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The role of calpain in an *in vivo* model of oxidative stress-induced retinal ganglion cell damage

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ABSTRACT

Purpose: In this study, we set out to establish an *in vivo* animal model of oxidative stress in the retinal ganglion cells (RGCs) and determine whether there is a link between oxidative stress in the RGCs and the activation of calpain, a major part of the apoptotic pathway.

Materials and methods: Oxidative stress was induced in the RGCs of C57BL/6 mice by the intravitreal administration of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH, 30 mM, 2 μl). Control eyes were injected with 2 μl of vehicle. Surviving Fluorogold (FG)-labeled RGCs were then counted in retinal flat mounts. Double staining with CellROX and Annexin V was performed to investigate the co-localization of free radical generation and apoptosis. An immunoblot assay was used both to indirectly evaluate calpain activation in the AAPH-treated eyes by confirming α-fodrin cleavage, and also to evaluate the effect of SNJ-1945 (a specific calpain inhibitor: 4% w/v, 100 mg/kg, intraperitoneal administration) in these eyes.

Results: Intravitreal administration of AAPH led to a significant decrease in FG-labeled RGCs 7 days after treatment (control: 3806.7 ± 575.2 RGCs/mm^2, AAPH: 3156.1 ± 371.2 RGCs/mm^2, $P < 0.01$). CellROX and Annexin V signals were co-localized in the FG-labeled RGCs 24 h after AAPH injection. An immunoblot assay revealed a cleaved α-fodrin band that increased significantly 24 h after AAPH administration. Intraperitoneally administered SNJ-1945 prevented the cleavage of α-fodrin and had a neuroprotective effect against AAPH-induced RGC death (AAPH: 3354.0 ± 226.9 RGCs/mm^2, AAPH+SNJ-1945: 3717.1 ± 614.6 RGCs/mm^2, $P < 0.01$).

Conclusion: AAPH administration was an effective model of oxidative stress in the RGCs, showing that oxidative stress directly activated the calpain pathway and induced RGC death. Furthermore, inhibition of the calpain pathway protected the RGCs after AAPH administration.

1. Introduction

Optic nerve fiber degeneration resulting in retinal ganglion cell (RGC) death, which occurs in diseases such as glaucoma, can threaten visual function and lead to blindness. Various studies have investigated possible treatments aimed at preventing this process, but the pathological mechanism of optic nerve degeneration and RGC loss is not yet well understood. Thus, viable neuroprotective strategies have not yet been found.

Many neuroprotective strategies have been considered so far. Among the most promising are those targeting oxidative stress, which is associated with aging. Oxidative stress has a role in the pathogenesis of various systemic diseases, and is known to induce neuronal cell death in neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease [1–3]. Oxidative stress has also been implicated in glaucoma, and it is necessary to better understand the underlying mechanism of oxidative stress-induced dysfunction in order to more effectively treat this disease [4,5]. Recently, a number of studies demonstrated that optic nerve crush (NC), a commonly used model of the axonal injury that occurs in ocular diseases such as glaucoma, could help elucidate the cytotoxic role of oxidative

Abbreviations: RGC, retinal ganglion cell; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; FG, Fluorogold; NC, optic nerve crush; Ca^{2+}, calcium ion; DPBS, Dulbecco's phosphate-buffered saline; PFA, paraformaldehyde; CMC, carboxymethylcellulose; ROS, reactive oxygen species; AIF, apoptosis-inducing factor.

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stress in the process of RGC death [6,7]. In a previous study, we showed that mice deficient in Nrf2, a key transcription factor regulating the expression of anti-oxidant genes, had significantly fewer surviving Fluorogold (FG)-labeled RGCs than wild-type mice 7 days after NC. This result suggested that the regulation of oxidative stress signaling was a potential neuroprotective treatment for diseases affecting the optic nerve fiber and the RGCs.

Our previous research showed that the activation of calpain played a key role in the process of RGC death after NC [7]. Calpain is a member of the cysteine protease family that is regulated by increased intracellular calcium ion (Ca^{2+}) levels and is raised locally through calcium channels. A large Ca^{2+} influx into the cytosol leads to calpain activation and cell death in various pathological conditions [8–10]. However, although our previous studies showed that NC caused oxidative stress and calpain activation in the retina *in vivo*, NC is a model of axonal injury generally, not oxidative stress specifically, and therefore cannot serve as a method of directly evaluating the efficacy of neuroprotective strategies against oxidative stress.

Therefore, to directly explore the effect of oxidative stress on calpain activation, we established a new *in vivo* mouse model of RGC degeneration caused by oxidative stress, using the intravitreal administration of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) [11]. In this report, we use this model to attempt to determine whether the calpain pathway has a causative role in oxidative damage-induced RGC death, and whether inhibiting oxidative stress-induced calpain activation has potential as a neuroprotective treatment for ocular diseases involving oxidative stress.

2. Materials and methods

This study used adult (10–12-week old) male C57BL/6 mice (SLC, Shizuoka, Japan). All animals were maintained and handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines from the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals. All experimental procedures described in the present study were approved by the Ethics Committee for Animal Experiments at Tohoku University Graduate School of Medicine.

2.1. Induction of oxidative stress in the retina

Intravitreal administration of AAPH was performed as previously described [12–14]. Briefly, each animal was anesthetized with pentobarbital (50 mg/kg). Two microliters of AAPH (30 mM) was injected intravitreally using a Hamilton syringe with a 32 G needle under surgical stereomicroscopy. The same volume of Dulbecco's phosphate-buffered saline (DPBS) was injected in the control group. If lens injuries or vitreous hemorrhaging were observed after the injection, the animals were excluded from the study.

2.2. Retrograde labeling of the RGCs and cell counting

Retrograde labeling was performed as described previously using a fluorescent tracer, FG (Fluorochrome, LLC, Denver, CO, USA), 7 days before the intravitreal injection of AAPH or DPBS [12]. Briefly, the mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (9 mg/kg) prepared at room temperature. Under full anesthesia, two small holes were drilled into the skull at sites corresponding to the superior colliculi, and 2 μl of 2% FG with 1% dimethylsulfoxide were injected using a Hamilton syringe with a 32 G needle. Seven days after the intravitreal injection of AAPH or DPBS, the retinas were harvested and fixed with 4% paraformaldehyde (PFA) for three hours. The retinas were then placed on glass slides with the ganglion cell layer facing up. Vectashield mounting medium (Vector Laboratories) and a cover glass were also applied. RGC survival was determined by counting FG-labeled RGCs in 12 distinct areas under the microscope [12]. Our investigation of the effect of calpain used the calpain inhibitor SNJ-1945 (Senju Pharmaceutical Co., Ltd). One day before administration of AAPH, the FG-labeled mice received an intraperitoneal administration of 4% w/v SNJ-1945 (100 mg/kg) in carboxymethylcellulose (CMC). The control group received CMC without SNJ-1945. Administration of SNJ-1945 continued daily until 7 days after the AAPH injection, when the retinas were harvested. Fixation of the retinas and RGC counting were then performed as described above.

2.3. Immunoblot assay

Immunoblotting was performed as previously described with minor modifications [13,14]. Isolated retinas were obtained surgically 3, 6 and 24 h after the intravitreal injection of AAPH or DPBS and placed in a lysis buffer (25 mM Tris–HCl; pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% protease inhibitor cocktail; Sigma–Aldrich, 1% phosphatase inhibitor cocktail 2; Sigma–Aldrich). Each sample was separated with SDS–PAGE and electroblotted to polyvinylidene fluoride membranes (Immobilon-P, Millipore). After blocking the membrane with 4% BlockAce, (Yukijirushi), they were incubated with primary mouse monoclonal antibodies against α-fodrin (1:750, Abcam), and β-actin (1:5000, Sigma–Aldrich) overnight at 4 °C. The membranes were then incubated with a horseradish peroxidase-conjugated goat immunoglobulin secondary antibody. Detection of the antigen–antibody complex was performed with the ECL Prime Western Blotting Detection System (GE Healthcare). Signals were measured in Image Lab statistical software (Bio-Rad, Hercules, CA, USA) and normalized to β-actin.

2.4. Detection of free radicals and RGC apoptosis

Staining with CellROX Green Reagent (Life Technologies) and an Annexin V-Cy3 detection kit (PromoKine) were used to evaluate the generation of oxidative stress and apoptosis. One week after FG labeling, 2 μl of AAPH 30 mM was injected in the mice intravitreally. Twenty-two hours later, 1 μl of 100 μM CellROX and 25×10^{-3} μg of Annexin V-Cy3 were injected intravitreally under general anesthesia (performed with ketamine and xylazine).

The retinas were harvested 24 h after the injection of AAPH, fixed with 4% PFA for 2 h and flat-mounted. The fluorescence of the reagents was evaluated with a confocal laser microscope (LSM780, Carl Zeiss, Oberkochen, Germany).

2.5. Detection of AAPH-induced calpain activation in the RGCs

To investigate the localization of calpain activation, we stained the retinas with a fluorogenic substrate [15]. To identify the RGCs, retrograde labeling was performed as described above, with DiI (CellTracker CM-DiI, Life Technologies). One week after retrograde labeling, the left eye of each mouse received an intravitreal injection of 2 μl of 30 mM AAPH, under anesthesia. As a control, the contralateral eye was injected with 2 μl DPBS. Twenty-one hours later, both eyes received intravitreal injections of fluorogenic cell-permeable calpain substrate ((DABCYL)-TPLK∼SPPPSPRE (EDANS)-RRRRRRR-NH_2 calpain substrate IV, Millipore). The retinas were harvested 24 h after AAPH injection, immediately fixed with 4% PFA for 2 h and flat mounted. The fluorescence of the fluorogenic substrate, representing the activation of calpain, was evaluated with an Axiovert 200 microscope (Carl Zeiss, Oberkochen, Germany).

2.6. Statistical analysis

Statistical analysis was performed using JMP pro 10.02 (SAS Institute Inc.) software for Windows. Continuous variables were expressed as mean values ± standard deviation. The *t*-test was used to analyze differences between pairs of groups. Multiple comparisons were done using ANOVA with Dunnett's post hoc analysis. The level of significance was 0.05 in all statistical tests.

3. Results

3.1. The effect of AAPH treatment on RGC survival

To investigate the effect of AAPH-induced oxidative stress on RGC survival, we performed the intravitreal injection of AAPH (30 mM, 2 μl) or DPBS (2 μl) in the mice. Seven days after the administration of AAPH, we counted the number of FG-labeled RGCs and found that they had significantly decreased (control: 3806.7 ± 575.2 RGCs/mm^2, AAPH: 3156.1 ± 371.2 RGCs/mm^2, $P < 0.01$, $n = 8$, Fig. 1). This finding indicated that the administration of AAPH could induce significant RGC death.

3.2. Detecting oxidative stress, calpain activation and apoptosis in the RGCs after AAPH treatment

In the control group, CellROX and Annexin V signals in the retina were not co-localized. However, in the AAPH-treated group, CellROX and Annexin V signals were co-localized with the FG-labeled RGCs 24 h after AAPH injection (Fig. 2A). This result indicated that oxidative stress occurred in the RGCs after AAPH treatment. Next, in order to confirm the activation of calpain in the RGCs, we used a fluorogenic calpain substrate to visualize calpain activation (Fig. 2B). The expression of the fluorogenic calpain substrate was co-localized with the DiI signal. This result indicated that calpain activation occurred in the RGCs 24 h after the administration of AAPH. In the control eyes, by contrast, we did not observe calpain activation in the RGCs.

3.3. Blocking calpain activation prevents RGC death caused by oxidative stress

To investigate the relationship between calpain activation and AAPH treatment, we performed a immunoblot analysis. This analysis showed that the band of cleaved α-fodrin, a substrate of calpain, was significantly detectible 24 h after AAPH administration, but not after 3 or 6 h (Fig. 3A). The intensity of the band increased significantly, by more than twofold. It is known that activated calpain can cleave α-fodrin to a size of 145 kDa. This result indicated that AAPH was able to induce RGC death through oxidative stress, and suggested that it could be related to calpain activation. We confirmed this result by treating the AAPH-injected eyes with SNJ-1945, a specific calpain inhibitor (Fig. 3B). SNJ-1945 prevented the cleavage of α-fodrin that occurred 24 h after AAPH injection (SNJ-1945 treatment: 0.6 ± 0.3-fold change vs. no treatment, $P < 0.05$, $n = 4$).

These results showed that AAPH-induced oxidative stress in the RGCs caused calpain activation and subsequent RGC death. In order to obtain further confirmation of these results, we performed the intraperitoneal administration of SNJ-1945, and found that it significantly reduced RGC death in our animal model (AAPH: 3369.8 ± 216.0 RGCs/mm^2, AAPH+SNJ-1945: 3817.8 ± 469.6 RGCs/mm^2, $P < 0.05$, Fig. 4).

4. Discussion

In this study, we used the intravitreal administration of AAPH, a free radical generator, to establish an animal model of the oxidative stress that occurs in the RGCs in neurodegenerative diseases of the eye. We found that oxidative stress in this model was associated with calpain activation in the retina and RGC death after 7 days. Moreover, AAPH administration successfully induced oxidative stress, confirmed by the finding that CellROX and Annexin V-Cy3 signals were co-localized in FG-labeled RGCs. AAPH administration also led to the activation of calpain in the retina after 24 h, confirmed by an immunoblot analysis. Moreover, the intraperitoneal administration of SNJ-1945 had a neuroprotective effect *in vivo*, preventing the cleavage of α-fodrin and RGC death after AAPH administration. These results indicate that oxidative stress-induced RGC death is mediated in part through the calpain pathway.

Previously, AAPH has been used to induce oxidative stress in non-RGC retinal cells, such as the photoreceptors, *in vitro* [16,17]. Furthermore, it has been reported that oxidative stress can induce apoptosis of the RGCs *in vitro* [18,19]. Our previous work has also demonstrated the importance of oxidative stress, by showing that mice deficient in Nrf2, a key transcription factor regulating antioxidant genes, had significantly fewer surviving FG-labeled RGCs after NC than wild-type mice [6], and that CDDO-Im, an extremely potent Nrf2 activator, prevented NC-induced RGC death *in vivo* [20]. Therefore, we expected that oxidative stress induced by AAPH would cause equivalently significant damage to the RGCs *in vivo*. Our results confirmed these expectations, showing that AAPH administration reduced the number of RGCs by 17% after 7 days.

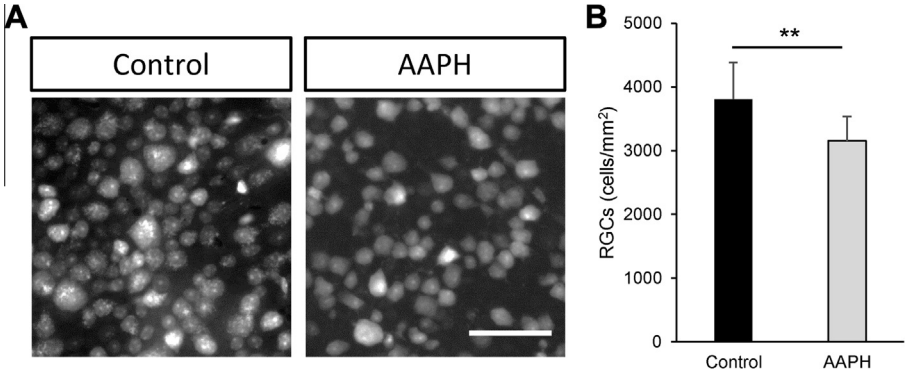

Fig. 1. Comparison of RGC density in the control and AAPH-treated groups. (A) Representative appearance of RGCs in flat-mounted retinas with quantitative data on RGC density 7 days after the intravitreal administration of AAPH. (B) Comparison of RGC density, determined by RGC counting, in the control and AAPH-treated groups. RGC density decreased significantly, by 17%, in the AAPH treatment group compared to the control group. RGC: retinal ganglion cell; scale bar: 50 μm, error bar: standard deviation; *t*-test, $^{**}P < 0.01$.

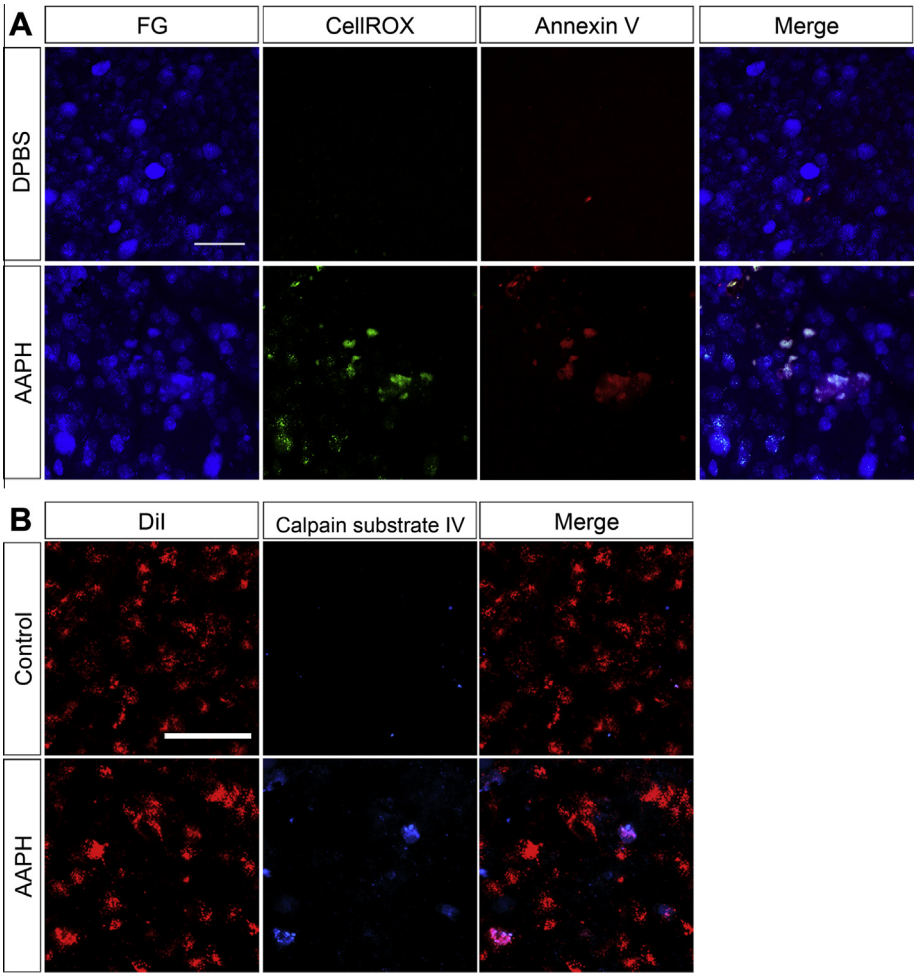

Fig. 2. Oxidative stress, calpain activation and apoptosis 24 h after AAPH administration. (A) Representative images of flat-mounted retinas showing CellROX and Annexin V signals, used to detect oxidative stress and apoptosis in the RGCs. CellROX and Annexin V signals were co-localized in the AAPH-treated retinas, while the DPBS-treated retinas showed no CellROX or Annexin V signals. (B) Representative appearance of DiI-labeled RGCs in flat-mounted retinas, with calpain substrate IV signals showing calpain activation. Twenty-four hours after AAPH administration, the control retinas showed no calpain substrate IV signals, while the AAPH-treated retinas showed co-localized calpain substrate IV signals and DiI-labeled RGCs. Scale bar: 50 μm.

Previous studies have reported that mitochondrial-derived death signaling is a major pathway of axonal damage-induced RGC death [21,22]. Mitochondria play an important role in the regulation of cell viability [23]. Oxidative stress induces mitochondrial-derived reactive oxygen species (ROS) generation and disturbs the function of calcium buffering [24–26]. To explore the mechanism of RGC death in the current investigation, we therefore assessed CellROX and Annexin V-positive RGCs in the AAPH-treated retinas, and obtained results suggesting that apoptosis of the RGCs was induced by ROS.

ROS induce the processing of mitochondrial apoptosis-inducing factor (AIF) and increase sensitivity to mitochondrial calpain, resulting in AIF cleavage and apoptosis [27]. Using immunoblotting, we investigated the AAPH-induced activation of calpain by testing for cleaved α-fodrin. Our results indicated that AAPH treatment induced fragmentation of α-fodrin as a result of calpain activation in the overall retina. Moreover, the co-localization of calpain substrate IV signals, which represent calpain activation, and DiI-labeled RGCs confirmed that calpain was activated in the RGCs. This suggests that the calpain pathway may be activated by an increased inflow of Ca^{2+} through Ca^{2+} channels under pathological conditions such as oxidative stress. Moreover, previous studies have shown that oxidative stress increases intracellular free Ca^{2+} levels, and activates Ca^{2+}-dependent enzymes [27–29]. These data suggest that oxidative stress and dysfunctional calcium buffering in the mitochondria may be major links with calpain activation in the retina, a finding that is consistent with our results.

Additionally, SNJ-1945 administration demonstrated a potent neuroprotective effect against AAPH-induced RGC death. Indeed, the number of RGCs after AAPH administration in the mice that received SNJ-1945 remained stable, in contrast with the mice that underwent the administration of AAPH and vehicle. SNJ-1945 reduced calpain activation and led to reduced cleaved α-fodrin intensity 24 h after AAPH administration. This result is consistent with past reports showing that oxidative stress caused calpain activation in RGCs that had been isolated *in vitro* [30] and that SNJ-1945 had a neuroprotective effect in the RGCs [7,31]. Here, using our *in vivo* model, we showed that the activation of calpain occurs downstream of oxidative stress, and that the inhibition of calpain activation was an effective way of preventing oxidative stress-induced RGC damage.

The animal model of oxidative stress described here may be appropriate for some purposes, such as investigating drug effects in cells that are sensitive to oxidative stress, but for other purposes, the intensity of AAPH-induced damage may be insufficient. Opportunities to refine this animal model therefore include the use of other ROS generators, such as rotenone [32] and paraquat, which

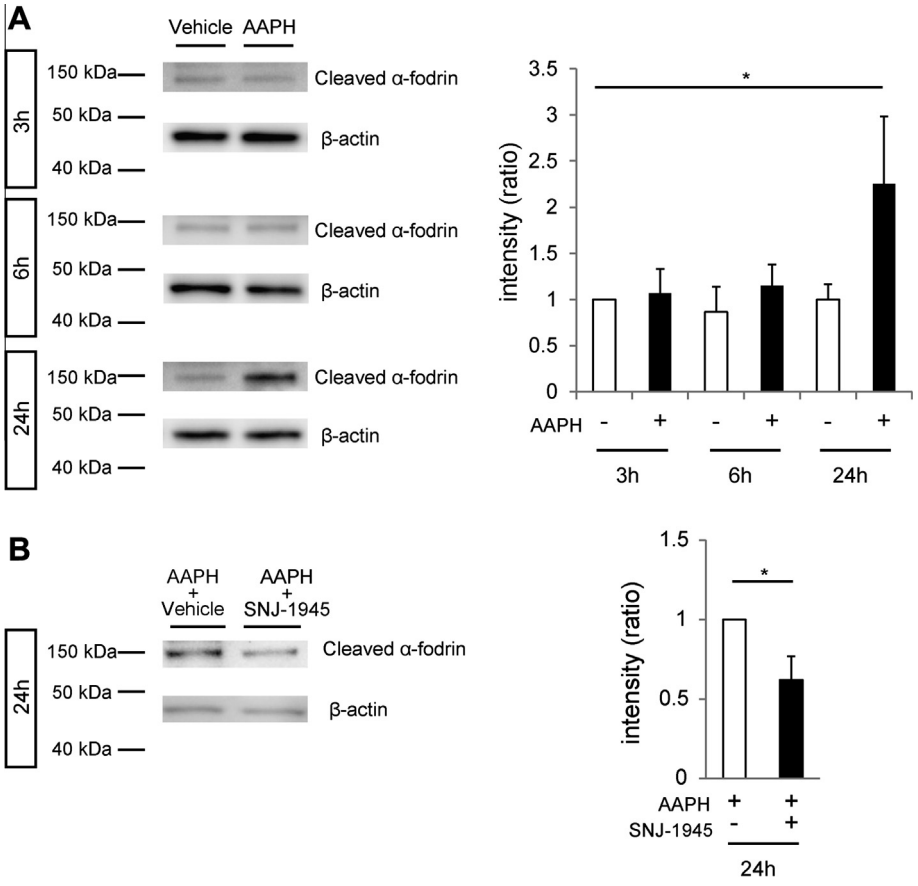

Fig. 3. Immunoblot analysis of calpain activation in mouse retinas after AAPH treatment. (A) Representative immunoblots and bar graphs showing the expression level of cleaved α-fodrin at each time point, compared with cleaved α-fodrin expression after 3 h in vehicle-treated retinas. β-Actin was used as an internal standard for the assessment of α-fodrin. The intensity of cleaved α-fodrin, i.e., of the 145-kDa fragments produced by activated calpain, was significantly higher 24 h after AAPH administration than after vehicle. Error bar: standard deviation ANOVA with Dunnett's post hoc analysis; $^{*}P < 0.05$. (B) Representative immunoblots showing calpain activation 24 h after AAPH administration, with or without SNJ-1945. The bar graphs show the expression level of cleaved α-fodrin in the SNJ-1945-treated and -untreated retinas. These results show that SNJ-1945 attenuated the intensity of α-fodrin cleavage. Error bar: standard deviation; *t*-test, $^{*}P < 0.05$.

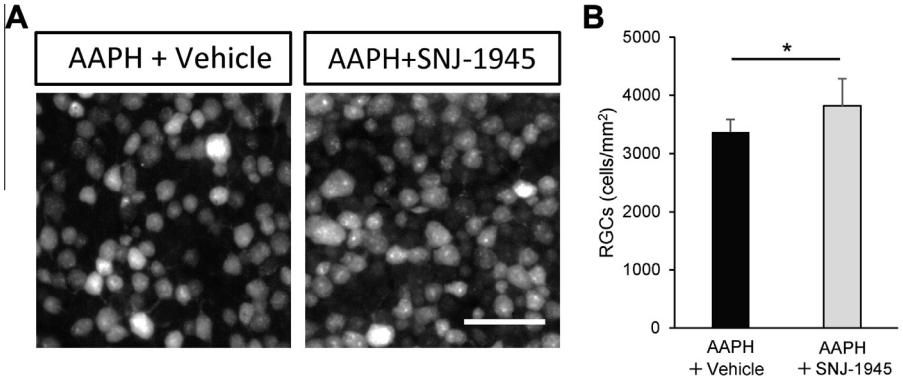

Fig. 4. Comparison of RGC density with and without SNJ-1945. (A) Representative appearance of RGCs in flat-mounted retinas 7 days after the intravitreal administration of AAPH. (B) Comparison of RGC density, determined by RGC counting, with and without SNJ-1945. RGC density was significantly higher with SNJ-1945, suggesting that SNJ-1945 protected the RGCs from AAPH-induced damage. Scale bar: 50 μm. Error bar: standard deviation; *t*-test, $^{*}P < 0.05$.

have a cytotoxic effect in retinal cells and dopaminergic cells [33,34].

In conclusion, we successfully established an *in vivo* model of oxidative stress in the RGCs with AAPH, and used this model to demonstrate that the calpain pathway is activated downstream of oxidative stress. Furthermore, suppressing calpain activation reduced RGC death in our model, suggesting that it may be a good candidate for neuroprotection therapy. Our findings may also be useful in future investigations of oxidative stress-related ocular diseases, such as glaucoma and diabetic axonal atrophy.

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