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ORIGINAL ARTICLE

Metipranolol promotes structure and function of retinal photoreceptors in the *rd10* mouse model of human retinitis pigmentosa

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Abstract

Metipranolol is a β-adrenergic receptor antagonist that is given orally for the treatment of hypertension and also applied topically to the cornea for treating glaucoma. It also inhibits nitrosative stress which has previously been shown to be the cause of cone photoreceptor death in retinitis pigmentosa. In this study, we tested the hypothesis that metipranolol protects photoreceptor structure and function in the mouse model *rd10*. At P35, compared with vehicle-treated *rd10* mice in which rod degeneration was nearly complete, *rd10* mice given daily subcutaneous injections of 40 mg/kg of metipranolol had reduction in markers of nitrosative stress, fewer TUNEL-positive cells, increased outer nuclear layer thickness, and substantially more staining for rhodopsin. This was accompanied by significantly higher mean scotopic and photopic electroretinogram b-wave amplitudes indicating improved photoreceptor function. At P50, metipranolol-treated *rd10* mice had decreased 3-nitrotyrosine staining in the retina, increased immunostaining for cone arrestin, a marker for cone photoreceptors, and significantly higher scotopic and photopic b-wave amplitudes at the highest stimulus intensity compared with vehicle-treated mice. At P65, cone density was significantly higher in metipranolol-treated versus vehicle-injected *rd10* mice. Metipranolol applied as eye drops promoted cone photoreceptor function in retinas of *rd10* mice greater than subcutaneously injected metipranolol. The reduced nitrosative damage and rescue of functional loss of photoreceptors in *rd10* mice suggests that metipranolol, a drug with established ocular safety and tolerability, may have potential for treating patients with retinitis pigmentosa.

Keywords: electroretinogram, nitrosative damage, retinal degeneration.

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Retinitis pigmentosa is a group of genetic diseases in which one of many mutations causes rod cell death resulting in night blindness. With so many different pathogenic mutations, the rods die by several different mechanisms. Regardless of the mutation and the mechanism of rod cell death, after rods die, cone photoreceptors undergo gradual degeneration resulting in gradual constriction of visual fields and eventual blindness. Understanding the mechanism of cone cell death is critical for development of sight-saving therapeutics.

Mutations causing RP can also occur in animals, providing us valuable animal models. The Royal College of Surgeons rat (RCS) was described by Bourne *et al.* in 1938 (Bourne *et al.* 1938). Many years later it was determined by linkage analysis that a mutation in the *Mertk* gene was causative of the disease (D'Cruz *et al.* 2000) and subsequently candidate gene studies showed that mutations in *Mertk* caused RP in humans (Gal *et al.* 2000). Likewise, the phenotype of the

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Abbreviations used: ANOVA, analysis of variance; CCD, charge-coupled device; ERG, electroretinogram; INL, inner nuclear layer; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; ONL, outer nuclear layer; P, postnatal day, *rd1* mouse, retinal degeneration 1 mouse; RCS rat, royal college of surgeons rat; *rd10* mouse, retinal degeneration 10 mouse; RP, retinitis pigmentosa; RRID, research resource identifier; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Retinal Degeneration 1 (*rd1*) mouse, studied by Keeler in 1924 (Keeler 1924) was used for numerous studies before it was discovered that the degeneration is caused by a mutation in *Pde6b* (cGMP phosphodiesterase 6B, rod receptor, beta polypeptide) (Bowes *et al.* 1990). There is rapid degeneration of rods in *rd1* mice with almost complete loss by postnatal day (P) 21 followed by the death of 80% of cones over the subsequent 2 weeks (Carter-Dawson *et al.* 1978). The *rd10* mouse has a separate mutation in *Pde6b* which results in slower photoreceptor degeneration with loss of majority of rods by P35 and cones by P50 (Chang *et al.* 2002; Gargini *et al.* 2007). Mutations in *Pde6b* are also implicated in RP in humans (McLaughlin *et al.* 1993).

These animal models have been extremely valuable for studies investigating the mechanism of cone cell death in RP. Rod photoreceptors greatly outnumber cone photoreceptors numbers in humans (95:5) and in rodents (98:2). In RCS rats or rats with rod degeneration from a mutation in *rhodopsin*, the degeneration of rods markedly reduces oxygen consumption in the retina resulting in a large excess of oxygen in the outer retina (Yu *et al.* 2004). The oxygen excess results in an excess of superoxide radicals and progressive oxidative damage to cones (Shen *et al.* 2005; Komeima *et al.* 2006, 2007). In humans, rod cell density is highest in the midperiphery of the retina and cone cell density is highest in the fovea and surrounding posterior retina. After rods die in RP, remaining cell density is lowest in the midperipheral retina and this is the location of initial cone cell death resulting in a ring scotoma. Cone cell death occurs predominantly at the anterior and posterior margins of remaining cones, resulting in contiguous enlargement of the scotoma, both anteriorly and posteriorly. Cones in the less dense anterior regions are eliminated first, leaving a constricted central field because of the surviving posterior cones. As cones at the outer margin die as a result of oxidative damage, oxidative stress spreads posteriorly leading to more cone death, reducing the area of remaining cones, and thereby shrinking the remaining visual field. Cones in the fovea survive the longest because, this is the region of highest cone cell density. Eventually, these cones also die eliminating central vision resulting in blindness.

Stimulation of NADPH oxidase contributes to the increase in oxidative damage because of generation of superoxide radicals (Usui *et al.* 2009b) which generate many other free radicals causing more damage. There are high levels of NO in the retina and the interaction of NO with superoxide radicals results in peroxynitrite which is very toxic and hard to detoxify. Inhibition of nitric oxide synthase (NOS) by multiple drugs reduces nitrosative damage and promotes cone function and survival in *rd10* mice (Komeima *et al.* 2008). Metipranolol has previously been shown to inhibit the nitrosative damage caused by sodium nitroprusside-induced lipid peroxidation in rat brain homogenates and in the mouse retina (Melena and Osborne 2003). In this study, we sought to determine the effect of metipranolol on retinal degeneration in *rd10* mice.

Methods

Study design

Mice were treated in accordance with the Association for Research in Vision and Ophthalmology and the protocol was approved by the Johns Hopkins Animal Care and Use Committee (approval number MO17M150). This study was not preregistered. The animals were housed in core barrier housing in barrier plastic cages with corn cob bedding and a wire bar lid that serves as a food hopper and handled in biosafety cabinets under aseptic conditions using protective clothing to prevent the spread of infections. Food and water are provided *ad libidum*. Not more than five mice were maintained in each cage. A veterinarian looked after the well-being of all the animals. The primary objective of this study was to determine if metipranolol promotes survival and function of cone photoreceptors in the *rd10* model of RP (RRID: IMSR_JAX:004297, Jackson Laboratory, Bar Harbor, ME, USA). The prespecified primary endpoint was the mean photopic b-wave amplitude at postnatal day (P) 50 in metipranolol-treated versus vehicle treated *rd10* mice. Important secondary outcome measures were mean scotopic b-wave amplitudes at P35, mean scotopic a-wave amplitudes at P35, cone density at P65, and outer nuclear layer thickness (ONL) at P35. Exploratory outcomes were nitrotyrosine and nitrite levels in retina, immunohistochemistry for nitrotyrosine and cone arrestin, and TUNEL. In order to achieve sufficient numbers of mice to guarantee reproducibility for all secondary outcome measures, seven litters of *rd10* mice were used. Arbitrary assignment method was used where, littermates from a breeding pair were arbitrarily divided into two groups (metipranolol and vehicle control) with approximately equal number of animals in each group where no considerations were taken regarding the sex of the animals. This method was done for all litters used in this study.

All mice had electroretinography at P35 and then were arbitrarily subdivided into smaller groups for each of the other outcome measures. There was no sample size differences between the beginning and end for most of the experiments except for the eye drop treatment because one mouse from the vehicle control group died because of unknown cause between P35 and P50. It was predetermined that no data would be excluded. No sample size calculations were done in this study, however, we used sample sizes similar to our previously published papers. The timeline of the experimental procedures conducted in shown in Fig. 1. No test for outliers was conducted in these studies.

Injections of metipranolol

Male and female r*d10* mice were maintained in a 12 h light/dark cycle and arbitrarily assigned to receive daily subcutaneous injections of 40 mg/kg metipranolol (British Pharmacopoeia Commission Laboratory, Tddington, UK, Catalog no. 642) or vehicle [phosphate-buffered saline (PBS) containing 5.8% ethanol] at approximately 10 am between P14 and P65. This concentration was similar to the concentration used in previously published studies (Zivný *et al.* 1983). The route of injection was into the loose skin

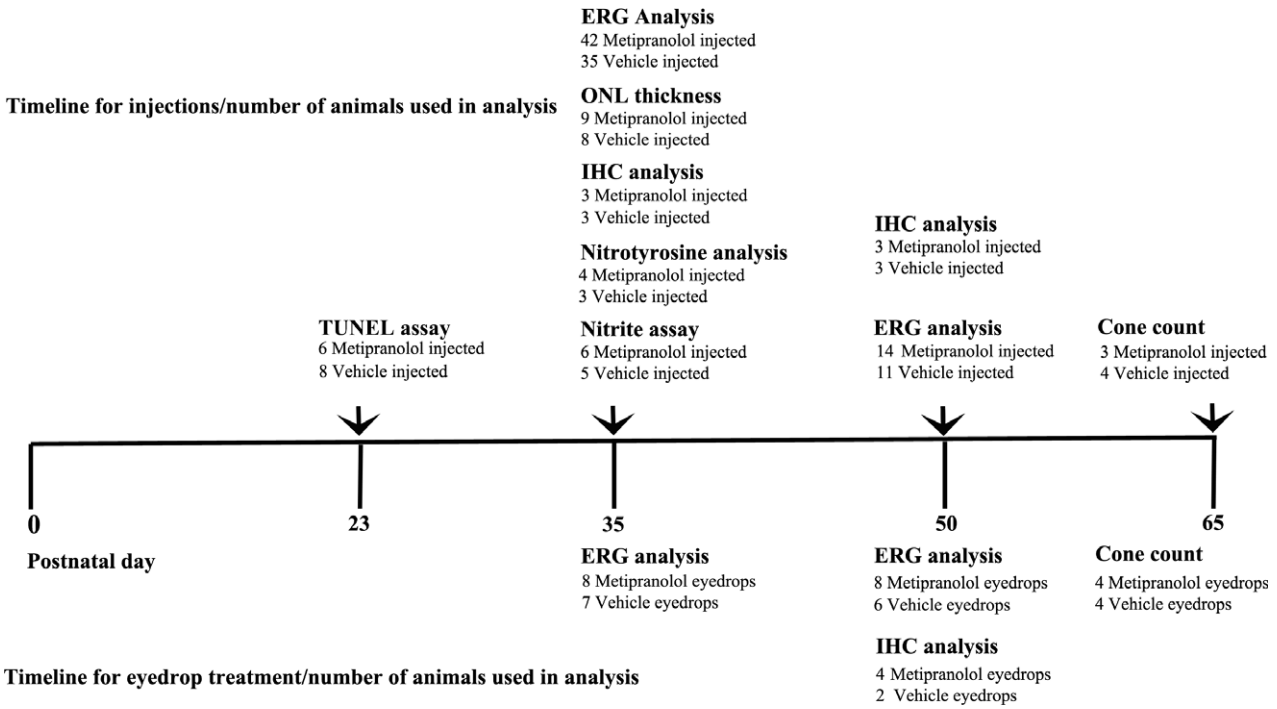


Fig. 1 Experimental timeline. Timeline of experimental design showing all the studies done with number of animals used in each study.

over the neck. The weight of mice at P14 was between 6 and 8 g. By P50 the mice weighed 16–20 g.

Metipranolol eye drops

A 0.06% solution of metipranolol in water was topically applied to the eyes of *rd10* mice three times a day (10 am, 2 pm, and 6 pm) from P14 to P65. Water was used as a vehicle control. A 0.06% concentration of metipranolol was used because it is the maximum solubility of metipranolol in water. ERGs were done on these animals at P35 and P50. The sample size for this study at the start of the experiment was eight animals for metipranolol and seven animals for vehicle controls. One animal from the vehicle control died because of unknown cause after P35 bringing a sample size of six for vehicle control.

Electroretinogram recordings (ERG)

An investigator masked with respect to treatment group performed electroretinograms (ERGs) using a Diagnosys instrument (Espion, Littleton, MA, USA) with custom made platinum electrodes as previously described (Komeima *et al.* 2006, 2007). For scotopic recordings, *rd10* mice were dark adapted overnight, anesthetized with a mixture of ketamine (100 mg/kg, Henry Schein®, Melville, NY, USA, Catalog no. 1049007) and xylazine (5mg/kg, Lloyd Laboratories Inc., Shenandoah, IA, USA, Catalog no. sc-362950Rx) by intraperitoneal injections, and pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Santen Pharmaceutical Co., Osaka, Japan, Catalog no. 005728). Mice were placed on a heating pad and platinum electrodes were placed on the corneas after application of gonioscopic prism solution (Alcon Labs, Fort Worth, TX, USA, Cat. no. 17478-064-12). Reference electrode was attached subcutaneously between the eyes on the scalp and a ground electrode was attached to the tail. The Ganzfeld illuminator was then placed over the head in a position to ensure equal illumination to both eyes. Scotopic ERGs were recorded at 11 flash intensities (−4, −2.5, −2.2, −1.79, −1.39, −1, −0.6, −0.39, −0.20, 1.0, 1.39 log cd-s/m^2). For each flash intensity, five readings were taken and averaged. For photopic ERGs, mice were initially light adapted for 7 min with 30 cd/m^2 background light and flashed at three light intensities (0.6, 1.0, 1.39 log cd-s/m^2). For each flash intensity, five readings were taken and averaged.

Measurement of outer nuclear layer (ONL) thickness

Measurements of ONL thickness were done as previously described.(Cronin *et al.* 2010) Three separate litters at P35 were used consisting of 9 metipranolol-treated and eight vehicle-treated mice to assure reproducibility of findings. The mice were anesthetized by inhalation of Isoflurane (Baxter, Deerfield, IL, USA, Catalog no. NDC10019-360-60) followed by cervical dislocation and removal of the eyes. Eyes were frozen in OCT compound Tissue-TEK® (Sakura Finetek USA, Torrance, CA, USA, Catalog no. 4583). Ten μm frozen ocular sections were cut in the 12:00–6:00 meridian, and then stained with hematoxylin solution (Sigma Aldrich, St. Louis, MO, USA, catalog no. HHS32) for 3 min. This was followed by rinsing in tap water to remove excess stain and staining with eosin Y solution (Sigma Aldrich, catalog no. E4009) for 2 min followed by rinsing with tap water to remove excess stain. The sections were dehydrated in 95% ethanol and 100% ethanol for 2 min followed by xylene (Sigma Aldrich, catalog no. 214736) for 5 min, air-dried and visualized with an Axioskop microscope (Zeiss, Thornwood, NY, USA) and digital images were obtained. Images of sections through the optic nerve were used to measure ONL

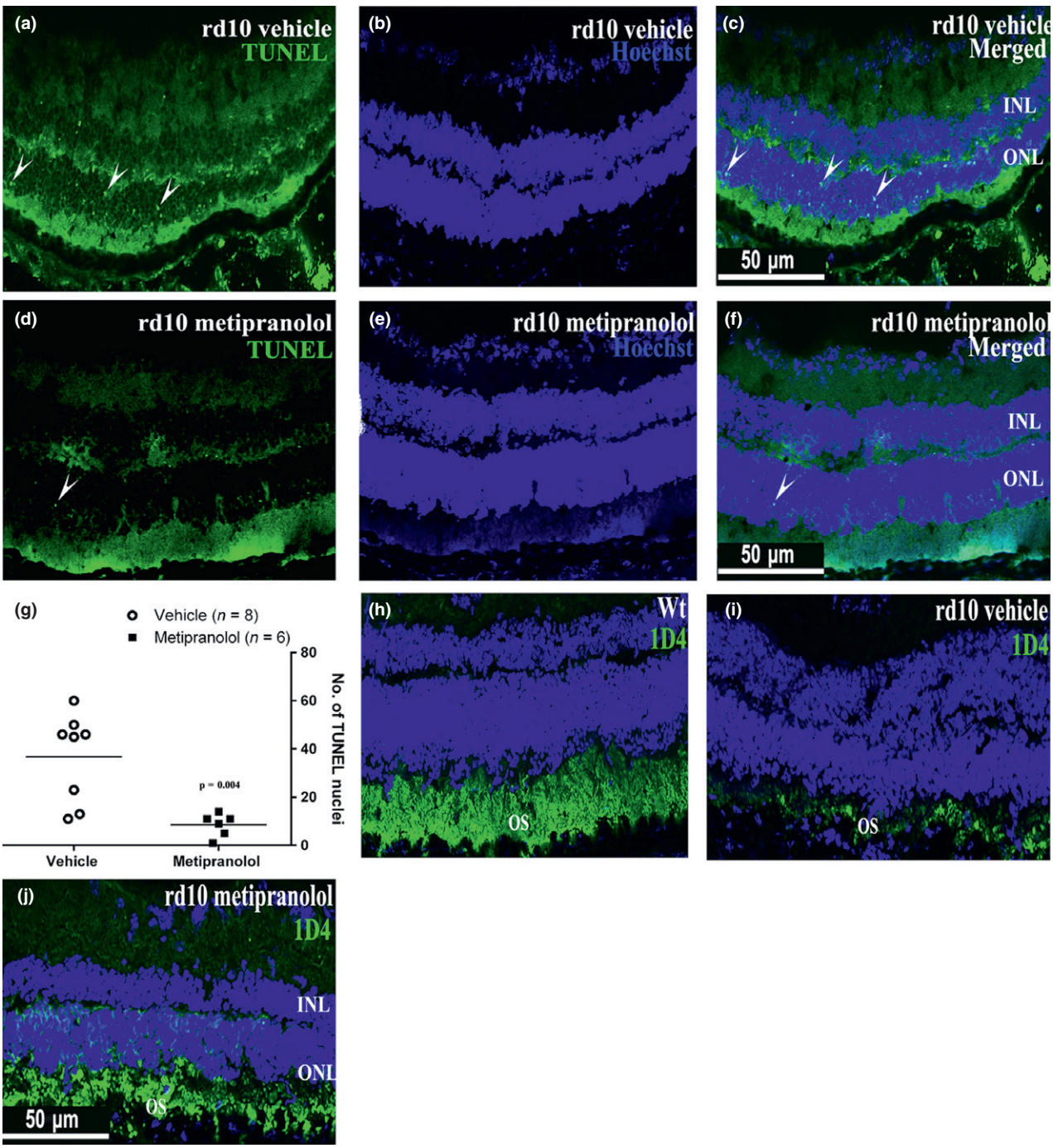


Fig. 2 Metipranolol reduces rod cell death and promotes retention of rod outer segments in P35 *rd10* mice. A TUNEL-stained retina from a P23 vehicle-treated *rd10* mice (a) shows many apoptotic cells (arrowheads) in the outer nuclear layer (ONL) and inner nuclear layer (INL). Hoechst staining of nuclei on the same section helps to identify the ONL and INL (b). Merged image of vehicle-treated *rd10* mice show the apoptotic cells (arrowheads) predominantly in Hoechst-stained ONL layer (c). A section from a corresponding location in a P23 metipranolol-treated *rd10* mouse shows few apoptotic cells (arrowhead) (d) and a thicker ONL and INL, best seen with Hoechst staining (e) and merged image (f). Quantification of TUNEL staining between vehicle and metipranolol injected *rd10* mice shows a statistically significant increase in mean number of apoptotic cells in vehicle-injected mice compared with those injected with metipranolol by Mann–Whitney test (g). Immunohistochemical staining with ID4 anti-rhodopsin antibody (h–i) shows a prominent band of rhodopsin-positive outer segments (OS) in P35 wild type (Wt) mice (h), almost no rhodopsin-positive outer segments in vehicle-treated P35 *rd10* mice (i), and a substantially larger band of rhodopsin-positive outer segments in metipranolol-treated *rd10* mice (j). *n* = number of mice used in the experiment.

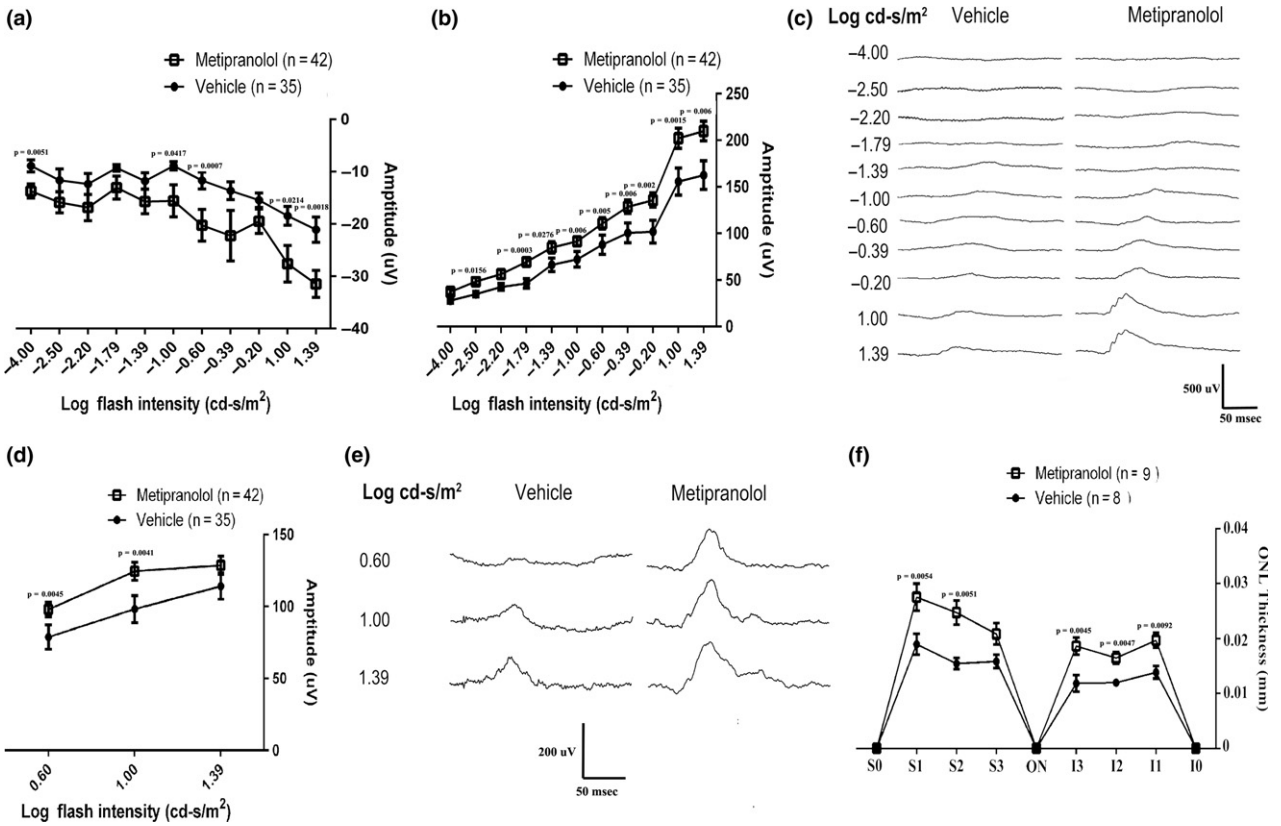


Fig. 3 Metipranolol promotes rod and cone photoreceptor survival and function in P35 *rd10* mice. (a) Mean (±SEM) scotopic a-wave amplitudes, a measure of rod photoreceptor function, were significantly greater in metipranolol-injected *rd10* mice compared with those injected with vehicle. (b) Mean (±SEM) scotopic b-wave amplitudes, a measure of the function of second order neurons in the rod pathway, were significantly greater in metipranolol-injected *rd10* mice compared with those injected with vehicle at multiple flash intensities. (c) Representative wave forms for scotopic electroretinogram (ERG) are shown for a mouse injected with vehicle and one injected with metipranolol. (d) Mean (±SEM) photopic b-wave amplitude, a measure of cone photoreceptor function, was significantly greater in metipranolol-injected *rd10* mice compared with those injected with vehicle at 0.6 and 1.0 log cd-s/m^2. (e) Representative wave forms for photopic ERG are shown for a mouse injected with vehicle and one injected with metipranolol. (f) Mean (±SEM) outer nuclear layer (ONL) thickness, a measure of rod photoreceptor survival, was significantly greater in five of six locations in the vertical meridian of retinas from metipranolol-injected *rd10* mice compared with retinas from *rd10* mice injected with vehicle. Mann–Whitney nonparametric analysis were done for all data and p values ≤0.05 were considered significant and exact p values shown for significant data points. n = number of mice used in the experiment.

thickness at six locations: 25% (S1), 50% (S2), and 75% (S3) of the distance between the superior pole and the optic nerve and 25% (I1), 50% (I2), and 75% (I3) of the distance between the inferior pole and the optic nerve. Three separate sections were used for each mouse and averaged.

Measurement of cone cell density

Cone cell density was analyzed as previously described (Komeima *et al.* 2006, 2007). Briefly, at P65 three metipranolol-treated and four vehicle-treated mice were anesthetized with isoflurane followed by cervical dislocation. Eyes were removed and fixed in 4% PFA (Santa Cruz Biotechnology, Dallas, TX, USA, Catalog no. 281692) for 1 h followed by dissection to remove cornea, iris, and lens. The retina was carefully isolated from the eyecup and a small incision was made at the nasal position for future orientation. The retinas were incubated in 10% normal goat serum in PBS for 1 h at room temperature followed by overnight treatment at 4°C in 1 : 100 rhodamine-conjugated peanut agglutinin (Vector Laboratories, Burlingame, CA, USA, Catalog no. RL-1072) in PBS containing 1% normal goat serum (Vector Laboratories, Catalog no. S-1000). The retinas were washed three times in PBS for 10 min each and flat mounted and visualized by fluorescence microscopy using an excitation wavelength of 543 nm to detect rhodamine fluorescence of peanut agglutinin (PNA). The number of cones within four 230 × 230 μm (512 × 512 pixels) squares located 1 mm superior, temporal, inferior, and nasal to the center of the optic nerve was determined. The results were plotted using GraphPad Prism 6 (La Jolla, CA, USA) as box plots.

TUNEL assay

At P23, *rd10* mice were euthanized as described above. Eyes were removed, frozen in OCT compound Tissue-TEK® (Sakura

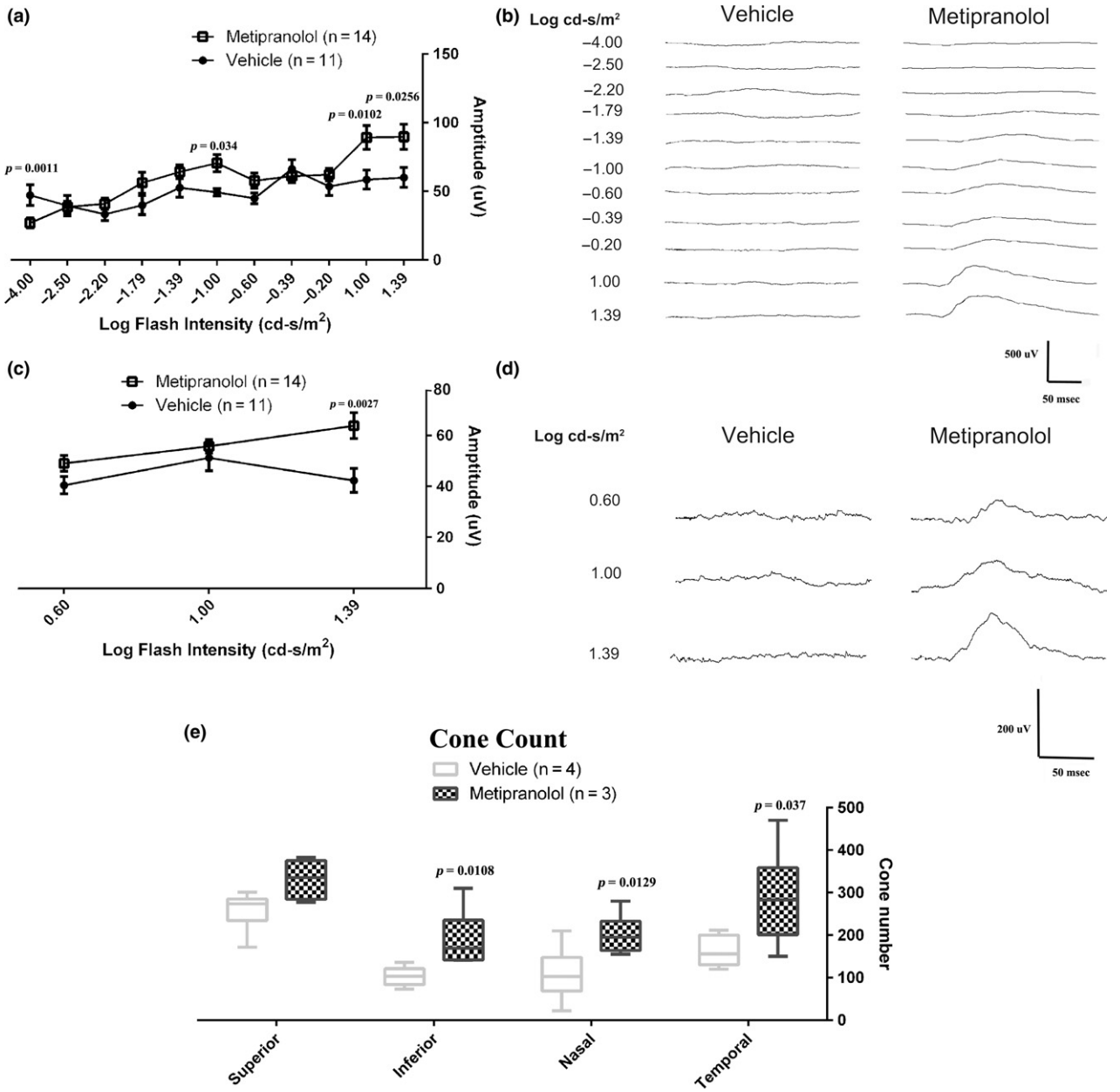


Fig. 4 Metipranolol promotes second order neuron and cone function and survival in cones of *rd10* mice. (a) At P50, mean (±SEM) scotopic b-wave amplitude was significantly greater in metipranolol-treated *rd10* mice compared with those treated with vehicle at light intensities of −4, −1, 1.0, and 1.39 log cd-s/m^2. (b) Representative wave forms for scotopic electroretinogram (ERG) at P50 are shown for a mouse injected with vehicle and one injected with metipranolol. (c) At P50, mean (±SEM) photopic b-wave amplitude, a measure of cone photoreceptor function, was significantly greater in metipranolol-treated *rd10* mice compared with those treated with vehicle at the highest light intensity of 1.39 cd-s/m^2. (d) Representative wave forms for photopic ERG at P50 are shown for a mouse injected with vehicle and one injected with metipranolol. (e) Mean (±SEM) cone cell density at P65 was significantly greater in the nasal, temporal and inferior quadrants of retinas from metipranolol-injected *rd10* mice compared to those injected with vehicle. No statistical differences were found in cone number at the superior quadrant. Mann–Whitney non parametric analysis were done for all data and p values ≤ 0.05 were considered significant and exact *p* values shown for significant data points. *n* = number of mice used in the experiment.

Finetek USA, Torrance, CA, USA, Catalog no. 4583) and TUNEL was done on 10 µM sections with the *In Situ* Cell Death Detection Kit (Sigma Aldrich, Catalog no. 11684795910) using the manufacturer's instructions. TUNEL positive cells in the ONL layer were counted on sections from eight vehicle-injected and 6 metipranolol-injected *rd10* mice using Image-Pro® plus (Media Cybernetics, Rockville, MD, USA). The results were plotted using GraphPad Prism 6 as scatter plots.

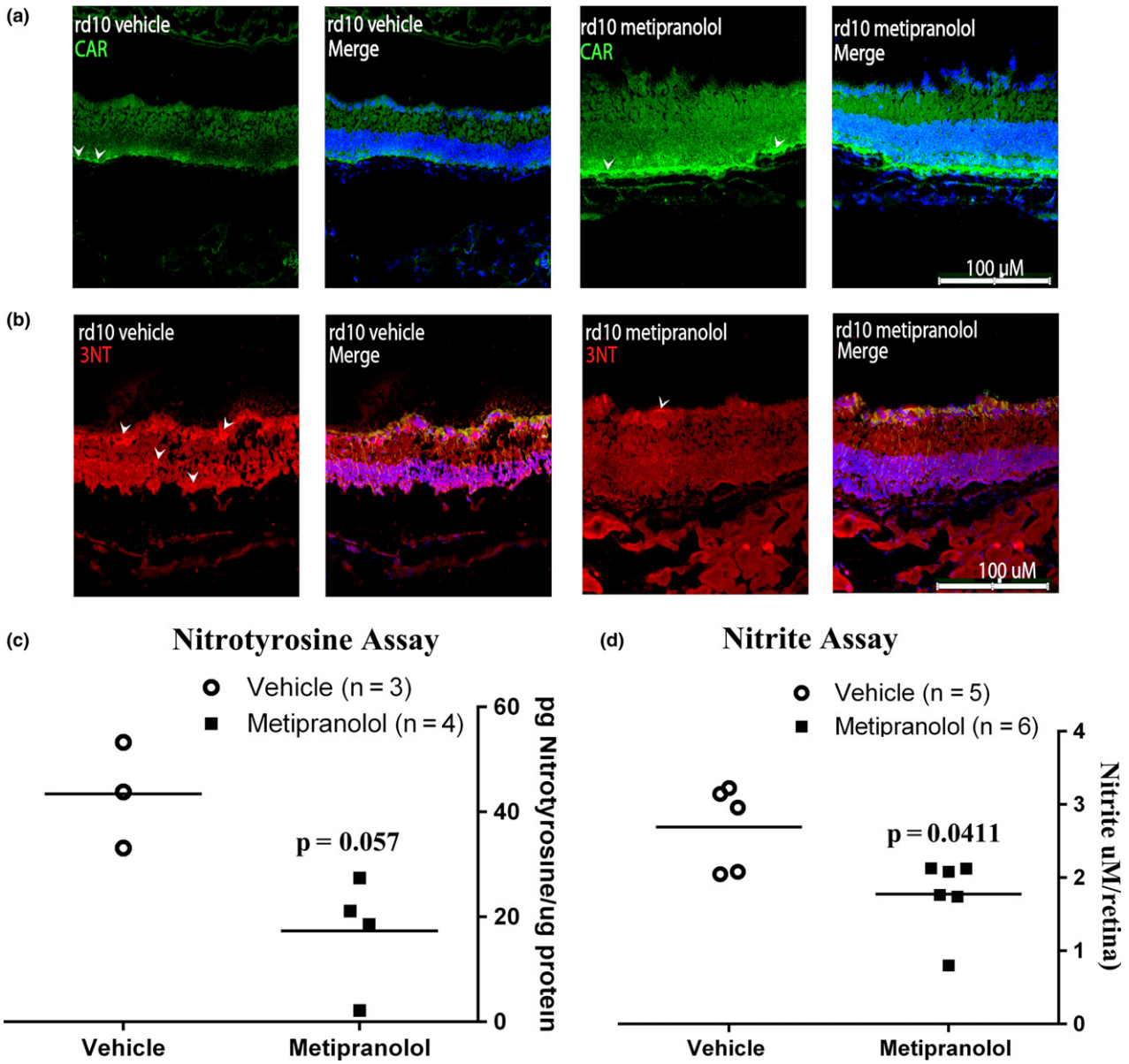


Fig. 5 Increased levels of cone arrestin and attenuated nitrosative damage in metipranolol-injected *rd10* mice. (a) Immunohistochemistry with an antibody specific for cone arrestin showed significantly reduced staining in retinas from P50 vehicle-treated *rd10* mice (arrowheads, first panel) and Hoechst staining showed marked thinning of the outer and inner nuclear layers (second panel). In contrast, there was robust staining for cone arrestin in P50 metipranolol-treated *rd10* mice (arrowhead, third and fourth panels). (b) Staining with anti-nitrotyrosine antibody (3NT) showed nitrotyrosine present in all layers of the retinas from P50 vehicle-injected *rd10* mice (arrowheads, first panel no counterstain, second panel Hoechst counterstain). There was much less nitrotyrosine staining in retinas from P50 metipranolol-treated mice (arrowhead, third panel no counterstain, fourth panel Hoechst counterstain). (c) Nitrotyrosine ELISA did not show significantly less nitrotyrosine-containing protein in retinal homogenates from metipranolol-treated P35 *rd10* mice compared with vehicle-treated mice. (d) Nitrite assay showed significantly lower nitrite levels in retinal homogenates from metipranolol-treated P35 *rd10* mice compared with vehicle-treated mice. Mann–Whitney nonparametric analysis were done for all data and p values ≤ 0.05 were considered significant and exact p values shown for significant data points. n = number of mice used in the experiment.

Nitrite and nitrotyrosine assays

At P35, *rd10* mice (n = 6 metipranolol-treated and n = 5 vehicle-treated) were euthanized as described above, and nitrite concentration was measured using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA, Catalog no. 780001) according to the manufacturer's instructions.

Nitrotyrosine assay was performed on retinas from P35 rd10 mice (n = 4 metipranolol-treated and n = 3 vehicle-treated) using the 3-Nitrotyrosine ELISA kit (Abcam, Cambridge, MA, USA, Catalog no. 116691). The results were plotted using GraphPad Prism 6 as scatter plots.

Immunohistochemistry

At P50, metipranolol-treated and vehicle control mice were euthanized as described above and 10 μm ocular frozen sections were permeabilized in 0.2% Triton X in PBS and blocked for 1 h at 26°C with blocking buffer (5% donkey serum, 1% bovine serum albumin and 0.2% Triton X in PBS). The sections were stained with validated antibodies (http://antibodyregistry.org/), anti-rhodopsin antibody 1D4 (1 : 200, Cat# MA1-722, RRID:AB_325050, ThermoFisher Scientific, Waltham, MA, USA), anti-3-nitrotyrosine antibody (10 μg/mL, EMD Millipore, Temecula, CA, USA, Catalog no. 06-284) or anti-cone arrestin antibody (1 : 10 000, Catalog no. AB15282, RRID: AB_1163387 EMD Millipore, Temecula, CA, USA) in blocking buffer overnight at 4°C. The sections were washed three times for 10 min in wash buffer (PBS + 0.2% Triton X) and incubated with the following secondary antibodies, goat anti-rabbit Alexa Fluor 594, Catalog no. A-11037, goat anti-rabbit 488, Catalog no. A32731, and goat anti-mouse Alexa Fluor 488, Catalog no. A32723 in blocking buffer for 1 h followed by three additional washes in wash buffer and mounted with DAP1 mounting media (Vector Laboratories, Catalog no. H-1200).

Statistical analysis

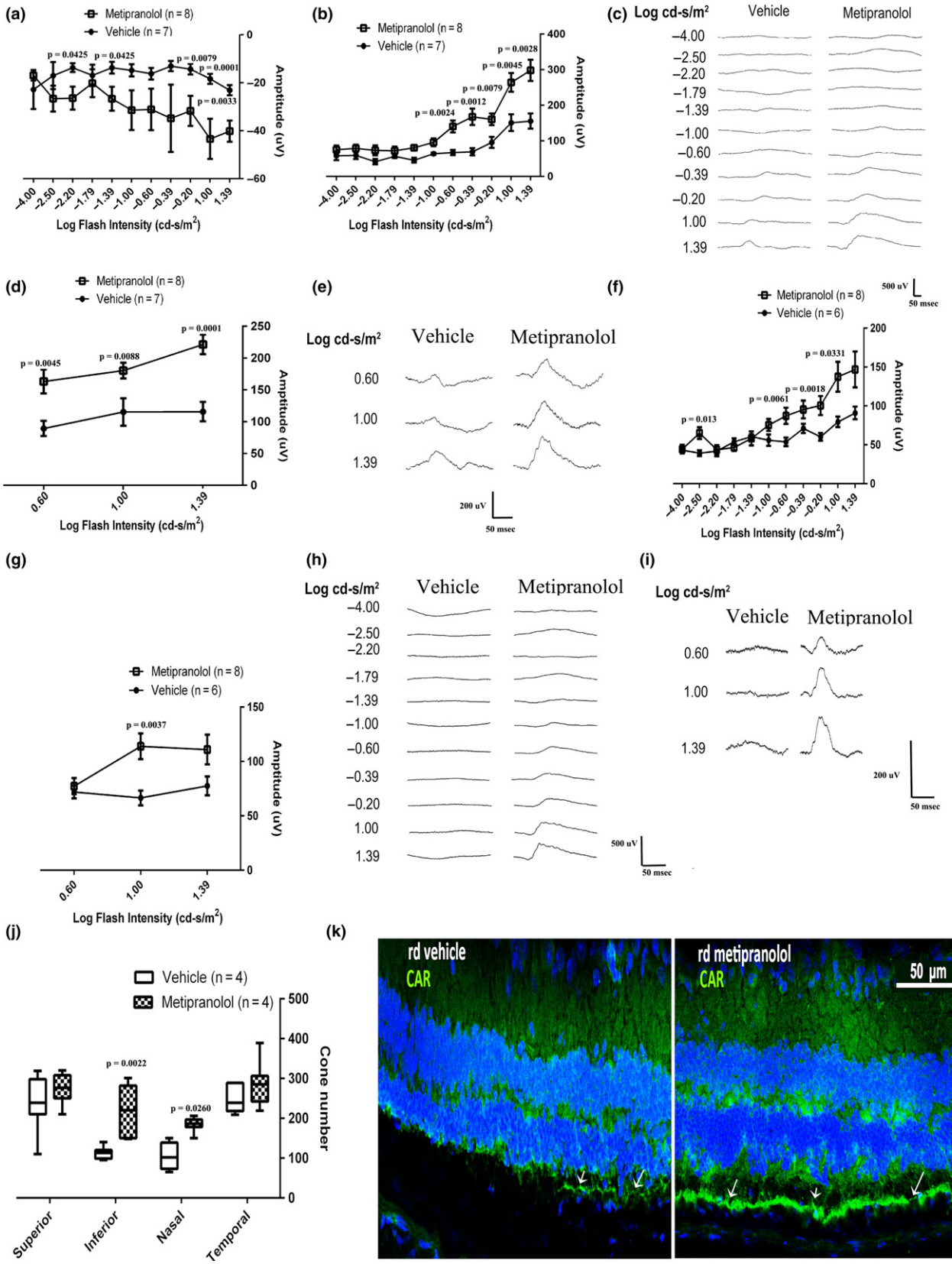
For differences in scotopic a, scotopic b, and photopic b wave values between metipranolol and vehicle treated animals, amplitudes at the same flash intensities were compared. Test for normality of variables using the Shapiro-Wilk test showed that the data did not have normal distribution and therefore a Mann–Whitney nonparametric statistical comparison was used for all data. p values below 0.05 were considered statistically significant and exact p values for significant data points are shown in the figures. N = number of animals used in the studies. All graphs were plotted using GraphPad Prism 6.

Results

Metipranolol promotes rod photoreceptor survival and function in P35 *rd10* mice

The peak of rod photoreceptor cell death in *rd10* mice is between P18 and P25 (Fig. 1) and most rods have been eliminated by P35 (Chang *et al.* 2002; Gargini *et al.* 2007). Starting at P14, *rd10* mice were given daily subcutaneous injections of 40 mg/kg metipranolol or vehicle. At P23 there were many TUNEL-positive cells in the ONL and inner nuclear layer (INL) of retinas from vehicle-treated *rd10* mice (arrowheads, Fig. 2a and c), but few TUNEL-positive cells in retinas from metipranolol-treated *rd10* mice (arrowhead, Fig. 2d and f) indicating suppression of rod cell death. This increase in apoptotic cells in vehicle- treated mice was quantified (Fig. 2g). Healthy rod photoreceptors transport rhodopsin into outer segments which form a thick band at the outer border of the ONL that stains intensely for rhodopsin (Fig. 2h). Rhodopsin staining was drastically reduced in retinas from vehicle-treated P35 *rd10* mice (Fig. 2i), indicating almost complete loss of rod outer segments. In retinas from metipranolol-treated P35 *rd10* mice, rhodopsin staining was less than that seen in wild type mice, but still robust indicating substantial retention of rod outer segments (Fig. 2j). At P35, compared with vehicle-treated *rd10* mice, mean scotopic a-wave amplitude, which is generated by rod photoreceptors, was significantly greater in metipranolol-treated *rd10* mice at a few light intensities (Fig. 3a). Mean scotopic b-wave amplitude, which is generated by second order neurons in the rod pathway, was significantly higher in metipranolol-treated mice at multiple flash intensities (Fig. 3b). Sample wave forms for scotopic a- and b-wave from an *rd10* mouse injected with vehicle and one injected with metipranolol are shown in Fig. 3c. Mean photopic b-wave amplitude, which is generated by second order neurons in the cone photoreceptor pathway, was significantly higher in

Fig. 6 Metipranolol eye drops preserve second order neuron function and cone photoreceptor number and function at P35 and P50 in *rd10* mice. (a) Mean (± SEM) scotopic a-wave amplitudes were significantly different in metipranolol eye drop treated P35 *rd10* mice compared with those treated with vehicle. (b) Mean (± SEM) scotopic b-wave amplitudes were significantly greater in metipranolol eye drop-treated P35 *rd10* mice compared with those treated with vehicle. (c) Representative wave forms for scotopic electroretinogram (ERG) at P35 are shown for a mouse treated with vehicle and one treated with metipranolol. (d) Mean (± SEM) photopic b-wave amplitudes were significantly greater in metipranolol eye drop-treated P35 *rd10* mice compared with those treated with vehicle. (e) Representative wave forms for photopic ERG at P35 are shown for a mouse treated with vehicle and one treated with metipranolol. (f) Mean (±SEM) scotopic b-wave amplitudes were significantly greater in metipranolol eye drop-treated P50 *rd10* mice compared with those treated with vehicle. (g) Mean (±SEM) photopic b-wave amplitudes were significantly greater in metipranolol eye drop-treated P50 *rd10* mice compared with those treated with vehicle at 1.0 log cd-s/m^2. (h) Representative wave forms for scotopic ERG at P50 for a mouse treated with vehicle and one treated with metipranolol. (i) Representative wave forms for photopic ERG at P50 for a mouse treated with vehicle and one treated with metipranolol. (j) Mean (±SEM) cone cell density at P65 was significantly greater in the nasal and inferior quadrants of retinas from metipranolol eye drop-treated *rd10* mice compared to those treated with vehicle eye drops. (k) Immunohistochemistry with an antibody specific for cone arrestin showed significantly reduced staining in retinas from P50 vehicle-treated *rd10* mice (k, left panel, arrows). In contrast, there was robust staining for cone arrestin in P50 metipranolol-treated *rd10* mice (k, right panel, arrows). Mann–Whitney nonparametric analysis were done for all data and p values ≤ 0.05 were considered significant and exact p values shown for significant data points. n = number of mice used in the experiment.


(a)
Metipranolol (n = 8)
Vehicle (n = 7)
p = 0.0425
p = 0.0425
p = 0.0079
p = 0.0001
p = 0.0033
Amplitude (uV)
Log Flash Intensity (cd-s/m2)
(b)
Metipranolol (n = 8
Vehicle (n = 7)
p = 0.0028
p = 0.0045
p = 0.0079
p = 0.0012
p = 0.0024
Amplitude (uV)
Log Flash Intensity (cd-s/m2)
(c)
Log cd-s/m2
Vehicle
Metipranolol
(d)
Metipranolol (n = 8)
Vehicle (n = 7)
p = 0.0001
p = 0.0088
p = 0.0045
Amplitude (uV)
Log Flash Intensity (cd-s/m2)
(e)
Log cd-s/m2
Vehicle
Metipranolol
200 uV
50 msec
(f)
Metipranolol (n = 8)
Vehicle (n = 6)
500 uV
50 msec
p = 0.0331
p = 0.0018
p = 0.0061
p = 0.013
Amplitude (uV)
Log Flash Intensity (cd-s/m2)
(g)
Metipranolol (n = 8)
Vehicle (n = 6)
p = 0.0037
Amplitude (uV)
Log Flash Intensity (cd-s/m2)
(h)
Log cd-s/m2
Vehicle
Metipranolol
500 uV
50 msec
(i)
Log cd-s/m2
Vehicle
Metipranolol
200 uV
50 msec
(j)
Vehicle (n = 4)
Metipranolol (n = 4)
p = 0.0022
p = 0.0260
Cone number
Superior
Inferior
Nasal
Temporal
(k)
rd vehicle
CAR
rd metipranolol
CAR
50 µm

metipranolol-treated mice at stimulus intensities of 0.6 and 1.0 log cd-s/m^2 (Fig. 3d). Representative photopic b-waves from a vehicle-injected mouse and one from a metipranolol-injected mouse are shown in Fig. 3e. These data indicate that metipranolol partially prevented loss of rod, second order neuron, and cone photoreceptor function in the *rd10* retina at P35. The thickness of the ONL was measured at six locations along the longitudinal meridian of the retina and mean ONL thickness was significantly greater at five of the six locations in retinas of P35 metipranolol-treated *rd10* mice compared to those from mice treated with vehicle (Fig. 3f).

Metipranolol promotes cone photoreceptor survival and function in P50 *rd10* mice

In *rd10* mice, rod degeneration is usually completed and cone degeneration is very advanced by P50 (Gargini *et al.* 2007). Therefore, there was no scotopic a-wave measurable at P50. However, compared with vehicle-treated P50 *rd10* mice, those treated with metipranolol had significantly higher mean scotopic b-wave amplitudes at flash intensities of, −4, −1.0, 1.0, and 1.39 log cd-s/m^2 (Fig. 4a and b). Mean photopic b-wave amplitude was significantly greater in metipranolol-treated mice at the highest flash intensity of 1.39 log cd-s/m^2 (Fig. 4c and d). At P65, mean cone photoreceptor cell density was significantly greater in 3 of 4 quadrants of metipranolol-treated *rd10* mice compared with vehicle-treated mice (Fig. 4e). Immunostaining for cone arrestin, an essential protein in the cone visual transduction cascade, was significantly reduced in vehicle-treated P50 *rd10* mice (arrowheads, Fig. 5a, first panel) and the ONL and INL were very thin (Fig. 5a, second panel). In contrast, at the same location in retinas from metipranolol-treated P50 *rd10* mice, staining for cone arrestin was robust (arrowheads, Fig. 5a, third panel), and the ONL and INL were not as thin as in vehicle-treated mice (Fig. 5a, fourth panel). Nitrotyrosine is a specific marker for nitrosative damage that can be assessed with specific anti-nitrotyrosine antibodies. There was greater staining for nitrotyrosine in retinas from P50 vehicle-treated *rd10* mice (arrowheads, Fig. 5b, first and second panels), than in P50 metipranolol-treated *rd10* mice (arrowhead, Fig. 5b, third and fourth panels). Measurement of the level of nitrotyrosine modifications in retinal homogenates by ELISA, however, did not show a significant reduction at P35 in *rd10* mice treated with metipranolol compared with those treated with vehicle (Fig. 5c). The mean concentration of nitrite, another marker of nitosative damage (van't Hof and Ralston 2001), however, showed significant reduction in retinal homogenates from P35 metipranolol-treated versus vehicle-treated *rd10* mice (Fig. 5d).

We also tested the effects of metipranolol applied as eye drops on *rd10* mice. Topical metipranolol resulted in significant differences in mean scotopic a-wave amplitudes compared with vehicle (Fig. 6a), and caused significantly higher mean scotopic b-wave amplitudes at higher flash intensities compared with vehicle-treated mice at P35 and P50 *rd10* mice (Fig. 6b, c, f, and h). In addition, mean photopic b-wave amplitude was significantly greater in metipranolol-treated mice at P35 (Fig. 6d and e) and at flash intensity 1.0 log cd-s/m^2 at P50 in *rd10* mice (Fig. 6g and i). Mean cone photoreceptor cell density was significantly greater in 2 of 4 quadrants of topical metipranolol-treated P65 *rd10* mice compared with vehicle-treated mice (Fig. 6j). In addition, immunostaining for cone arrestin was nearly undetectable in vehicle-treated P50 *rd10* mice (Fig. 6k left panel), but was strong in metipranolol-treated P50 *rd10* mice (Fig. 6k, right panel). Comparison of ERG scotopic and photopic b-wave amplitudes obtained in *rd10* mice with subcutaneous injections of metipranolol showed significantly greater benefit with topical metipranolol at multiple stimulus intensities at P50 (Fig. 7).

Discussion

Rod photoreceptors are the major oxygen consumers in the retina being that they constitute 95% of cells in the outer retina and have extremely high metabolic activity required to maintain the dark current (Braun *et al.* 1995). Measurements of oxygen levels in the outer retina before and after rod photoreceptor degeneration in RCS rats or rats with a mutation in rhodopsin, showed that loss of rods resulted in a dramatic increase in oxygen levels in the outer retina (Yu *et al.* 2000; Yu and Cringle 2005). In *rd10* mice, rods degenerate earlier than cones and by P35, compared with wild-type C57 mice, there is only 12% remaining scotopic a-wave amplitude, 24% remaining scotopic b-wave amplitude, and 44% remaining photopic b-wave amplitude. As rod photoreceptors drop out, the increasing levels of oxygen result in progressive oxidative damage to cones, remaining rods, and inner retinal cells (Shen *et al.* 2005). Increased levels of oxidative stress and damage have been demonstrated in numerous models of RP and humans with RP and occurs regardless of which of the many different mutations is responsible for rod photoreceptor death (Campochiaro *et al.* 2015). The oxidative stress leads to cone cell death and by P50, scotopic a-wave is extinguished and there is only 10% scotopic b-wave amplitude and 20% photopic b-wave amplitude remaining. Reduction in oxidative stress with drugs or by increasing expression of components of the antioxidant defense system reduces the decline in cone photoreceptor function and cone cell death (Komeima *et al.* 2006, 2007; Usui *et al.* 2009a,b, 2011; Cronin *et al.* 2010; Xiong *et al.* 2015). Because of the high nitric oxide levels in the retina, free radicals generate peroxynitrite which is highly reactive and difficult to detoxify making it an important exacerbating factor; therefore, nitric oxide synthase inhibitors also reduce cone cell death in models of RP (Komeima *et al.* 2008). We have previously shown protection of cone

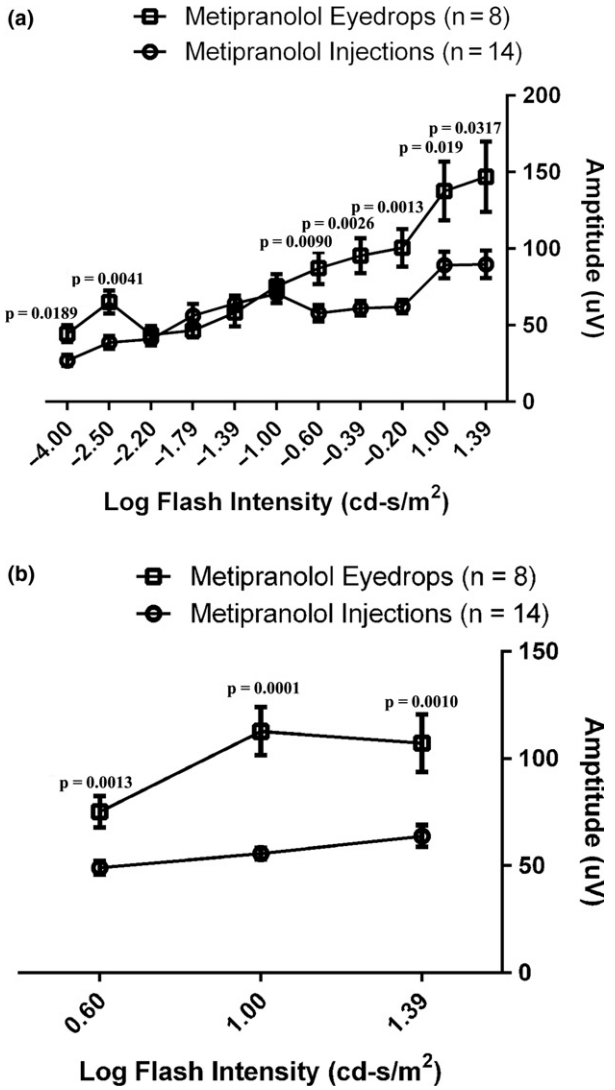


Fig. 7 Comparison of subcutaneous injections of metipranolol vs. metipranolol eye drops in P50 *rd10* mice. (a) Mean (±SEM) scotopic b-wave amplitudes were significantly greater in mice treated topically with metipranolol compared with mice given subcutaneous injections. (b) Mean (±SEM) photopic b-wave amptitudes were significantly greater in mice treated topically with metipranolol compared with mice given subcutaneous injections. Mann–Whitney nonparametric analysis were done for all data and p values ≤ 0.05 were considered significant and exact p values shown for significant data points. n = number of mice used in the experiment.

photoreceptors in *rd1* mice using a mixture of nitric oxide synthase (NOS) inhibitors: N-monomethyl-L-arginine (L-NMMA), NG-nitro-L-arginine, N(omega)-nitro-L-arginine methyl ester (L-NAME), and aminoguanidine bicarbonate (400mg/kg).(Komeima *et al.* 2008) Treatment with 7-nitroindazole, a specific inhibitor of neuronal NOS was able to protect cone photoreceptors from cell death in *rd1* mouse, while aminoguanidine, an inhibitor of inducible nitric oxide synthase was not.

Metipranolol is a nonselective β-adrenergic receptor antagonist given orally for the treatment of arterial hypertension that is fourfold more potent than propranolol (Neuvonen *et al.* 1978). Topical administration of metipranolol to the cornea reduces intraocular pressure by slowing the rate of aqueous humor production and is used for treatment of glaucoma (Krieglstein *et al.* 1987). Metipranolol, timolol, another nonselective β-adrenergic receptor antagonist, and betaxolol, a selective β_1-adrenergic receptor antagonist, all preserve retinal ERG function after ischemia-reperfusion injury (Wood *et al.* 2003). To investigate the mechanism, rat brain homogenates were treated with iron/ascorbate or sodium nitroprusside, a nitric oxide donor that promotes nitrosative stress, in the presence of metipranolol, timolol, betaxolol, and several other glaucoma drugs, and only metipranolol reduced lipid peroxidation (Melena and Osborne 2003). Likewise, only metipranolol reduced retinal cell death after intraocular injection of sodium nitroprusside a nitric oxide donor that promotes nitrosative stress (Osborne and Wood 2004). In this study, we investigated the effect of metipranolol, in the *rd10* model of RP and found that it slowed rod and second order neuron cell death, and more substantially slowed cone cell death and loss of function. We show that, metipranolol injected subcutaneously or applied topically was able to increase scotopic and photopic ERG values at P35 and P50. Mechanistic studies in *rd10* mice showed that metipranolol reduced retinal nitrites which are known markers for nitrosative stress, a major contributor to cone cell death (Komeima *et al.* 2008). In addition, comparisons between subcutaneous injections and topical administration of eye drops showed that the eye drops provided greater protection at P50 (Fig. 7). Expressed in equivalent units, subcutaneous injections involved injections of ~0.24–0.58 mg of metipranolol as the animals grew from P14 to P50. Topical application involved approximately 0.083 mg per dose three times a day for a total of 0.25 mg of metipranolol per day. Although at young ages an equivalent daily dose was given by the different routes (0.24 mg via subcutaneous injections vs. 0.25 mg via eyedrops) and at higher ages, a higher dose was given by subcutaneous injection (0.58 mg via subcutaneous injections vs. 0.25 mg via eyedrops), only a small percentage of the subcutaneously administered drug enters the eye because of the blood-retinal barrier. In addition, accounting for the half-life and clearance rate of the drug, there is greater bioavailability when the drug is applied three time a day, as opposed to injections once a day and this explains the greater efficacy seen with topical administration.

These data suggest that metipranolol may provide benefit in patients with RP by slowing cone degeneration. It is also possible that improvement in rod survival by metipranolol as shown by increased ONL thickness at P35 (Fig. 2j and Fig. 3f) and increased rod function (Fig. 3a), may contribute to cone survival through rod-derived cone viability factor which promotes cone survival (Mei *et al.* 2016).

Metipranolol is delivered topically to the cornea for treatment of glaucoma. Future studies should test whether topical administration can provide sufficient levels of metipranolol to suppress nitrosative stress in the retina. If not, development of a sustained delivery formulation that provides therapeutic levels to the retina for several months after intravitreous injection would be a valuable next step.

Acknowledgments and conflict of interest disclosure

YK, KG, and PAC have filed a use patent for Metipranolol in Retinitis Pigmentosa. Supported by R01EB016121 and the Altsheler-Durell Foundation.

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