

Altered Transport Velocity of Axonal Mitochondria in Retinal Ganglion Cells After Laser-Induced Axonal Injury In Vitro

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PURPOSE. Axonal damage precedes retinal ganglion cell (RGC) apoptosis in glaucomatous optic neuropathy. Because mitochondria in RGC axons are damaged before cell death, we examined axonal mitochondrial transport dynamics after axonal injury.

METHODS. Mitochondria in rat-cultivated RGCs were stained with rhodamine 123. After axonal injury induced using a laser microdissection system, axonal transport was evaluated by time-lapse imaging. The RGC apoptosis was detected using ethidium homodimer-1 on day 3 after axonal injury.

RESULTS. The rate of stationary mitochondria in RGC axons significantly increased from 37.6% before axonal injury to 70.6% at 5 minutes ($P < 0.001$) and 63.6% at 18 hours ($P < 0.001$) after axonal injury. The mean axonal mitochondrial transport velocity in RGCs transiently deteriorated from $0.48 \pm 0.01 \mu\text{m/s}$ before axonal injury to $0.37 \pm 0.02 \mu\text{m/s}$ at 5 minutes after axonal injury ($P < 0.001$). However, 23.5% of RGCs showed recovered axonal transport velocity at 18 hours after injury. On day 3 after axonal injury, RGCs with the recovery of axonal transport did not undergo apoptosis, whereas 69.2% of RGCs without the recovery of axonal transport underwent apoptosis ($P = 0.029$).

CONCLUSIONS. Axonal injury disrupts mitochondrial transport in RGC axons. Irreversible decreased axonal mitochondrial transport velocity may be useful to predict RGC apoptosis after axonal injury.

Keywords: apoptosis, axonal injury, axonal transport, mitochondria, retinal ganglion cell

Optic nerve injury (eg, due to axotomy or intraocular pressure [IOP] elevation) causes retinal ganglion cell (RGC) apoptosis.¹⁻³ Animal optic nerve histology using radioactive tracers has shown that IOP-dependent injury is associated with disrupted axonal transport.⁴ Our previous in vitro time-lapse imaging study found that disrupted axonal transport of brain-derived neurotrophic factor causes RGC apoptosis.⁵ Thus, axonal transport plays a critical role in RGC survival, and its failure may be associated with glaucomatous optic neuropathy.⁶

Mitochondria are an essential energy source for cellular activity.⁷ Indeed, mitochondrial dysfunction causes various ocular diseases such as Leber's hereditary optic neuropathy, dominant optic atrophy, and normal tension glaucoma.⁸ Furthermore, Radius and Anderson⁹ reported that elevated IOP in primate eyes interrupts axonal mitochondrial transport. Three-dimensional reconstructions of mitochondrial images at glial lamina of DBA/2J obtained by an electron microscope revealed that alteration of mitochondrial dynamics induced by elevated IOP is involved in glaucoma.¹⁰ Moreover, disrupted axonal mitochondrial transport is critical for some diseases.¹¹ Neurons have unique morphology and are highly polarized cells with extended axons and dendrites; therefore, disruption of mitochondrial transport leads to inadequate mitochondrial distribution within the axon and an inability to meet local ATP

demands and/or toxic exchanges in calcium buffering.¹² Recently, we have shown disturbed mitochondrial transport in the RGC axon of experimental glaucoma in vivo.¹³ Consequently, mitochondrial transport in RGC axons assessed by live imaging may be a biomarker for prediction of RGC death after axonal damage. However, to date, the relationship between RGC axonal damage and change in mitochondrial transport in a single RGC is still not well known. In the present study, we investigated the dynamics of axonal mitochondrial transport in cultivated RGCs after laser-induced axonal injury.

METHODS

RGC Culture

All experimental procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the guidelines were approved by the Committee on the Use and Care of Animals from the University of Fukui. As previously described,¹⁴ RGCs from 3-day-old Sprague-Dawley rats were purified by a two-step immunopanning procedure using anti-macrophage antibodies (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) and anti-Thy 1.1 antibodies derived from T11D7E2 cells (American Type Culture Collection, Manassas, VA, USA). This method is reported to yield RGCs with

>99.5% purity in the literature¹⁴ and 94.5% purity in our laboratory. RGCs were plated at a low density (2000 cells/cm²) on 35-mm grid-lined dishes (ibidi, Martinsried, Germany) coated with poly-D-lysine (Sigma-Aldrich Corp., St. Louis, MO, USA) and laminin (Thermo Fisher Scientific, Inc., Waltham, MA, USA). As previously described,⁵ RGCs were cultured in serum-free medium (Neurobasal; Thermo Fisher Scientific, Inc.) without phenol red but with brain-derived neurotrophic factor (50 ng/mL; Pepro-Tech, Rocky Hill, NJ, USA), ciliary neurotrophic factor (50 ng/mL; PeproTech), basic fibroblast growth factor (50 ng/mL; Pepro-Tech), forskolin (10 μ M; Sigma-Aldrich Corp.), B27 supplement (Thermo Fisher Scientific, Inc.), glutamine (Sigma-Aldrich Corp.), insulin (Sigma-Aldrich Corp.), sodium pyruvate (Thermo Fisher Scientific, Inc.), Sato Supplement, triiodothyronine (Sigma-Aldrich Corp.), and *N*-acetyl-cysteine (Sigma-Aldrich Corp.). To prevent bacterial contamination, penicillin/streptomycin (Thermo Fisher Scientific, Inc.) was added to the medium. To support RGC culture, one-half of the medium was replaced at day 4 after seeding.

Time-Lapse Imaging

Mitochondria were stained using 1 μ M rhodamine 123 (Thermo Fisher Scientific, Inc.) at 37°C in a CO₂ incubator for 15 minutes. Mitochondrial dynamics were recorded using a confocal microscope (FV-10i; Olympus, Tokyo, Japan) before and after axonal injury (5 minutes and 18 hours after injury). Sequential images were acquired using a 60 \times objective from the inverted microscope, at 3-second intervals for 3 minutes in a chamber maintained at 37°C and 5% CO₂. Mitochondrial dynamics were quantified using the kymograph macro program (EMBL, Heidelberg, Germany)¹⁵ and image analysis software (ImageJ software v1.49, <http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). An axonal distance of 150 μ m from the cell body was used for further analysis. Velocity was calculated from the kymograph; the length of horizontal axis was divided by the length of vertical axis.

Classification of Mitochondrial Transport

Depending on the movement of mitochondrial vesicles within the axon during the 3-minute recordings, vesicles were classified into four groups, as previously described⁵: vesicles that moved in an anterograde direction for >10 μ m (anterograde), vesicles that moved in a retrograde direction for >10 μ m (retrograde), vesicles that did not move (stationary), and vesicles that fluttered \leq 10 μ m (fluttering).

Laser-Induced Axonal Injury

To study changes in mitochondrial dynamics induced by axonal injury, a laser microdissection system was used (AS LMD; Leica, Wetzlar, Germany). On day 6 after seeding, an RGC and its axon (which crossed the imprinted 500- μ m relocation grid line on the culture dish) were selected. Laser power was set as down below in "Laser Control" section of integrated software; power was 99, speed was 20, and specimen balance was 0. Under this setting, laser power and duration theoretically calculated were 0.46 mW and 4 ns/pulse, respectively. Laser shots traced the relocation grid with the range of approximately 150 μ m repeatedly to induce axonal injury.

Comparison of Apoptosis in Recovered and Unrecovered Mitochondrial Transport Groups After Axonal Injury

RGCs were categorized into the recovered group if the mean mitochondrial transport velocity in the axon at 18 hours after

injury recovered to the mean velocity at 5 minutes after injury plus 2 SEs or faster. RGCs were categorized into the unrecovered group if the velocity was less than the mean velocity at 5 minutes after injury plus 2 SEs. Three days after axonal injury, cell viability was evaluated 20 minutes after application of ethidium homodimer-1 (1 μ M) from a live/dead cell assay kit (Thermo Fisher Scientific, Inc.). Fluorescent RGCs were deemed to be apoptotic cells. Seventeen RGCs were examined to compare apoptosis between the recovered and unrecovered groups.

Statistical Analysis

Statistical analyses were performed using the χ^2 test to compare numbers of stationary mitochondrial vesicles before and after axonal injury, the Tukey-Kramer test to compare velocity at each time point, and Fisher's exact test to compare the number of ethidium homodimer-1 positive cells between the recovered and nonrecovered groups at 3 days after axonal injury. *P* values less than 0.05 were considered statistically significant.

RESULTS

Dynamics of Axonal Mitochondrial Transport in Purified RGCs

After rhodamine 123 administration, fluorescein-positive mitochondria were distributed in cell bodies, dendrites, and axons (Fig. 1A). Long static bright rhodamine 123-positive regions were observed in axons. Between these rhodamine 123-positive axonal regions, rhodamine 123-positive vesicles moved in anterograde and retrograde directions. In total, we counted 834 rhodamine 123-positive vesicles in 17 axons. During 3 minutes of time-lapse imaging, 23.4% of vesicles moved in an anterograde direction, 21.0% moved in a retrograde direction, 37.6% were stationary, and 18.0% were fluttering (Fig. 1B; Supplementary Movie S1).

Distribution of mitochondrial transport velocity in RGC axons is shown (Fig. 1C). The velocity of mitochondria moving within axons was 0.48 ± 0.01 μ m/s (mean \pm SE). Anterograde and retrograde movement velocities were 0.46 ± 0.02 and 0.50 ± 0.02 μ m/s, respectively. Retrograde movement was significantly faster than anterograde movement (*P* = 0.04).

Mitochondrial Transport in RGC Axons After Injury

Laser shots successfully injured RGC axons (Figs. 2A, 2B). At 5 minutes after axonal injury, a total of 642 mitochondrial vesicles were observed in the 17 axons. Of these mitochondria, 12.3% moved in an anterograde direction, 7.6% moved in a retrograde direction, 70.6% were stationary, and 9.5% were fluttering. Compared with before laser injury, the rate of stationary mitochondria significantly increased at 5 minutes after laser injury (*P* < 0.001, χ^2 test for stationary versus anterograde + retrograde + fluttering). At 18 hours after axonal injury, 544 vesicles were observed in the 17 axons, with 11.0% moving in an anterograde direction, 15.6% in a retrograde direction, 63.6% stationary, and 9.8% fluttering. The rate of stationary mitochondria at 18 hours after axonal injury was significantly higher than before axonal injury (*P* < 0.001) but significantly lower than at 5 minutes after axonal injury (*P* = 0.010; Fig. 2C). Mean mitochondrial transport velocities in the anterograde and retrograde groups decreased to 0.37 ± 0.02 μ m/s (mean \pm

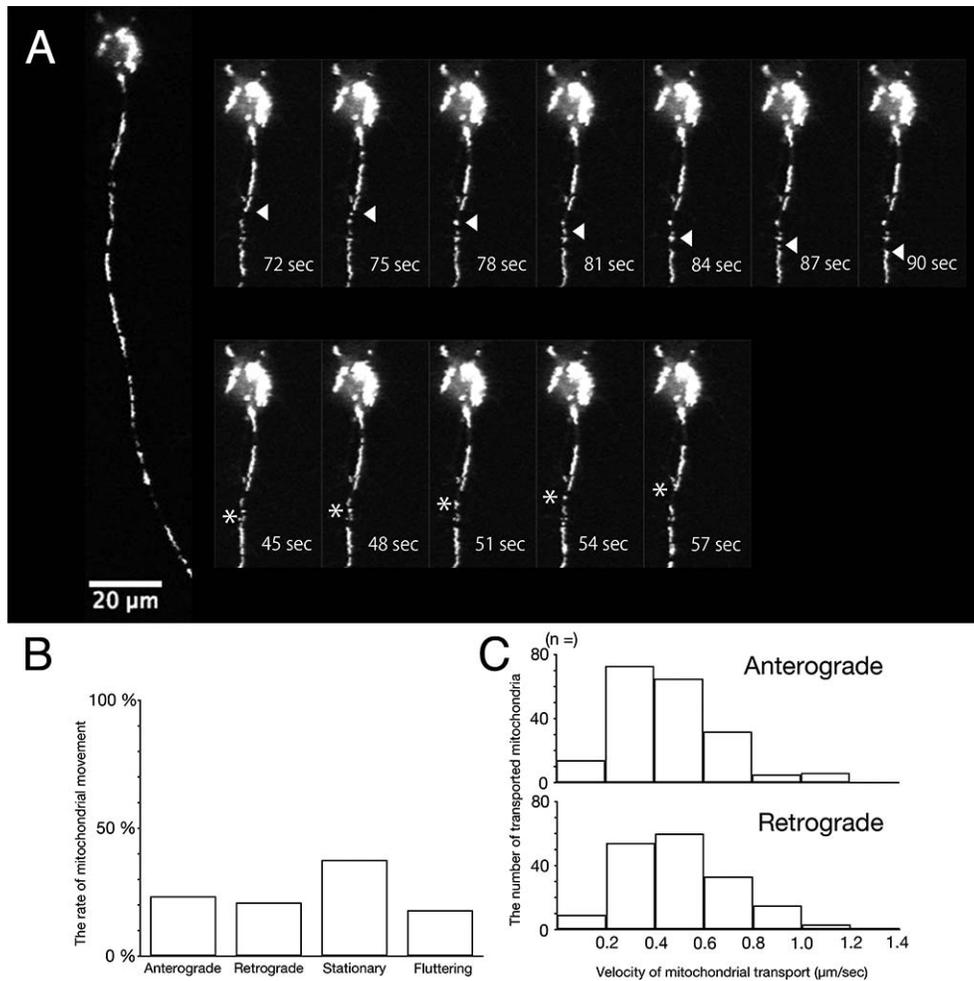


FIGURE 1. Time-lapse imaging for visualized mitochondrial movement. **(A)** Mitochondrial movement was detected by time-lapse imaging. The *arrowhead* shows anterograde movement and the *asterisk* shows retrograde movement. **(B, C)** Axonal mitochondrial transport characteristics in retinal ganglion cells before laser treatment. **(B)** The percentage of mitochondria moving in each direction, stationary, and fluttering. $n = 834$, from 17 axons. **(C)** Histograms showing mitochondrial movement velocities in the anterior and retrograde directions. Axonal mitochondrial transport was faster in the retrograde group than in the anterograde group ($P = 0.04$). $n = 195$ for anterograde movement, and $n = 175$ for retrograde movement, each from 17 axons. The results are from 17 independent experiments.

SE) at 5 minutes after axonal injury ($P < 0.001$, compared with before laser treatment) but partially recovered to $0.46 \pm 0.02 \mu\text{m/s}$ (mean \pm SE) at 18 hours after axonal injury ($P = 0.002$, compared with 5 minutes after laser-induced axonal injury; Fig. 2D).

Comparison of Apoptosis in the Recovered and Unrecovered Mitochondrial Transport Groups After Axonal Injury

As described in the Methods, RGCs were classified into two groups according to the mean velocity of axonal mitochondrial transport at 18 hours after laser-induced axonal injury (Fig. 2E). The recovered group included four RGCs (23.5%; Fig. 2F), whereas the unrecovered group included 13 RGCs (76.5%; Fig. 2G). In the recovered group, no cells were stained with ethidium homodimer-1, whereas 9 of the 13 RGCs in the unrecovered group were stained with ethidium homodimer-1 on day 3 after laser injury (Figs. 3A, 3B). The unrecovered group had a significantly greater rate of ethidium homodimer-1-positive RGCs than the recovered group ($P = 0.029$).

DISCUSSION

In the present study, we used live imaging to quantify axonal mitochondrial transport dynamics in living RGCs. We demonstrated the increase in the rate of stationary mitochondria and the decrease in mitochondrial velocity by laser-induced axonal injury. In 23.5% of RGCs, disrupted axonal mitochondrial transport velocity due to laser-induced injury recovered by 18 hours after axonal injury. No RGCs in the recovered group underwent apoptosis, whereas 69.2% of RGCs in the non-recovered group underwent apoptosis. Therefore, adequate axonal mitochondrial transport is critical for RGC survival.

A previous study demonstrated axonal transport dysfunction in animal glaucoma models *in vivo*⁴ but was performed by histologic analysis. Using time-lapse imaging, we previously found that colchicine-induced microtubule disruption causes RGC apoptosis *in vitro*.⁵ However, we did not exclude the possibility of microtubule damage in RGC dendrites and axons. Kunik et al.¹⁶ introduced the laser-assisted single-axon transection system and compared RGC morphology. The present study shows a relationship between axonal transport dynamics after single-axon injury and RGC apoptosis.

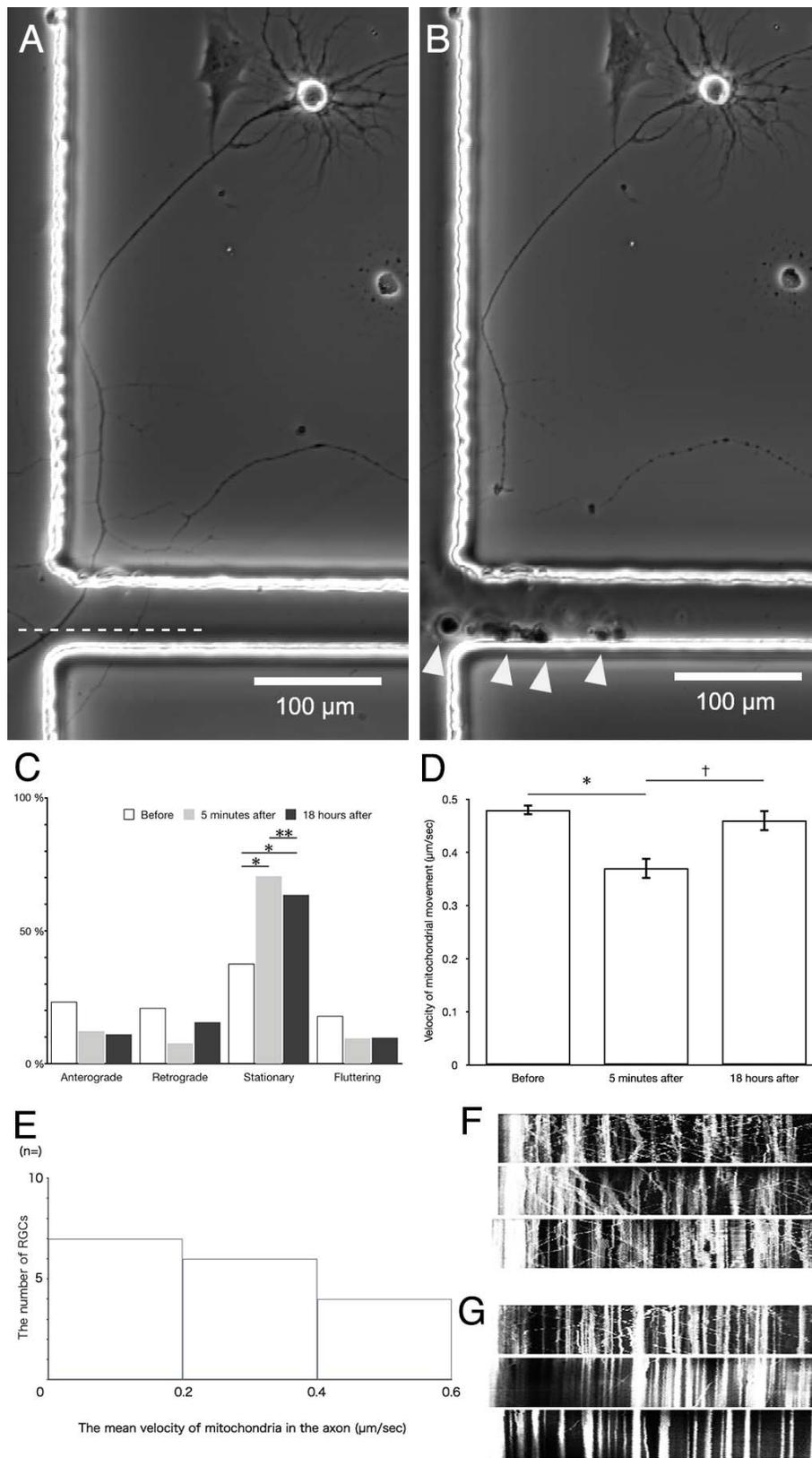


FIGURE 2. Axonal mitochondrial transport changes in retinal ganglion cells after axonal injury. Axon was injured by the laser microdissection system. The targeted axon was damaged along the imprinted grid line. The figure demonstrates the culture dish before (A) and after (B) laser treatment. (A) The dashed line indicates where the laser was introduced. (B) The arrowheads indicate the crack on the bottom of the culture dish made by the laser. (C) At each time point, mitochondrial vesicles were classified into four categories (anterograde, retrograde, stationary, and fluttering). $n = 834, 642,$ and 544 for before, 5 minutes, and 18 hours after laser treatment, respectively, from 17 axons. The rate of stationary mitochondria at 5 minutes after axonal injury was significantly higher than before axonal injury ($^*P < 0.001$). The rate of stationary mitochondria at

18 hours after axonal injury was significantly higher than before axonal injury ($*P < 0.001$) but significantly lower than at 5 minutes after axonal injury ($**P = 0.010$). (D) The mean axonal mitochondrial transport velocity in the anterograde and retrograde directions decreased at 5 minutes after axonal injury and partially recovered at 18 hours after axonal injury ($*P < 0.001$, $\dagger P = 0.002$). $n = 370$, 128, and 145 for before, 5 minutes, and 18 hours after laser treatment, respectively, from 17 axons. (E-G) Axonal mitochondrial transport in retinal ganglion cells at 18 hours after axonal injury. At 18 hours after axonal injury, mitochondrial movement recovered in some cells. (E) The distribution of mean mitochondrial transport in the each of 17 axons is shown in the histogram. A cutoff point of $0.41 \mu\text{m/s}$ was determined from the velocity mean plus 2 times SE at 5 minutes after axonal injury. (F) Change in mitochondrial movement in the recovered group is shown in the kymograph. (G) Change in mitochondrial movement in the nonrecovered group is shown in the kymograph. In (F) and (G), *top*, before; *middle*, 5 minutes after; and *bottom*, 18 hours after axonal injury. The results are from 17 independent experiments.

Axonal mitochondrial transport velocity can differ because of cell type or culture conditions. In embryonic hippocampal neurons, Ligon and Steward¹⁷ reported that the majority of “instantaneous” velocities were below $0.5 \mu\text{m/s}$ in both

directions. In N2A cells, De Vos et al.¹⁸ reported velocities of $0.61 \mu\text{m/s}$ in the anterograde direction and $0.67 \mu\text{m/s}$ in the retrograde direction. However, no statistical comparison was performed. In this study, we found faster retrograde transport

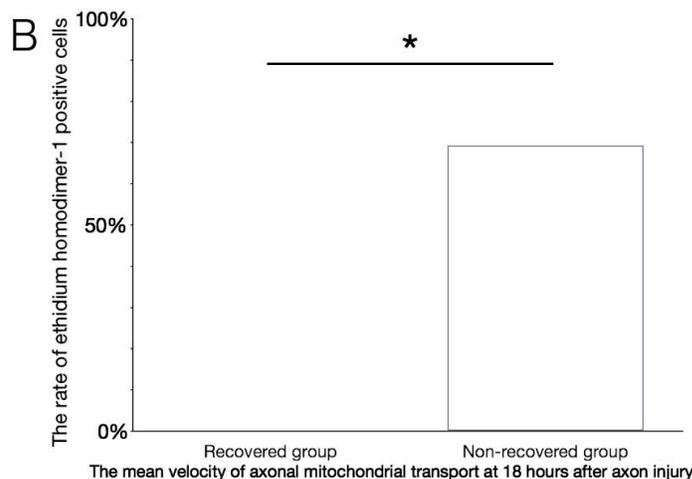
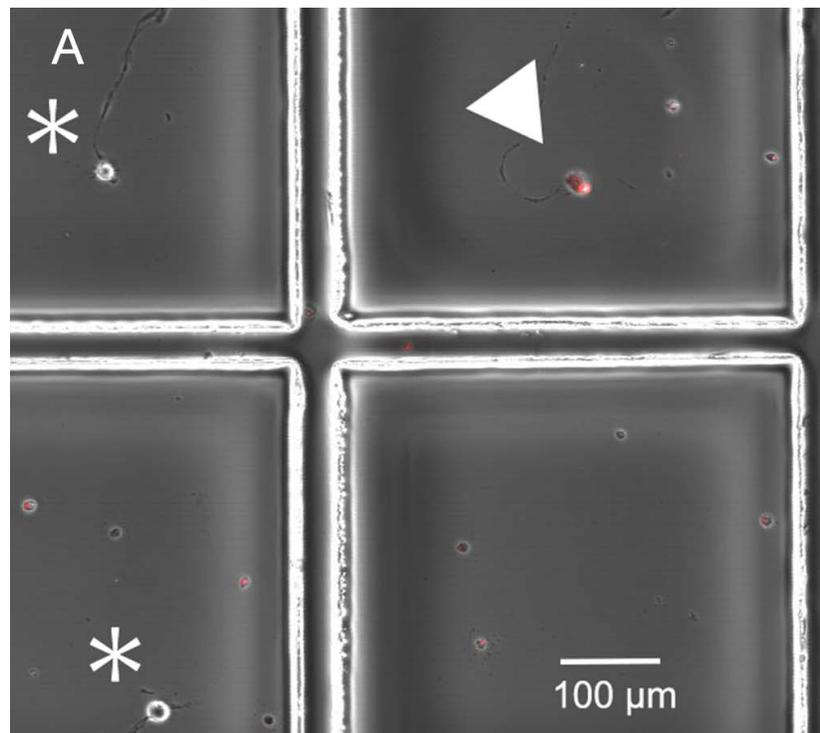


FIGURE 3. The viability of retinal ganglion cells. (A) For the only RGC indicated by the *arrowhead*, laser-induced axonal injury was conducted. This RGC was positive for ethidium homodimer-1 staining, indicating that apoptosis was induced by laser-induced axonal injury. By contrast, the other RGCs (*asterisks*) that extended axons without laser-induced injury were negative for ethidium homodimer-1. (B) The rate of ethidium homodimer-1-positive cells was significantly higher in the nonrecovered group (9 of 13) than the recovered group (0 of 4) ($*P = 0.029$). The results are from 17 independent experiments.

in RGC axons, being 0.46 $\mu\text{m/s}$ in the anterograde direction and 0.50 $\mu\text{m/s}$ in the retrograde direction. Mitochondria are transported by the motor proteins kinesin and dynein in axons.¹⁹ The velocity in this study is consistent with previously reported mammalian kinesin and dynein velocities, ranging from 0.5 to 1.0 $\mu\text{m/s}$.²⁰ The velocity difference between anterograde and retrograde transports may come from differential motor protein activity.

Axonal damage contributes to the pathophysiology of glaucoma.⁶ Furthermore, axonal damage induced by elevated IOP disrupts axonal transport, resulting in RGC apoptosis.²¹ Therefore, we used live-cell imaging to determine whether disrupted axonal mitochondrial transport could be detected before RGC death after single-axon damage. Before laser-induced axonal injury, the mean axonal mitochondrial transport velocity in RGCs was similar to that previously reported in hippocampal neurons in vitro.¹⁷ Although the laser system was different, as indicated by Kunik et al.,¹⁶ laser shots can injure RGC axons. In this report, we show that axonal mitochondrial transport velocity decreases immediately after laser-induced axonal injury. This is consistent with the previous report showing decreased axonal transport velocity after colchicine administration.⁵ The reason why axonal damage disrupts axonal transport has not been determined in this study. A possible mechanism is that laser-induced axonal injury results in calcium influx via calcium-specific ion transport mechanism.²² Elevated intracellular calcium arrests mitochondrial motility via the mitochondrial Rho GTPase.²⁰ Indeed, after intraretinal laser axotomy, mitochondrial transport was interrupted at the site of axon injury in vivo.²³ Mitochondrial Ca^{2+} content correlated inversely with the speed of mitochondrial movement.²⁴ In some RGCs, axonal mitochondrial transport velocity recovered by 18 hours after laser-induced axonal injury and the recovery was associated with future RGC survival. More ATP is required for repairing damaged axons. Active mitochondrial transport is essential for local energy demands. To survive after laser-induced axonal injury, mitochondria have to support axons to buffer calcium concentration and to meet energy demand for repair. In axons which can maintain normal calcium concentration after mitochondrial support, mitochondrial transport velocity would recover. However, in axons that cannot maintain normal calcium concentration, calcium-dependent enzymes including calpain would be strongly activated, resulting in ultimate RGC death. Moreover, excessive mitochondrial fission is suggested to be involved in glaucoma.²⁵ Kim et al.¹⁰ have shown accumulated dynamin-related protein 1 (DRP1) immunoreactivity in the cytosolic and perinuclear regions of RGCs in the glaucomatous DBA/2J mice. In mammals, DRP1 participates in the mitochondrial fission process. In RGCs, axonal injury may lead to an increase in DRP1 at the cell bodies, resulting in excessive mitochondrial fission. Live-cell imaging of axonal mitochondrial transport in RGCs may be useful for predicting cell fate after axonal damage.

A limitation of our study is that in vitro mitochondrial dynamics may be different from those in vivo. In vivo mitochondrial transport imaging in the optic nerve may be useful for predicting future RGC loss in glaucomatous optic neuropathy. Thus, dynamic axonal transport imaging may help determine whether further IOP reductions or other treatments are desirable in glaucoma patients. Further in vivo studies are required to determine the clinical application of dynamic axonal mitochondrial transport imaging for glaucomatous optic neuropathy.

In conclusion, time-lapse imaging has clarified mitochondrial transport dynamics in RGC axons. Axonal injury causes deterioration in mitochondrial transport velocity, and irrevers-

ible deterioration in velocity is associated with future RGC death.

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