As glia–glia and glia–neuron interactions play important roles in neuroprotection, it would be interesting to investigate if spermidine mediates its neuroprotective effects also through regulation of various systems within Müller glia.

Interestingly, spermidine is known to act on the neural N-methyl-D-aspartate receptor (NMDAR). Excessive activation of the NMDAR has been implicated in the pathogenesis of glaucoma. Previously, it was reported that polyamines potentiate NMDA-triggered excitotoxicity. However, we show that spermidine ameliorates retinal degeneration in the GCL. This discrepancy may be explained by various factors concerning experimental procedures including the route of polyamine administration, the nature of the molecules used (spermidine, putrescine, or spermine) and the dose. Indeed, spermidine acts on the NMDAR in a biphasic concentration-dependent manner: At submillimolar doses it blocks the receptors, but at submicromolar levels it activates the receptors. Although we did not examine the spermidine concentration in the retina in this study, it is possible that spermidine inhibited the NMDAR activity. We have previously shown that the NMDAR antagonist memantine suppresses glaucomatous retinal degeneration in GLAST KO mice. These findings suggest that effects of spermidine in EAAC1 KO mice may include reduced NMDAR activity. Further studies will be required to confirm this and determine the dose for the best outcome from spermidine treatment.

We recently showed that dedicator of cytokinesis 3 (Dock3), a member of atypical guanine exchange factors, protects RGCs from oxidative stress and glutamate neurotoxicity. In addition, overexpression of Dock3 suppressed retinal degeneration in GLAST KO mice. Interestingly, Dock3 signaling is enhanced by brain-derived neurotrophic factor (BDNF), which induces neuroprotection, axonal outgrowth and neurogenesis. A recent study has shown that a novel phosphine-borane complex promotes RGC protection through the induction of BDNF and activation of the extracellular signal-regulated kinases (ERK) 1/2.

![Figure 5](https://image-url.com)

**Figure 5.** Spermidine reduces oxidative stress levels in the EAAC1 KO mouse retina. (A) Representative images of 4-HNE in the retina at 8 weeks old. Scale bar: 100 μm. (B) Quantitative analyses of (A). Data are normalized to the 4-HNE intensity at the GCL in control WT mice (100%). n = 6 in each group. (C) Representative images of immunoblot analyses of 4-HNE in the retina at 8 weeks old. (D) Quantitative analyses of (C). Data are normalized to the 4-HNE intensity in control WT mice (1.0). n = 6 in each group. **P < 0.01, *P < 0.05.
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...is also activated by valproic acid and this pathway seems to play important roles in valproic acid-induced neuroprotection in GLAST KO mice. 15 We have also reported that the orally active antagonist of angiotensin II type 1 receptor (AT1-R) suppressed Toll-like receptor 4 and lipopolysaccharide-induced inducible nitric oxide synthase expressions in EAAC1 KO mouse retina. 16 Valproic acid and AT1-R antagonists are widely prescribed drugs for the treatment of epilepsy and high blood pressure, respectively. 13, 15, 49 These findings raise intriguing possibilities for the management of glaucoma by utilizing spermidine, in combination with Dock3 overexpression and existing drugs for neuroprotection, as well as conventional treatments to lower IOP. 13–15, 31

In conclusion, we report that spermidine exerts neuroprotective effects on retinal degeneration in a mouse model of NTG. Spermidine is a natural component of our diet, and several foods are known to contain high levels of spermidine, including soy beans, tea leaves, and mushrooms. 24, 25 Thus, beneficial effects of spermidine can be easily attained from daily food 26 and polyamines including spermidine may be good therapeutic candidates for retinal degeneration, such as glaucoma and traumatic optic neuropathy.

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References


