Heliyon 5 (2019) e01936

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

Vision evaluation by functional observational battery, operant behavior test, and light/dark box test in retinal dystrophic RCS rats versus normal rats

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ARTICLE INFO

Keywords:
Bioengineering
Neuroscience
Physiology
Zoology

ABSTRACT

Background: Vision plays a key role in some behavior tests for rats. Okayama University-type retinal prosthesis (OURePP) is a photoelectric dye-coupled polyethylene film which generates electric potential in response to light and stimulates nearby neurons. This study aims to assess vision in retinal dystrophic (RCS) rats, in comparison with normal rats, by selected behavior tests. We also examined whether the tests could detect vision changes in RCS rats with dye-coupled film implantation.

Methods: Data sets were 5 normal rats, 4 untreated RCS rats, 7 RCS rats with dye-coupled films implanted at the age of 7 weeks after excluding unsuccessful implantation at autopsy. Behavior tests chosen were landing foot splay and visual forelimb-placing response in the menu of functional observational battery, operant-conditioning lever-press response and light/dark box test.

Results: Normal visual placing response was significantly less frequent in untreated RCS rats at the age of 9 and 11 weeks, compared with normal rats ($P = 0.0027$, chi-square test) while normal response was significantly more frequent at the age of 9 weeks in RCS rats with dye-coupled film implantation, compared with untreated RCS rats ($P = 0.0221$). In operant-conditioning lever-press test, the correct response rate was significantly lower in untreated RCS rats than in normal rats at the age of 9 weeks ($P < 0.05$, Tukey-Kramer test) while the rate was not significantly different between normal rats and RCS rats with dye-coupled film implantation. In light/dark box test, the time to enter dark box was significantly shorter in normal rats, compared with untreated RCS rats or RCS rats with dye-coupled film implantation ($P < 0.05$, Tukey-Kramer test).

Conclusions: Behavior tests of functional observational battery, operant-conditioning lever-press response and light/dark box test discriminated vision between normal rats and RCS rats. The visual placing response and operant-conditioning lever-press test might have sensitivity to detect vision recovery in RCS rats with OURePP implantation.

1. Introduction

Hereditary retinal dystrophy has been known in humans and animals such as mice [1], rats [2, 3], and dogs [4]. Visual cells (photoreceptors) become dead due to hereditary molecular errors, and hence the vision has been lost gradually. Blind patients and blind animals with hereditary retinal diseases, such as retinitis pigmentosa [5, 6], have dead photoreceptor cells, but the other retinal neurons, retinal bipolar cells and ganglion cells, the latter of which send axons to the brain, remain alive [7]. The basic concept of retinal prostheses is to stimulate surviving retinal neurons with electric current outputted from artificial devices in response to light. These living retinal neurons in the degenerative retina are expected to send signals to the brain, and thus, the vision would be restored [8].

Okayama University-type retinal prosthesis (OURePP) is a novel type of retinal prostheses, so called photoelectric dye-coupled thin film retinal prosthesis [9, 10, 11, 12, 13, 14]. Stable photoelectric dye molecules with absorption spectrum of visible light [15, 16, 17] were chemically coupled

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https://doi.org/10.1016/j.heliyon.2019.e01936
Received 18 January 2019; Received in revised form 5 May 2019; Accepted 6 June 2019

to polyethylene film surface. The dye-coupled film generates electric potential in response to light, and displacement current stimulates nearby neuronal cells to induce action potential [18]. The dye-coupled film, implanted in subretinal space of the eye globe, serves as a light-receiver and a potential-generator, and thus, replaces the function of dead photoreceptor cells in retinal dystrophy to send signals to the brain via living retinal bipolar cells, ganglion cells and their axons as optic nerve fibers.

In our previous study, the dye-coupled films were implanted subretinally in retinal dystrophic rats, Royal College of Surgeons (RCS) rats [2, 3], to prove vision recovery by a behavior test of head-turning to the same direction in response to a rotating black-and-white striped drum around each rat [19, 20]. The vision recovery in RCS rats with dye-coupled film implantation was also shown by electroretinographic [20] and visual evoked potential recording [21].

In this study, we selected different behavior tests in rats from the viewpoint that the vision would be used as a clue: landing foot splay test and visual forelimb-placing response test in the menu of functional observational battery, operant-conditioning lever-press response test, and light/dark box test. These common behavior tests were used to examine the vision in RCS rats which were confirmed to have no vision by flat waves at electroretinographic recording, in comparison with normal rats. In addition, we tried to assess whether these behavior tests would detect vision changes in RCS rats with dye-coupled film implantation.

2. Methods

2.1. Preparation of dye-coupled polyethylene film

Thin films were made from polyethylene powder and exposed to fuming nitric acid to introduce carboxyl moieties on the film surface. Photoelectric dye molecules, 2-[2-[4-(dibutylamino)phenyl]ethenyl]-3-carboxymethylbenzothiazolium bromide (NK-5962, Hayashibara, Inc., Okayama, Japan), were coupled to carboxyl moieties of the polyethylene film surface via ethylenediamine, as described previously [11, 12, 19]. The fuming nitric acid-treated only polyethylene film and the photoelectric dye-coupled polyethylene film were designated as the plain film and the dye-coupled film, respectively. Films were manufactured in quality management system at a clean-room facility in Okayama University Incubator. No toxicity of the dye-coupled film was proven in all tests for biological evaluation of medical devices, based on International Standard ISO 10993.

2.2. Animals and surgery

Five male normal Wistar rat (RCS/jcl-+/+, specific pathogen-free, CLEA Japan, Tokyo) and 16 male retinal dystrophic rats (RCS/jcl-rdy/rdy) were obtained at the age of 3 weeks. The study consisted of 4 groups of rats: 5 normal rats, 4 retinal dystrophic rats with no intervention, 4 dystrophic rats with plain films implanted in both eyes, and 8 dystrophic rats with dye-coupled films implanted in both eyes. Films were implanted in eyes of rats at the age of 7 weeks. This study was approved by the Animal Care and Use Committee at Ina Research, Inc., and also by the Committee in Okayama University, based on the Animal Welfare and Management Act in Japan. Ina Research, Inc. was accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International.

Rats were anesthetized by intraperitoneal injection of a mixture (5 ml/kg of body weight) of medetomidine (0.15 mg/kg of body weight, Domitor 1 mg/ml, Nippon Zenyaku Kogyo Co., Koriyama, Japan), midazolam (2 mg/kg of body weight, Dormicum 5 mg/ml, Astellas Pharma Inc., Tokyo, Japan), and butorphanol tartrate (2.5 mg/kg of body weight, Betorphal, 5 mg/ml, Meiji Seika Pharma, Tokyo). Mydriasis in both eyes was induced by 0.5% tropicamide and 0.5% phenylephrine eye drops (Mydrin-P, Santen Pharmaceutical, Osaka, Japan) on the day of surgery. Topical anesthesia was further obtained with 4% lidocaine (Xylocaine Ophthalmic Solution, AstraZeneka, London, UK).

Under a dissecting microscope, the conjunctival incision was made on the temporal side of the eye and the sclera was tapped with a microsurgery knife (Straight/Stab 22.5°, Kai Medical, Seki, Japan). Drops of 0.5% levofloxacin (Cravit, Santen Pharmaceutical) were instilled to the scleral-choroidal puncture to induce a bleb retinal detachment [14]. A sheet of either dye-coupled film or plain film in the size of 1 × 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. 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. Sedation was reversed with subcutaneous injection of x10-saline-diluted atipamezole (0.1 ml/body, Antisedan 0.5 mg/ml, Nippon Zenyaku Kogyo Co, Koriyama, Japan). Rats were given subcutaneous injection of meloxicam (0.01 ml/body, Metacam 0.5%, Boehringer Ingelheim, Ingelheim am Rhein, Germany) as a non-steroidal anti-inflammatory drug for 3 days after surgery, and subcutaneous injection of enrofloxacin (0.03 ml/body, Baytril 2.5%, Bayer Animal Health) on surgery day and for 5 days after surgery. Postoperative instillation of 0.5% levofloxacin (Cravit, Santen Pharmaceutical) and 1% atropine (Nitten Pharmaceutical, Nagoya, Japan) once daily was continued postoperatively until sacrifice. Each rat was housed in a standard rat cage in a room with the 12-hour-each light and dark cycle, and was observed to be healthy after the surgery, based on eating and drinking habit.

After the experiments, all animals were sacrificed with bleeding after inhalation of isoflurane (Mylan Pharmaceuticals, Canonsburg, PA, USA), and the eyes were enucleated, fixed with phosphate-buffered 1% formaldehyde and 2.5% glutaraldehyde, stored in 10% neutral-pH formalin, and embedded in paraffin. Paraffin sections were cut to examine whether films were implanted in the subretinal space of each eye.

2.3. Electroretinographic recording

Electroretinographic recording was performed to confirm the presence of vision in normal rats and the absence of vision in RCS rats. Electroretinograms in both eyes of all rats were recorded at 6 weeks of the age before film implantation, at 9 weeks and 11 weeks of the age, 2 weeks and 4 weeks, respectively, after film implantation [20]. Rats were placed overnight in a dark room for dark adaptation. Rats were anesthetized as above and placed on a heating pad, set at 37 °C. After mydriasis, a contact lens electrode with white light-emitting diode (LED) was placed on the corneal surface, with no air bubble trapped between the cornea and the contact lens, a reference electrode was put into the mouth, and an earth clip was placed along the tail. Rod response (dark-adapted 0.01 ERG with 1,000 cd/m^2 x 10 μsec), maximal response (dark-adapted 10 ERG with 5,000 cd/m^2 x 2 msec), and single-flash cone response (light-adapted 3.0 ERG with 1,000 cd/m^2 x 3 msec) were sequentially recorded at the interval of 90 seconds, based on the International Society for Clinical Electrophysiology of Vision (ISCEV) standards (PuREC and LED Visual Stimulator LS-100, Mayo Corporation, Aichi, Japan).

2.4. Functional observational battery

Visual forelimb-placing response and landing foot splay were tested at three time points: 4 weeks of the age (before surgery), 9 weeks of the age (2 weeks after film implantation), and 11 weeks of the age (4 weeks after film implantation) [22, 23, 24, 25, 26, 27, 28]. As visual placing response, with the tail of a rat was held up, the rat was neared to the flat surface of a table from about 15 cm (Fig. 1A). The response of a rat was observed whether to raise the head up and to straighten the forelimbs forward before the beard had contact with the table surface. At landing foot splay test, the palms of hind limbs of a rat were stained with dye and a rat was fallen from 30 cm above the table surface (Fig. 1B). At the

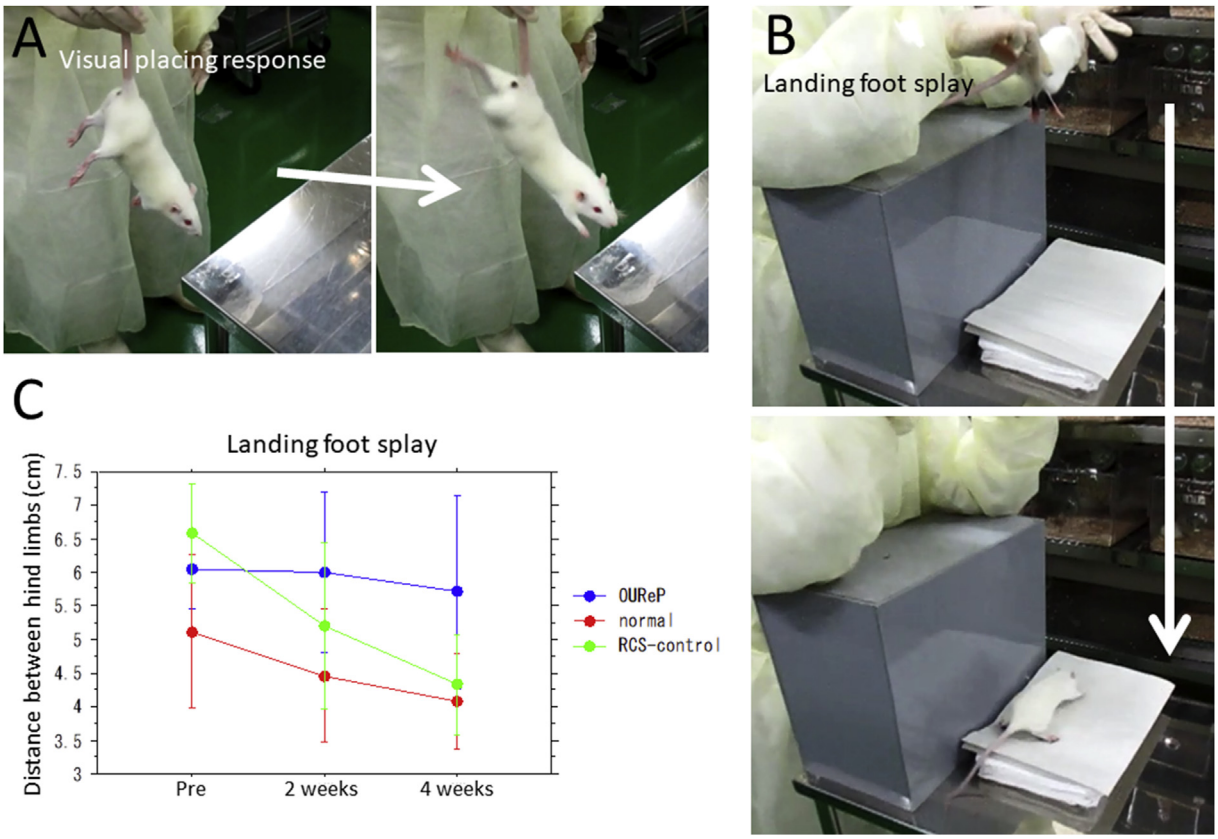

Fig. 1. Visual forelimb-placing response and landing foot splay in functional observational battery. A. Visual forelimb-placing response. A Rat held with the tale is neared from 15 cm away toward the edge of a table and the response of head positioned up and forelimbs straightened is observed. B. Landing foot splay test. A Rat with hind limb palms stained with dye is fallen from 30 cm high and the distance between the palms are measured. C. Landing foot splay measurements at 4 weeks of the age (pre-surgery), 9 weeks of the age (2 weeks after film implantation), and 11 weeks of the age (4 weeks after film implantation) in 5 normal rats (normal), 4 untreated retinal dystrophic (RCS) rats (RCS-control), and 7 retinal dystrophic (RCS) rats with dye-coupled film implantation (OUReP). Normal rats had tendency to show narrower distance between hind limbs, compared with untreated RCS rats or RCS rats with dye-coupled film implantation, at the time points of pre-implantation and 4 weeks after film implantation ($P = 0.0558$ and $P = 0.0514$, respectively, one-factor ANOVA, no significance at post-hoc test by Tukey-Kramer test).

landing, the distance between the hind limbs was measured.

2.5. Operant-conditioning lever-press response test

The operant behavior tests [29, 30] were done at the age of 7 weeks (before surgery), 9 weeks (2 weeks after film implantation), and 11 weeks (4 weeks after film implantation). An operant test chamber (Fig. 2A, 241 mm width x 305 mm depth x 292 mm height, ENV-007, Med Associates Inc., Fairfax, VT, USA) was placed inside a soundproof chamber (Fig. 2B, 670 mm width x 600 mm depth x 560 mm height) with 100 lux light and a ventilating fan. Two sets of a lever associated with a lamp, and a dish for food pellet (Dustless Precision Pellets, Rodent, Purified, 45 mg, Bio-Serv, Flemington, NJ, USA) were placed on the inner wall of the test chamber, and a pellet dispenser was placed outside the chamber (Fig. 2A). Experimental control and data recording were accomplished by a software (ATTN, rat visual, Ina Research, Inc.).

A rat was placed inside the test chamber and 5 food pellets were dispensed on a food dish. After 15 minutes, the rat was confirmed to eat all pellets. One set of a lever with a lamp was placed on either right side or left side of the inner wall for training. At reinforcement schedule of a fixed-ratio 1 (FR1), one pellet was dispensed when a rat pressed a lever with a lamp on. After the rat got the pellet, the lamp was turned off for a time-out period of 20 seconds in which lever-pressing got nothing. A session of training ended when a rat got 20 pellets or the time passed for 15 minutes. Repeat sessions of training were allowed within a day. The position of a lever with a lamp was changed reciprocally either on the right side or on the left side of the wall after each session of training. Some rats were at first presented to a pellet which was placed directly on the lever. After a rat got 20 pellets within 15 minutes, a fixed-ratio was increased from 1 to 3 (three times lever-pressing to get one pellet), and then to 5 (five times lever-pressing to get one pellet), and finally to 10 (10 times lever-pressing to get one pellet) to elucidate an optimal fixed-ratio for each rat. Criteria for acquisition of lever-pressing behavior was based on the fact that one session of getting 20 pellets within 15 minutes was repeated consecutively at least twice by pressing a lever with a lamp on, which was placed reciprocally either on the right side or on the left side of the wall.

Two sets of a lever with a lamp were placed on both sides of the wall for the operant behavior test. A lamp on either side was randomly put on for 30 seconds. As correct response, a rat could get a pellet when the rat pressed a lever with a lamp on for consecutive three times. As incorrect response, a rat could not get a pellet when the rat pressed a lever with a lamp off. In the case of incorrect response, a put-on lamp associated with another lever was turned off. As no response, rat could not get a pellet when the rat did neither press a lever with a lamp on nor press a lever with a lamp off. After a time-out period of 20 seconds, the lamp on either side was turned on for next trial. If a lever was pressed when the lamp was off, 20-second time-out period was reset and the lamp was put on further 20 seconds later. One session of the test consisted of 20 trials in which a lamp on the right side or a lamp on the left side was put on at an equal rate. One session of the test ended when a rat got 20 pellets or the time passed for 20 minutes. The maintenance training was done about 3 times in a week. Several sessions were allowed to be repeated within a day.

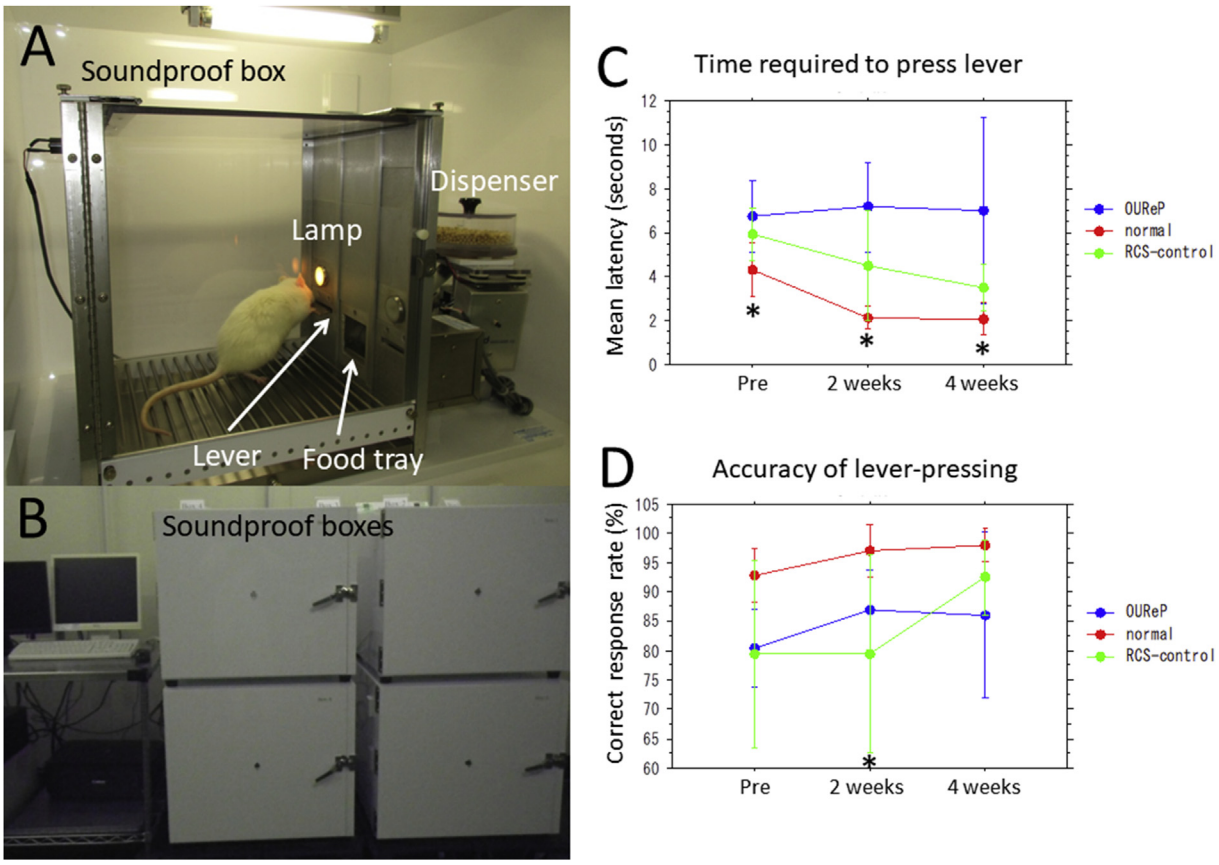

Fig. 2. Operant-conditioning lever-press response test. A. Operant test chamber (241 mm in width, 305 mm in depth, and 292 mm in height) is placed inside soundproof box (670 mm in width, 600 mm in depth, and 560 mm in height). B. Computer and soundproof boxes. C, D. Mean time (latency) required to press lever and correct response rates at the age of 7 weeks (pre-surgery), 9 weeks (2 weeks after film implantation), and 11 weeks (4 weeks after film implantation) in 5 normal rats (normal), 4 untreated retinal dystrophic (RCS) rats (RCS-control), and 7 retinal dystrophic (RCS) rats with dye-coupled film implantation (OUReP). The time required to press lever was significantly shorter in normal rats at three time points ($P = 0.0383$, $P = 0.0016$, and $P = 0.0338$). The correct response rate at 2 weeks after film implantation in RCS rats had no significant difference compared with normal rats while untreated RCS rats showed significantly lower correct response rates than normal rats ($P = 0.0519$, one-factor ANOVA, and $P < 0.05$, Tukey-Kramer test).

Parameters for analyses were the mean latency (seconds) from a lamp on to initial lever-pressing in the correct responses and the correct response rate (%) in all responses including correct responses, incorrect responses, and no responses.

2.6. Light/dark box test

The light/dark box test [31, 32] was done at the age of 11 weeks (4 weeks after film implantation). A rat was placed in a bright box, at 100 lux illumination, which was connected through an opening with a dark box of a step-through box system (Fig. 3A). The time required to put forelimbs or all 4 limbs on the floor of the dark box was measured, and used as parameters for analysis. The tests were repeated three times, and the observation was suspended in a cut off time of 5 minutes.

3. Results

3.1. Electroretinographic recording and surgical results

Electroretinography showed normal waves in all normal rats at 6 weeks, 9 weeks and 11 weeks of the age. In contrast, all RCS rats with no intervention, or plain film or dye-coupled film implantation had no apparent electroretinographic response at 6 weeks of the age, 9 weeks and 11 weeks of the age, namely, 2 weeks and 4 weeks after the plain film or dye-coupled film implantation, respectively.

At autopsy, plain films in both eyes of two RCS rats were not placed in the subretinal space. In contrast, plain films in the unilateral eye of the other two RCS rats, either in the right eye or in the left eye, were placed in the subretinal space while plain films in the contralateral eye of these rats were not placed in the subretinal space. Dye-coupled films in both eyes of one RCS rat were not placed in the subretinal space. Dye-coupled films in both eyes of four RCS rats were placed in the subretinal space. In contrast, dye-coupled films in the unilateral eye of the other three RCS rats, either in the right eye (two rats) or in the left eye (one rat), were placed in the subretinal space while dye-coupled films in the contralateral eye of these rats were not placed in the subretinal space.

Rats with plain films or dye-coupled films in both eyes which were not placed in the subretinal space were excluded from the following analyses. After the exclusion, data sets consisted of 5 normal rats, 4 untreated control RCS rats, and 7 RCS rats with subretinal dye-coupled film (OUReP) implantation either in both eyes or in the unilateral eye. Two RCS rats with subretinal plain film implantation in either eye could not be used for statistical analysis.

3.2. Functional observational battery

The visual forelimb-placing response was designated either as normal or subnormal. The normal response was marked when a rat raised the head up and straightened the forelimbs forward before the beard had contact with the table surface, as described in methods. The subnormal response was marked when a rat raised the head but did not align the body perpendicularly with the table and did not straighten the forelimbs.

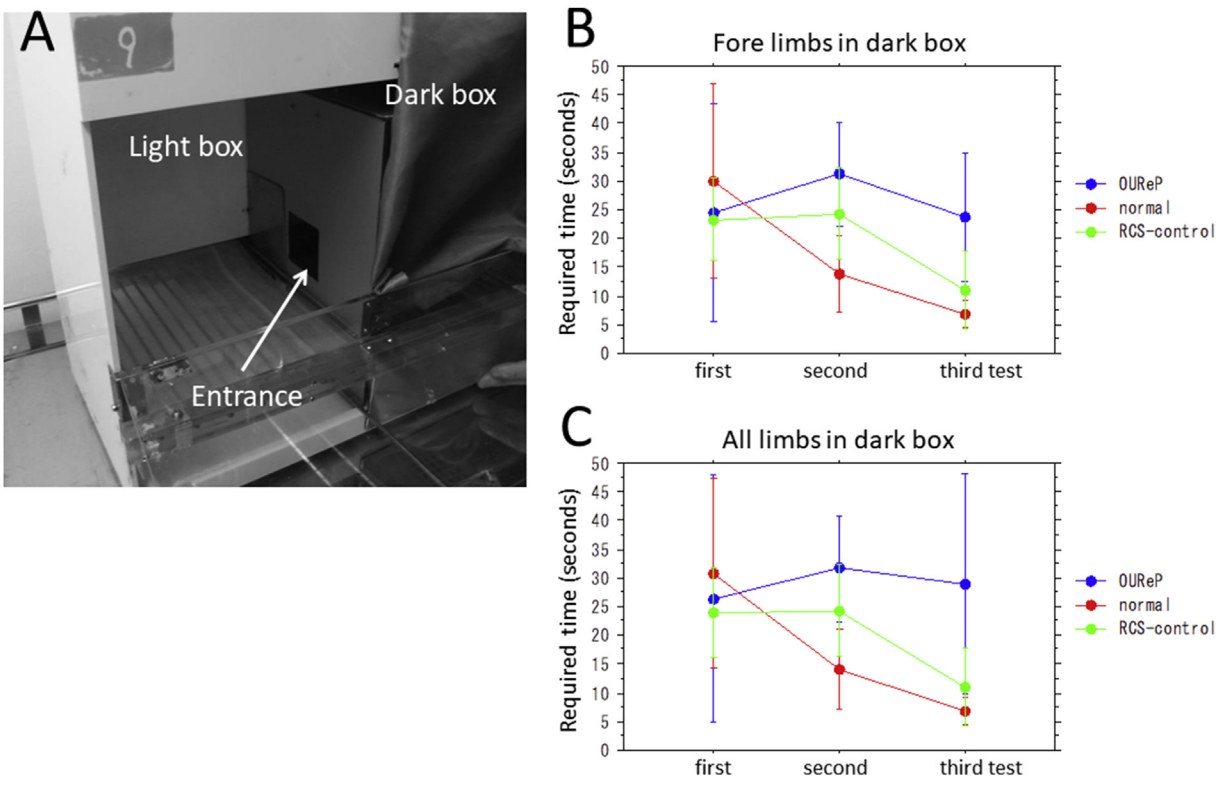

Fig. 3. Light/dark box test. A. Light box (18 cm in width, 20 cm in depth, and 22 cm in height) and dark box (20 cm in width, 20 cm in depth, and 17 cm in height) connected with each other by an entrance in 5 × 5 cm square size. B, C. The time (latency) required to put forelimbs or all 4 limbs on the floor of the dark box was measured at the age of 11 weeks (4 weeks after film implantation) in 5 normal rats (normal), 4 untreated retinal dystrophic (RCS) rats (RCS-control), and 7 retinal dystrophic (RCS) rats with dye-coupled film implantation (OUReP). By combining three repeat measurements of first, second, and third test in each rat all together, the time required to put forelimbs on the floor of the dark box was significantly shorter in normal rats, compared with untreated RCS rats or RCS rats with dye-coupled film implantation ($P = 0.046$, one-factor ANOVA, $P < 0.05$, Tukey-Kramer test). The time required to put all 4 limbs on the floor of the dark box tended to be shorter in normal rats ($P = 0.0681$).

All 5 normal rats showed the normal response at three time points of testing at the age of 4 weeks, 9 weeks, and 11 weeks. All 4 untreated RCS rats showed the normal response at the age of 4 weeks, but showed the subnormal response at both time points of testing at the age of 9 weeks and 11 weeks. All 7 RCS rats with dye-coupled film implantation showed the normal response at the age of 4 weeks before film implantation. At the age of 9 weeks, 2 weeks after film implantation, 5 rats showed the normal response while the remaining 2 rats showed the subnormal response. At the age of 11 weeks, 4 weeks after film implantation, only one rat showed the normal response while the remaining 6 rats showed the subnormal response.

The subnormal response was significantly more frequent at the age of 9 weeks and 11 weeks in untreated RCS rats, compared with normal rats ($P = 0.0027$ and $P = 0.0027$, respectively, chi-square test). The subnormal response was significantly less frequent at the age of 9 weeks in RCS rats with dye-coupled film implantation, compared with untreated RCS rats ($P = 0.0221$, chi-square test).

At landing foot splay test, the distance between hind limbs was significantly different among three groups of 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation ($P = 0.0087$), and became significantly narrower in the time course of pre-implantation to 2 and 4 weeks after film implantation ($P = 0.011$, repeat-measure analysis of variance (ANOVA), Fig. 1C). However, the narrowing trend of the distance between hind limbs was not significantly different among three groups of rats, 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation, at the age of 4 weeks before film implantation, at the age of 9 weeks or 11 weeks, 2 weeks or 4 weeks after film implantation ($P = 0.3328$).

Normal rats had tendency to show narrower distance between hind limbs, compared with untreated RCS rats or RCS rats with dye-coupled film implantation, at the time points of pre-implantation and 4 weeks after film implantation ($P = 0.0558$ and $P = 0.0514$, respectively, one-factor ANOVA, no significance at post-hoc test by Tukey-Kramer test).

3.3. *Operant-conditioning lever-press response test*

The mean latency (seconds) from a lamp on to initial lever-pressing was significantly different among three groups of 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation ($P = 0.0042$), and became shorter, although not significantly, in the time course of pre-implantation to 2 and 4 weeks after film implantation ($P = 0.0513$, repeat-measure ANOVA, Fig. 2C). However, the shortening trend of the latency was not significantly different among three groups of rats, 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation, at the age of 7 weeks before film implantation, at the age of 9 weeks or 11 weeks, 2 weeks or 4 weeks after film implantation ($P = 0.2005$).

The mean latency was significantly different among three groups of rats at the age of 7, 9, and 11 weeks ($P = 0.0383$, $P = 0.0016$, and $P = 0.0338$, respectively, one-factor ANOVA). In the post-hoc test, the latency was significantly shorter in normal rats compared with RCS rats with film implantation at all three time points of pre-implantation, 2 and 4 weeks after film implantation ($P < 0.05$, Tukey-Kramer test).

The correct response rate (%) was significantly different among three groups of 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation ($P = 0.0442$), and became significantly better in the time course of pre-implantation to 2 and 4 weeks after film implantation ($P = 0.0186$, repeat-measure ANOVA, Fig. 2D). However,

the improving trend of the correct response rate was not significantly different among three groups of rats, 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation, at the age of 7 weeks before film implantation, at the age of 9 weeks or 11 weeks, 2 weeks or 4 weeks after film implantation ($P = 0.3021$).

At the age of 9 weeks, the correct response rate was significantly better in normal rats, compared with untreated RCS rats ($P = 0.0519$, one-factor ANOVA, and $P < 0.05$, Tukey-Kramer test). In contrast, there was no significant difference in the correct response rate between the normal rats and RCS rats with dye-coupled film implantation. The correct response rate at the age of 7 weeks and 11 weeks was not significantly different among three groups of rats ($P = 0.065$ and $P = 0.1748$, respectively, one-factor ANOVA).

3.4. Light/dark box test

At the age of 11 weeks, the time required to put forelimbs on the floor of the dark box was significantly different among three groups of 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation ($P = 0.046$), and became significantly shorter in the time course of the first, second and third measurement ($P = 0.027$, repeat-measure ANOVA, Fig. 3B). In contrast, the time required to put all 4 limbs on the floor of the dark box was not significantly different among three groups of 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation ($P = 0.0681$), and did not change significantly in the time course of the first, second and third measurement ($P = 0.068$, repeat-measure ANOVA, Fig. 3C). The shortening trend in repeat measurements of the time required to put forelimbs and all limbs was not significantly different among three groups of rats, 5 normal rats, 4 untreated RCS rats, and 7 RCS rats with dye-coupled film implantation ($P = 0.1604$ and $P = 0.1483$, respectively).

All three repeat measurements of first, second, and third test in each rat were combined in each group of normal rats, untreated RCS rats, or RCS rats with dye-coupled film implantation, and compared among three groups of rats. The time required to put forelimbs on the floor of the dark box was significantly shorter in normal rats, compared with untreated RCS rats or RCS rats with dye-coupled film implantation ($P = 0.046$, one-factor ANOVA, and $P < 0.05$, Tukey-Kramer test). The time required to put all 4 limbs on the floor of the dark box tended to be shorter, although not significantly, in normal rats, compared with untreated RCS rats or RCS rats with dye-coupled film implantation ($P = 0.0681$, one-factor ANOVA).

4. Discussion

Behavior tests for small animals such as mice and rats have been developed mainly as screening tests for general safety of new drugs [29, 30, 31]. The set of behavior tests designated as functional observational battery can test physical and mental status in mice and rats [23, 24, 25, 26, 27, 28]. Some tests in functional observational battery certainly require the vision to be performed correctly [33]. We chose visual-forelimb placing response and landing hind limb foot splay test in the menu of functional observational battery [23, 24, 25, 26, 27, 28] to test the vision of rats in this study. Operant-conditioning lever-press response test is dependent on visual response to on-and-off of a lamp [29, 30, 34]. Light/dark box test is also dependent on rats' light-sensing and propensity for dark environment [31, 32]. The choice of these behavior tests in the present study was at our own discretion and would not have a specific meaning. The functional observational battery, indeed, aims to test sensory and motor aspects including the vision [25, 26].

The main goal of this study was to examine whether normal rats and retinal dystrophic rats would show the difference on these common behavior tests which were chosen from the standpoint of requiring the vision. In visual forelimb-placing response test, untreated RCS rats showed subnormal response, compared with normal response in normal rats. In landing foot splay test, RCS rats showed wider distance between hind limbs than normal rats. By careful observation in both tests, rats did not appear to use touch sensation by vibrissae. It is noteworthy that these simple tests in functional observational battery could detect the absence or the presence of vision in rats.

In operant-conditioning lever-press response test, untreated RCS rats showed longer latency and lower correct response rates than normal rats. By simply stating, untreated RCS rats were slower to press the lever and made more errors than normal rats. In light/dark box test, RCS rats had longer latency or required more time to put fore limbs or all limbs on the floor of dark box than did normal rats. All these results showed that these behavior tests could certainly discriminate RCS rats from normal rats.

Normal rats showed a trend of narrower distance of hind limbs on landing foot splay test in the time course of repeat measurements at 4, 9, and 11 weeks of the age. Normal rats showed a trend of shorter latency and better correct response rates on operant-conditioning lever-press response test in the time course of repeat measurements at 7, 9, and 11 weeks of the age. In repeat measurements at the age of 11 weeks, normal rats showed a trend of shorter latency to put forelimbs and all limbs on the floor of the dark box. Retinal dystrophic RCS rats shared a similar trend, although not so apparent, with normal rats in all these tests. These results suggest that normal rats and retinal dystrophic rats would have learning effect on all the tests done in this study.

Under the circumstances, visual forelimb-placing response is a simple test to detect the vision roughly. It should be noted that normal response was certainly observed in retinal dystrophic RCS rats at the age of 4 weeks when the rats are known to keep the vision. In contrast, the RCS rats lost normal response at the elder ages of 9 and 11 weeks when the vision had been lost.

The second goal of this study was to assess the vision of retinal dystrophic rats with dye-coupled film implantation. In our previous study, we assessed the vision of RCS rats with dye-coupled film implantation by a behavior test to observe head-turning in direction of rotation of a black-and-white-striped drum around the rats [19, 20]. We proved vision recovery by this behavior test in RCS rats with dye-coupled film implantation, compared with control plain film implantation [19, 20]. This behavior test, using a rotating drum, however, is laborious in experimental procedures, and thus a simpler behavior test would be desirable.

In visual forelimb-placing response test of this study, RCS rats with dye-coupled film implantation showed normal response at a significantly higher rate, at 2 weeks after the implantation, compared with untreated RCS rats. In operant-conditioning lever-press response test, RCS with dye-coupled film implantation showed no significant difference in the correct response rate at 2 weeks after the implantation, compared with normal rats, while untreated RCS rats showed significantly lower correct response rates than normal rats. However, there was no significant difference between untreated RCS rats and dye-coupled film-implanted RCS rats at 4 weeks after the implantation in visual forelimb-placing response and operant-conditioning lever-press response.

As a major drawback in the present study, we had to exclude a group of RCS rats with plain film implantation in statistical analyses for different behavior tests because the group had only two rats which were confirmed to have the film implanted properly in the subretinal space of the eye. Therefore, in the present study, we could not compare behavioral results between the rats with dye-coupled film implantation and the rats with plain film implantation. Previous studies have suggested that subretinal implantation of any material would have a neuroprotective effect on the sensory retina in RCS rats [35]. A better behavioral response in RCS rats with dye-coupled film implantation in the present study might be attributed to beneficial impact which was exerted on the sensory retina by subretinal implantation in itself. Another limitation would be that the group of RCS rats with dye-coupled film implantation was, indeed, the mixture of rats with the implantation either in both eyes or in the unilateral eye. Due to the limited number of rats, we could not stratify the rats with either bilateral or unilateral implantation.

In our previous study, we showed the recovery of visual evoked

potential amplitudes in monkey eyes with macular degeneration by subretinal dye-coupled film implantation in the period of 6 months [36]. The dye-coupled film was also implanted in eyes of dogs [37] and rabbits [38, 39] to show the safety and surgical feasibility. The present results, indeed, suggest that the common behavior tests, chosen in this study, might be used also for screening the vision in rats. However, a narrow window in each behavior test might exist to detect the difference among rats with different levels of vision. For instance, careful observation is required to differentiate subnormal response from normal response in visual forelimb-placing response test of functional observational battery. In light/dark box test, the latency to put fore limbs on the floor of the dark box appeared to be a better indicator than the latency to put all limbs on the floor of the dark box. Under the circumstances, we have to be cautious about interpretation of the outcome in these behavior tests.

5. Conclusions

We showed visual forelimb-placing response and landing foot splay test in functional observational battery could be used for screening the vision in rats. Operant-conditioning lever-press response test and light/dark box test could be also used to assess the vision in rats. To the best of our knowledge, this study is the first to evaluate behavior tests from the standpoint of vision assessment in rats. On the other side of the coin, care must be taken in behavior tests to consider whether or not rats have normal vision [33]. Out of these behavior tests which were evaluated in the present study, the visual forelimb-placing response in functional observational battery and the operant-conditioning lever-press response test might have experimental sensitivity to detect vision changes induced by dye-coupled film implantation in RCS rats.

Declarations

Author contribution statement

Toshihiko Matsuo: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Tetsuya Uchida, Koichiro Yamashita: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Shigiko Takei, Daisuke Ido, Atsushi Fujiwara, Masahiko Iino, Masao Oguchi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by a grant (Seeds C in 2016) for the Translational Research Network Program from the Japan Agency for Medical Research and Development (AMED).

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

Acknowledgements

We thank Chie Matsuo, DDS, PhD, visiting scientist in Okayama University Graduate School of Interdisciplinary Science and Engineering in Health Systems, for statistical analysis and preparation of figures.

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