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Retinal Ganglion Cell Death of Guinea Pig is Slower Than Rat as Optic Nerve Transection Animal Model

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ABSTRACT. *Glaucoma is a progressive optic neuropathy that leads to retinal ganglion cell (RGC) apoptosis and is a leading cause of acquired blindness. Recently, lack of clinical protective treatment for optic nerve in glaucoma thus inhibitor drugs towards progression of RGC degeneration are urgently needed. In this study, we established a guinea pig optic nerve transection model and investigated the conditions under which RGC death is evaluated in the development of retinal neuroprotective drugs for the treatment of glaucoma. Guinea pig optic nerve axotomy induces RGC death similarly to other rodent models; however, the time to progress to significant RGC death is 21 days after transection and is longer than in the rat, 10 days after optic nerve axotomy. The results are useful for evaluating species-specific optic neuroprotective agents against rats and mice.*

Keywords: *glaucoma, guinea pig, optic nerve axotomy*

INTRODUCTION

According to a systematic review and meta-analysis of eye disease surveys, glaucoma is the second leading cause of blindness (11%) after cataract (45%) [1] and 5% of people over the age of 40 have glaucoma [2]. With a global prevalence of 2.2%, primary open-angle glaucoma, the most common type of glaucoma, is estimated to affect 57.5 million people worldwide in 2017 [3]. In principle, glaucoma can be defined as a progressive optic neuropathy that leads to cupping of the optic nerve head and apoptosis of the retinal ganglion cells (RGCs) [4]. Because intraocular pressure (IOP) is the only known factor associated with the progression of visual field loss [5], current glaucoma treatment focuses on lowering IOP.

When the aqueous humor flow in the anterior chamber is blocked and aqueous humor accumulates, the ocular pressure increases, putting pressure on the optic nerve and causing symptoms. Glaucoma medications improve symptoms by lowering IOP by stimulating the outflow of aqueous humor and reducing the production of aqueous humor.

IOP-lowering medications are classified into those that enhance aqueous humor outflow through the trabecular meshwork and uveoscleral way (sympathomimetics [α-blockers], parasympathomimetics, and prostaglandin-related drugs) and those that suppress aqueous humor production from the ciliary body (sympathomimetics [β-blockers], carbonic anhydrase inhibitors) [6]. In addition, drugs with novel mechanisms of action, such as EP2 receptor agonists [7] and Rho kinase inhibitors [8, 9], as well as combinations of these drugs, have recently become available, greatly expanding the drug treatment options.

In cases where the IOP response is inadequate or the deficiency progresses despite various eye drop treatments, aqueous humor drainage by surgical procedures such as trabeculotomy or trabeculectomy may be attempted. Many minimally invasive glaucoma surgery (MIGS) devices have been developed to safely and easily perform these surgical procedures [10], providing the most options for glaucoma treatment of any eye disease.

However, drugs that directly protect the optic nerve have not yet reached clinical application. Therefore, optic neuroprotective agents are considered an unmet medical need in the treatment of glaucoma. Because RGCs are reduced in glaucoma, optic nerve transection and optic nerve crush models are used as preclinical

models [11, 12]. Although rodents such as mice and rats are commonly used as animal models, species differences in target proteins and compounds can make rodent models ineffective for general drug development. To address this issue, the present study was conducted to establish a guinea pig model of optic nerve transection as an alternative to rats and mice, and to determine whether this model could be used for the development of optic neuroprotective drugs.

RESEARCH METHODS

Animals

Eight- to thirteen-week-old male Sprague-Dawley (SD) rats (Charles River Laboratories Japan, Yokohama, Japan) and 8- to 19-week-old male Hartley guinea pigs (Japan SLC, Shizuoka, Japan) were used in the study. In the time course study, the animals were divided into two groups: sham surgery group and axotomy group. In the study to evaluate the neuroprotective effect of brain-derived neurotrophic factor (BDNF), animals were divided into three groups: sham surgery group and axotomy group with vehicle treatment and axotomy group with BDNF treatment. All experimental protocols for the use of laboratory animals followed our laboratory guidelines for animal experimentation. Animals were housed in the controlled environment of an animal room (temperature 23 ± 3°C, humidity 55 ± 15%, 12-hour light cycle) with free access to food (CRF-1 for rats and RC-4 for guinea pigs; Oriental Yeast, Tokyo, Japan) and water.

Optic Nerve Axotomy

Optic nerve was transected according to previous report [11] with some modifications. To prevent the corneas from drying out, saline was applied to both eyes. Under isoflurane anesthesia, the right eyelid was grasped with forceps and incised with scissors. The tissue was cut along the eye to expose the optic nerve and a small incision was made in the longitudinal direction of the sheath and the incision was widened with 45° forceps. Only the optic nerve was cut, taking care not to cut the vessels surrounding the optic nerve. We confirmed that there was no abnormality in fundus blood flow after surgical procedures. In the sham-operated eye, surgery was performed until the optic nerve was exposed and the optic nerve sheath was cut. Antibiotics (Tarivid® ophthalmic ointment; 0.3% ofloxacin, Santen Pharmaceutical, Osaka, Japan) were applied to both eyes, and the animals were returned to the cage. The cage was placed on a warm plate set at 37°C until the animals woke up. The experimental schedule is shown in Figure 1.

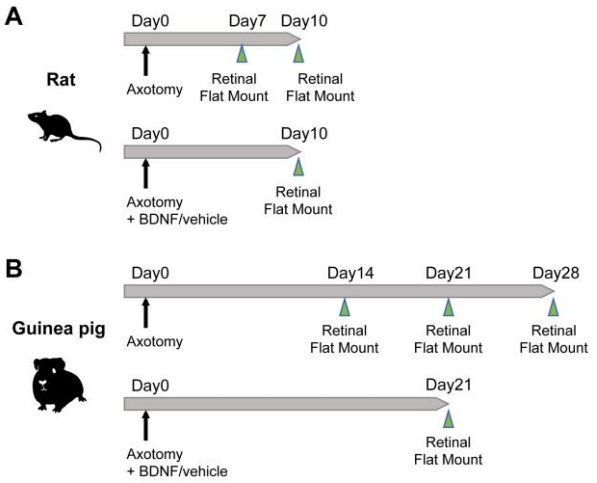


Figure 1. Experimental schedule of optic nerve axotomy and RGC evaluation for rat (A) and guinea pig (B).

Intravitreal Administration of BDNF

BDNF, purchased from PeproTech (Cranbury, NJ, USA), was dissolved in vehicle (0.15% BSA/PBS) to a concentration of 0.33 mg/mL and sterilized by filtration through a 0.22 µm filter. Intravitreal administration was performed under isoflurane anesthesia with mydriatic drops (Mydrin-P® ophthalmic solution; 0.5% tropicamide/0.5% phenylephrine hydrochloride, Santen Pharmaceutical). 1 µg/3 µL/eye of BDNF or vehicle was administered intravitreally by microsyringe within 30 min after optic nerve axotomy. After administration, the intraocular area was observed under a stereomicroscope to confirm the absence of hemorrhage [13].

Preparation of Retinal Flat Mounts and Immunostaining of RGCs

Animals were exsanguinated by transection of the abdominal aorta under isoflurane anesthesia at the indicated day after axotomy (Fig. 1), and the eyeballs were fixed in 4% paraformaldehyde to prepare retinal flat mounts. The prepared specimens were stained with an antibody against RNA binding protein with multiple splicing (RBPMS), a selective marker for ganglion cells in the mammalian retina. Flatmounted retinas were blocked with 10% donkey serum and incubated with rabbit polyclonal anti-RBPMS antibody (Abcam, Cambridge, UK; dilution 1:200) followed by goat anti-rabbit IgG (Alexa Fluor® 488; Thermo Fisher Scientific, Waltham, MA, USA; dilution 1:1000) [14].

Measurement of the Number of Viable RGCs

Flatmount specimens were photographed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan), and the number of anti-RBPMS antibody-positive RGCs was counted using image analysis software (WinROOF Ver.

5.01; Mitani Corporation, Tokyo, Japan). Twelve views were counted per specimen, and the number of anti-RBPMS antibody-positive cells per 1 mm^2 was calculated [14].

Statistical Analysis

All results are expressed as mean ± SEM. Statistical differences between the number of RGCs in the sham-operated group and the optic nerve transection group at the same time point were analyzed by *F*-test followed by Student's *t*-test for equal variances or Aspin-Welch *t*-test for unequal variances. Statistical analysis was performed using GraphPad Prism 6.07 (GraphPad Software, La Jolla, CA, USA), and differences were considered significant at $p<0.05$ (2-sided).

RESULTS AND DISCUSSIONS

First, the optic nerve axotomy model was established in SD rats (Fig. 2). On day 7, the density of RGCs in the optic nerve axotomy group was 1563±228 cells/mm^2 compared with 1966±49 cells/mm^2 in the sham group, a 20% decrease that was not significant. The density of RGCs in the optic nerve axotomy group was 928±125 cells/mm^2 compared to 2036±51 cells/mm^2 in the sham group, a 54% reduction ($p<0.01$) at day 10.

Next, we attempted to establish a guinea pig model of optic nerve axotomy in case evaluation in mice or rats was not possible due to species differences in neuroprotective drugs (Fig. 3). Preliminary studies in guinea pigs showed no RGC death at 10 days after axotomy, so we attempted to study a longer period in guinea pigs (data not shown). The guinea pig is larger than the rat, and the procedure was more difficult than in the rat because of the long distance to the optic nerve at the back of the eye and the large number of blood vessels before reaching the optic nerve. The average RGC density of all guinea pigs used in this study was 1155±40 cells/mm^2, which was 42% lower than the average for rats (2001±12 cells/mm^2). However, as in rats, axotomy reduced RGCs in guinea pigs: Day 14 sham; 1102±13 cells/mm^2, axotomy; 952±151 cells/mm^2, Day 21 sham; 1185±98 cells/mm^2, axotomy; 572±75 cells/mm^2 ($p<0.01$), Day 28 sham; 1190±103 cells/mm^2, axotomy; 681±298 cells/mm^2. The number of RGCs at 14, 21, and 28 days after optic nerve axotomy was 86%, 48%, and 57% of the sham group, respectively. Compared with rats, it took longer for the number of RGCs to decrease after optic nerve axotomy; approximately three weeks for the number of RGCs to decrease sufficiently. Therefore, it would take approximately one month to evaluate drug efficacy in the guinea pig optic nerve axotomy model.

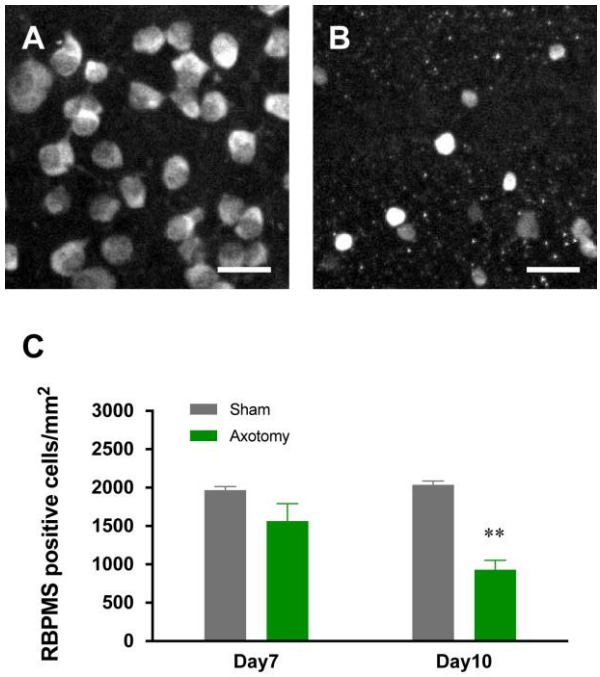


Figure 2. RGCs in the rat optic nerve axotomy model. Typical fluorescence microscopy image of sham (A) and axotomized (B) rat RGCs immunostained with anti-RBPMS antibody at 10 days after surgery. (C) Number of RBPMS-immunostained RGCs at 7 and 10 days after axotomy. A significant decrease in viable RGCs was observed 10 days after axotomy. Bar=50 μm. Each value represents the mean±SEM (n=3-4). **$p<0.01$ vs. Sham group (Student's t-test).

Some drugs are known to show neuroprotective effect on axotomized rat RGCs [11]. In the present study, BDNF was used to test whether an optic neuroprotective drug could be evaluated using the optic nerve transected model. In rats treated with intravitreal BDNF immediately after optic nerve transection, the density of RGCs was 1616 ± 61 cells/mm^2 at 10 days after treatment, a significant 62% increase compared to the vehicle-treated group (928 ± 125 cells/mm^2). Based on these results, it was considered appropriate to evaluate the drug at the 10th day of optic nerve axotomy (Fig. 4). On the other hand, the effect of BDNF at day 21, when RGC density is reduced, was limited (+25%) in guinea pigs (vehicle-treated group; 658 ± 58 cells/mm^2, BDNF-treated group; 797 ± 142 cells/mm^2) (Fig. 4). Although further validation is needed due to the small number of experiments, higher concentrations or multiple doses of neuroprotective drugs may be required in the guinea pig model of optic nerve axotomy compared to rats. Also, the vitreous volume in rats is reported to be approximately 50 μL [15], whereas it is reported to be approximately 300 μL in guinea pigs [16], so the vitreous concentration of BDNF was 1/6 of that in rats, which may have contributed to the low efficacy of the drug.

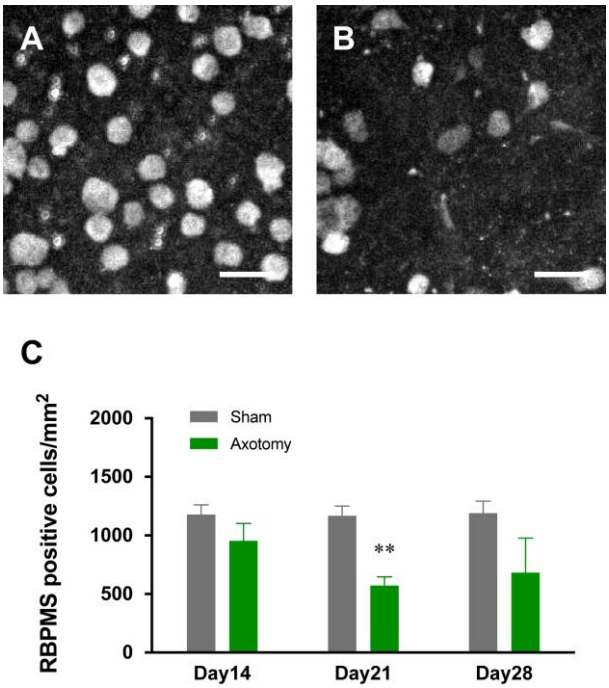


Figure 3. RGCs in the guinea pig optic nerve axotomy model. Typical fluorescence microscopy image of sham (A) and axotomized (B) retina 28 days after axotomy. (C) Number of RBMPS-immunostained RGCs at 14, 21 and 28 days after axotomy. Viable RGCs were significantly reduced 21 days after axotomy. Bar=50 μm. Each value represents the mean±SEM (n=2-3). **p<0.01 vs. Sham group (Student's t-test).

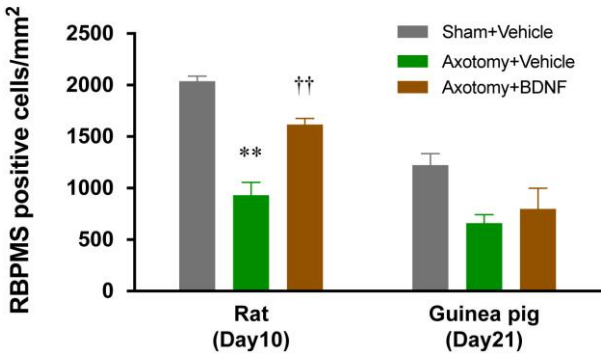


Figure 4. Neuroprotective effect of BDNF on axotomized RGCs. Intravitreal BDNF treatment significantly prevented RGC death in the rat on day 10, but the effect was weak in the guinea pig on day 21. Each value represents the mean±SEM (n=2-4). **p<0.01 vs. Sham+Vehicle group (Student's *t*-test), ††p<0.01 vs. Axotomy+Vehicle group (Student's *t*-test).

Since there is no treatment option to directly protect the optic nerve in glaucoma, new drugs that inhibit the progression of RGC degeneration are urgently needed. BDNF is a known neurotrophic factor that potently prevents axotomized RGC degeneration [17]. Intravitreal delivery of adenoviral vectors expressing the human BDNF gene from a cytomegalovirus promoter/enhancer (Ad-BDNF) is being investigated as a therapeutic approach [18]. Some compounds such as bis (3-propionic acid methyl ester) phenylphosphine borane complex 1 (PB1) is reportedly promotes RGC survival through a significant increase in retinal BDNF expression and downstream activation of the ERK1/2 pathway in rat optical nerve axotomized model [19]. In the development of new drugs, some targets or compounds may not be effective in rats/mice due to species differences [20, 21]. In addition, it is generally difficult to evaluate efficacy in primates, necessitating the use of alternative species.

Morphologically, guinea pig fundus images show dense blood vessels in the optic nerve head, but do not show retinal vascular structures extending radially from the optic nerve head as in humans, rats, and mice [22]. On the other hand, the guinea pig optic nerve head has been shown to have a well-organized collagenous lamina cribrosa similar to that in humans. In addition, optical coherence tomography (OCT) imaging identified a nerve fiber layer, although the cup is barely visible on fundus images [23]. These morphological observations suggest that the guinea pig is a promising alternative to primate models of human ocular diseases known to affect the optic nerve head, such as myopia and glaucoma. Therefore, it would be useful to establish a guinea pig model of optic nerve transection and explore the conditions under which RGC death is evaluated in the development of retinal neuroprotective drugs for the treatment of glaucoma.

CONCLUSION

This study showed that guinea pig optic nerve axotomy induces RGC death similar to other rodent models; although the time to RGC death is longer than in the rat, the results are useful for evaluating species-specific optic neuroprotective agents against rats and mice.

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