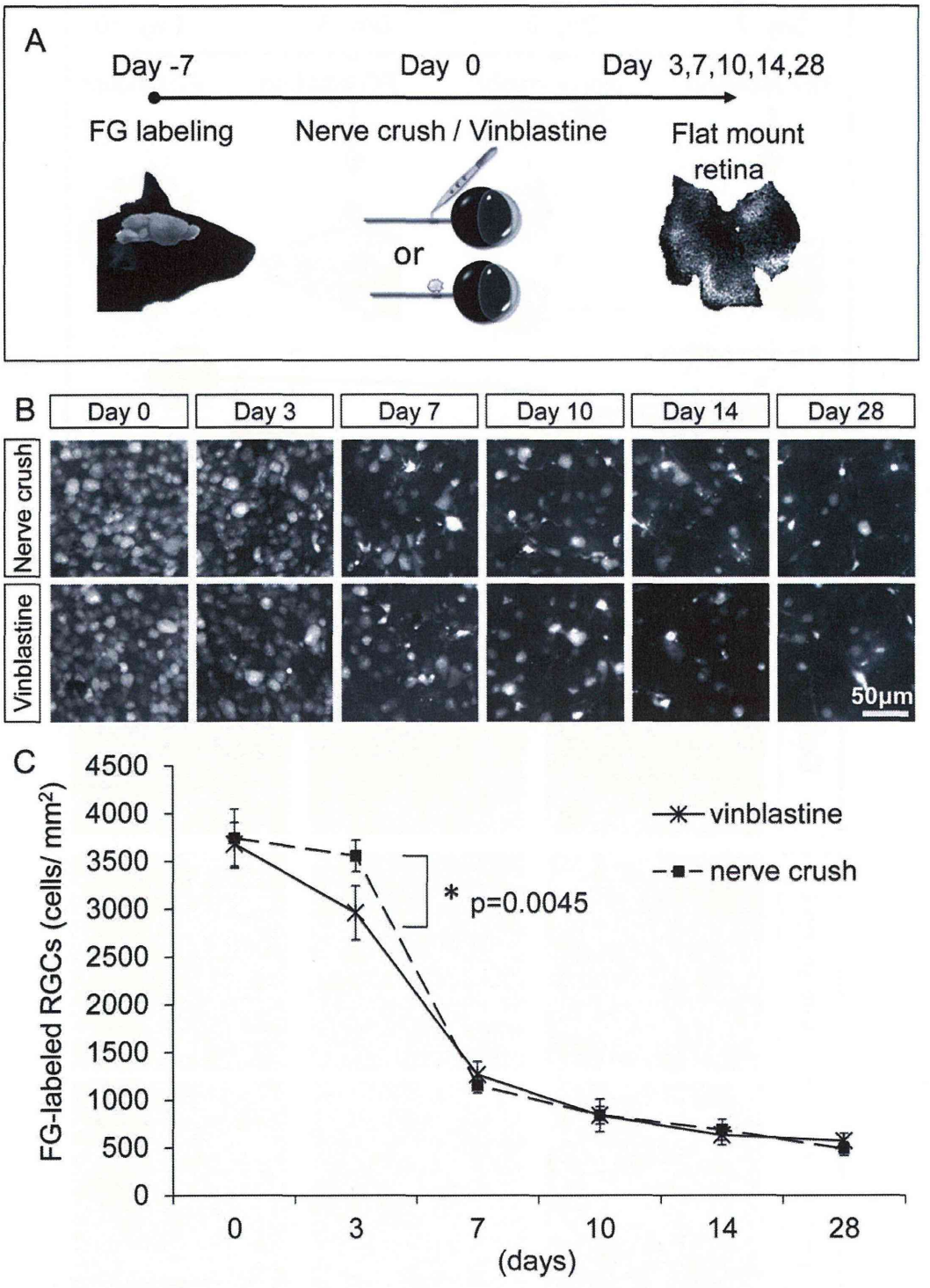

Fig. 3. Time course of RGC death induced by axonal damage. **A:** Schematic diagram of axonal injury-induced RGC death. **B:** Representative appearances of FG-labeled RGCs on the flat-mounted retina at various time points (0, 3, 7, 10, 14, and 28 days) after nerve crush (upper panels) or vinblastine treatment (lower panels). **C:** Quantitative data on FG-labeled RGC following axonal injury.

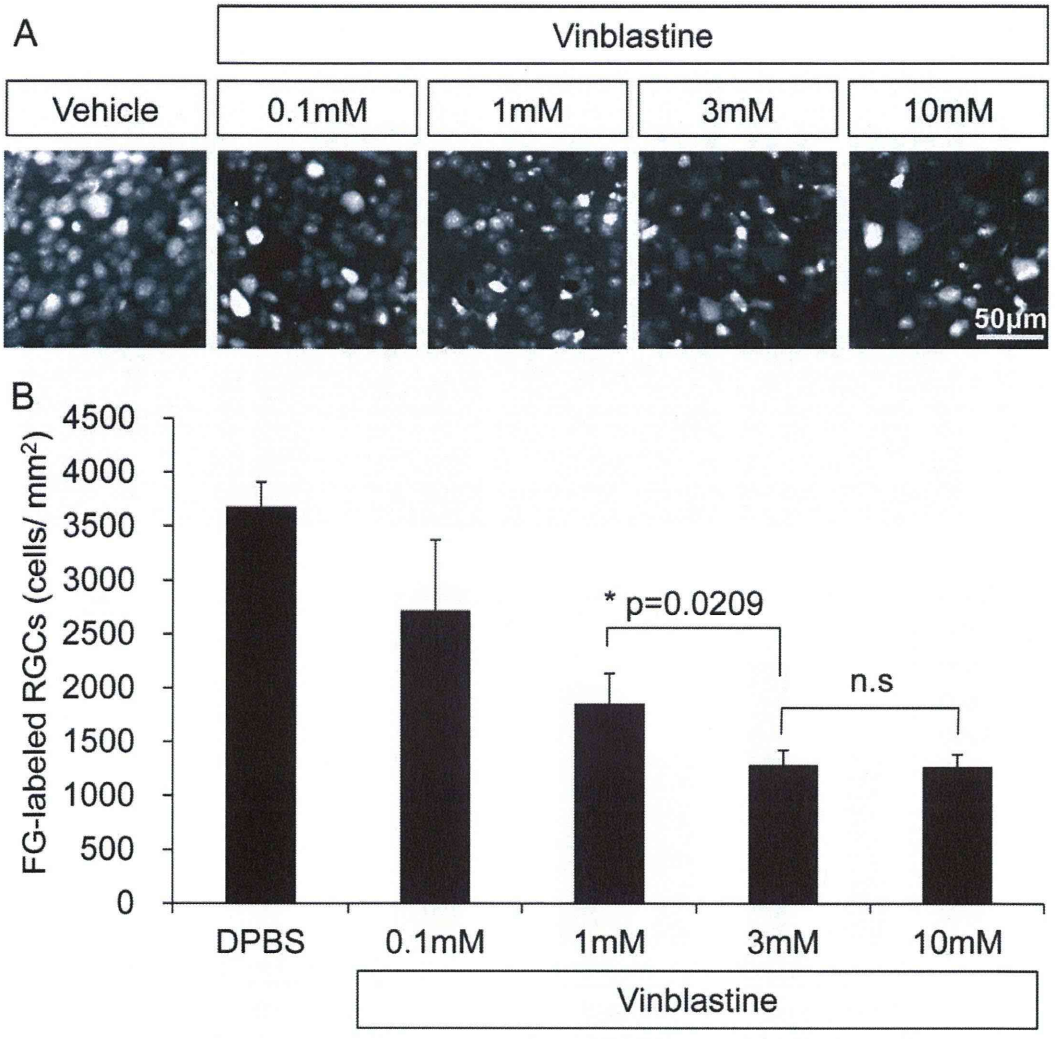

Fig. 4. Dose dependence of vinblastine-induced RGC death. **A:** Representative appearances of FG-labeled RGCs on the flat-mounted retina 7 days after vinblastine treatment at various concentrations (0, 0.1, 1, 3, 10 mM). **B:** Quantitative data on FG-labeled RGC following vinblastine treatment.

compared with the vehicle control (control: 3,623 ± 199 cells/mm^2, 0.1 mM: 2,721 ± 656 cells/mm^2, 1 mM: 1,855 ± 281 cells/mm^2, 3 mM: 1,287 ± 136 cells/mm^2, 10 mM: 1,267 ± 117 cells/mm^2). The RGC density in the 3 mM vinblastine group was significantly lower than that in the 1 mM group ($P = 0.019$). However, there was no significant difference in density noted between the 3 and 10 mM vinblastine groups ($P = 0.631$; Fig. 4B). Therefore, to model axonal injury, 3 mM vinblastine was used for further investigations.

BDNF retrograde transportation blockage is thought to be a trigger for RGC apoptosis (Quigley et al., 2000). Many other studies, including our previous study (Nakazawa et al., 2002), demonstrate that BDNF is a potent neurotrophic factor for RGC survival. We investigated whether intravitreal administration of BDNF could prevent RGC loss in these axonal damage models (Fig. 5A,B). The density of surviving RGCs following axonal injury was significantly higher in the BDNF-treated group than that in the vehicle control group (Fig. 5B; nerve crush without BDNF; 1,153 ± 79 cells/mm^2, nerve crush with BDNF: 1,618 ± 175 cells/mm^2, $P = 0.002$; vinblastine without BDNF: 1,056 ± 104 cells/mm^2, vinblastine with BDNF: 1,477 ± 249 cells/mm^2, $P = 0.001$). Our results indicate that BDNF is neuroprotective for preventing axonal damage-induced RGC death.

Tat-BH4, a potent inhibitor for mitochondria-derived apoptosis, was administered intraperitoneally to investigate the contribution of mitochondria in these

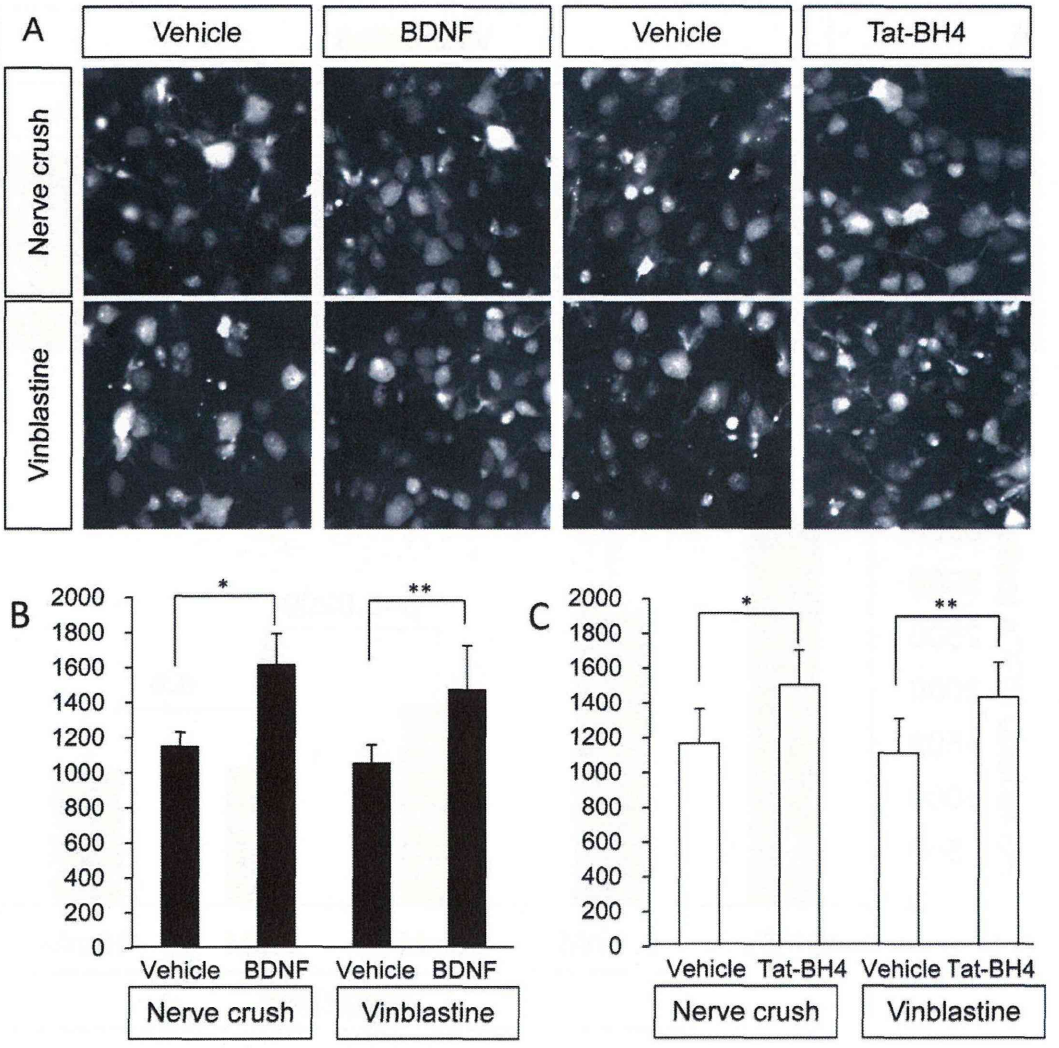

Fig. 5. Brain-derived neurotrophic factor (BDNF) and Tat-BH4 protect RGCs in axonal damage models. **A:** Representative appearances of RGCs on flat mounted retinas. **B:** Quantitative data on the density of RGCs treated with BDNF. **C:** Quantitative data on the density of RGCs treated with Tat-BH4. $*P < 0.05$, $**P < 0.01$.

axonal damage models (Fig. 5A,C). Tat-BH4 is a fusion protein of the protein transduction domain (PTD) from HIV virus and the BH4-domain peptide from Bcl-xL. PTD has the function of transmembrane delivery of proteins or peptides, and the BH4 domain inhibits mitochondrial membrane permeability. Intraperitoneal administration of Tat-BH4 significantly suppressed axonal damage-induced RGC death (nerve crush without Tat-BH4: 1,166 ± 79 cells/mm^2, nerve crush with Tat-BH4: 1,505 ± 139 cells/mm^2, $P < 0.001$; vinblastine without Tat-BH4: 1,112 ± 200 cells/mm^2, vinblastine with Tat-BH4: 1,434 ± 206 cells/mm^2, $P = 0.030$; Fig. 5C). These data suggest that mitochondria-derived signaling is involved in these axonal damage models.

Next we investigated the role of calpain in axonal damage-induced RGC death. Immunoblotting with the antibody for α-fodrin, one of the known calpain substrates, indicated that cleaved α-fodrin (145 kDa) was detectable 3 days after axonal damage either by nerve crush or by vinblastine treatment (Fig. 6). These data suggest that axonal damage induced the fragmentation of α-fodrin as a result of calpain activation in the retina.

Calpastatin (CAST) tightly regulates calpain status, and mice deficient for CAST are susceptible to calpain-dependent neuronal degeneration (Takano et al., 2005). To investigate whether calpain activation following axonal injury was involved in the pathogenesis of RGC death, axonal damage was induced in CAST KO mice.

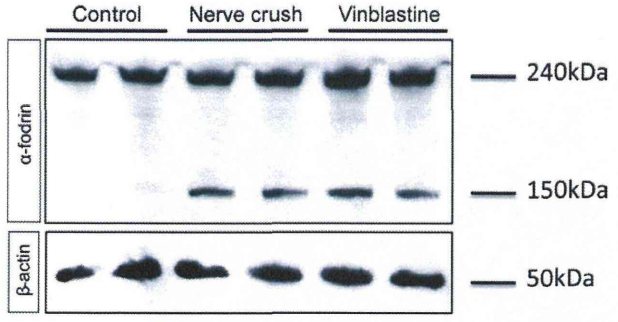

Fig. 6. Cleaved α-fodrin, a calpain substrate, after axonal damage. Representative data on immunoblotting with an antibody for α-fodrin (upper panels), one of the known calpain substrates, and internal control β-actin (lower panels) at day 3 following axonal damage. Note that the cleaved α-fodrin (145 kDa) was detectable with nerve crush or vinblastine treatment.

At first, the densities of RGCs in the uninjured CAST KO mice were not different from those observed in heterozygous and wild-type mice aged between 12 and 24 weeks (Fig. 7A). However, the density of RGCs following nerve crush in CAST KO mice was significantly decreased compared with that observed in wild-type mice (day 3: 3,545 ± 174 cells/mm^2, $P = 0.001$; day 7: 1,153 ± 85 cells/mm^2, $P < 0.001$; day 10: 839 ± 92 cells/mm^2, $P < 0.001$; day 14: 685 ± 110 cells/mm^2, $P < 0.002$; day 28: 488 ± 71 cells/mm^2, $P = 0.006$; Fig. 7B). The trend of susceptibility in CAST KO mice was also evident with the vinblastine treatment (day 3: 2,900 ± 311 cells/mm^2, $P = 0.462$; day 7: 1,266 ± 139 cells/mm^2, $P = 0.001$; day 10: 837 ± 171 cells/mm^2, $P = 0.045$; day 14: 629 ± 101 cells/mm^2, $P = 0.015$; day 28: 570 ± 78 cells/mm^2, $P = 0.007$; Fig. 7C). These data suggest that calpastatin, an endogenous inhibitor, plays a neuroprotective role in the axonal damage-induced RGC.

Next we investigated the therapeutic potential of SNJ-1945, a potent calpain inhibitor, in both in vitro and in vivo systems of RGC injury. In vitro, we administered SNJ-1945 to retinal mixed cells from wild-type mice and CAST KO mice for 24 hr (Fig. 8A). In the vehicle group, the survival rate (RGC/total percentage) of RGCs from the CAST KO mice was significantly lower than that from wild-type mice (wild: 4.2 ± 0.9%, CAST KO: 2.9 ± 0.4%, $P = 0.033$; Fig. 8B). When the retinal mixed culture cells were treated with SNJ-1945 (4 and 40 μM), the survival rate significantly increased, and the difference between wild-type and CAST KO mice vanished. In vivo, orally administered SNJ-1945 significantly delayed RGC death (Fig. 9A) induced by either nerve crush (vehicle: 1,153 ± 85 cells/mm^2, SNJ-1945: 1,650 ± 107 cells/mm^2, $P = 0.002$) or vinblastine treatment (vehicle: 1,299 ± 134 cells/mm^2, SNJ-1945: 1,825 ± 168 cells/mm^2, $P = 0.014$; Fig. 9B) on day 7. According to the results shown in Figure 7B,C, calpain activation in CAST KO mice accelerated the apoptosis of axon-damaged RGCs,

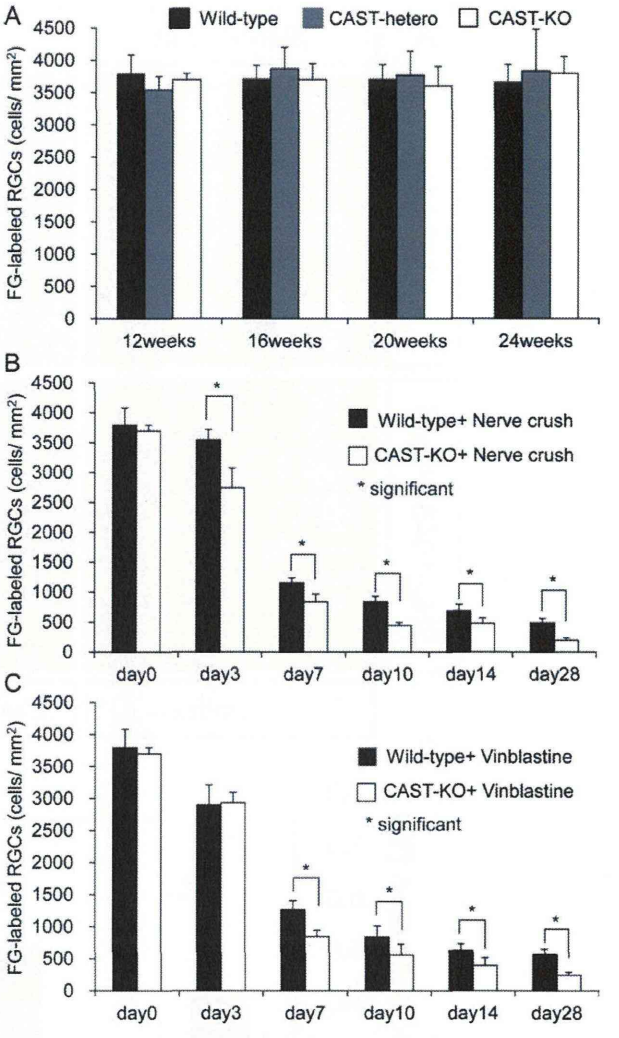

Fig. 7. Susceptibility of calpastatin KO mice to the axonal damage-induced RGC death. **A:** Quantitative data on RGC density without any damage in wild-type, CAST heterozygous, and CAST KO mice between 12 and 24 weeks. **B:** Quantitative data on RGC density damaged by nerve crush. **C:** Quantitative data on RGC density damaged by vinblastine treatment. Note that the susceptibility of CAST KO mice was also evident following nerve crush and vinblastine treatment. *$P < 0.05$.

but the calpain inhibitor could not protect the RGCs after day 14. The retinal transition of orally administered SNJ1945 might be not enough, contracting the neuroprotective effect from day 14. These data suggest that SNJ-1945 has a neuroprotective effect in both in vivo and in vitro models of RGC injury, even in the neurons of the CAST KO mice.

DISCUSSION

This study reveals a critical role for calpain activation in axonal damage-induced RGC death. First, we

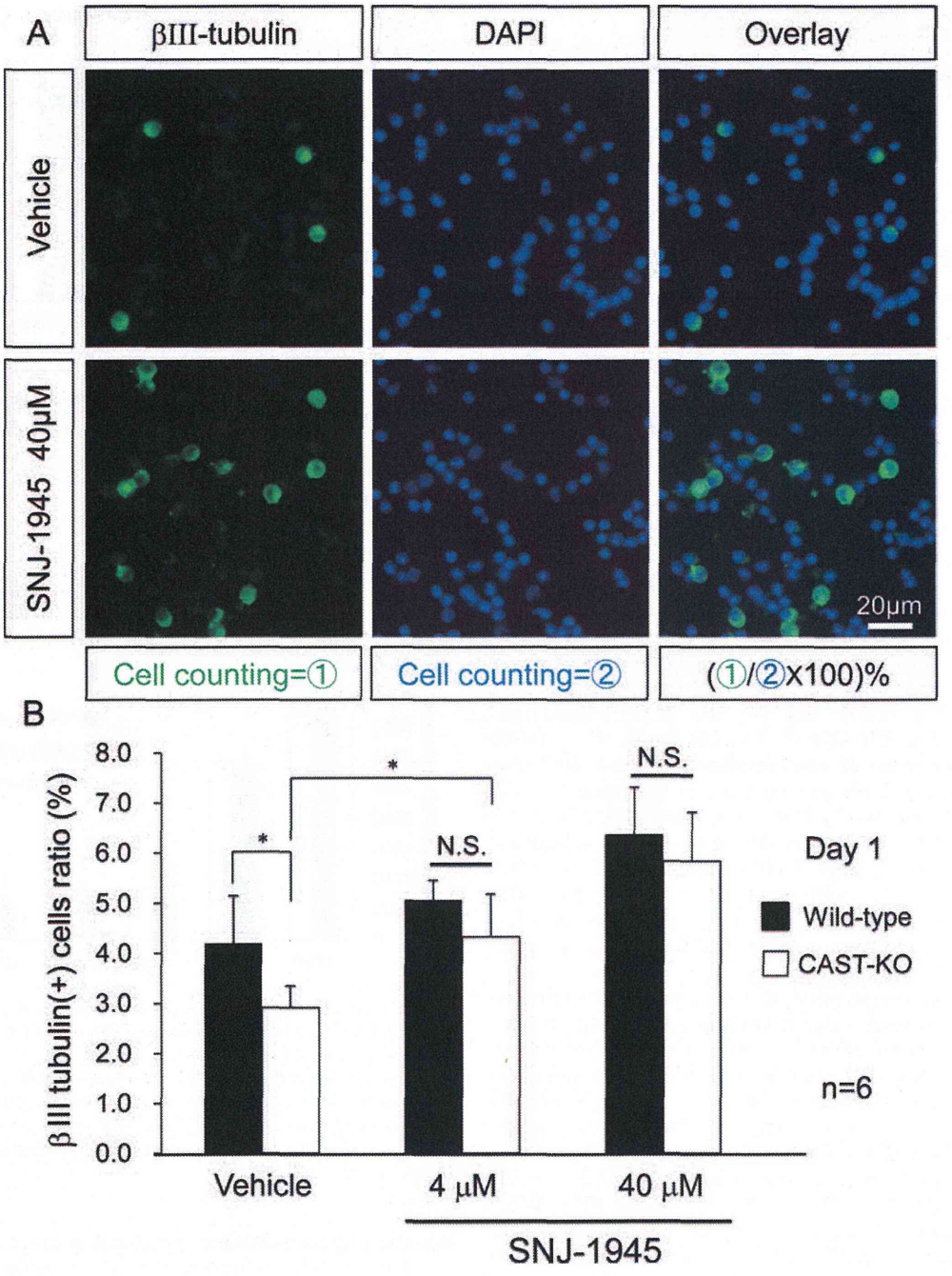

Fig. 8. Neuroprotective effects of SNJ-1945 in the cultured RGCs in vitro. **A:** Representative photographs of ICC with βIII-tubulin, a RGC marker, with (lower panels) or without (upper panels) SNJ-1945 treatment for 24 hr. **B:** Quantitative data on percentage (counting of βIII-tubulin$^+$ RGCs/counting of total DAPI$^+$ cells × 100%) from adult wild-type mice or CAST KO mice with vehicle or SNJ-1945 (4 or 40 mM) on day 1 (24 hr incubated). Note that the susceptibility of CAST KO mice and SNJ-1945 had significant neuroprotective effects in vitro. *$P < 0.05$.

developed a novel model of axonal damage by leaving a gelatin sponge around an optic nerve soaked in vinblastine, a tubulin-disassembly drug. We confirmed that the axoplasmic flow was prevented both in the nerve crush and in the vinblastine models with a double labeling technique. These types of axonal damage induced rapid

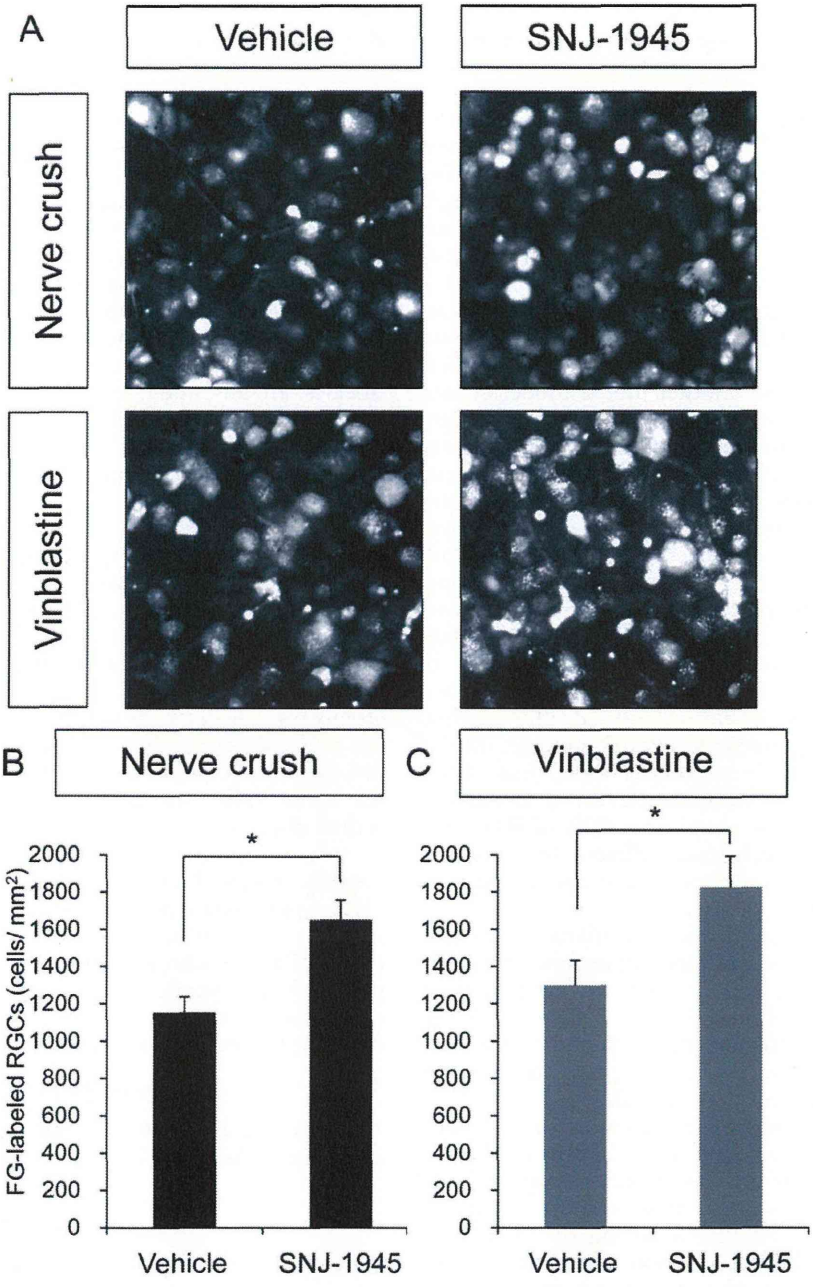

Fig. 9. Neuroprotective effects of SNJ-1945 in the axonal injury-induced RGC death in vivo. **A:** Representative appearances of FG-labeled RGCs on the flat-mounted retina 7 days after nerve crush (upper panels) or vinblastine-treatment (lower panels) with (right panels) or without (left panels) oral administration of SNJ-1945 (100 mg/kg/day). **B:** Quantitative data on FG-labeled RGCs 7 days (upper panels) or 14 days (lower panels) following nerve crush (left panels) and vinblastine treatment (right panels) with or without oral administration of SNJ-1945. Note that SNJ-1945 significantly suppressed RGC death on day 7 induced by either nerve crush or vinblastine treatment. $*P < 0.05$.

RGC loss during the first 7 days in part through a mitochondria-dependent cell death pathway. Using these axonal damage models, we found that calpain was activated in the damaged retina. To investigate whether the calpain activation was involved in the axonal damage-induced RGC death, we induced axonal damage in mice deficient for the calpastatin gene (CAST KO), an endogenous inhibitor protein for calpain. After axonal

damage, the number of surviving FG-labeled RGCs of the CAST KO mice was significantly lower than the number observed in wild-type and heterozygous mice. SNJ-1945, a potent calpain inhibitor, significantly prevented the RGC death in wild-type mice and CAST KO mice in vitro. SNJ-1945 also demonstrated a potent neuroprotective effect against nerve crush- and vinblastine-induced RGC death in vivo. These data strongly suggest that axonal damage-induced calpain activation plays a critical role in axonal damage-induced RGC death.

Nerve crush and axotomy of the optic nerve are often used as models of RGC death to mimic glaucoma or as an experimental model for axon regeneration. Here, we tried to establish another mouse model of axonal damage by preventing axoplasmic flow in the optic nerve. Vinblastine is a microtubule-disassembly drug and has been used in clinical settings for cancer treatment. We left a gelatin sponge soaked with vinblastine at various concentrations around the optic nerve and observed that 10 mM vinblastine destroyed the axon significantly in the treated area, and the axoplasmic flow of FG was almost completely suppressed. After the treatment with vinblastine, the density of surviving RGCs rapidly decreased to approximately 20% of that observed in the untreated control. The time course of RGC death was similar to that of NC-induced RGC death. Furthermore, in these two models of axonal damage, BDNF and Tat-BH4 significantly prevented RGC loss. These data suggest that vinblastine-induced RGC death is a novel model in which the axoplasmic flow of RGC was prevented through a mechanism different from what is observed in traditional mechanical axon damage models, such as nerve crush or axotomy.

Calpastatin is an endogenous inhibitor of calpain. Calpastatin clearly inhibits calpain-dependent proteolysis of cytoskeletal proteins, which results in the prevention of neurodegeneration (Takano et al., 2005). Therefore, we used calpastatin-deficient mice (CAST KO) as a model in which the mice are susceptible in damage-induced calpain activation. We hypothesized that, if the RGCs of CAST KO mice were vulnerable to the axonal damage, we could evaluate the contribution of the calpain-calpastatin pathway to the axonal damage-induced death of RGCs. Interestingly, there was no change in the density of FG-labeled RGCs during the aging process, suggesting that the contribution of calpain was not strong physiologically. On the other hand, orally administered SNJ-1945, a calpain inhibitor, suppressed the activation of calpain pathway in the retina and prevented RGC death in the in vivo mouse model of axonal damage. Taken together, these results demonstrate that the activation of calpain had a strong contribution to the axonal damage-induced RGC death.

Calpain is activated by increased intracellular Ca^{2+}. Ca^{2+} is raised locally through calcium channels and stressed intramitochondrial and endoplasmic reticulum (ER) storage (Azuma and Shearer, 2008). SNJ-1945 was able to inhibit calpain-1 (IC50 = 62 nM) and calpain-2 (45 nM), and orally administered SNJ-1945 (10 mg/kg) accumulated in the retinal tissue at 300 nM (1 hr, at peak) and 100 nM (8 hr; Shirasaki et al., 2006). The data suggest that oral administration allowed SNJ-1945 penetration into the neural retina and that the concentration was enough for the inhibition of calpain-1 and calpain-2.

Axonal and dendritic degeneration, including synapse degeneration, precedes cell body death in CNS diseases such as Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease, HIV dementia, and multiple sclerosis (Coleman, 2005; Stys, 2005; Koike et al., 2008). Hence, an axonoprotection strategy is required for the treatment of patients suffering from chronic CNS diseases. In this study, we found a significant protective effect against axon degeneration-induced RGC death, suggesting that SNJ-1945 is a strong potential drug candidate for the treatment of diseases sharing a similar pathogenesis.

In retinal diseases, the calpain pathway has been shown to be involved in ischemia (Azuma and Shearer, 2008), glutamate excitotoxicity (Nakazawa et al., 2009; Shimazawa et al., 2010), glaucoma (Huang et al., 2010; Qu et al., 2010), photoreceptor degeneration (Mizukoshi et al., 2010; Paquet-Durand et al., 2010), and diabetic retinopathy (Harris et al., 2006). Furthermore, SNJ-1945 attenuates cell death in cultured human retinal endothelial cells (Ma et al., 2009). Based on our data, SNJ-1945 has therapeutic potential as a new intervention not only for axonopathy but also for treatment of pathological retinal diseases.

In conclusion, orally administered SNJ-1945 successfully reversed axonal damage-induced RGC loss in vivo. Suppression of calpain activation is an important strategy for preventing axonal damage-induced RGC death. These findings suggest that SNJ-1945 is a novel potential therapeutic drug that could be used in the treatment of vision-threatening diseases, such as glaucoma and diabetic axonal atrophy.

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Clinical Ophthalmology

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ORIGINAL RESEARCH

Reproducibility of retinal circulation measurements obtained using laser speckle flowgraphy-NAVI in patients with glaucoma

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Background: Laser speckle flowgraphy (LSFG) enables noninvasive quantification of the retinal circulation in glaucoma patients. In this study, we tested the intrasession reproducibility of LSFG-NAVI, a modified LSFG technique.

Methods: Sixty-five eyes from 33 subjects (male (M):female (F) = 17:16) with a mean age of 49.4 ± 11.2 years were examined in this study. Two parameters indicating reproducibility – the coefficient of variation (COV) and the intraclass correlation coefficient (ICC) – were analyzed three times on the same day that mean blur rate (MBR) was measured using LSFG-NAVI. The sites analyzed were the retinal artery and vein, the optic disk, and the choroid. Following classification according to the Glaucoma Hemifield Test (GHT; SITA-Standard 30-2 program), the COV and ICC were examined in patients with (GHT+; 38 eyes, M:F = 20:18, average age 48.9 ± 12.8 years) and without (GHT–; 27 eyes, M:F = 13:14, average age 50.1 ± 8.7 years) abnormal glaucomatous visual fields.

Results: For all subjects, the intrasession reproducibility of MBR in the optic disk (COV: 3.4 ± 2.0; ICC: 0.95) and choroid (COV: 4.7 ± 3.4; ICC: 0.98) was excellent. The reproducibility for the retinal vein (COV: 8.4 ± 5.6, ICC: 0.90) and retinal artery (COV: 10.9 ± 9.9, ICC: 0.9) was moderate. MBRs in the optic disk had good reproducibility in both the GHT+ group (COV: 3.8 ± 2.0; ICC: 0.97) and the GHT– group (COV: 2.9 ± 2.1; ICC: 0.95). Local assessment of the optic disk in normal or glaucoma patients showed that the COVs of the quadrant optic disk areas were best in the temporal area of MBR (3.4%, 4.2%, respectively).

Conclusion: LSFG-NAVI showed favorable reproducibility in evaluation of retinal circulation of glaucoma patients, particularly in the optic disk and choroid.

Keywords: ocular circulation, reproducibility, optic nerve, retina

Introduction

Glaucoma affects more than 70 million people worldwide[1,2] and is the second most frequent cause of blindness.[1] The increase in lifespan worldwide has increased the number of individuals presenting with glaucoma and blindness.[3] Although the pathogenesis of glaucoma remains unclear, one population-based study has suggested that the important risk factors for glaucoma are intraocular pressure (IOP), senescence, and myopia.[4] Other factors such as vascular components may also contribute to optic nerve damage in glaucoma. An increasing body of evidence suggests that dysfunction of ocular microcirculation in the optic nerve influences the progression of glaucoma.[5–7] The association between the degree of optic nerve damage and the defect area has also been demonstrated using fluorescein angiography (FAG).[8–12] Decreased microcirculation in the optic nerve induced by intravitreal administration of endothelin-1 in the

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