The Rat With Oxygen-Induced Retinopathy Is Myopic With Low Retinal Dopamine

Nan Zhang,^{1,2} Tara L. Favazza,¹ Anna Maria Baglieri,¹ Ilan Y. Benador,¹ Emily R. Noonan,¹ Anne B. Fulton,^{1,2} Ronald M. Hansen,^{1,2} P. Michael Iuvone,³ and James D. Akula^{1,2}

¹Department of Ophthalmology, Boston Children's Hospital, Boston, Massachusetts

²Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts

³Departments of Ophthalmology and Pharmacology, Emory University School of Medicine, Atlanta, Georgia

Correspondence: James D. Akula, Department of Ophthalmology, 300 Longwood Avenue, Fegan 4, Boston, MA 02115; xImtc@yahoo.com.

Submitted: June 5, 2013

Accepted: October 14, 2013

Citation: Zhang N, Favazza TL, Baglieri AM, et al. The rat with oxygen-induced retinopathy is myopic with low retinal dopamine. *Invest Ophthalmol Vis Sci.* 2013;54:8275–8284. DOI:10.1167/iovs.13-12544

PURPOSE. Dopamine (DA) is a neurotransmitter implicated both in modulating neural retinal signals and in eye growth. Therefore, it may participate in the pathogenesis of the most common clinical sequelae of retinopathy of prematurity (ROP), visual dysfunction and myopia. Paradoxically, in ROP myopia the eye is usually small. The eye of the rat with oxygen-induced retinopathy (OIR) is characterized by retinal dysfunction and short axial length. There have been several investigations of the early maturation of DA in rat retina, but little at older ages, and not in the OIR rat. Therefore, DA, retinal function, and refractive state were investigated in the OIR rat.

METHODS. In one set of rats, the development of dopaminergic (DAergic) networks was evaluated in retinal cross-sections from rats aged 14 to 120 days using antibodies against tyrosine hydroxylase (TH, the rate-limiting enzyme in the biosynthesis of DA). In another set of rats, retinoscopy was used to evaluate spherical equivalent (SE), electroretinography (ERG) was used to evaluate retinal function, and high-pressure liquid chromatography (HPLC) was used to evaluate retinal contents of DA, its precursor levodopamine (DOPA), and its primary metabolite 3,4-dihydroxyphenylacetic acid (DOPAC).

RESULTS. The normally rapid postnatal ramification of DAergic neurons was disrupted in OIR rats. Retinoscopy revealed that OIR rats were relatively myopic. In the same eyes, ERG confirmed retinal dysfunction in OIR. HPLC of those eyes' retinæ confirmed low DA. Regression analysis indicated that DA metabolism (evaluated by the ratio of DOPAC to DA) was an important additional predictor of myopia beyond OIR.

CONCLUSIONS. The OIR rat is the first known animal model of myopia in which the eye is smaller than normal. Dopamine may modulate, or fail to modulate, neural activity in the OIR eye, and thus contribute to this peculiar myopia.

Keywords: retinopathy of prematurity, myopia, dopamine, rat, development, ocular

In a recent magnetic resonance imaging (MRI) study, Chui et al.¹ found the eyes of rats with oxygen induced retinopathy (OIR) to be characterized by short axial length, increased corneal power and lens power, and proportionally shallow anterior segment. Furthermore, using an untested approach, they calculated, but did not measure, the mature rat eye with a history of OIR to be relatively myopic. Dopamine (DA) has been implicated as a mediator of ocular growth, mostly as a stop signal. In form-deprivation myopia models, eye enlargement is accompanied by decreased retinal DA and DA agonists prevent the myopia.^{2–7} Thus, DA might participate in attenuating the growth of the OIR eye, and possibly (via downstream paracrine messengers) in the development of the anterior segment.

In the retina, DA is produced by a single class of amacrine cell^{8–13} that constitutes less than 0.1% of the population of retinal neurons.^{6,14,15} Stimulation of the retinal ON pathway by light induces DA release from these cells.⁶ Defects in retinal ON pathway signaling are associated with anomalous eye growth,¹⁶ and OIR in the rat produces persistent dysfunction of the neurosensory retina.¹⁷ OIR-induced changes in DA release might participate in this retinal dysfunction since DA regulates

many aspects of retinal signaling. For example, DA alters retinal circuitry to favor cone-driven, contrast-sensitive pathways in bright light^{6,18} and rod-driven, light-sensitive pathways in dim light,¹⁹ in part by inducing nitric oxide (NO) release.^{20,21} Dopamine also modulates connexin-36 gap junctions,^{22–24} glutamatergic synapses,^{25,26} voltage-gated potassium channels in ON-cone bipolar cells,^{22,27} Na⁺,K⁺-ATPase channels in photoreceptors,²⁸ and cGMP-gated channels in cones.²⁹ It is, therefore, plausible that DA participates both in the retinal dysfunction and the prevalent ametropia recently documented in OIR rats' eyes.

In the present study of the OIR rat, the development of retinal dopaminergic (DAergic) neurons and the association of refractive state, retinal function, and DA metabolism are investigated. Herein is reported that (1) despite having recently been found to be characterized by a small eye,¹ the OIR rat is myopic, (2) the OIR rat is characterized by delayed and attenuated development of retinal DAergic networks, and (3) diminished DA metabolism is associated with the magnitude of the myopia. Provocatively, human eyes with a history of retinopathy of prematurity (ROP) are, on average, also

FIGURE 1. Dopamine (DA) synthesis and metabolism. Inhibition of aromatic L-amino acid decarboxylase (AAAD) by *m*-hydroxybenzylhydrazine (NSD-1015) prevents the decarboxylation of LL-dihydroxyphenylalanine (DOPA) into DA. *Shadowed* metabolites were assessed in isolated retinæ by high-pressure liquid chromatography (HPLC). Other abbreviations: TH, tyrosine hydroxylase; MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; DBH, dopamine β -hydroxylase; DHPG, 3,4-dihydroxyphenylethylene glycol; MHPG, 3-methoxy-4-hydroxyphenyl glycol; PNMT, phenylethanolamine N-methyltransferase.

characterized by steep corneas, thick lenses, and shallow anterior segments³⁰ that render them myopic in spite of short axial length.^{30–42} Thus, the rat with OIR may provide insight into the development of some of the ocular features of human ROP eyes.

METHODS

Experimental Design

All experiments were performed in Sprague-Dawley rats (Charles River Laboratories, Worcester, MA). In the first set of experiments, DAergic development in the retina was studied immunohistochemically (IHC) in retinal cross sections. Prior to these IHC studies, the antibodies used were validated by Western blot and by comparison of labeled cell structures to published literature. In the second set of experiments, individual rats were refracted, tested by electroretinography (ERG), and then their retinal DA content was evaluated by high-pressure liquid chromatography (HPLC). These data were analyzed for significant relations using regression analyses. All experiments were conducted with the approval of the Institutional Animal Care and Use Committee at Boston Children's Hospital.

Animals and Induction of Retinopathy

Retinopathy was induced in rats following the “50/10” method of Penn et al.⁴³ Neovascularization and an avascular peripheral retina characterize this retinopathy.^{44–46} Although the rat is not born prematurely, the rat pup's eyes are in a state of maturity that corresponds to approximately human midgestation. On the day of birth until postnatal day (P) 14, newborn pups and dam were placed in an oxygen-controlled environment (Oxy-Cycler; Biospherix Ltd., Redfield, NY) where the ambient oxygen concentration alternated every 24 hours between $50 \pm 1\%$ and $10 \pm 1\%$. This oxygen regimen targeted the rats' retinae during a period of rhodopsin and sensitivity development similar to the timing of the supplemental oxygen used to manage very prematurely born human infants. In the OIR rat,

FIGURE 2. Validation of anti-TH monoclonal antibody. (A) Western-blot analysis revealed a tight band with an apparent molecular weight predicted by the amino acid sequence of TH. (B) Image of a whole-mounted retina labeled with the monoclonal antibody. A bright soma and the tell-tale pattern of dopaminergic processes is clearly visible, confirming appropriate immunoreactivity of the antibody.

and the human infant with active ROP, retinal sensitivity and vessel tortuosity are about the same.⁴⁷ Room-air-reared animals served as controls (“RAR rats”). A total of 87 animals were studied. Numbers of OIR and RAR rats used in each experiment are given in the respective section of the Results. The light cycle was 12 hours dark, 12 hours of 75 lux (or less) light. All samples and measurements were obtained during the light period, at least 2 hours after lights on and 2 hours before lights off, because synthesis and turnover of DA are known to follow a circadian rhythm.^{48,49} All procedures were carried out in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

Immunohistochemistry

Validation of Antibodies by Western Blot. A mouse monoclonal antibody (MAB318; Millipore, Billerica, MA) that targets tyrosine hydroxylase (TH, the rate-limiting enzyme in the biosynthesis of DA) was tested for quality by Western blot on one rat's retinae. Homogenization was performed at 4°C with a lysis buffer containing phosphatase inhibitor cocktail and protease inhibitor according to the instruction of the vendor. Lysates were centrifuged at 14,000g at 4°C for 10 minutes. Protein concentration was assayed by the Bradford⁵⁰ method. The samples were boiled for 5 minutes in 2× Laemmli sample buffer (Bio-Rad, Hercules, CA). Protein (15–50 μ g) was subjected to electrophoresis on 10% Tris-HCl ready gel (Bio-Rad), followed by transfer to a nitrocellulose membrane that was blocked for 1 hour in a 5% suspension of dried milk in washing buffer and incubated overnight at 4°C with monoclonal anti-TH (1:1000). Visualization was performed using goat anti-mouse secondary antibody conjugated to horseradish peroxidase (GxM-HRP; Millipore). The band was developed with a chemiluminescence detection system (X-OMAT 2000A; Eastman Kodak, Rochester, NY).

Validation of Antibodies by Target Features. An enucleated eyeball was punctured through the cornea and fixed in 4% paraformaldehyde for 1 hour. The retina was flatmounted⁴⁶ and the mouse monoclonal primary antibody (MAB318, 1:500; Millipore) was applied. Then, the retina was incubated with 1:1000 secondary antibody (Alexa Fluor 954-conjugated goat anti-mouse), washed, and photographed (40×; DM5500 microscope; Leica, Wetzlar, Germany). The labeled cells were compared with descriptions of DAergic cells in the literature.^{6,8,12,51–56}

Development of Dopaminergic Amacrine Cell Networks. Eyecups for cyrosectioning were fixed as described above, then placed in 30% sucrose at 4°C overnight, embedded

FIGURE 3. Immunohistochemical analyses of retinal sections. *Upper panels (A–D)* show retinal sections, the IPL and INL are noted. **(A)** Monoclonal anti-TH antibody in the red channel. The *yellow arrow* indicates a dopaminergic (DAergic) cell soma. **(B)** Polyclonal anti-TH antibody in *green* channel with the same soma labeled. **(C)** All cell bodies labeled with DAPI in *blue* channel. **(D)** A tricolored overlay. The *yellow* coloring of the DAergic cell and profiles indicates that the two antibodies label the same structures in the retina. The *scale bar* applies to **(A–D)** and is 25 μm . **(E)** Representative tricolor sections of the region of interest (IPL–INL) from RAR (*left*) and OIR (*right*) central retinas at the postnatal days indicated. The *scale bar* in the lower right is 25 μm .

in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA) and frozen. Retinal cross-sections (16 μm) were cut with a cryostat and kept frozen until immunostaining. The monoclonal (MAB318, 1:500; Millipore) and a rabbit polyclonal (AB512, 1:500; Millipore) antibodies against TH were used to detect DAergic cells. The retinal sections were then incubated with 1:1000 respective secondary antibodies (Alexa Fluor 954-conjugated goat anti-mouse and 488-conjugated goat anti-rabbit; Millipore). After washing, each section was counterstained with 4',6-diamidino-2-phenylindole (DAPI) and treated with Antifade (Life Technologies, Grand Island, NY) to prolong immunofluorescence.

Digital images (40 $\times$) of each section were obtained using respective filters and overlaid to generate a tricolor (pseudo-RGB) image. To quantify TH+ labeling, a region of interest (ROI) along the border between the inner nuclear layer (INL) and the inner plexiform layer (IPL) was identified and circumscribed by an operator (NZ). Automated segmentation was then performed on the ROI in ImageJ,⁵⁷ and the pixels

above threshold were counted. The operator then traced the length of the INL–IPL boundary and divided total the number of counted pixels by this length.

Retinoscopy

Two experienced retinoscopists (AMB, ABF) performed streak retinoscopy two times in both eyes, at least 15 minutes after instillation of 0.2% cyclopentolate hydrochloride and 1% phenylephrine hydrochloride (Cyclomydril; Alcon, Fort Worth, TX). Each retinoscopist was masked as to the other's results. Because the “small eye artifact”⁵⁸ results in relatively hyperopic estimates of spherical equivalent (SE), the SE in OIR rats was expressed relative to that in RAR rats.

Electroretinography

ERG were recorded as previously described.⁵⁹ In brief, rats were anesthetized with an intraperitoneal injection of 75 $\text{mg}\cdot\text{kg}^{-1}$ ketamine and 10 $\text{mg}\cdot\text{kg}^{-1}$ xylazine. Mydriasis was

FIGURE 4. Development of dopaminergic (DAergic) processes in the retina as assessed by quantitative immunohistochemistry against TH+. TH+ at the boundary of the INL and IPL increased through P57 before decreasing at P120 in both central retina (within 1 mm of the optic disk) and peripheral retina (within 1 mm of the ora serrata). TH+ was significantly lower in OIR rats than in RAR controls.

induced using Cyclomydril (Alcon). Body temperature was maintained with a warming pad. Gold loop electrodes were placed on both eyes, the reference was placed in the mouth, and the ground was affixed to the tail or hind foot. ERG stimuli were delivered using an Espion e^2 with Colordome Ganzfeld stimulator (Diagnosys LLC, Lowell, MA). Responses were elicited using a series of flashes of doubling intensity, ranging from one that elicited a small b-wave to one that saturated the a-wave (green light emitting diode [LED] 0.000125 – 2.05 $\text{cd}\cdot\text{s}\cdot\text{m}^{-2}$ then white xenon arc 8.20 – 524 $\text{cd}\cdot\text{s}\cdot\text{m}^{-2}$). The saturating amplitude (Rm_{P_3}) and sensitivity (S) of the rod photoresponse were estimated by fit of a model (P_3) of the biochemical processes involved in the activation of phototransduction to the ERG a-waves.^{60–63} P_2 , a putatively purely postreceptoral potential, was derived by digitally subtracting P_3 from the intact ERG responses. The saturating amplitude (Rm_{P_2}) and sensitivity ($1/K_{P_2}$) of the dark-adapted postreceptor retina were derived from the Naka-Rushton⁶⁴ equation fit to the response versus intensity relationship of P_2 .⁶⁵ The oscillatory potentials (OPs), which characterize activity in retinal cells distinct from those that generate P_3 and P_2 , such as inner-retinal amacrine and ganglion cells,⁶⁶ were also studied. Their sensitivity ($1/K_{OPs}$) and saturating amplitude, estimated by the square root of saturating OP energy ($Em^{1/2}$), were also measured.⁶⁷ Thus, P_3 , P_2 , and the OPs are doubly thought

of as including predominating contributions from cells with respectively deeper retinal positions: photoreceptors, bipolars, and amacrine and ganglion cells. All ERG data were recorded as the log change from normal ($\Delta\text{LogNormal}$). By expressing the data in log values, changes in observations of fixed proportion become linear, consistent with a constant fraction for physiologically meaningful changes in parameter values.⁶⁸

Detection of DAergic Activity

The utilization of DA can be estimated by monitoring the decline in levels of DA and its metabolites when its synthesis is interrupted. As shown in Figure 1, DA is synthesized from L-tyrosine by a two-step process: (1) hydroxylation of L-tyrosine by TH to produce levodopamine, the carboxylated form of dihydroxyphenylalanine (DOPA), and (2) decarboxylation of DOPA by aromatic L-amino acid decarboxylase (AAAD) to produce DA. The primary metabolite of DA is 3,4-dihydroxyphenylacetic acid (DOPAC); DA can also be converted to norepinephrine by dopamine β -hydroxylase, though not significantly in retina. *m*-Hydroxybenzylhydrazine (NSD-1015) inhibits AAAD, resulting in accumulation of DOPA, which can be used to estimate in situ TH activity/DA synthesis; the decline in DA and its metabolites in the presence of NSD-1015 is a measure of DA utilization.⁶⁹ In order to analyze DA turnover in OIR rat retinæ as compared with RAR rat retinæ, NSD-1015 (150 $\text{mg}\cdot\text{kg}^{-1}$) was administered intraperitoneally. Approximately 30 minutes later, animals were killed with CO_2 and their retinæ were removed and flash frozen in liquid nitrogen; elapsed time was carefully noted. Uninjected rats served as controls. The retinal samples were later homogenized with 0.1 N HClO_4 containing 0.1% sodium metabisulfite to prevent the oxidation of catecholamine, and retinal levels of DA, DOPA, and DOPAC were assayed using HPLC with coulometric detection.⁷⁰ Regression on elapsed time versus metabolite concentration provided an estimate of the rate of change in each metabolite following injection of NSD-1015. The coefficients of this regression enabled us to estimate the steady-state levels of each metabolite.

Data Analysis

Parametric statistical analyses were performed in SPSS Statistics 21 (SPSS, Inc., An IBM Company, Chicago, IL). The TH+ labeling in retinal sections was studied as a function of age by

FIGURE 5. Spherical equivalent in RAR and OIR rats measured by retinoscopy. Spherical equivalent in RAR rats was normalized to Plano; OIR rats were significantly more myopic relative to RAR rats.

TABLE. Mean (SD) Key Parameters of Refracted Rats

Parameter	Unit	RAR	OIR
Weight at P55–59	G	356 (107)	287 (62)
Mean refraction	D	1.82 (2.59)	0.19 (3.00)
Rm_{P3}	$ \mu V $	470 (148)	259 (64)
S	$m^2/cd \cdot s^3$ (xenon arc)	3420 (1260)	2490 (770)
Rm_{P2}	μV	946 (295)	321 (79)
$1/K_{P2}$	$m^2/cd \cdot s$ (green LED)	954 (439)	367 (187)
$Em^{1/2}$	μV	201 (49)	40 (26)
$1/K_{OPs}$	$m^2/cd \cdot s$ (green LED)	6.10 (7.85)	3.27 (1.11)
DOPA	pg/mL	159 (60)	148 (39)
DA	pg/mL	2130 (160)	1330 (300)
DOPAC	pg/mL	1140 (370)	640 (310)

ANOVA. Refractive state in OIR and RAR rats was compared by a second ANOVA. Within-subjects comparisons of ERG parameters and HPLC data obtained from the refracted rats were performed by additional, respective ANOVA, and the relation of ERG and HPLC parameters to refractive state (SE) was studied by multiple regression.

RESULTS

Normal Postnatal Development of Retinal DAergic Networks Is Disrupted in OIR

One RAR rat's retinae were lysed for Western blot analysis of the monoclonal anti-TH antibody, another's retinae were flat mounted for inspection of the structure of the cells labeled by this antibody, and 32 RAR and 32 OIR rats' retinae were sectioned for evaluation of the development of the DAergic retinal cell networks. First, the specificity of the monoclonal anti-TH antibody in the lysate was confirmed: the results of the Western blot showed a single band at approximately 59 kDa (Fig. 2A), consistent with the description provided by the vendor. Second, the monoclonal anti-TH antibody was used to label the flat-mounted retina. As shown in Figure 2B, the telltale features of DAergic amacrine cells were revealed: a dense plexus of fibers was seen when the plane of focus was at

the IPL, there were numerous varicosities along the fibers, and characteristic ring-like profiles were noted. These unique, ring-like formations arise because DAergic amacrine cell networks surround AII amacrine cells and innervate them and, thus, anatomic confirmation of retinal DAergic cell labeling was found.^{6,13,51,71,72} Third, to confirm the second (polyclonal) antibody, cells in retinal sections from a RAR rat at P20 were double-labeled and counterstained with DAPI (so that the retinal laminae could be easily recognized). As shown in Figure 3, both the mono- and polyclonal antibodies labeled the same retinal structures: Figure 3A shows the monoclonal antibody labeling (red channel); Figure 3B shows the polyclonal antibody labeling (green channel); Figure 3C shows DAPI labeling (blue channel); Figure 3D shows the composited RGB overlay. A representative DAergic amacrine cell can easily be observed, with its soma located in the INL and close to the IPL and its fiber processes ramifying in the IPL, mainly in sublamina 1 along the border between the INL and IPL. Some fiber processes also ramified in sublamina 3 in the IPL. That the labeling is in yellow confirms that the same regions were immunoreactive to both the mono- and polyclonal antibodies. Thus, both antibodies labeled DAergic cells.

In additional retinal sections, obtained from the 32 RAR and 32 OIR rats of selected ages from infancy (P14) to adulthood (P120), the development of DAergic processes along the IPL (Fig. 3E) was studied systematically. From each animal, at least six central retinal sections and six peripheral retinal sections were studied. Qualitatively, at P14, little TH+ staining was noted in either control retinae ($n = 3$) or OIR retinae ($n = 3$). At P16, TH+ processes were readily detected in the IPL of control retinae ($n = 3$), but not OIR retinae ($n = 3$). Even at P18, when there was robust TH+ labeling along the IPL-INL boundary in control retinae ($n = 6$), there was only very little TH+ in OIR retinae ($n = 6$). At P19, the number of observed TH+ processes along the IPL were abundant in control retinae ($n = 5$) and well-stained somata of DAergic amacrine cells were observed from time to time, but TH+ staining was much less prominent in control retinae ($n = 5$). The number of stained profiles along the IPL continued to rise from P20 ($n = 5$ RAR, $n = 5$ OIR) through P57 ($n = 3$ RAR, $n = 3$ OIR), but declined at the final, P120, test ($n = 5$ RAR, $n = 5$ OIR). This decline has not, to the authors' knowledge, been previously reported in rat.

Quantitative analysis of these same sections, using ImageJ,⁵⁷ on at least six slices per animal, demonstrated the same pattern of results both centrally and peripherally (Fig. 4): a waxing and

FIGURE 6. Response amplitude and sensitivity in rod photoreceptors (Rm_{P3} , S), postreceptor bipolar cells (Rm_{P2} , $1/K_{P2}$), and inner retinal amacrine and ganglion cells ($Em^{1/2}$, $1/K_{OPs}$) derived from the dark-adapted ERG are plotted on the abscissa; the ordinate value 0 represents normal. At every retinal depth, response amplitude and sensitivity was significantly subnormal in ROP rats.

FIGURE 7. High-pressure liquid chromatography results. DA and DOPAC levels were significantly lower in OIR than control retinæ. Intraperitoneal injection of NSD-1015 caused significant accumulation of DOPA and depletion of DA in both OIR and RAR rat retinæ, but did not much alter DOPAC levels.

waning of anti-TH labeling in the IPL ($F_{\text{age}} = 51.2$; $df = 6, 46$; $P < 0.001$) that appeared delayed in its onset and was diminished at its peak in OIR rats ($F_{\text{group}} = 132$; $df = 1, 46$; $P < 0.001$). Overall, there was slightly ($\sim 24.5\%$ decrease at P57) but significantly ($F_{\text{eccentricity}} = 60.9$; $df = 1, 46$; $P < 0.001$) less labeling in peripheral retina (within 1 mm of the ora serrata) than in central retina (within 1 mm of the optic nerve head).

OIR Eyes Have Lower Spherical Equivalents Than Control Eyes

Retinoscopy was performed on 10 RAR and 11 OIR rats at P55; results were analyzed by repeated-measures ANOVA with factors group (RAR, OIR) and eye (left, right). As shown in Figure 5 and the Table, the mean SE in OIR rats was 1.63 diopters (D) lower than in RAR rats ($F_{\text{group}} = 13.2$; $df = 1, 19$; $P = 0.0018$). The OIR rats were also markedly smaller than the RAR rats in this study. There was no systematic left versus right eye anisometropia ($F_{\text{eye}} = 0.120$; $df = 1, 19$; $P = 0.73$).

The OIR Retina Is Dysfunctional

Following retinoscopy, retinal function was tested in the same rats by ERG. The respective amplitude and sensitivity ERG parameters for the photoreceptors (Rm_{p3} , S), postreceptor cells (Rm_{p2} , $1/K_{p2}$), and inner retina ($Em^{1/2}$, $1/K_{OPs}$) were analyzed by repeated-measures ANOVA with factors group, category (amplitude, sensitivity), depth (photoreceptor, postreceptor inner retina), and eye (left, right). As shown in Figure 6 and the Table, ERG response amplitude and sensitivity were both significantly reduced in OIR rats at every retinal depth ($F_{\text{group}} = 7.56$; $df = 1, 19$; $P = 0.013$). Differences in other factors (depth, category, and eye) and their interactions were all insignificant (all $P \geq 0.39$).

Steady-State Levels of Retinal DA Are Reduced in OIR Without a Defect in DA Turnover

In the same rats refracted and tested by ERG, DA level was studied using HPLC. Five of the RAR rats and six of the OIR rats were injected with NSD-1015 and killed 19 to 48 minutes later, along with the remaining rats who did not receive an injection.

The results of HPLC of DOPA, DA, and DOPAC in excised retinæ are shown in Figure 7 and given (for rats not treated with NSD-1015) in the Table. DOPA, DA, and DOPAC were all measurable in both RAR and OIR retinæ and were analyzed by repeated-measures ANOVA with factors group, metabolite (DOPA, DA, DOPAC), and injection (none, NSD-1015). In rats not treated with NSD-1015, compared with control retinæ, OIR retinæ had similar levels of DOPA, but significantly decreased levels of DA and DOPAC ($F_{\text{group} \times \text{metabolite}} = 3.63$; $df = 2, 34$; $P = 0.037$). NSD-1015 had very similar effects on both OIR and control retinæ: marked increase in DOPA, marked decrease in DA, and no significant change in DOPAC ($F_{\text{metabolite} \times \text{injection}} = 84.9$; $df = 2, 34$; $P < 0.001$). These results are evidence that enzymatic conversion of L-tyrosine to DA is normal in OIR retina, but that instead there are fewer DAergic cells or processes.

The coefficients for regression on elapsed time since NSD-1015 injection versus metabolite were 1.01 for DOPA, -0.20 for DA, and -0.028 for DOPAC, suggesting that, following NSD-1015 injection, DOPA levels rise rapidly, DA levels fall moderately, and DOPAC levels fall very slowly.

DA Cell Networks May Contribute to OIR Myopia

In order to evaluate what factors could contribute to the OIR myopia, forward-stepwise multiple regression using information criterion for parameter entry and removal with parameters group (RAR = 0, OIR = 1), photoreceptor amplitude (Rm_{p3}) and sensitivity (S), postreceptor amplitude (Rm_{p2}) and sensitivity ($1/K_{p2}$), inner-retinal amplitude ($Em^{1/2}$) and sensitivity ($1/K_{OPs}$), and log-levels of DOPAC/DA were run; utilization of DA is commonly estimated by the ratio of DOPAC to DA.^{69,73} As shown in Figure 8A, the resulting model ($P = 0.031$) included only group (RAR or OIR) and ratio (DOPAC/DA). As shown in Figure 8B, these two parameters alone were sufficient to produce a model with excellent agreement between predicted and observed SE ($r^2 = 0.71$). To ascertain whether the predictions regarding the steady-state levels of DA and DOPAC in the NSD-1015-injected animals were reasonable, these animals' predicted refractions, as calculated from the regression model, were added to the plot. The agreement remained excellent.

FIGURE 8. Linear regression analyses. **(A)** The model resulting from a forward-stepwise multiple linear regression with predictor removal by information criterion included only group and DOPAC/DA (a measure of DA metabolism). The contribution to the final model of both is shown. **(B)** The final model predicted actual refraction with good accuracy. It also predicted refraction in NSD-1015-injected animals after correcting their DA and DOPAC values as described in the text. The line is an orthogonal regression through all the data.

DISCUSSION

Dopamine released by DAergic amacrine cells reaches target neurons either locally at the synaptic terminals or via paracrine diffusion. Thus, DA is poised to regulate a wide range of retinal functions, and loss of DA will have sundry ocular effects. In the present study, despite the fact that the OIR rats' axial lengths were presumably low,¹ development of DAergic processes was delayed and, when the retina was mature, remained less prominent than in RAR retinæ (Figs. 3, 4). In addition, as summarized in the Table, the mature eye of the OIR rat is characterized by neurosensory retinal dysfunction and myopia. Some of these features may have been, in part, consequences of altered retinal DA.

Maturation of Retinal DAergic Cells

Development of TH+ processes in the normal retina proceeded steadily from P14 through P20; TH+ immunoreactivity was higher still at P57 and then decreased by P120 (Fig. 4). In the rat retina, most progenitor cells destined to become DAergic neurons reach their cell fate within a prenatal period of amacrine cell differentiation between embryonic days 16 and 20.⁷⁴ Although cell number is approximately fixed, DAergic perikarya increase in size at least through P21 and their processes become greater in density at even older ages.⁷⁵ In these sections, dendritic and axonal ramifications were not distinguished. The elaboration of DAergic dendritic processes seems to be complete by approximately P15, but axonogenesis is only just beginning: there is little evidence of axon terminals in the form of rings until approximately P17 and, while the rings are mostly in place at P21, maturation of DAergic axons continues into adulthood.⁷⁵ These results are consistent with postnatal maturation of DAergic networks, but also indicate a regression in the mature eye. Since soma were not counted, cell death, decreased ramification per cell, or simply loss of TH immunoreactivity could not be distinguished.⁷⁶ Interestingly, there is a decrease in many ERG parameters at ages older than 30 to 60 days in rats⁷⁷ and mice.⁷⁸ Notably, the induction of OIR occurs from P0 to P14, a timeframe during which DAergic cells are mainly undergoing development instead of differentiation, meaning there may well be a full complement of

DAergic cells, but with altered morphology in OIR. In particular, it is likely that the development of dendritic processes is impacted and, thus, the persistently low TH+ might be mostly associated with loss of DAergic dendrites.

DA and OIR

The postnatal development and maturation of DAergic amacrine cell networks was found to be delayed and diminished. Dopamine released by these cells presumably acts on the RPE to regulate eye growth.^{79,80} Dopamine production is decreased in form-deprivation myopia in both monkeys and chicks.^{81,82} Thus, DA is a stop signal in myopia. However, complete depletion of DA causes an overall reduction of eye size in fish.⁸³ Refractive development in OIR rat eyes, being myopic but small,¹ would seem to depend on mechanisms beyond DA.

Regression analysis suggested a relationship between refractive state and DA metabolism (Fig. 8). While DA released in the retina could reach the anterior segment via vitreal or uveal routes, more likely (if it indeed plays a role at all), it would regulate anterior segment development via alternative pathways. For example, NO has been reported to inhibit DA release from DAergic neurons,^{84,85} whereas DA has been reported to induce release of NO in retina.^{20,21} Other data suggest that there is altered nitric oxide synthase (NOS) activity in OIR.⁸⁶ These data point to the possibility that altered NO production is an important step during OIR pathogenesis, perhaps leading to the alterations in postnatal development and maturation of DAergic amacrine cells in OIR retina.

Retinal Function

The neural retina was dysfunctional in the OIR rats (Table; Fig. 6). Significant attenuation was observed in responses originating in photoreceptors, bipolar cells, and inner retinal neurons, with the most marked dysfunction in the inner retinal response amplitudes (OPs). The OIR rats were approximately four-fifths the size of the RAR rats, and it is possible that the size of the rat (or its eye) could cause the observed change in response parameters, rather than residual retinal pathology. However,

within each group (RAR, OIR), there was no straightforward association between weight and ERG amplitude. Furthermore, evidence suggests that the association between eye size and ERG amplitude is quite small and in the opposite direction: smaller eyes tend to have larger amplitudes.^{87–89} Thus, any effect of size would likely have led to an underestimation of the impact OIR on retinal function.

Relationship to ROP Myopia

The axial length of the eye of the OIR rat has been previously shown to be short.¹ These data confirm that the OIR rat is also myopic (Table; Fig. 5). The OIR rat's eye has some provocative similarities to the eye with a history of ROP¹; it is, therefore, possible that similar processes are at work in OIR and ROP eyes, although the relevance to human ROP myopia is equivocal. On the one hand, in both ROP myopia and OIR rat myopia, the dioptric power of the anterior segment is high, more than offsetting the short axial length. On the other hand, the OIR rat is not born prematurely and does not suffer from cicatricose consequences when left untreated,⁴⁶ these are the features most feared in severe ROP (i.e., ROP that would be treated), and instead OIR seems to produce vascular consequences, which resolve without scarring, similar to milder ROP (i.e., ROP that would not be treated). In mild ROP, the incidence of myopia is not much increased,⁹⁰ but myopia does seem to be a feature of the OIR rat. Furthermore, the rats in this study were born full term, and thus, did not suffer any effects from premature externalization. Nevertheless, further study of the OIR rat may provide insights into ocular development difficult or impossible to obtain using conventional (axial) myopia models.

Acknowledgments

Supported by National Institutes of Health (NIH) Grants RC1EY020308 (JDA), R01EY004864 and P30EY006360 (PMI), Research to Prevent Blindness, Inc. (PMI), and the Massachusetts Lions Eye Research Fund (RMH).

Disclosure: **N. Zhang**, None; **T.L. Favazza**, None; **A.M. Baglieri**, None; **I.Y. Bendor**, None; **E.R. Noonan**, None; **A.B. Fulton**, None; **R.M. Hansen**, None; **P.M. Iuvone**, None; **J.D. Akula**, None

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