

The Role of Retinal Dopamine in C57BL/6 Mouse Refractive Development as Revealed by Intravitreal Administration of 6-Hydroxydopamine

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PURPOSE. Although retinal dopamine (DA) has been long implicated in myopia development, current studies demonstrate that retinal DA levels are unaltered in C57BL/6 mice with form-deprivation myopia. This work was undertaken to explore whether and how refractive development is perturbed in this mouse strain when retinal DA levels are reduced by 6-hydroxydopamine (6-OHDA) administration.

METHODS. On two successive days, 6-OHDA was injected into the vitreous of P18 mice. Retinal DA levels were measured by HPLC and TH levels analyzed by quantitative Western blotting. To choose appropriate 6-OHDA doses that significantly reduce retinal DA levels, but cause minimal disturbance of overall retinal physiology, ERG analysis was performed. Refractive errors were measured using a photorefractor, and ocular biometry performed with optical coherence tomography and photokeratometry.

RESULTS. Administration of 6-OHDA of 6.25 μ g and 12.5 μ g significantly reduced retinal levels of DA and TH, but without affecting ERG a- and b-wave amplitudes. With normal visual experience, 6-OHDA induced myopic refractive shifts in a dose-dependent fashion. Form deprivation induced further myopic shifts in 6-OHDA-injected eyes, but did not cause further decline in retinal DA. Furthermore, 6-OHDA administration resulted in a shorter axial length and a steeper cornea, whereas form deprivation led to a longer axial length, without changing the corneal radius of curvature.

CONCLUSIONS. Reducing retinal DA levels led to myopic refractive shifts in C57BL/6 mice, which mainly resulted from a steeper cornea. In addition to the DA-independent mechanism for form-deprivation myopia, there is a DA-dependent mechanism in parallel that underlies myopic refractive shifts under normal laboratory conditions in this mouse strain.

Keywords: refractive development, form-deprivation myopia, dopamine, C57BL/6 mouse, 6-hydroxydopamine

In a variety of vertebrates, deprivation of sharp retinal images by placing translucent occluders in front of growing eyes leads to exaggerated axial enlargement, which is known as form-deprivation myopia (FDM).¹⁻¹⁶ In form-deprived chicks,^{17,18} monkeys,¹⁹ tree shrews,²⁰ and guinea pigs,^{21,22} the daytime retinal dopamine (DA) levels are reduced, and such changes in DA levels are regarded as evidence that DA may work as a key messenger molecule in the signaling cascade that underlies myopic eye growth. In these species, this working hypothesis seems to be supported by results obtained via several distinct approaches. For instance, systemic or intraocular administration of DA and its agonists effectively suppresses FDM formation.^{18,21-31} Moreover, consistent with this hypothesis, several epidemiologic investigations show that outdoor activity and strong illumination, both enhancing retinal DA levels, protect children from myopia development.³²⁻³⁵

Recently, FDM has been successfully induced in the mouse.³⁶⁻⁴¹ Because of its easy breeding and maintenance, and its accessibility of genetic manipulations, this species provides a promising model for investigating the mechanisms underlying refractive development.⁴² However, unlike most previous results obtained in other animal models, several lines of evidence suggest that retinal DA in the C57BL/6 mouse, a strain that is commonly used in experimental myopia research, plays a minor, if any, role in FDM development. In form-deprived C57BL/6 mice, both retinal and vitreal DA/3,4-dihydroxyphenylacetic acid (DOPAC) levels remain unchanged.⁴³⁻⁴⁵ In addition, retinal protein/transcript levels of tyrosine hydroxylase (TH) and DA transporter (DAT) also are unchanged, and dopaminergic amacrine cells remain intact during FDM development.⁴⁵ Form-deprivation myopia development seems not to be associated with retinal DA levels in this



mouse strain, although it is not clear whether other aspects of the retinal dopaminergic system (e.g., DA receptor sensitivity) may be modified by form deprivation.

The present work was undertaken to further investigate the possible role of retinal DA in refractive development in this mouse strain. For this purpose, the refractive development process of C57BL/6 mice was monitored when retinal DA levels were reduced by intravitreal injections of the catecholaminergic toxin 6-hydroxydopamine (6-OHDA). The appropriate doses of 6-OHDA were so chosen such that retinal DA levels were clearly reduced but overall retinal function was minimally affected. At these appropriate doses, 6-OHDA induced myopic refractive shifts under normal visual conditions in a dose-dependent manner. Ocular dimension measurements further revealed that, in contrast to the longer axial length (AL) and unchanged corneal radius of curvature (CRC) seen in form-deprived eyes, 6-OHDA administration resulted in a shorter AL and a steeper cornea, as compared with fellow eyes. These results suggest that, in addition to the DA-independent mechanism responsible for myopic shifts induced by form deprivation, a DA-dependent mechanism operates for mediating myopic shifts due to reduced DA levels.

MATERIALS AND METHODS

Animals

Eighteen-day-old male C57BL/6 mice were used in the present study. They were raised in a 12-hour light/dark cycle (light on at 8:00 AM) with ad libitum access to food and water. Illumination was provided by cool white fluorescent bulbs, which produced an ambient illumination of approximately 200 lux. All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the regulations of Fudan University for animal experimentation.

Intravitreal Injection

The mice were deeply anesthetized with 0.6% pentobarbital sodium (15 μ L/g). Four doses of 6-OHDA (3 μ g, 6.25 μ g, 12.5 μ g, and 50 μ g), all dissolved in 0.8 μ L saline solution (containing 1 mg/mL ascorbic acid), were injected into the vitreous of the right eyes using a Nanoject II microinjector (3-000-205/206; Drummond Scientific Company, Broomall, PA, USA) with a glass micropipette (10–30 μ m in tip size); left eyes were left intact. To minimize the effect on IOP, injections were made twice, on two successive days (postnatal day 18 [P18] and P19), 0.4 μ L each time (both freshly made), through the nasal and temporal part of the sclera, respectively. It is known that 6-OHDA has a half-life of approximately 0.5 to 2.0 hours under in vivo conditions,⁴⁶ and is capable of rapidly producing a long-lasting lesion of dopaminergic systems, with even a single dose.^{47–49} Control groups received the same volume of drug vehicle.

Myopia Induction

The procedure of FDM induction in C57BL/6 mice has been previously described in detail.^{38,45} In brief, handmade translucent occluders were attached carefully to the fur around right eyes by glue for 4 weeks. To prevent the removal of occluders by animals, plastic collars were fitted around the neck. Data were discarded if frequent loss of occluder or eye diseases (such as cataracts, corneal opacity) occurred. Mice wearing occluders were housed singly, and the others were housed in groups of five to six. A pilot experiment showed that housing animals differently did not influence their refractive development.

Refraction Assessment

Refraction measurements by a calibrated eccentric infrared photorefractor were carried out as previously described,⁴⁵ and three sets of measurements were averaged for each eye. To minimize nonspecific effects, mice with an initial interocular refractive difference greater than 3 diopters (D) were not used in subsequent experiments. Mice receiving injections of 6-OHDA or vehicle were refracted at postinjection days 3, 10, 17, 24, and 31 (D3, D10, D17, D24, and D31), whereas those receiving form-deprivation treatment were refracted only at D31.

Ocular Biometry

Ocular dimensions, including corneal thickness (CT), anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD), retina thickness (RT), and AL, were measured at D31, using a custom-built, ultra-long depth, high-resolution (10 μ m) spectral-domain optical coherence tomography (SD-OCT) system.^{50,51} Similar measurements were performed on age-matched animals with 4 weeks of form-deprivation treatment. The measurement procedure was described in detail in a previous study.³¹ In brief, mice were anesthetized with a mixture of 1.5% ketamine and 0.2% xylazine (1 mg/kg body weight, dissolved in sterile saline), and then mounted on a positioning stage in front of the optical scanning probe. Final orientation and positioning of the eye were determined by a computer-assisted video viewing system (LifeCam Cinema 720p HD Webcam; Microsoft, Redmond, WA, USA). The raw OCT data were exported and analyzed by custom-designed software to obtain axial components. Each eye was scanned along the entire AL three times to obtain a mean value. The CRC was measured by a keratometer (OM-4; Topcon, Tokyo, Japan)⁵² equipped with a +20 D achromatic doublet lens and calibrated by a series of stainless-steel ball bearings (diameters from 2.318 mm to 4 mm). The measurement was replicated three times for each eye, and the average value was used.

Immunohistofluorescence

Tissue Preparation. The retinas were prepared as previously described.⁴⁵ Briefly, mice were deeply anesthetized with 25% ethyl carbamate (1 g/kg) and each eye was quickly enucleated. After anterior segments of the eyes were removed, the posterior eyecups were immediately fixed in fresh 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 20 minutes. The retinas were dissected from the pigment epithelium, and then attached, ganglion cell side up, to a piece of filter paper (Millipore, Billerica, MA, USA). Four radial cuts were made to flatten the retina. Whole-mount retinas were blocked in 0.1 M PBS (pH 7.4) containing 6% normal donkey serum, 1% normal BSA and 0.2% Triton X-100 for 6 hours at 4°C. Mouse anti-TH monoclonal antibody (1:10000, 3 days at 4°C, T1299; Sigma-Aldrich Corp., St. Louis, MO, USA) was used to label dopaminergic amacrine cells. Immunoreactivity was detected with donkey anti-mouse IgG tagged with Alexa Fluor 555 (1:200, 2 hours, room temperature; Invitrogen, Carlsbad, CA, USA). In control experiments conducted on intact retinas from naïve eyes, this immunofluorescence protocol revealed strong TH staining (positive control), but no detectable positive signals when the primary antibody was omitted (negative control).

Counting of TH⁺ Cell Bodies. The number of TH⁺ cell bodies was counted using a fluorescence microscope (Axioskop 40; Carl Zeiss, Inc., Oberkochen, Germany) under a \times 20 objective. Eight distinct microscopic fields (520 \times 520 μ m) of flat-mounted retinas were chosen for counting: two zones in

each eye quadrant (dorsal, ventral, nasal, and temporal) located at 0.6 (corresponding to central retina) and 1.5 mm (corresponding to peripheral retina) from the optic nerve head. The number of TH⁺ cells in each microscopic field was counted, and pooled to calculate the mean cell density of the retina.

Electroretinographic Recording

To assess overall retinal function, electroretinographic responses (ERGs) were recorded using a custom-made system. In brief, mice were dark-adapted for at least 4 hours before the experiments. Under weak red light (approximately 1.4 lux at the cornea) provided by a light-emitting diode (LED) (XL-2003; Xuelang Illumination, Ningbo, Zhejiang, China), mice were anesthetized with a mixture of 1.5% ketamine and 0.2% xylazine (1 mg/kg body weight, dissolved in sterile saline), with pupils being dilated by compound tropicamide eye drops (Mydrin-p; Santen Pharmaceutical, Osaka, Japan). Gold wire ring electrodes (3104RC; Roland, Berlin, Germany) were positioned on the surface of both corneas for binocular ERG recordings. The animals were further dark-adapted for 30 minutes and then ERGs were acquired in darkness by a preamplifier (FZG-81; Jia Long Educational Instruments, Shanghai, China) and band-pass filtered (0.1–100 Hz). A multi-data acquisition card (PCIe 6321; National Instruments, Austin, TX, USA) was adapted to digitize signals and to control the circuits driving light stimuli, with a program coded by LabVIEW (National Instruments). Amplitudes of a-waves were measured from the baseline; b-wave amplitudes were measured from the baseline or the preceding troughs, depending on whether a-waves were absent or present. Data obtained from three to five recordings under the same condition were averaged. White light flashes of 3 ms, generated from an LED light source (6000 K in color temperature, CQ-LU9079; Qianhan Lighting, Shanghai, China), were presented by a custom-built Ganzfeld dome at two different intensities: 0.01 cd·s/m² (dim) and 4 cd·s/m² (bright), for evoking rod-dominant responses and mixed rod- and cone-driven responses, respectively. The flashes were always presented in order of increasing intensity. During all the experiments, mice were laid on a thermostatic plate (36–38°C) to maintain body temperature.

Analysis by HPLC

Because mouse retinal DA contents are light-dependent and under diurnal regulation,^{53,54} all samples for HPLC analysis were harvested under strictly controlled illumination levels at zeitgeber time (ZT) 1 (1 hour into the light period), when retinal DA levels are reported to reach their daily peak.⁵³ As described previously,⁴⁵ each frozen sample was homogenized into 100 μ L (for the retina) or 15 μ L (for the vitreous body) of ice-cold 0.1M perchloric acid containing 10 μ M ascorbic acid, 0.1 mM EDTA disodium salt, and 0.02 μ M 3,4-dihydroxybenzylamine (as internal standard). Dopamine and DOPAC levels were assessed with the Agilent 1200 series neurotransmitter analyzer (Agilent Technologies, Santa Clara, CA, USA) consisting of a G1367B autosampler, a G1312A binary pump, a G1322A degasser, the ANTEC DECRARD SDC electrochemical detector (Antec, Zoeterwoude, The Netherlands) equipped with a Sencell with a 2-mm glassy carbon working electrode, and an Acclaim C18 column (2.2 μ m, 2.1 \times 100 mm; Thermo Fisher Scientific, Waltham, MA, USA). This system has worked very well with high reliability, as demonstrated by previous studies,^{55–57} and is sensitive enough to detect significant changes in mouse retinal DA/DOPAC levels. Separations were performed at a flow rate of 0.2 mL/min using a mobile phase of PB, containing (in mM) 0.05 EDTA, 1.7 orthosilicic acid (OSA),

90.0 Na₂HPO₄, 50.0 citric acid, and 5% acetonitrile, with the detection cells set at +700 mV. The columns and detector cells were kept at 35°C in a column oven. The data were collected and analyzed by ChemStation (Agilent Technologies). Example chromatographs and retention times for reference standards, and retinal and vitreal extracts of the HPLC system are given in Supplementary Figure S1.

Western Blot Analysis

The procedure for Western blot analysis was previously described in detail.⁵⁸ The protein extracts of mouse retinas were loaded, subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 2 hours at room temperature in blocking buffer, consisting of 20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 0.1% Tween-20, and 5% nonfat milk. Following incubation in a buffer containing the antibody against TH (1:10,000) overnight at 4°C, the membranes were treated by horseradish peroxidase-conjugated donkey anti-mouse IgG (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The blots were probed with a mouse anti-actin monoclonal antibody (1:50000, A5441; Sigma-Aldrich Corp.) as loading control, and finally visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA).

Statistical Analysis

Data, which were all verified to be normally distributed, were presented as mean \pm SEM. Statistical significance was determined by 1-way ANOVA, 2-way ANOVA (followed by Sidak's multiple comparisons test) or *t*-test, using OriginPro 2015 (OriginLab Corporation, Northampton, MA, USA). Values of *P* less than 0.05 were taken to be significant.

RESULTS

In a Dose-Dependent Fashion, 6-OHDA Chronically Reduces Retinal DA Levels

6-OHDA of each of four doses (3 μ g, 6.25 μ g, 12.5 μ g and 50 μ g, all in 0.8 μ L saline solution) was injected into right eyes of 18-day-old C57BL/6 mice, which were raised in normal visual environment. Age-matched control animals received the same volume of vehicle solution. These mice were killed at various intervals over a 31-day period (D1, D3, D10, D17, D24, and D31) and their retinal DA levels were measured by HPLC assay (Fig. 1A). Because retinal DA levels show large individual differences,^{29,59,60} the ratios of DA concentrations obtained in 6-OHDA-treated and fellow eyes were calculated and used for quantitative analysis. Analysis by HPLC revealed that retinal dopamine levels were reduced by 6-OHDA injection in a dose-dependent manner (2-way ANOVA main effect of dose, $F_{4,251} = 27.43$, $P < 0.0001$). Injections of 3 μ g 6-OHDA did not change retinal DA levels (versus the vehicle group, $P = 0.9996$, Sidak's multiple comparisons test), but reductions of DA levels were found at the other three doses (6.25 μ g, 12.5 μ g, 50 μ g) even at the first sampling day (D1). The reduction clearly depended on the dose of 6-OHDA injected, and it was approximately 20% (6.25 μ g, $P = 0.041$), 40% (12.5 μ g, $P < 0.0001$), and 60% (50 μ g, $P < 0.0001$), respectively, as compared with the vehicle group (Sidak's multiple comparisons test). It is noteworthy that the DA levels in the 6.25- and 12.5- μ g groups declined at D1 and then leveled off. In the 50- μ g group, however, the DA levels, although below those seen in any other groups, partially recovered in a progressive manner over the entire 31-day period, following a sharp drop at D1.

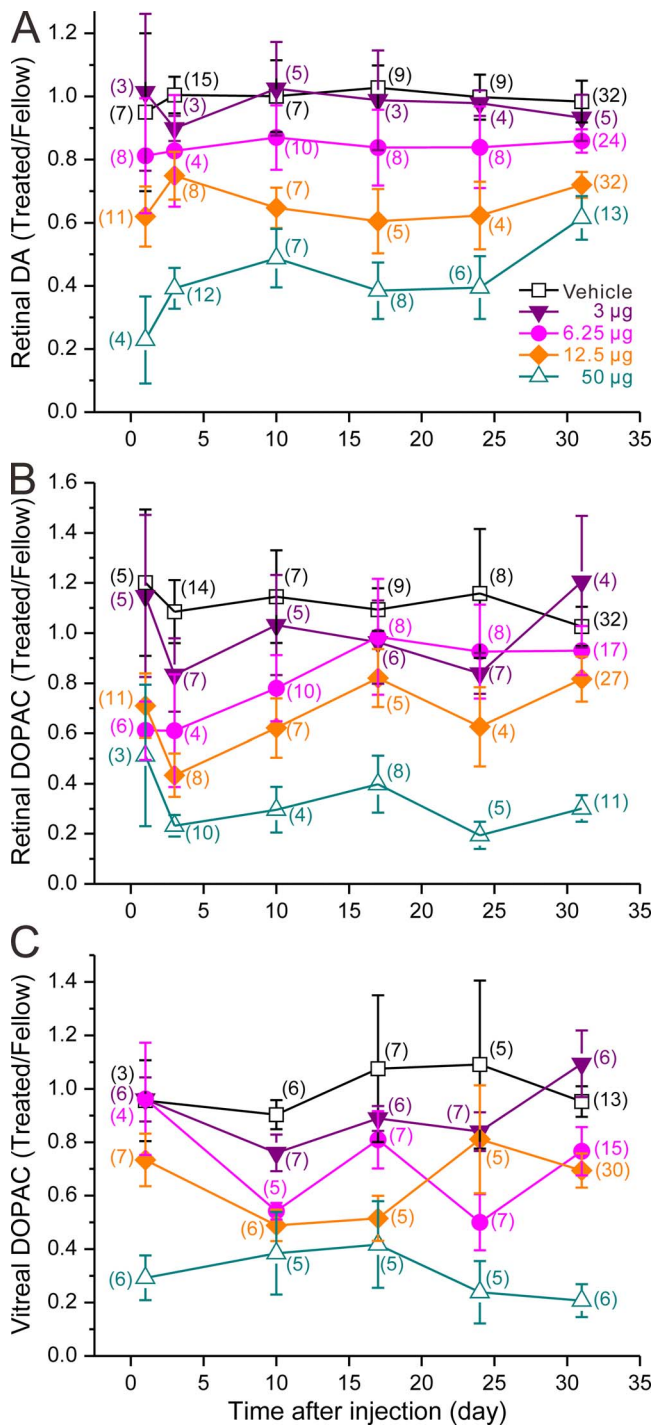


FIGURE 1. In a dose-dependent fashion, 6-OHDA reduces retinal DA levels. Retinal DA levels (A), retinal DOPAC levels (B), and vitreal DOPAC levels (C) measured by HPLC are plotted as a function of time after 6-OHDA of different doses and vehicle were injected. Data are presented as the ratios of the values obtained in injected eyes, relative to those in fellow eyes. No significant changes in retinal DA, DOPAC, and vitreal DOPAC were detected for 3 µg 6-OHDA. In the range between 6.25 µg and 50 µg, 6-OHDA reduced these three levels in a dose-dependent fashion. Note that in the 50-µg group, the retinal DA levels partially recovered with time following a sharp drop at the first postinjection day (D1), but such a recovery was not observed for retinal or vitreal DOPAC levels. Error bars represent 1 SEM. Sample sizes are given in parentheses. All underlying raw data are shown in Supplementary Raw Data (Parts S1–S3).

Retinal levels of the principal DA metabolite DOPAC and vitreal DOPAC levels, a robust index of DA release,⁶¹ also were assessed using HPLC analysis. Figures 1B and 1C show the changes in retinal and vitreal levels of DOPAC induced by 6-OHDA of four doses, respectively. It is clear that 6-OHDA injection reduced both retinal (2-way ANOVA main effect of dose, $F_{4,235} = 20.15$, $P < 0.001$) and vitreal levels of DOPAC (2-way ANOVA main effect of dose, $F_{4,159} = 18.64$, $P < 0.001$) in a dose-dependent manner. These results indicated that 6-OHDA administration significantly perturbed not only DA stores but also DA release/use.

To confirm that the effect on refractive development is specifically due to the reduction of retinal DA by 6-OHDA injections, nonspecific toxic effect of 6-OHDA must be avoided as much as possible. For this purpose, we first determined the 6-OHDA doses, at which limited reduction in retinal DA could be detected but overall retinal function was hardly influenced. Electroretinogram and immunohistochemical assays were used for evaluating effects of 6-OHDA of different doses on overall retinal function and dopaminergic amacrine cells, respectively.

Figures 2A1 through 4 show representative ERGs to dim and bright flashes recorded in dark-adapted animals, treated with vehicle, 6.25 µg, 12.5 µg, and 50 µg 6-OHDA, respectively. The responses to dim flashes were only b-waves, whereas those evoked by bright flashes consisted of both a- and b-waves with superimposed oscillatory waves. It is noteworthy that, for 6.25 µg and 12.5 µg 6-OHDA-treated animals, the responses recorded from treated and fellow eyes were quite comparable (Figs. 2A2, 2A3), but for 50 µg 6-OHDA-treated eyes, no response could be recorded at all (Fig. 2A4), even at the first sampling day (D3), indicating a severe disturbance of retinal function. Pooled data of average relative amplitudes (treated/fellow) of a- and b-waves obtained in these groups are shown in Figures 2B through 2D. The data obtained in the 6.25-µg or 12.5-µg group were not significantly different from those in the vehicle group (Sidak's multiple comparisons test: dim flash, $P = 0.951$ for 6.25 µg and 0.172 for 12.5 µg [for b-wave]; bright flash, $P = 0.793$ for 6.25 µg and 0.301 for 12.5 µg [for a-wave], $P = 0.985$ for 6.25 µg and 0.903 for 12.5 µg [for b-wave]). This result implies that 6-OHDA of these two doses barely perturbed retinal functions in general. However, oscillatory potentials, which are reportedly highly sensitive to disruption of dopaminergic pathways,⁶² were significantly decreased in amplitudes in the 12.5-µg group (see Supplementary Fig. S2 for details).

To determine whether 6-OHDA at these two doses caused a reduction of retinal DA levels by ablating dopaminergic amacrine cells, the sole neuronal population secreting DA in mouse retina, we assessed the densities of these cells, labeled by a mouse anti-TH antibody, in 6-OHDA-treated eyes (Figs. 3A–C). Cell counting in eight retinal regions, four in central and four in peripheral retina, revealed no significant difference in average densities among vehicle, 6.25-µg, and 12.5-µg groups (2-way ANOVA main effect of dose, $F_{2,112} = 0.728$, $P = 0.485$, Fig. 3D). This is consistent with previous studies showing that TH⁺ retinal cells were hardly changed in number when mice were treated with reagents that deplete DA, such as 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).^{63,64} It should be noted that in the 12.5-µg group the TH fluorescence signal intensity seemed weaker than that observed in the vehicle group (Fig. 3C). In a few samples, we also tried to determine whether 50 µg 6-OHDA changed dopaminergic cell density, but found that the retinas were paler and thinner than vehicle-treated ones, and almost no TH staining could be detected, thus making cell counting impossible (Supplementary Fig. S3).

To determine whether reduced retinal DA levels by 6-OHDA may be due to a disturbance of the retinal DA synthesis

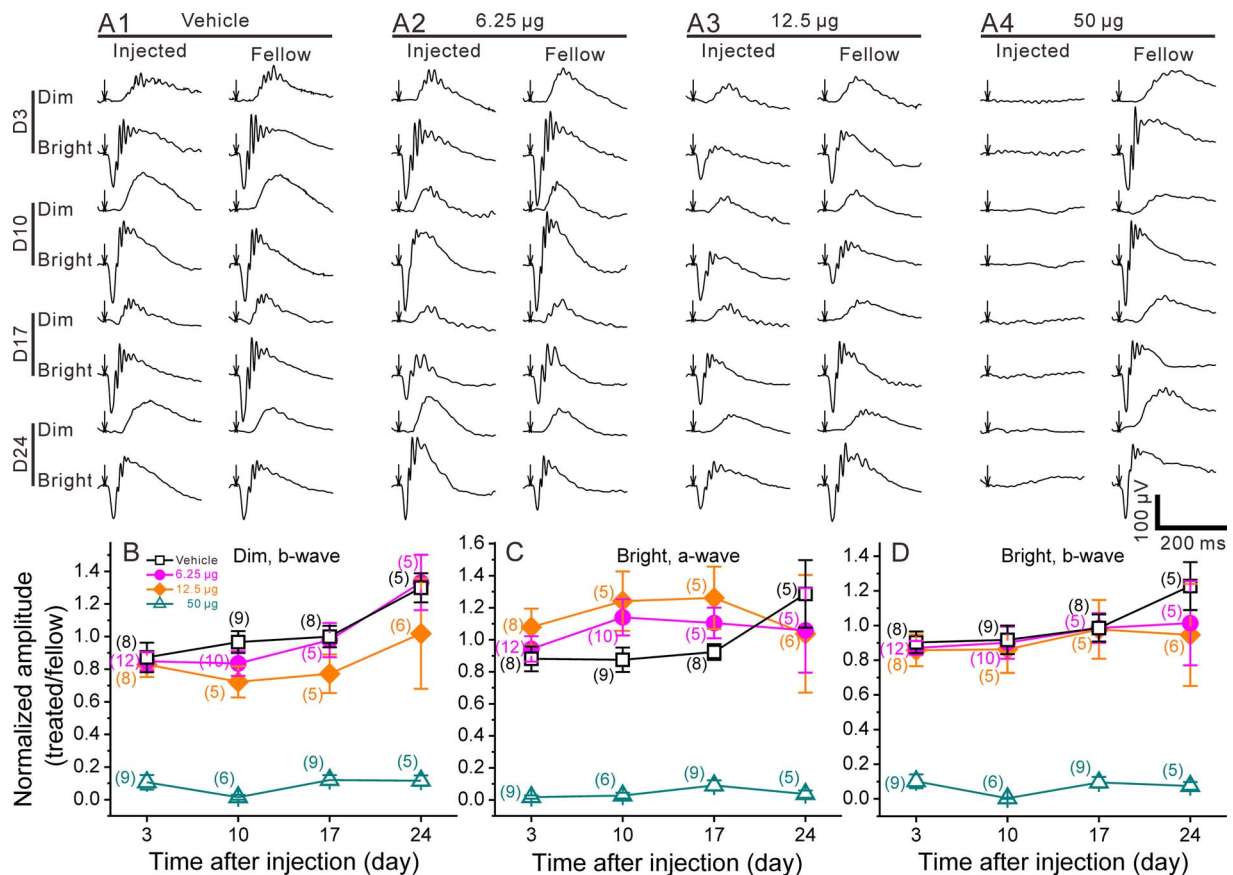


FIGURE 2. Effects of 6-OHDA of different doses on dark-adapted ERG. (A1–A4) Representative dark-adapted ERG recordings from animals with 6-OHDA (6.25, 12.5, and 50 μg) injections at various sampling time points, as compared with those recorded from fellow eyes. Electroretinograms were evoked by both dim (0.01 $\text{cd}\cdot\text{s}/\text{m}^2$) and bright (4 $\text{cd}\cdot\text{s}/\text{m}^2$) flashes (3 ms, arrows). Note that ERG responses (both a- and b-waves) were eliminated in animals treated with 50 μg 6-OHDA. In contrast, neither a- nor b-wave was significantly changed when 6.25 or 12.5 μg 6-OHDA was administered. (B–D) Pooled data comparing average ERG amplitudes of a- and b-waves, which were normalized relative to those obtained in fellow eyes among groups across various time points. For all three sets of data analyzed (b-wave to dim flash [B], a-wave to bright flash [C], and b-wave to bright flash [D]), statistical analysis showed that 50 μg 6-OHDA significantly reduced ERG amplitudes, whereas 6.25 μg or 12.5 μg 6-OHDA did not. At each postinjection time point, the ERG waves are from different mice with typical responses. Error bars represent 1 SEM. Sample sizes are given in parentheses.

machinery, Western blot analysis was performed to assess retinal expression levels of TH, a rate-limiting enzyme in the biosynthesis of DA, at D3, D17, and D31. A single band of approximately 60 kDa, corresponding to the molecular weight of TH,⁶⁵ was detected in all the retinal protein extracts (Figs. 4A–C, top). Densitometric analysis revealed that 6-OHDA of both the doses significantly reduced retinal TH protein levels at the three time points (treated versus fellow eyes, paired *t*-test: at D3, $P = 0.002$ for 6.25 μg and 0.004 for 12.5 μg ; at D17, $P = 0.0009$ for 6.25 μg and 0.005 for 12.5 μg ; at D31, $P = 0.012$ for 6.25 μg and 0.0009 for 12.5 μg) (Figs. 4A–C, bottom). There are two points that should be noted. First, the TH levels obtained at the three time points were similar in both the 6.25- μg (1-way ANOVA, $F_{2,13} = 0.350$, $P = 0.711$) and 12.5- μg (1-way ANOVA, $F_{2,13} = 2.544$, $P = 0.117$) groups, implying that the reduction of TH protein levels caused by 6-OHDA had reached a maximum before D3. Second, the reduction in TH protein levels was dose-dependent (2-way ANOVA main effect of dose, $F_{2,39} = 23.07$, $P < 0.0001$), as indicated by a larger reduction for the 12.5- μg group than that for the 6.25- μg group ($P = 0.013$, Sidak's multiple comparisons test). These findings suggest that reduced retinal DA levels might have been a consequence of reduced TH protein levels caused by 6-OHDA administration.

Based on the above results, it seems reasonable to deduce that the changes in refractive development following intravit-

real injections of 6-OHDA at these doses may be largely generated by the 6-OHDA-induced reduction in retinal DA levels. These two doses of 6-OHDA were therefore chosen for all subsequent experiments.

In a Dose-Dependent Manner, 6-OHDA Induces Myopic Refractive Shifts

We then investigated effects of 6-OHDA injections on refractive development with normal laboratory visual experience. Figure 5A shows how the refractive power of vehicle-treated animals changed as a function of time. The refractive power was steadily increased from 1.930 ± 0.275 D at D0 to 6.203 ± 0.908 D at D31 in a progressive manner, reflecting how the refractive state of the eye changes with age. Following intravitreal injections of 6.25 μg 6-OHDA, a sharp drop in refractive power from 2.917 ± 0.374 D to 0.712 ± 0.360 D was seen as early as at D3 (Fig. 5B). The refractive power was gradually increased in injected eyes thereafter, but the refractive state was invariably more myopic than that in fellow eyes in the subsequent 4 weeks and the differences in refractive power between injected and fellow eyes were statistically significant (paired *t*-test, $P < 0.05$). Experiments with 12.5 μg 6-OHDA yielded a similar result (Fig. 5C). Again, at each postinjection time point, the refractive powers of injected

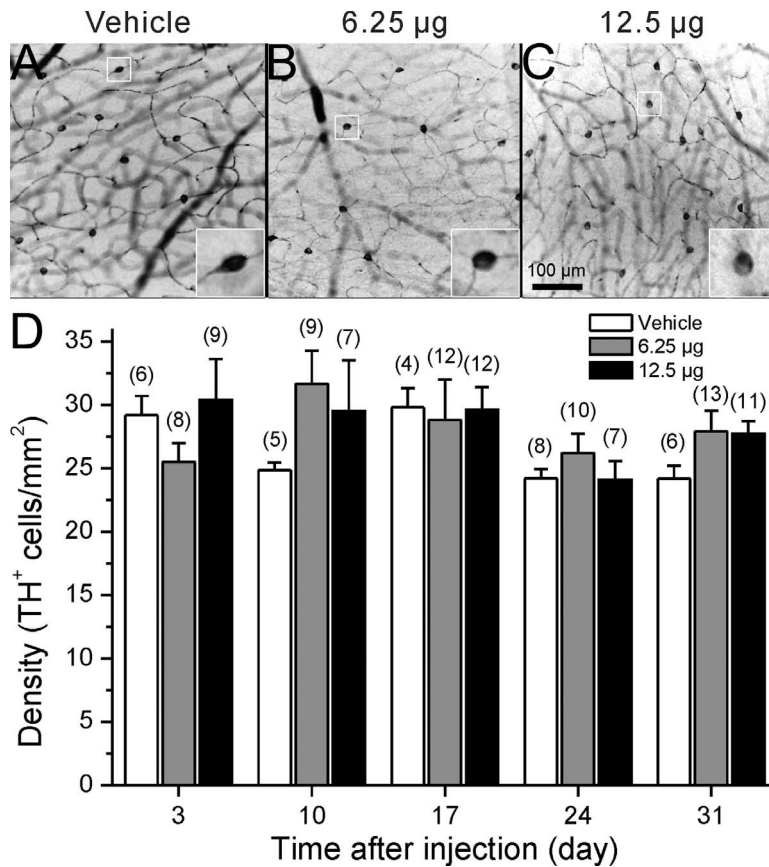


FIGURE 3. At lower doses, 6-OHDA (6.25 µg, 12.5 µg) did not cause dopaminergic amacrine cell loss. (A–C) Representative photomicrographs of the retinal whole-mount showing TH-immunopositive dopaminergic amacrine cells following vehicle or 6-OHDA treatment. Contrast of the micrographs is reversed so that TH fluorescence is represented by dark-on-light. The thicker branching structures are blood vessels, of which IgG-rich contents are labeled by the secondary antibody (to mouse IgG). Images in the targeted areas in all three panels are shown in the insets with a higher magnification. Note that in the 12.5-µg group, the fluorescence intensity is weaker than that observed in other two groups. (D) Bar charts showing the average TH⁺ cell densities of the 6.25 µg, 12.5 µg, and vehicle groups at various postinjection time points. There was no significant difference in TH⁺ neuron density among the three groups. Numbers of retinas are given in parentheses. Error bars represent 1 SEM.

eyes were invariably lower than those in fellow eyes, and the differences between injected eyes and fellow eyes were more significant than that seen in the 6.25-µg group (paired *t*-test, $P < 0.05$). The effect of 6-OHDA doses is more clearly seen in

Figure 5D, in which the refractive shifts obtained in the three groups are plotted as a function of time after 6-OHDA injections. Significant differences were found among the three groups (2-way ANOVA main effect of dose, $F_{2,638} = 36.89$, $P <$

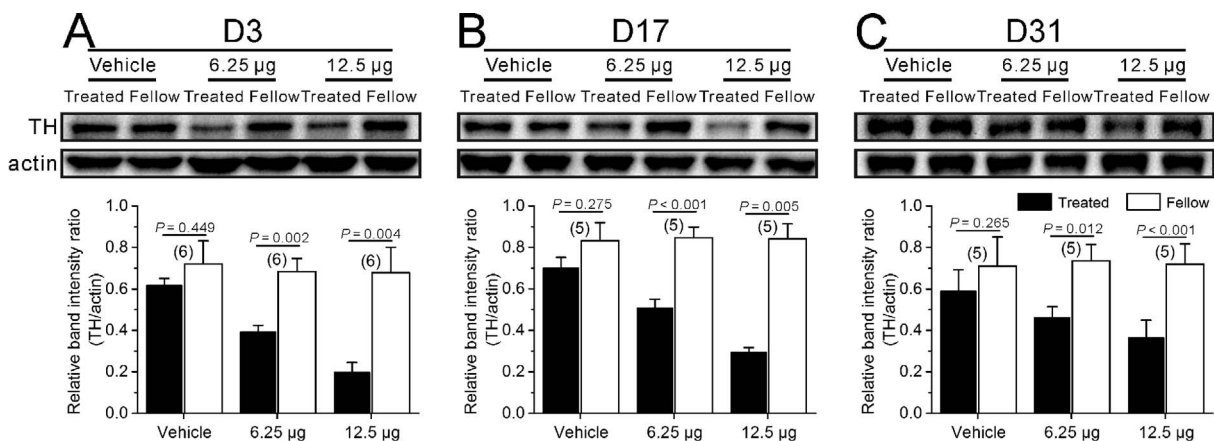


FIGURE 4. TH levels are significantly reduced by 6-OHDA. (A–C) Top, representative TH staining on Western blots of retinal protein extracts from vehicle and 6-OHDA-injected animals. For each treatment group, samples were collected from both eyes at D3 (A), D17 (B), and D31 (C). Bottom, TH protein expression levels, normalized as a ratio of actin levels, were found to be always significantly lower in 6-OHDA-injected eyes, as compared with fellow eyes, suggesting that 6-OHDA reduced retinal DA levels by suppressing TH expression. Error bars represent 1 SEM. Sample sizes are given in parentheses.

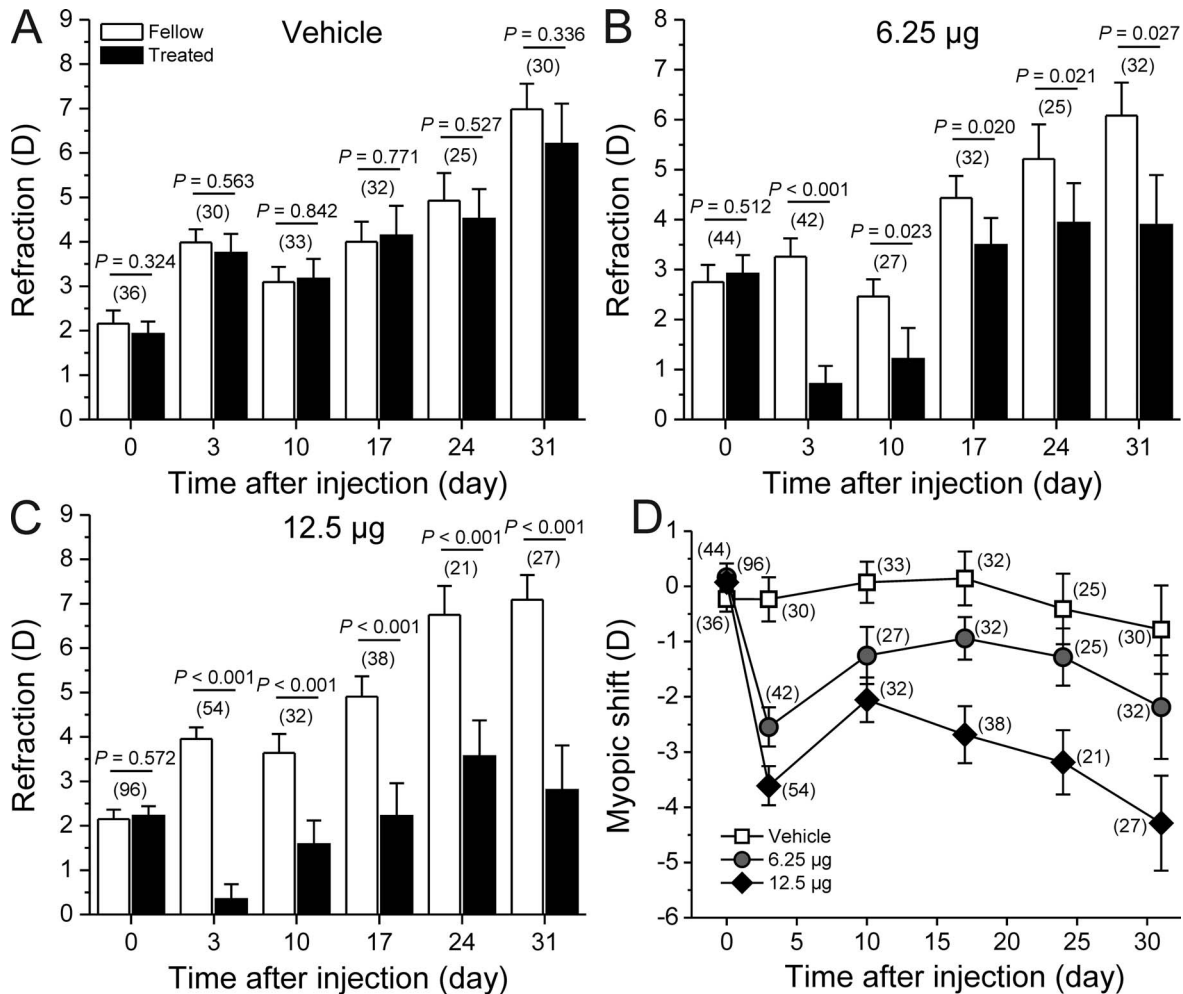


FIGURE 5. In a dose-dependent fashion, 6-OHDA induces myopic refractive shifts. (A) No significant differences in refractive error were detected over a 31-day postinjection period between vehicle-injected eyes and their fellow eyes. (B–C) Comparison of refractive errors obtained at different postinjection time points between 6-OHDA-injected eyes (6.25 µg in [B]) and 12.5 µg in [C]) and fellow eyes. Note that for both the doses, the refractive errors obtained at different postinjection times in 6-OHDA-injected eyes were all significantly smaller than those in fellow eyes. (D) Comparison of myopic refractive shifts obtained at different times in vehicle-injected eyes and 6-OHDA-injected eyes (6.25 µg and 12.5 µg). The shifts induced by 12.5 µg 6-OHDA were larger than those induced by 6.25 µg 6-OHDA. Error bars represent 1 SEM. Sample sizes are given in parentheses. All raw data are shown in Supplementary Raw Data (Part S4).

0.001), with both the 6.25-µg group ($P = 0.0003$) and 12.5-µg group ($P < 0.001$) being more myopic as compared with the vehicle group (Sidak's multiple comparisons test). Given that form deprivation leads to myopic shift in refraction in the C57BL/6 mouse without altering retinal DA levels,^{43–45} these results suggest an intriguing possibility that another mechanism, which is DA-dependent, may exist to mediate myopic refractive shifts in normal refractive development, in addition to the DA-independent mechanism responsible for FDM development.

Axial Length Is Reduced by 6-OHDA

Whether form deprivation and 6-OHDA may differentially influence ocular biometrics was examined by SD-OCT (Fig. 6). As observed previously in mice,^{38,40,41} form deprivation for 4 weeks resulted in a significantly excessive axial elongation of form-deprived eyes (2.742 ± 0.010 mm versus 2.717 ± 0.010 mm for fellow eyes, $P = 0.030$, paired t -test). In age-matched animals, however, the AL obtained in 6-OHDA-injected eyes (6.25 µg) was shorter than that obtained in fellow eyes (2.714 ± 0.008 mm versus 2.734 ± 0.009 mm, $P = 0.040$, paired t -

test). This effect was unlikely due to an elongation of fellow eyes, because no significant difference (1-way ANOVA, $F_{2,51} = 0.713$, $P = 0.495$) in the AL was found among fellow eyes of the three groups (treated with 6-OHDA, form deprivation, and vehicle, respectively). The changes in the ocular components measured are summarized in the Table. In addition to the change in AL, a deeper ACD and a larger RT were commonly detected in 6-OHDA-injected eyes (Table). No significant changes in any ocular dimensions were found in vehicle-injected eyes.

Corneal Radius of Curvature Is Reduced by 6-OHDA

A shorter AL should have resulted in a hyperopic refractive shift, but in 6-OHDA-injected eyes, the myopic refractive shift was obtained. This strongly suggests that, among others, the radius of curvature of the cornea, which makes major contribution to the refractive state of the eye, might have been shortened. This was experimentally demonstrated. At D31, photokeratometry showed that the CRC of eyes injected with 6.25 µg 6-OHDA was 1.523 ± 0.006 mm, significantly

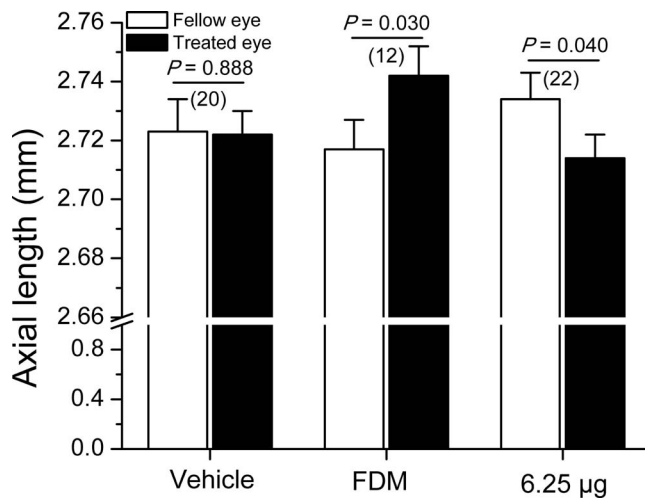


FIGURE 6. Axial lengths of form-deprived eyes and 6-OHDA-injected eyes (6.25 µg). Axial lengths were measured by SD-OCT. Although form deprivation for 4 weeks resulted in a significantly longer AL (*middle*), intravitreal injection of 6.25 µg 6-OHDA reduced the AL (*right*). Vehicle injection showed no effects on AL (*left*). Error bars represent 1 SEM. Sample sizes are given in parentheses.

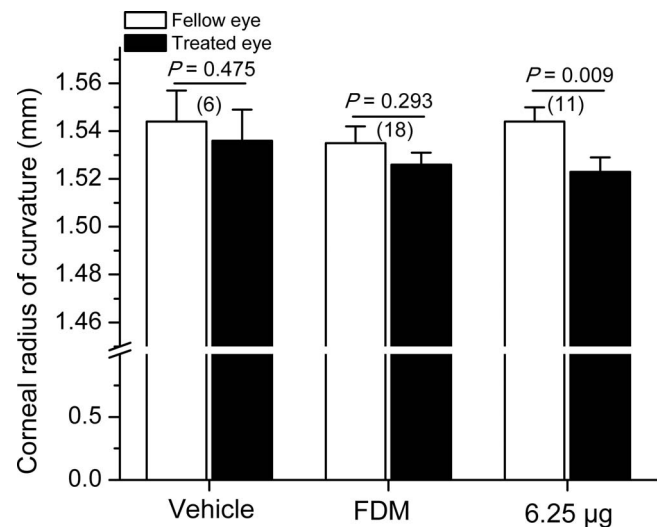


FIGURE 7. Changes in CRC in form-deprived eyes and 6-OHDA-injected eyes (6.25 µg). After 4 weeks of treatment, no significant difference in CRC was found between form-deprived eyes and fellow eyes (*middle*), but the CRC in 6-OHDA-injected eyes was significantly shorter than that of fellow eyes (*right*). Error bars represent 1 SEM. Sample sizes are given in parentheses.

shorter ($-21 \mu\text{m}$) than that of their fellow eyes ($1.544 \pm 0.006 \text{ mm}$, $P = 0.009$, paired *t*-test) (Fig. 7). In other words, 6-OHDA injections made the cornea steeper, thus leading to a myopic refractive shift. Such changes in CRC may be an important factor that compromises the hyperopic changes due to the shorter AL, and eventually resulted in the myopic shifts observed (see the Discussion section). In contrast, in age-matched mice, there was no significant change in CRC in form-deprived eyes ($1.526 \pm 0.005 \text{ mm}$ versus $1.535 \pm 0.007 \text{ mm}$ for fellow eyes, $P = 0.293$, paired *t*-test).

To what extent such changes in the CRC (approximately 21 µm at D31, Fig. 7) could compromise the shorter AL-induced hyperopic effect was further examined. Based on the schematic mouse eye developed by Remtulla and Hallett,⁶⁶ we derived that changes in refractive power caused by a 21-µm reduction in the CRC could indeed compromise the hyperopic effect due to the shorter AL, eventually making the eyes myopic of approximately 1.5 D (see the Supplementary Methods for details), a value quite close to the experimentally observed myopic shifts seen in 6-OHDA-injected eyes (approximately 2.2 D; Fig. 5D).

Form Deprivation Induces Additional Myopic Shifts in 6-OHDA-Injected Eyes but Without Changing Retinal DA Levels

We then continued to explore whether form deprivation could induce additional myopic shifts in 6-OHDA-injected eyes. In a set of experiments, animals were divided into two groups

(groups I and II). Group I animals were treated with intravitreal injections of 6-OHDA (6.25 µg) alone, whereas group II animals with 6-OHDA (6.25 µg) combined with 4-week form deprivation started at D3, respectively. Myopic shifts and retinal DA levels were determined at D31. As shown in Figure 8A, myopic shifts in group II ($-5.846 \pm 0.727 \text{ D}$) were much larger (by approximately -3.7 D) than those obtained in group I ($-2.185 \pm 0.938 \text{ D}$) ($P = 0.019$, unpaired *t*-test), implying that form deprivation induced additional myopic shifts in 6-OHDA-injected eyes. However, no significant difference in retinal DA levels was found between the two groups ($P = 0.551$, unpaired *t*-test), indicating that form deprivation did not further change retinal DA levels in 6-OHDA-treated eyes (Fig. 8B).

DISCUSSION

Mouse models of experimental myopia, which were recently developed, have at least two advantages over avian and fish models. First, as compared with the chick, a widely used animal model for refractive research, the mouse exhibits eyes, to a large extent, similar in structure and biochemistry to human eyes. Second, a great deal has been discovered about the biology and genetics of mouse eyes, and genetic manipulations have been available to modify the eyes morphologically and physiologically, even though genetic manipulation is achievable in more and more other species

TABLE. Effects of Form Deprivation and 6-OHDA Injection on Ocular Axial Dimensions (SD-OCT; Mean \pm SEM, mm)

Group	n	Eye	CT	ACD	LT	VCD	RT	AL
Vehicle	20	Right	0.114 \pm 0.004	0.376 \pm 0.003	1.601 \pm 0.004	0.630 \pm 0.005	0.254 \pm 0.005	2.722 \pm 0.008
		Left	0.119 \pm 0.002	0.368 \pm 0.003	1.609 \pm 0.005	0.631 \pm 0.005	0.255 \pm 0.006	2.723 \pm 0.011
Form deprivation	12	Right	0.109 \pm 0.006	0.371 \pm 0.006	1.618 \pm 0.004	0.649 \pm 0.005*	0.242 \pm 0.004	2.742 \pm 0.010*
		Left	0.107 \pm 0.002	0.363 \pm 0.005	1.618 \pm 0.005	0.619 \pm 0.011	0.252 \pm 0.004	2.717 \pm 0.010
6.25 µg 6-OHDA	22	Right	0.106 \pm 0.004	0.381 \pm 0.003*	1.621 \pm 0.008	0.615 \pm 0.011	0.250 \pm 0.005*	2.714 \pm 0.008*
		Left	0.111 \pm 0.004	0.371 \pm 0.003	1.624 \pm 0.009	0.622 \pm 0.012	0.237 \pm 0.004	2.734 \pm 0.009

* $P < 0.05$, paired *t*-test, right versus left eye.

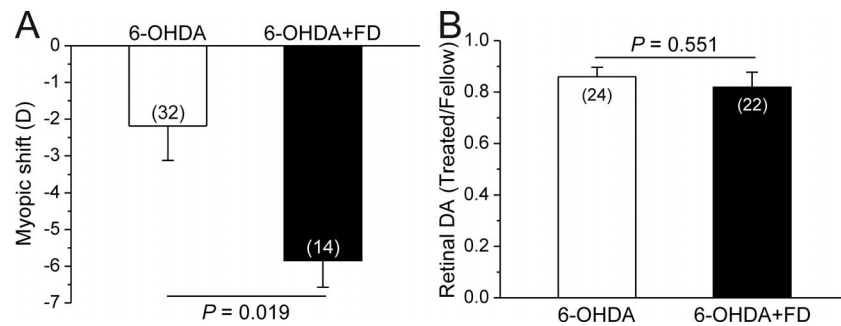


FIGURE 8. Comparison of myopic shifts (**A**) and retinal DA levels (**B**) when the animals were treated with 6-OHDA (6.25 μ g) alone or along with form deprivation beginning at D3. Myopic shifts in (**A**) are refractive errors of treated eyes relative to those of fellow eyes. Retinal DA levels in (**B**) are represented as ratios of the data obtained in treated eyes in relation to those in fellow eyes. Error bars represent 1 SEM. Sample sizes are indicated inside the bars.

with the rapid progression of molecular techniques such as RNA interference. Mouse models are therefore expected to be widely used for exploring the mechanisms underlying myopia development.⁴² Unlike in several other species,^{17–19,21,22} however, it was recently found in the C57BL/6 mouse strain that retinal DA levels are unchanged during the development of FDM.^{43–45} In the present work, we tried to address whether retinal DA plays any role in eye growth and FDM development in this mouse strain by reducing retinal DA levels through intravitreal injections of 6-OHDA. Our results demonstrated that reduced retinal DA levels did induce a myopic shift in refraction, suggesting the involvement of retinal DA in refractive development. Ocular biometrics further showed that the myopic shift induced by 6-OHDA was generated by a mechanism different from that underlying the myopic shift induced by form deprivation.

Reduced Retinal DA by 6-OHDA Induces Myopic Refractive Shifts

Intravitreal injections of 6-OHDA provide an effective neurotoxicologic method for exploring the role of DA in refractive development by specifically lesioning dopaminergic neurons. For this purpose, appropriate doses of 6-OHDA should be chosen to avoid nonspecific toxic effects induced by higher doses of 6-OHDA.^{59,67} As previously shown in several species, retinal DA levels are reduced by approximately 20% to 30% during FDM.^{17–19} In mice, retinal DA levels fluctuate daily by this extent under physiological conditions.⁵³ It seems meaningful to explore the role of retinal DA in refractive development when retinal DA levels are changed by a comparable extent. Our experiments showed that intravitreal injections of 6-OHDA could reduce retinal DA levels in a dose-dependent manner (Fig. 1A), and a 6.25- μ g dose of 6-OHDA reduced retinal DA levels by approximately 20%. It was further demonstrated that 6-OHDA of this dose influenced retinal TH protein content, thus leading to a reduction of retinal DA levels, but produced minimal perturbation of overall retinal function, as suggested by normal ERGs. At this dose, 6-OHDA also did not cause a loss of dopaminergic amacrine cells. Therefore, it seems likely that the effect of 6-OHDA at this dose is due to the reduction in retinal DA levels, although other mechanisms (e.g., 6-OHDA-induced noradrenergic system lesion) cannot be excluded. This dose could be therefore regarded appropriate for examining effects of reduced retinal DA levels on refractive development. When 6-OHDA at a higher dose (50 μ g) was administered, in addition to a considerable reduction of retinal DA levels, nonspecific toxic effects were induced, as suggested by the complete extinction of ERG responses (Fig. 2), remarkable histologic changes of the

retina, and severely diminished TH⁺ cells (Supplementary Fig. S3). Similar severe disturbance of overall function, as reflected by significant changes in ERG responses and contrast sensitivity, for example, has been reported in Cre-mediated retinal-specific TH-knockout mice, in which retinal DA levels and dopaminergic neuron number are reduced by approximately 90%.⁶⁸ If such inappropriate dose of 6-OHDA was used, then the conclusion that can be drawn should be dramatically altered. Interestingly, retinal DA was still detectable in 50 μ g 6-OHDA-treated eyes (Fig. 1), suggesting that residual TH, in combination with some unknown compensatory mechanisms, may still work to produce an amount of DA measurable for HPLC analysis.

Effects of 6-OHDA on refractive development appeared to vary from species to species. In chicks, 6-OHDA suppressed FDM by retarding the excessive axial elongation associated with occluder wear, but it did not affect regular eye developments in normal visual environment, nor did it have any effects on optical properties of either occluder-treated or otherwise normal eyes.^{59,60,69–73} In Cichlidae, 6-OHDA treatment reduced the overall eye size, which was likely to induce a hyperopia-like effect, but the focal length of the eyes was reduced by similar amounts, so that no apparent effects on the refractive state of the eye were reported.⁷⁴ In young quail, 6-OHDA induced relative myopic shift, as compared with age-matched nontreated quail.⁷⁵ This result, at first sight, seems comparable to our results, but the dose of 6-OHDA used in that study significantly affected ERG amplitudes, suggesting that it could be a consequence of the nonspecific action of 6-OHDA on retinal functions.

Two Distinct Mechanisms Underlying Myopic Refractive Shifts Coexist in Mouse Retina

Intravitreal injections of 6.25 μ g or 12.5 μ g 6-OHDA induced dose-dependent myopic refractive shifts in C57BL/6 mice. This result prompts us to deduce that myopia development under normal visual conditions may be associated with retinal DA levels, a deduction that is seemingly contradictory to the recent finding that retinal DA levels are unaltered in this mouse strain with FDM. This paradox raises an intriguing possibility that there coexist two distinct mechanisms, DA-independent and DA-dependent, for myopic shifts in refraction in the C57BL/6 mouse strain. Form deprivation induces myopic shift in refraction through a DA-independent mechanism, but retinal DA reduction may trigger a DA-dependent mechanism, thus also causing myopic refractive shifts with normal visual input (the unchanged retinal DA levels, as we noted previously, do not necessarily imply that DA plays no role in FDM development in C57BL/6 mice, and other aspects of the

dopaminergic system, say DA receptor levels or sensitivity, may be associated with myopia development; more strictly, we should say “DA level-independent” mechanism instead of “DA-independent” mechanism). This possibility is largely strengthened by two sets of data. First, the biometric data obtained in the present work demonstrated that form deprivation resulted in a larger AL without changing the CRC, as reported previously,^{38,41} but reduced DA levels caused a shorter AL and reduced the CRC (Figs. 6, 7; Table). Second, form deprivation induced additional myopic shifts in 6-OHDA-treated eyes, without resulting in an additional decline in retinal DA levels (Fig. 8). In other words, different myopia shifts could be induced when retinal DA levels remain the same.

The refractive state of the eye is determined by a variety of parameters, including the AL, curvature of its various media, and other factors. The fact that 6-OHDA made the AL shorter strongly suggests that there must be some changes in ocular dimensions that make the 6-OHDA-injected eyes myopic. As the CRC is important in determining the refractive state of the eye,^{66,76} a possible explanation may be that 6-OHDA has made the cornea steeper. By photokeratometry, we did find that 6-OHDA (6.25 µg) caused the CRC to be shorter by 21 µm at D31, compared with fellow eyes (Fig. 7). Although it remains to be explored how 6-OHDA caused the cornea to be steeper, optical modeling showed that such changes in CRC could counteract the hyperopic effect caused by the shorter AL, eventually leading to a myopic shift (see the Supplementary Methods). Needless to say, we could not rule out a possibility that 6-OHDA causes some changes in other ocular dimensions that contribute to the myopic shift seen. It is of interest that the retinal-specific TH-knockout mice were found to be more myopic than wild-type animals and exhibited a shorter AL and a steeper cornea (Bergen M, et al. *IOVS* 2015;56:ARVO E-Abstract 2153).

Whether the coexistence of both DA-independent and -dependent mechanisms in nocturnal C57 mice could be generalized to other species, specifically to diurnal primates and humans, remains to be explored. This is especially because DA is light-driven in its expression, even though mice and diurnal species exhibit similar circadian/diurnal rhythms in retinal DA levels.^{53,54} Increasing evidence suggests that the mechanisms underlying myopic shifts in refraction may be species-dependent. In several species, administration of DA receptor agonists has been reported to affect myopia formation,^{18,21-31} and as observed in guinea pigs, activation of D1-like and D2-like receptors even could exert opposite effects on refractive development.⁷⁷ Unlike observed in chicks,^{29,59,69} 6-OHDA did not block FDM in 6-OHDA-treated C57 mouse eyes and instead induced further myopic shifts (Fig. 8). This is also consistent with the result that form deprivation induces similar magnitudes of myopic shift in retinal-specific TH-knockout mice as compared with wild-type animals, despite the 90% loss of retinal DA (Bergen M, et al. *IOVS* 2015;56:ARVO E-Abstract 2153). Moreover, in mice, as shown in the present work, either form deprivation or 6-OHDA treatment caused a relative myopic shift only in treated eyes, as compared with fellow eyes, with both eyes still being hyperopic. This is clearly different from human myopia in which the refractive powers become absolutely myopic, which could be due to the differences in ocular system between mice and humans. It is known that the ciliary muscle, which is well developed in humans, is rudimentary in mice.^{36,78} Based on the above discussion, an intriguing hypothesis could be proposed. That is, both DA-dependent and -independent mechanisms may ubiquitously coexist, but with one or another being predominant in a specific species. The coexistence of both the mechanisms in the retina could provide the organism with

more versatile ways to accommodate to the changing visual environment, by modifying development of the ocular refractive system in different ways (through changing the CRC by the DA-dependent mechanism and/or adjusting the AL by the DA-independent mechanism).

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