Behavior tests and immunohistochemical retinal response analyses in RCS rats with subretinal implantation of Okayama University-type retinal prosthesis

Short title: Behavior tests in rats with retinal prostheses

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Abstract

We have developed a photoelectric dye-coupled polyethylene film as a prototype of retinal prosthesis, named “Okayama University-type retinal prosthesis. The purposes of this study are to conduct behavior tests to assess vision in Royal College of Surgeons (RCS) rats which underwent subretinal implantation of the dye-coupled film, and are also to reveal the retinal response to the dye-coupled film by immunohistochemistry. Polyethylene films were made of polyethylene powder at refined purity, and photoelectric dyes were coupled to the film surface at higher density, compared with the prototype. The dye-coupled film or the dye-uncoupled plain film, as a control, was implanted subretinally from a scleral incision in both eyes of a RCS rat at 6 weeks of the age. Behavior tests, 2, 4, 6, and 8 weeks after the implantation, were conducted by observing head-turning or body-turning in the direction, consistent with clockwise or anticlockwise rotation of a black-and-white-striped drum around a transparent cage, housed with a rat. After the behavior tests at 8 weeks, rats’ eyes were enucleated to confirm the subretinal implantation of the films and were processed for immunohistochemistry. In the behavior tests, the number of head-turning, consistent with the direction of the drum rotation, was significantly larger in RCS rats with dye-coupled film implantation, compared with rats with plain film implantation ($P<0.05$,

repeated-measure ANOVA). The number of apoptotic neurons was significantly smaller in the eyes with the dye-coupled film implantation, compared to the eyes with the plain film (P<0.05, Mann-Whitney U-test). In conclusion, subretinal implantation of the photoelectric dye-coupled films restored the vision in RCS rats and prevented the remaining retinal neurons from apoptosis.

Keywords

Retinal prosthesis, photoelectric dye, polyethylene film, RCS rat, behavior test, apoptosis, immunohistochemistry

Introduction

A basic concept for retinal prosthesis is to replace the lost function of visual cells (photoreceptor cells) which receive light and transfer light energy to generate electric potentials of cell membrane.[1-3] The retinal prosthesis has not yet been used clinically and has been in the process of the development, aiming to treat patients, mainly, with retinitis pigmentosa, a hereditary disease with progressive loss of the visual field and visual acuity. Currently, various models of prototypes for the retinal prosthesis have been developed at different institutions all over the world. A main system of the prototypes is to generate electric currents to stimulate the remaining retinal neurons in the degenerated retina: digital camera-captured images are transferred to electric signals which are outputted as electric currents from a multi-electrode array placed around the neurosensory retina.[1-5] The multi-electrode array would be placed on the surface of the retina in the vitreous,[4] in the subretinal space between the retina and the retinal pigment epithelium,[2] or in the subchoroidal space between the sclera and choroid.[5]

Another system for the retinal prosthesis is to use photodiodes.[6] Numerous photodiodes are compacted to form a disc which is placed in the subretinal space. A major problem of this multi-photodiode array is that output electric currents from photodiodes are too small to stimulate the retinal neurons. We have designed a third

type of retinal prosthesis which utilizes photoelectric dye molecules which output electric potentials in response to light.[7,8] The prototype is a photoelectric dye-coupled polyethylene film.[9,10] Until now, we have proven the efficacy and the safety of the photoelectric dye in itself and also the dye-coupled film.[11,12]

In this study, we increased the density of photoelectric dyes on the surface of polyethylene film to aim at higher sensitivity, and we tested the efficacy of the upgraded dye-coupled films implanted in eyes of Royal College of Surgeons (RCS) rats by their behavior tests. We also studied the effects of the dye-coupled films on the remaining neurons by immunohistochemistry.

Materials and methods

Preparation of dye-coupled polyethylene film

High-density polyethylene powder was washed with xylene and placed between two square aluminum plates in the size of 100 mm x 100 mm with 2 mm thickness. The polyethylene was dissolved at 155 ℃ for 15 minutes between the plates set at a pressurized machine (IMC-11FD, Imoto Machinery, Co. Ltd., Kyoto, Japan). The dissolved polyethylene was pressed at the magnitude of 10 mega-Pascal by oil pressure for 3 minutes under a vacuum in the pressurized machine. The polyethylene film

between the aluminum plates was cooled by ice water and was rinsed further with distilled water.

A polyethylene film in the size of 2 x 4 cm was held by a gold string and placed in a fluororubber-plugged three-neck flask attached with a Dimroth condenser. The flask was placed in an oil bath kept at 80 ℃ and the polyethylene film was exposed to fuming nitric acid for 14 minutes to introduce carboxyl moieties. The film was washed by water until neutral pH, kept in distilled water for 24 hours, and then dried in the room atmosphere.

The fuming nitric acid-treated film was reacted with 1 or 4 x 10^{-5} mol of ethylenediamine and 1 or 4 x 10^{-5} mol of N, N-dicyclohexylcarbodiimide (DCC) in 70 mL of chlorobenzene in a glass-plugged flask which was placed in a water bath at 35 ℃ and rotated at 50 rounds per minute (rpm) for 48 hours. The film was then washed with chlorobenzene. The ethylenediamine-coupled film was reacted with 1 or 4 x 10^{-5} mol of a photoelectric dye, 2-[2-[4-(dibutylamino)phenyl]ethenyl]-3-carboxymethylbenzothiazolium bromide (NK-5962, Hayashibara, Okayama, Japan) and 1 or 4 x 10^{-5} mol of DCC in 75 mL of chlorobenzene in a glass-plugged conical flask in a water bath at 35 ℃ for 48 hours with rotation at 50 rpm. The film was washed with chlorobenzene and further soaked

with chlorobenzene for 24 hours. The film was washed with water and dried in the room atmosphere.

The dye has a molecular weight of 503.5 dalton and absorption spectra of 400-600 nm with peak absorption at 539 nm (Fig. 1B).[7,8] The two steps of chemical processes were monitored by infrared and visible light absorption spectra (Fig. 1B), respectively, to confirm the successful reactions. The fuming nitric acid-treated only polyethylene film and the photoelectric dye-coupled polyethylene film, as described above, were designated as the plain film and the dye-coupled film, respectively (Fig. 1A), in the following experiments.

Film implantation into subretinal space of rat eyes

Male RCS rats at 6 weeks of the age were anesthetized by intraperitoneal injection of ketamine (87 mg/kg body weight) and xylasine (13 mg/kg). Under a dissecting microscope, the conjunctival incision was made on the temporal side of the eye and the sclera was tapped with a 20-gauge needle. Saline was applied to the scleral-choroidal puncture to make a bleb retinal detachment. A sheet of either dye-coupled film or plain film in the size of 1 x 5 mm with one edge marked with black ink was inserted with a forceps to the bleb retinal detachment in both eyes of each rat.[11] The scleral incision was left without suture, and antibiotics eye drops were applied to the eyes. The fundus was

examined by indirect funduscopy with a 20 diopter lens to confirm the film insertion. Each rat was housed in a standard rat cage in a room with the 12-hour-each light and dark cycle at the Animal Center of Okayama University.

At 8 weeks after the film implantation, rats were deeply anesthetized with an over dose of ether to death. The eyes were enucleated and fixed with 4% paraformaldehyde. The eyes were cut with a razor blade circumferentially at the midperiphery and the posterior halves were observed with a dissecting microscope to confirm the subretinal implantation of the films. All the procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and also to the Animal Protection Act in Japan. This study was approved by the Animal Committee at Okayama University.

Behavior test

A rat was housed in a round cylindrical cage with a transparent wall with the internal diameter of 20 cm or 30 cm (Fig. 2). A drum at the diameter of 40 cm, with the inside painted with black-and-white stripes, was rotated around the cage at the speed of 2 or 4 rounds per minute (rpm).[13,14] Each black or white stripe on the drum had the width of 5 degrees, and the drum contained 36 black stripes and 36 white stripes in the entire 360-degree circumference. The behavior of rats was recorded by a digital videocamera

from above (Fig. 2A). The drum was rotated clockwise for 3 minutes, and anticlockwise for 3 minutes after the 3-minute interval. The sequence of the experiments was clockwise drum rotation at 2 rpm for 3 minutes, rest for 3 minutes, anticlockwise drum rotation at 2 rpm for 3 minutes, rest for 3 minutes, clockwise rotation at 4 rpm for 3 minutes, rest for 3 minutes, and anticlockwise rotation at 4 rpm for 3 minutes. The sequence of testing was done in the bright condition at 150 lux under the usual fluorescence ceiling light in a large transparent-walled round cage with the internal diameter of 30 cm on the first day, (Fig. 2C), and was repeated in the large cage in the dim condition at 50 lux, illuminated with a fluorescence light source placed on the floor, on the second day (Fig. 2B), and further repeated in a small cage with the internal diameter of 20 cm in the bright condition at 150 lux on the third day (Fig. 2D). The rest between the 2 rpm testing and the 4 rpm testing in a small cage at 150 lux on the third day was 30 minutes in place of 3 minutes in the other conditions.

The number of head movements (turning) and the number of body movements (turning) in the same direction of the rotation of the drum in 3 minutes were counted. In addition, the total time of head movements or body movements in the same direction of the drum rotation was measured. For statistical analysis, repeated-measure analysis of variance (ANOVA) was applied to two groups (control group versus experiment group)

at the time points of 2, 4, 6, and 8 weeks after the implantation of plain films in both eyes (control group) or dye-coupled films in both eyes (experiment group).

In total, 20 RCS rats were used in the study: 10 rats were implanted with dye-coupled films in both eyes and 10 rats were implanted with plain films in both eyes. Of these 20 rats, data obtained by behavior tests in 7 RCS rats with dye-coupled film implantation and 7 rats with plain film implantation were used for statistical analyses. Of the 7 rats with dye-coupled film implantation, 4 rats were confirmed to have the subretinal implantation in the right eye and 3 rats to have the subretinal implantation in the left eye. Of the 7 rats with plain film implantation, 4 rats were confirmed to have the subretinal implantation in the right eye and 3 rats to have the subretinal implantation in the left eye. Three rats with dye-coupled film implantation and 3 rats with plain film implantation were excluded from the study because the film in neither eye was inserted correctly in the subretinal space between the sensory retina and the retinal pigment epithelium.

Immunohistochemistry

The eyes implanted with the films were enucleated and immersed in 4% paraformaldehyde for 2-3 hours, cut into halves circumferentially in the midperiphery of the eye balls, and cryo-protected by immersion in 10% sucrose in 100 mM phosphate

buffer (pH7.4) for 3 hours. Each eyecup with the film was embedded in Tissue-Tek O.C.T. compound (Sakura Seiki, Tokyo, Japan), and frozen sections were sliced at the thickness of 16 μm. The sections were rehydrated with phosphate-buffered saline (PBS: 10 mM sodium phosphate, 150 mM sodium chloride, pH7.4) for 30 minutes, blocked with PBS containing 0.3% Triton X-100, 1% bovine serum albumin (BSA), and 10% normal goat serum (NGS) for 30 minutes, and incubated at 37 ℃ with primary antibodies in PBS containing 0.3% Triton X-100, 1% BSA , and 10% NGS for 4 hours. The primary antibodies used were: anti-calbindin D-28K in 1:500 dilution (rabbit polyclonal antibody, Millipore, Temecula, CA, USA), anti-protein kinase C (PKC)- α in 1:250 dilution (mouse monoclonal antibody, Sigma-Aldrich, St. Louis, MO, USA), anti-synaptophysin in 1:500 dilution (mouse monoclonal antibody, Sigma-Aldrich), and anti-opsin (rhodopsin) in 1:1000 dilution (mouse monoclonal antibody, Sigma-Aldrich). After being washed three times for 10 minutes each with PBS, the sections were incubated with second fluorescence-labeled antibodies, Alexa-350-labeled goat anti-mouse IgG antibody or Alexa-350-labeled goat anti-rabbit IgG antibody in 50 mM sodium phosphate (pH7.4) and 200 mM sodium chloride at room temperature for 30 minutes, and washed with PBS. The sections were finally incubated with propidium iodide (PI) at working concentration of 4μg/ml in PBS for 20 minutes at room

temperature for nuclei staining and washed with PBS. The images were captured by a CCD camera attached to a fluorescence microscope (Axiovert 200 M, Carl Zeiss Japan, Tokyo, Japan) with rhodamine and DAPI filter sets.

In situ cell death detection

The frozen sections were rehydrated with PBS for 20 minutes, permeabilized in PBS containing 0.3% Triton X-100 at room temperature for 30 minutes, and washed with PBS for 10 minutes. The sections were incubated with reaction mixture of enzyme solution (terminal deoxynucleotidyl transferase) and labeling solution (fluorescein-conjugated dUTP) at 37 ℃ for one hour in the dark, washed with PBS twice each for 10 minutes, and reacted with 4',6-diamidino-2-phenylindole 2HCL (DAPI) at the concentration of 250 ng/ml in PBS for 10 minutes for nuclei staining, and washed again with PBS for 10 minutes (In Situ Cell Death Detection Kit, Fluorescein, Roche Diagnostics, Mannheim, Germany). The images were captured by a CCD camera attached to a fluorescence microscope (Axiovert 200 M) with a FITC filter set.

The number of apoptotic cells was counted in the areas of the retina with 1 mm width, perpendicular to the vitreous-retinal pigment epithelial axis, on each section. Three areas were chosen for comparison of the number of apoptotic cells: the area apposed to the film, the area neighboring to the film, and the area opposite to the film

across the posterior pole of the eye (Fig. 5A). The number of apoptotic cells in each area was compared between 6 eyes of 6 RCS rats with dye-coupled film implantation and 6 eyes of 6 RCS rats with plain film implantation.

Results

Dye-coupling reaction

In the coupling reaction of the photoelectric dye to the carboxyl moiety-bearing polyethylene film surface, the 4-fold increase of ethylenediamine from 1 x 10^{-5} mol to 4 x 10^{-5} mol at the first step and the 4-fold increase of the dye from 1 x 10^{-5} mol to 4 x 10^{-5} mol at the second step resulted in about 2-fold increase of the conjugated dye molecules on the film surface, as revealed by visible light absorption spectrometry (Fig. 1C).

Behavior test

Table 1 summarizes the statistical results of behavior tests. We used, as a main indicator, the sum of the number (the combined number) or the sum of the total time of head or body turning both in the clockwise rotation and in the anticlockwise rotation of the drum. The combined number of head turning, consistent with the direction of the clockwise and anticlockwise rotation of the black-and-white-striped drum at 2 rpm, was

significantly larger in the RCS rats with the dye-coupled film implantation in the time course of 8 weeks of the observation, compared with the rats with the plain film implantation, both in the small cage under the bright light and in the large cage under the dim light (P=0.0261 and P=0.0227, respectively, repeated-measure ANOVA, n=7, Fig. 3). The sum of the total time of head turning, consistent with the clockwise and anticlockwise rotation at 2 rpm, was significantly longer in the RCS rats with the dye-coupled film implantation, compared with the rats with the plain film implantation, both in the bright condition and in the dim condition (P=0.0319 and P=0.0036, respectively, repeated-measure ANOVA, n=7, Fig. 3).

The combined number of body turning and the sum of the total time of body turning, in the same direction of the drum rotation, did not show significant and consistent changes in the 8-week observation period between the RCS rats with dye-coupled film implantation and the rats with plain film implantation.

Immunohistochemistry and apoptosis

Rhodopsin staining in photoreceptor cells was residual both at the site of the dye-coupled film implantation and at the site of the plain film implantation (Fig. 4). The layer of PKC-α-stained rod bipolar cells was in the same thickness between the dye-coupled film implantation and the plain film implantation (Fig. 4).

Calbindin-stained horizontal cells and amacrine cells were observed in the same number between the dye-coupled film implantation and the plain film implantation (Fig. 4). Synaptophysin staining was noted in the residual outer plexiform layer and was fully preserved in the inner plexiform layer at the same level between the dye-coupled film and the plain film implantation (Fig. 4). There was no inflammatory cell, necrotic cell, or apparent gliosis or fibrosis in the retina of the eyes with the dye-coupled film or plain film implantation.

The number of apoptotic cells (Fig. 5B) was significantly smaller in the retinal area of RCS rats at the site of dye-coupled film implantation, compared with the number in the retinal area of RCS rats at the site of plain film implantation (P=0.03737, Mann-Whitney U-test, n=6, Fig. 5C). The number of apoptotic cells was not different between the dye-coupled film and the plain film implantation in the other two areas of the retina, at the site neighboring to the film implantation and at the site opposite to the film implantation across the posterior pole (Fig. 5C).

Discussion

The goals of this study were two fold: 1) to study the efficacy of the photoelectric dye-coupled film implantation in RCS rats by assessing their vision with behavior tests,

and 2) to study the safety of the dye-coupled film by immunohistochemistry of the rats' eyes after the completion of the 8-week course of the behavior tests. To this end, we increased the sensitivity of the dye-coupled film by increasing the density of the photoelectric dye molecules on the surface of the polyethylene film. The density of the dye molecules on the film surface could be increased twice by optimizing the dye-coupling reaction which was performed after fuming nitric acid treatment to induce carboxyl moieties on the film surface. In our previous study, we finished to determine the optimal duration of fuming nitric acid treatment which caused no damage to the film strength.[15]

The timing of film implantation was set at 6 weeks of the age in RCS rats since the rats at this age were in the adulthood and their eyes were large enough to allow the microsurgical manipulation. In addition, photoreceptor outer segments at this age of the RCS rats have almost degenerated but the other neurons remain basically intact.[16] The film implantation in the right place between the sensory retina and the retinal pigment epithelium was confirmed histologically after the behavior tests were completed in the time course of 8 weeks. Immunohistochemical staining at the age of 14 weeks, resulting from the implantation at the age of 6 weeks and the observation for 8 weeks, showed residual rhodopsin in the outer retina in the RCS rats with either dye-coupled film or

plain film implantation.

The behavior tests to observe head-turning of rats in the direction of a rotating black-and-white striped drum is a well-established standard method to assess rats' vision.[13,14] In the behavior tests of this study, we demonstrated that the RCS rats with the dye-coupled film implantation restored the vision in the course of 8 weeks of the observation after the surgery at the age of 6 weeks, compared with the rats with the plain film implantation. In this study, two movements of rats were observed in the behavior tests: head-turning and body-turning in the direction, consistent with the clockwise and anticlockwise rotation of the black-and-white-striped drum. The number of each movement was counted and the total time of the specified movement was also calculated. Furthermore, we set, as a main indicator, the combined number and the sum of the total time for each movement both in the clockwise rotation and in the anticlockwise rotation of the drum. In these procedures, we aimed to elucidate which of the number or the total time of head-turning or body-turning would be a better indicator for assessing the vision in the behavior tests. In this study, the number and the total time of head-turning showed significant differences in the course of the behavior tests, suggesting that the number and the total time of head-turning would a good indicator for the vision assessment in rats. This finding was consistent with previous studies on the

behavior tests which utilized the number of head-turning as a sole indicator. [13,14]

The speed of the rotation of the black-and-white-striped drum was set at 2 rpm or 4 rpm, based on the previous reports.[13,14] The present results suggest that the slower speed at 2 rpm would be better, than the faster speed at 4 rpm, for assessing the rats' vision. We also did the behavior tests at two levels of brightness, at 150 lux under the ceiling light in a usual room and at 50 lux obtained with a light source placed lower on the floor (Fig. 2). We assumed that behavior tests in the dim condition might assess the vision of rats more effectively since the rat is a nocturnal animal. We also assumed that the dye-coupled film with higher density of dye molecules would have sensitivity enough to stimulate retinal neurons in response to the light intensity at 50 lux. The present results of the behavior tests supported these lines of assumption.

We used either a small cage or a large cage with the transparent wall in the behavior tests in the bright condition since the distance between the cage wall and the rotating drum would influence the behavior of rats. A larger space of the cage would allow rats to move around randomly and might lead to difficulty in determining the head-turning or body movement, consistent with the direction of the drum rotation. Based on our observation in the present study, the smaller cage in the bright condition would be better to assess the vision of rats by the behavior tests.

In our previous study, we demonstrated that dye-coupled film and plain film implantation in the subretinal space of rats' eyes did not induce inflammation or necrosis.[11] We also finished to design the surface of polyethylene films to cause less gliosis.[11] In this study with immunohistochemical analyses, antibodies to identify specific types of retinal neurons were used to study the effect of the dye-coupled film and the plain film on the integrity of the retinal layered structure of neurons. Neural remodeling is known to take place in the degenerated retina of RCS rats.[17] A hypothesis we made at the beginning of this study was that the dye-coupled film might influence the neural network in the degenerating retina. To test the hypothesis, synaptophysin, expressed at synaptic ends, was stained to reveal possible changes in the neural network in the degenerating retina. In the course of 8 weeks of the implantation, the retinal structures were not different between the eyes with dye-coupled film implantation and plain film implantation. A longer period of observation, more than 8 weeks, would be necessary to detect the supposed difference in neural connections.

The number of apoptotic cells at the site of the film implantation was surprisingly reduced in the eyes with the dye-coupled film implantation, compared with the plain film implantation. The results were consistent with our previous study in which the photoelectric dye in itself prevented retinal cells from death in mixed culture of retinal

neurons and glial cells in vitro.[12] Since the RCS rats were housed in the 12-hour-each light and dark cycle, electric output from the dye-coupled film in response to light would stimulate retinal neurons and would rescue the cells from apoptosis. The results of both the previous in vitro cell culture study[12] and the present in vivo rats study suggest that the photoelectric dye, used for our retinal prosthesis, might have a cell-rescuing effect by unknown mechanism. The difference in color, reddish versus whitish, between the dye-coupled film and the plain film, would not influence the results since no marked changes of neurons were noted immunohistochemically.

Conclusions

The density of photoelectric dyes coupled to the surface of polyethylene film was increased twice in comparison with the prototype. This high-density dye-coupled film was made of refined polyethylene powders, and thus, would be used for clinical studies in future. The vision of RCS rats with the dye-coupled film implantation became significantly better in the 8-week course of the behavior tests, compared with rats with the plain film implantation. The retinal layered structures were preserved after the implantation of the dye-coupled film and plain film. Surprisingly, apoptosis was significantly reduced at the site of the dye-coupled film implantation, compared with the

plain film implantation, suggesting that the photoelectric dye would have a protective effect on the retinal neurons.

These results in the present study, altogether, support the efficacy and safety of the photoelectric dye-coupled polyethylene film in RCS rats, and would serve as basic requirements for future clinical studies. A big advantage of the photoelectric dye-coupled film, as a medical device, is that the same film could be cut in a different size to fit with the size of the eyeball. In this context, experiments with larger animals are not necessarily required since the same machine or appliance, namely, the dye-coupled film, could be used for rats and also for patients. The indication of the photoelectric dye-coupled polyethylene film, as retinal prosthesis, has to be determined beforehand in future clinical studies involving patients with retinitis pigmentosa.[3,18,19]

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Fig. 1 **(A)** Plain polyethylene film (plain film, whitish) and photoelectric dye-coupled polyethylene film (dye-coupled film, reddish). **(B)** Ultraviolet and visible light absorption spectra of dye-coupled film obtained by reaction solvent containing 4×10^{-5} mol photoelectric dye molecules. The absorption ranges from 400 to 600 nm with the maximum absorption peak at 539 nm. **(C)** Relation between the maximum absorption of the dye-coupled film and the concentration of dye molecules in reaction solvent, chlorobenzene. The increase of dye concentration from 1 to 4×10^{-5} mol in 75 mL of chlorobenzene results in 2-fold increase of absorption by the film.

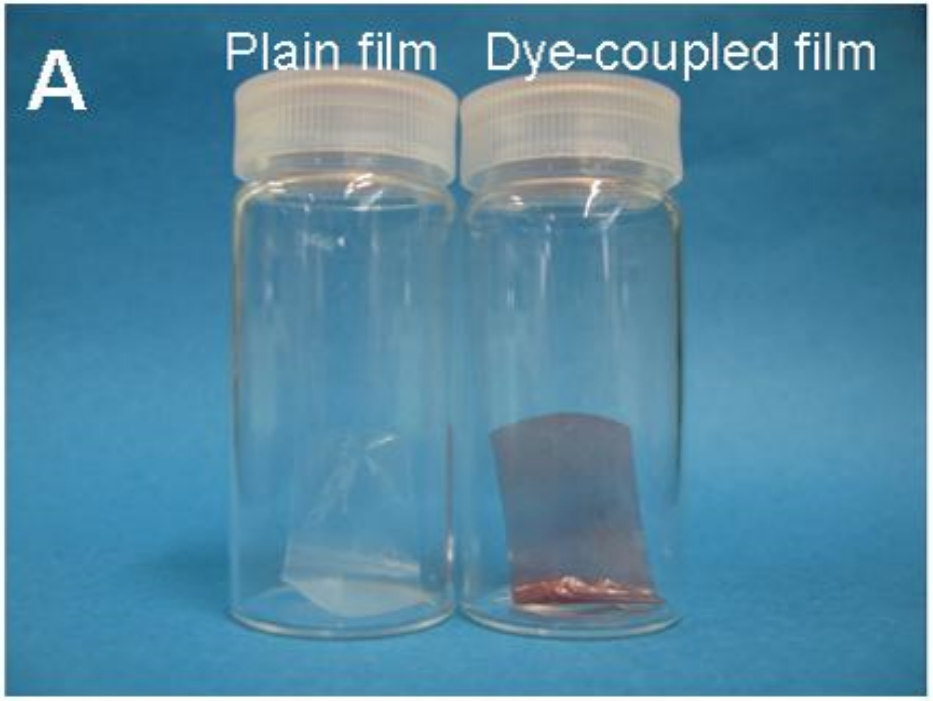
Fig. 2 Photographs of behavior tests in RCS rats. A black-and-white-striped drum with the diameter of 40 cm is rotated around a transparent-walled round cage with the diameter of 20 or 30 cm at the speed of 2 or 4 rounds per minutes (rpm) in the dim (50 lux) or the bright (150 lux) condition. The rat's behavior is recorded from above by a digital videocamera (A). Only a large cage is used in the dim condition (B) while a large cage (C) and a small cage (D) are used in the bright condition.

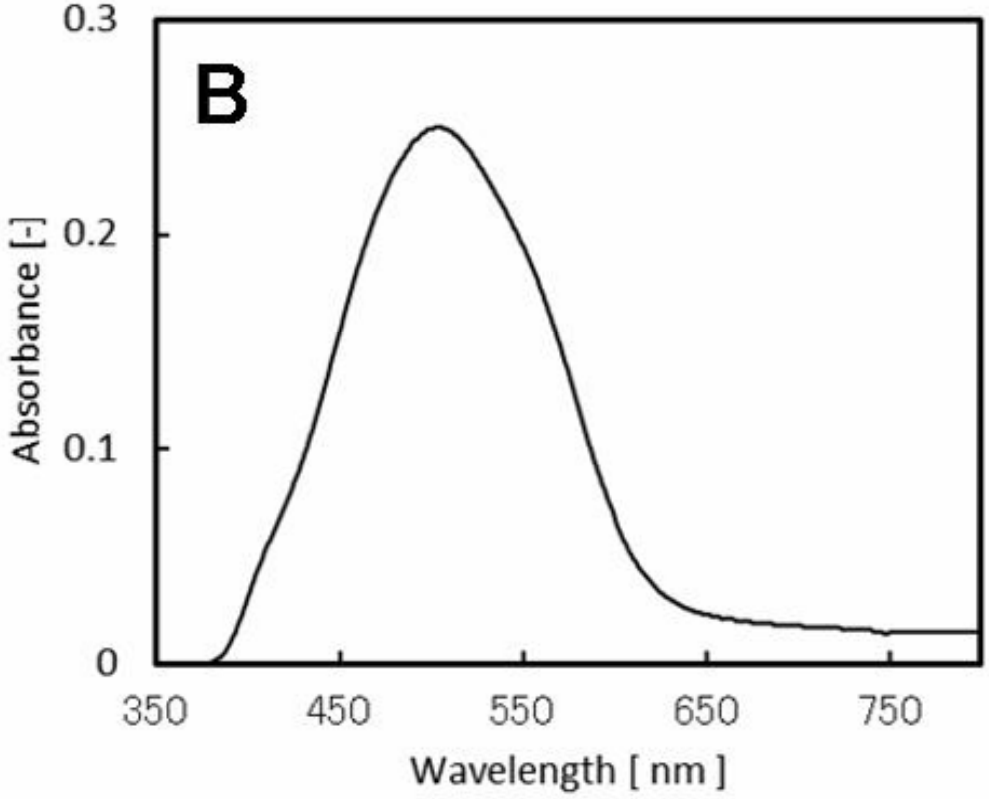
Fig. 3 Results of behavior tests in RCS rats with dye-coupled film or plain film implantation in the 8-week time course of observation. The combined number and the

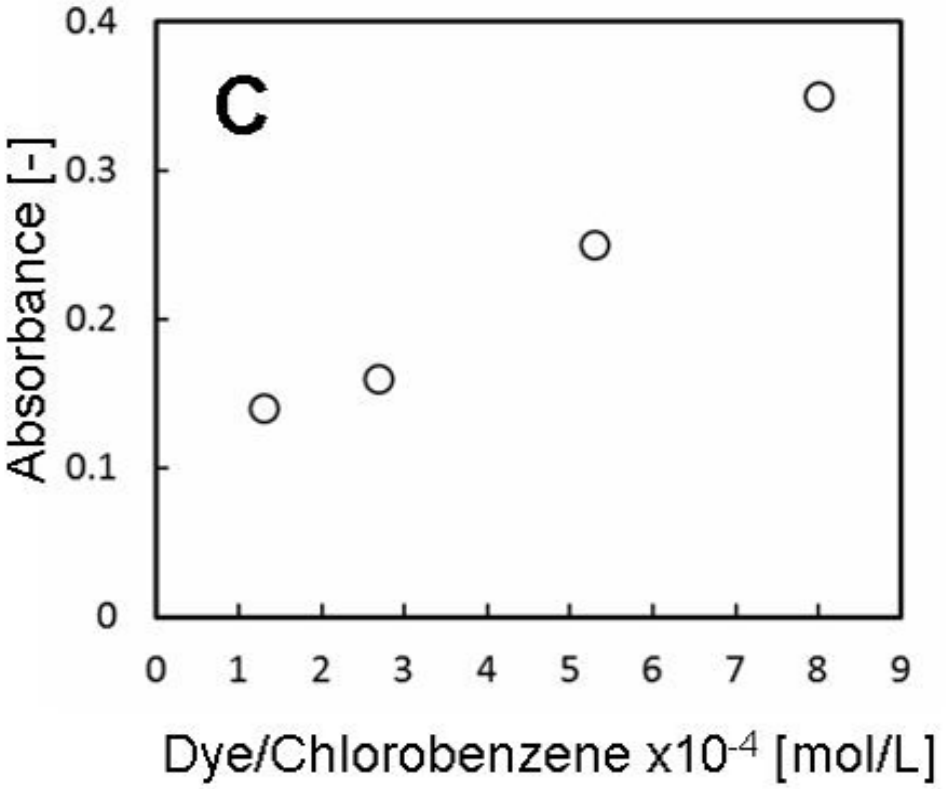
sum of the total time for head-turning, consistent with the direction of the clockwise and anticlockwise rotation of the black-and-white-striped drum at 2 rpm, are significantly larger and longer in RCS rats with dye-coupled film implantation, compared with rats with plain film implantation, in a small cage under the bright light at 150 lux (P=0.0261 and P=0.0319) and in a large cage under the dim light at 50 lux (P=0.0227 and P=0.0036, repeated-measure analysis of variance, n=7). T bars indicate standard deviation.

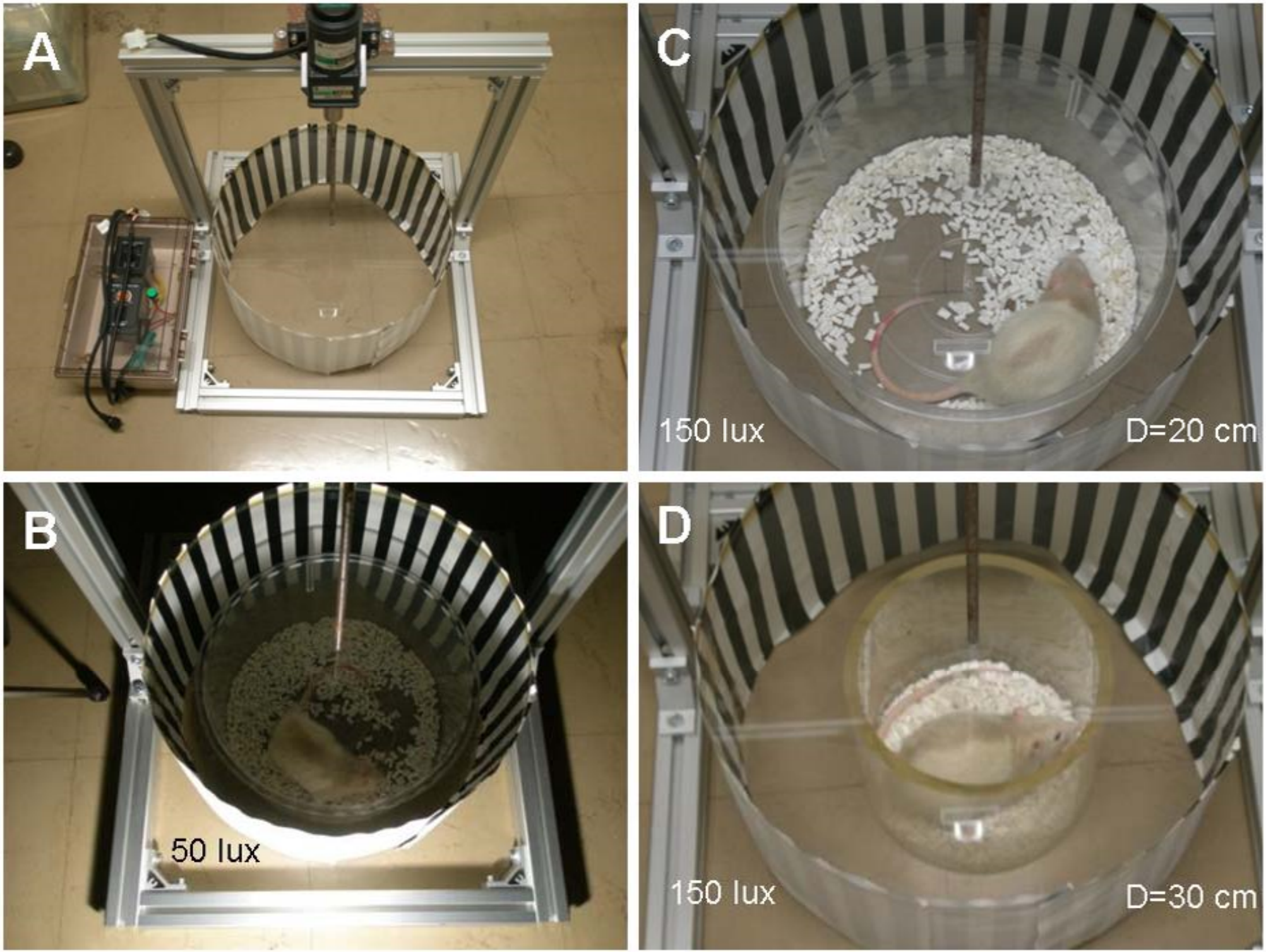
Fig. 4 Immunohistochemical staining in the retina of RCS rats' eyes with subretinal implantation of plain film or dye-coupled film. Rhodopsin to stain photoreceptor cells, protein kinase C-α (PKC-α) to stain rod bipolar cells, calbindin to stain horizontal cells and amacrine cells, and synaptophysin to stain inner plexiform and outer plexiform layer. Note autofluorescence of dye-coupled film. There is no difference in neuronal layers of retinal structure between the dye-coupled film and plain film implantation. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. DAPI filter color scale was converted to FITC filter color scale. DAPI filter color scale was used since autofluorescence of dye-coupled film was at a lower level with this filter than the other filter sets. Bar = 50 μm.

Fig. 5 Apoptotic cell detection in the retina of RCS rats' eyes with subretinal implantation of plain film or dye-coupled film. **(A)** Schematic drawing to show three areas in a retinal section to count apoptotic cells: area apposed to the film designated as "film", area neighboring to the film designated as "near", and area opposite to the film insertion across the posterior pole designated as "opposite". **(B)** Apoptotic cells (arrowheads) in retinal sections with dye-coupled film or plain film implantation. INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar = 50 μ m. **(C)** The number of apoptotic cells in each area. At the site of film implantation, the number of apoptotic cells is significantly smaller in dye-coupled film implantation, compared with plain film implantation (**P*=0.03737, Mann-Whitney U-test, n=6). There is no significant difference in the number of apoptotic cells at the sites neighboring or opposite to the film implantation between the dye-coupled film and plain film. A box with a bar indicates the upper 75 percentile and lower 25 percentile, with a median, maximum, and minimum.

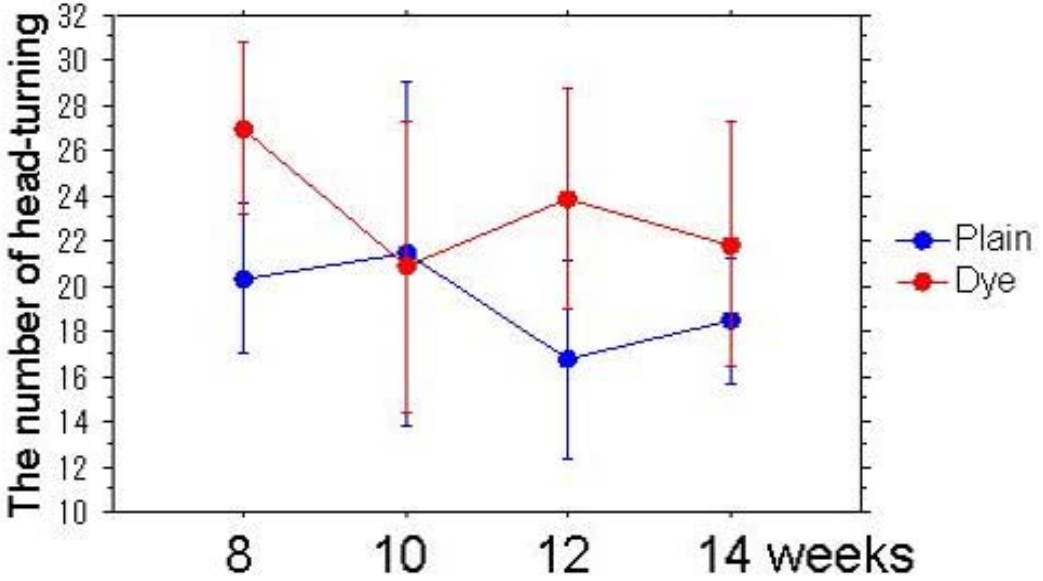

A
Plain film
Dye-coupled film


B
0.3
0.2
0.1
0
Absorbance [-]
350
450
550
650
750
Wavelength [nm]

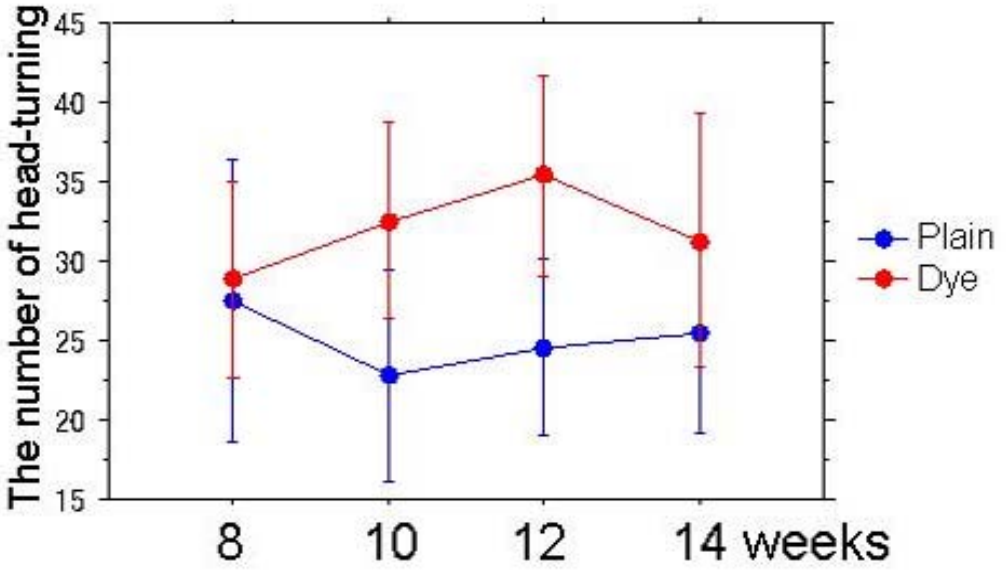

C
0.4
0.3
0.2
0.1
0
Absorbance [-]
0
1
2
3
4
5
6
7
8
9
Dye/Chlorobenzene x10-4 [mol/L]

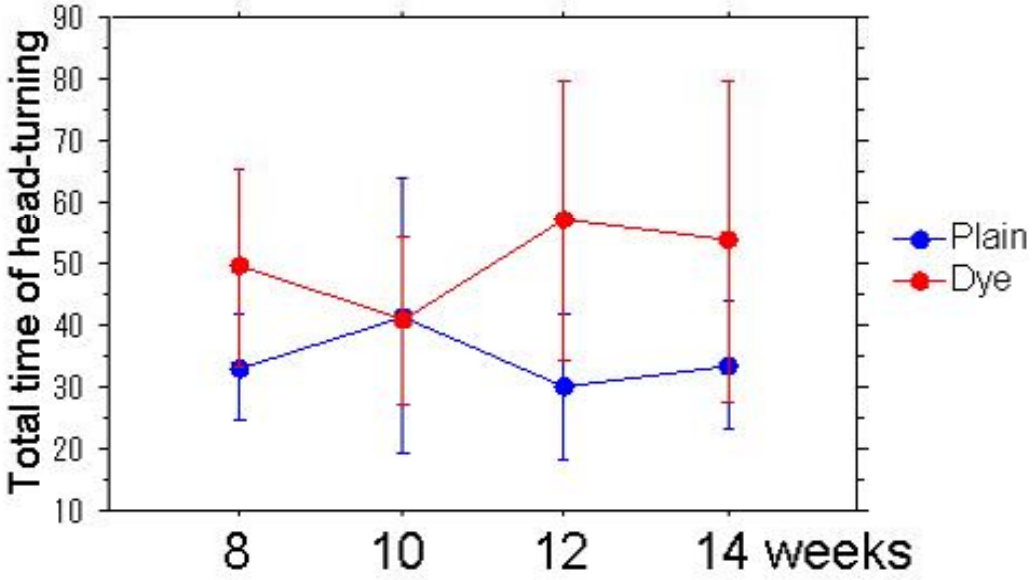

A
C
150 lux
D=20 cm
B
50 lux
D
150 lux
D=30 cm

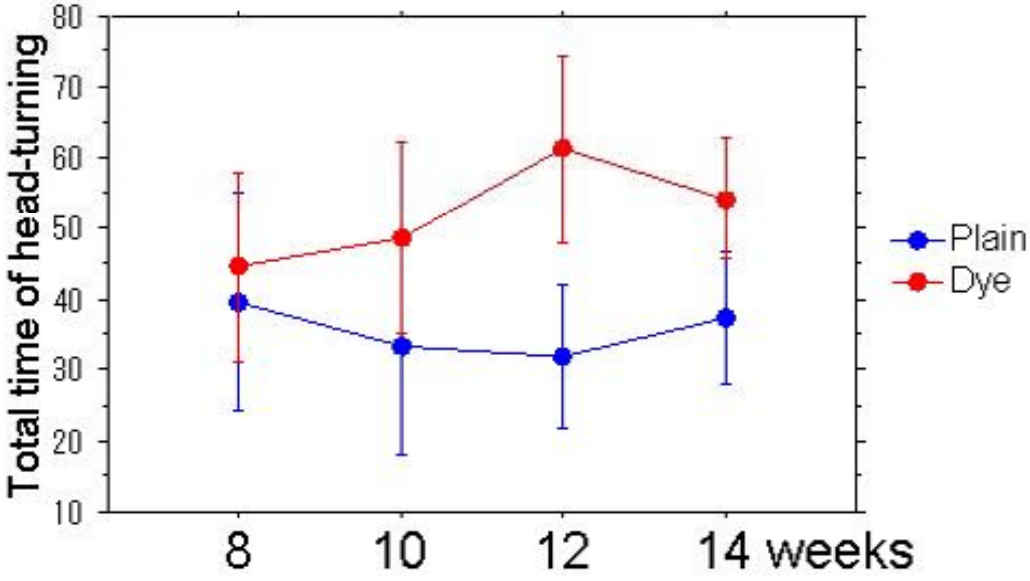
Bright in a small cage at 2 rpm

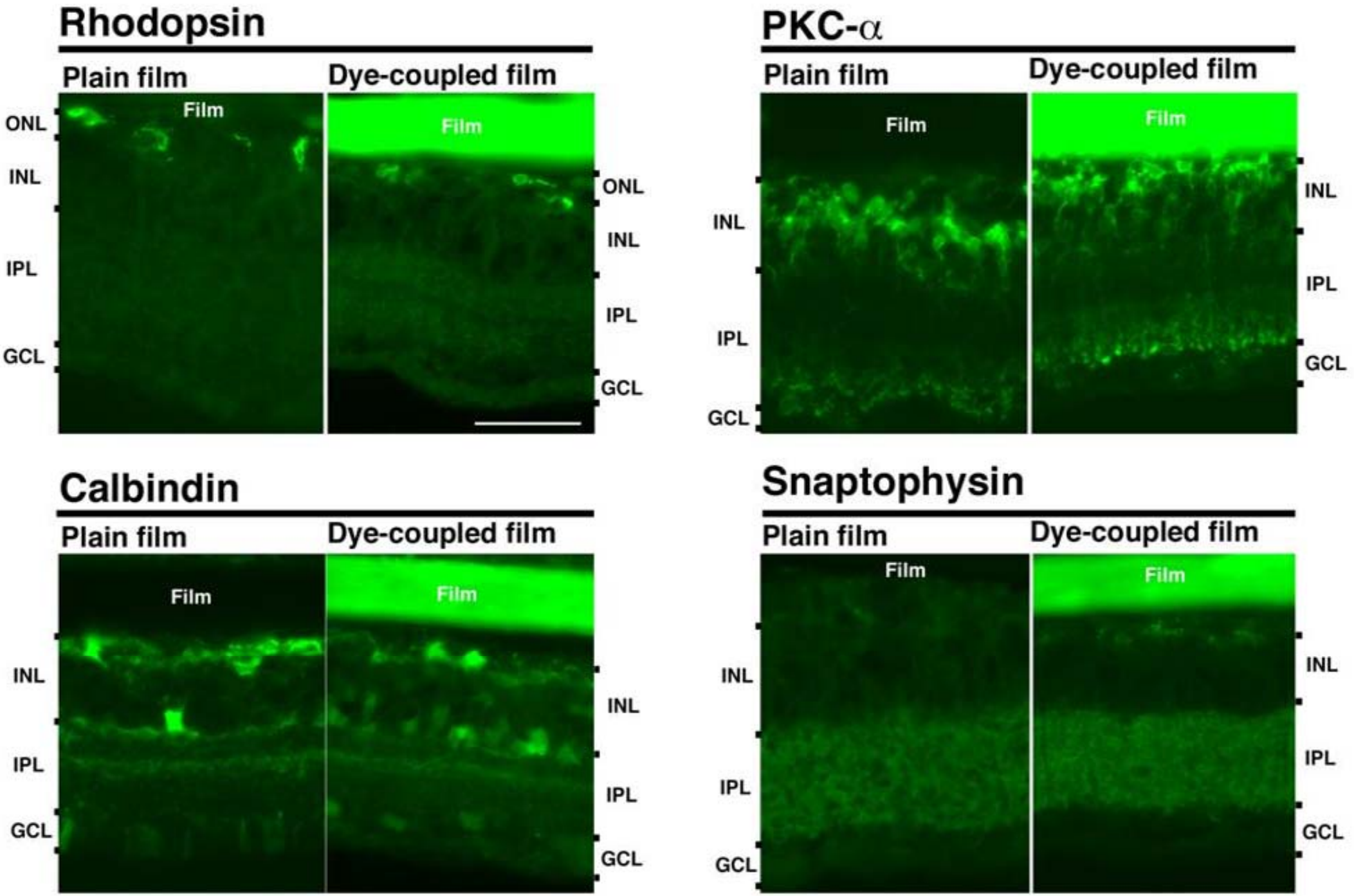


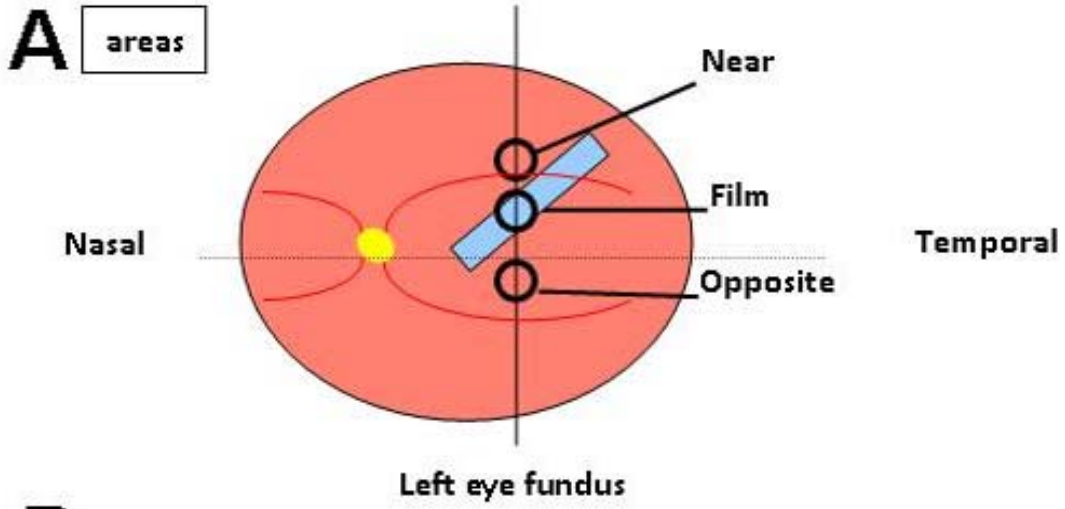
Dim in a large cage at 2 rpm

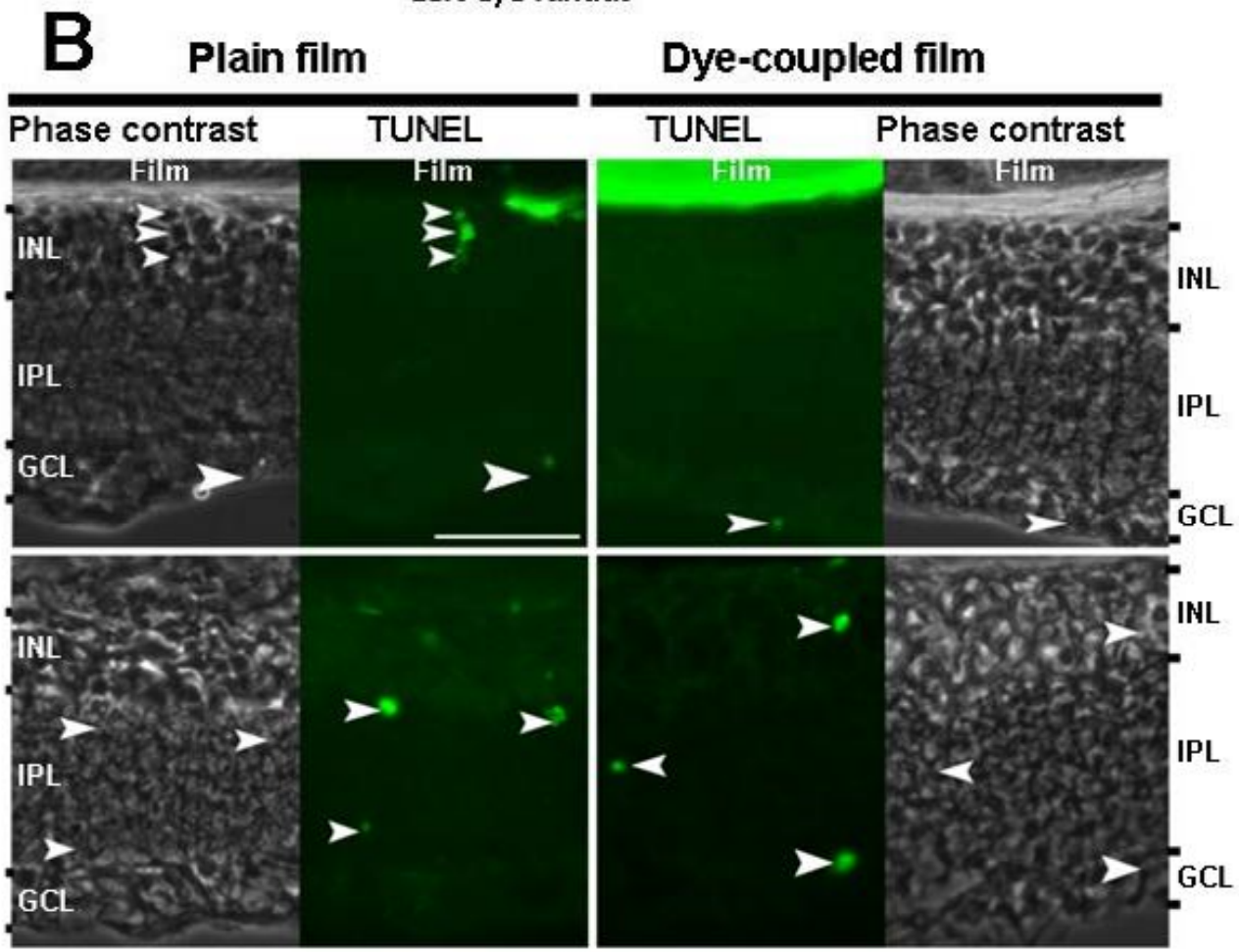







Rhodopsin
Plain film
Dye-coupled film
Film
Film
ONL
INL
IPL
GCL
ONL
INL
IPL
GCL
PKC-α
Plain film
Dye-coupled film
Film
Film
INL
IPL
GCL
INL
IPL
GCL
Calbindin
Plain film
Dye-coupled film
Film
Film
INL
IPL
GCL
INL
IPL
GCL
Snaptophysin
Plain film
Dye-coupled film
Film
Film
INL
IPL
GCL
INL
IPL
GCL


A
areas
Near
Film
Opposite
Nasal
Temporal
Left eye fundus


B
Plain film
Dye-coupled film
Phase contrast
TUNEL
TUNEL
Phase contrast
Film
INL
IPL
GCL

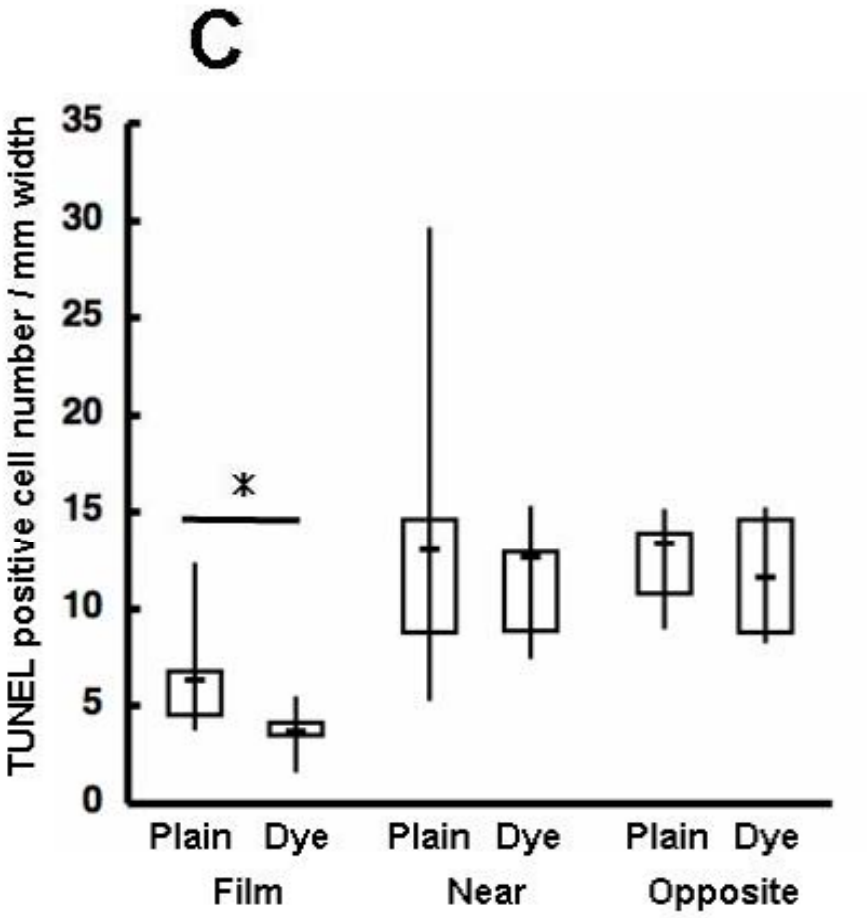

C
TUNEL positive cell number / mm width
35
30
25
20
15
10
5
0
*
Plain
Dye
Film
Near
Opposite

Table 1 Statistical analysis of behavior tests in the time course of 8 weeks, comparing RCS rats with dye-coupled film implantation with those with plain film implantation.

	Head turning				Body turning			
	The number		Total time		The number		Total time	
	P value for							
	Class	Time course	Class	Time course	Class	Time course	Class	Time course
Bright at 150 lux in a large cage								
Clockwise rotation at 2 rpm	0.3667	0.1083	0.3033	**0.0056**	0.8803	0.0902	0.7280	0.1871
Anticlockwise rotation at 2 rpm	0.2972	0.7249	**0.0462**	0.7172	0.9218	**0.0107**	0.6181	**0.0015**
Clockwise rotation at 4 rpm	0.6916	0.0718	0.5030	0.1935	0.9142	0.8625	0.7797	0.8005
Anticlockwise rotation at 4 rpm	0.7966	**0.0202**	0.3922	**0.0206**	0.2906	**0.0378**	0.8577	**0.0010**
Clockwise+Anticlockwise at 2 rpm	0.2007	0.2257	**0.0407**	0.1583	0.8321	**0.0040**	0.5345	**0.0083**
Clockwise+Anticlockwise at 4 rpm	0.7107	**0.0037**	0.3686	**0.0103**	0.5436	0.5906	0.7675	**0.0344**
Bright at 150 lux in a small cage								
Clockwise rotation at 2 rpm	**0.0428**	0.0572	0.0892	0.5324	0.9463	0.0639	0.5100	0.4356
Anticlockwise rotation at 2 rpm	0.1259	0.5807	**0.0390**	0.9515	0.8112	0.6519	0.5196	0.1075
Clockwise rotation at 4 rpm	0.1659	0.5452	0.1058	0.3364	0.0704	0.9763	0.6000	0.1620
Anticlockwise rotation at 4 rpm	0.4006	0.6436	0.5572	0.8226	0.2816	0.3133	0.2104	0.9212
Clockwise+Anticlockwise at 2 rpm	**0.0261**	0.1854	**0.0319**	0.9485	0.8458	0.1295	0.4644	0.1575
Clockwise+Anticlockwise at 4 rpm	0.1785	0.4639	0.2895	0.7257	0.0593	0.8545	0.2659	0.3608
Dim at 50 lux in a large cage								
Clockwise rotation at 2 rpm	0.1475	0.6097	**0.0404**	0.7864	0.8791	0.3398	0.9331	0.6260
Anticlockwise rotation at 2 rpm	**0.0297**	0.4053	**0.0092**	0.2044	0.8742	0.4271	0.9101	0.6587

Clockwise rotation at 4 rpm	0.6108	0.1781	0.7206	0.0804	0.1141	0.5915	0.0648	0.7881
Anticlockwise rotation at 4 rpm	0.8625	0.7860	0.1012	0.2246	0.0615	0.7026	**0.0047**	0.7321
Clockwise+Anticlockwise at 2 rpm	**0.0227**	0.7069	**0.0036**	0.4579	0.7639	0.1657	0.8478	0.4177
Clockwise+Anticlockwise at 4 rpm	0.6568	0.1446	0.3008	**0.0199**	**0.0060**	0.4279	**0.0016**	0.8624

P values for repeated-measure analysis of variance (ANOVA). “Class” means the difference between the RCS rats with dye-coupled film implantation and the rats with plain film implantation. “Time course” means the difference in the time course of 8 weeks with 4 time points of behavior tests. “Clockwise+anticlockwise” indicates the sum of the number or total time in clockwise rotation and anticlockwise rotation. Significant differences ($P<0.05$) are highlighted with bold font.