Dopamine D1 Receptors Contribute Critically to the Apomorphine-Induced Inhibition of Form-Deprivation Myopia in Mice

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Apomorphine (APO) can inhibit FDM development in animal models, including chickens,6,17,18 rabbits,19 monkeys,20 and guinea pigs.8,21 Although the stability of retinal dopamine during FDM development in mice is different from the decreased levels in other species,12–14 daily systemic APO injection in mice nevertheless offers protection against FDM development by at least 75%, in agreement with other species.22 Therefore, the mouse is not an outlier compared with other animal models with respect to the development of FDM and responses to APO. Collectively, the inhibitory effect of APO on FDM suggests that activation of dopamine receptors can prevent myopia development. APO, as well as dopamine, also produces more consistent and effective inhibition of FDM development in C57BL/6 mice.12–14 These results suggest that subtle, but as yet undetected, changes of the retinal dopamine levels and/or changes in other aspects of the dopaminergic system could be altered by FDM in mice. Administration of 6-hydroxydopamine (6-OHDA) significantly reduced the retinal dopamine level in mice and resulted in relative myopic refractive errors under normal visual environments and an enhanced FDM response.15 Additionally, reducing retinal dopamine by retinal-specific tyrosine hydroxylase knockout (KO) in mice also induced spontaneous myopia, although it did not alter the response to FDM.16 Therefore, dopamine clearly functions as an important neurotransmitter involved in the signaling cascade that controls ocular growth and vision in mice.

Myopia is a refractive error of the eye that is prevalent worldwide.1,2 It has become an important public health issue as evidenced by the increasingly high incidence in the past few decades and by the sight-threatening pathologies associated with high myopia.1,2 However, the pathogenesis of myopia still remains unknown; thus, there are no effective methods of preventing or curing myopia based on its pathogenesis.

Studies employing form-deprivation myopia (FDM) models have shown that the dopaminergic system plays an important role in refractive development and ocular growth.3,4 Retinal and vitreous dopamine and/or the dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) levels are reduced during the development of FDM in various animals, including chickens,5,6 monkeys,7 and guinea pigs.8 Furthermore, the decrease of retinal DOPAC was restricted only to the deprived area of the retina during partial deprivation.9,10 In chickens, DOPAC recovered to normal levels after removal of the form-deprivation goggle.11 Unlike FDM in chickens, recent studies showed that retinal dopamine levels remained unaltered in FDM C57BL/6 mice.12–14 These results suggest that subtle, but as yet undetected, changes of the retinal dopamine levels and/or changes in other aspects of the dopaminergic system could be altered by FDM in mice. Administration of 6-hydroxydopamine (6-OHDA) significantly reduced the retinal dopamine levels in mice and resulted in relative myopic refractive errors under normal visual environments and an enhanced FDM response.15 Additionally, reducing retinal dopamine by retinal-specific tyrosine hydroxylase knockout (KO) in mice also induced spontaneous myopia, although it did not alter the response to FDM.16 Therefore, dopamine clearly functions as an important neurotransmitter involved in the signaling cascade that controls ocular growth and vision in mice.

Consistent with the findings that retinal dopamine was reduced during the development of FDM in most of the species studied, local administration of dopamine, levodopa (a dopamine precursor), or the nonselective dopamine agonist apomorphine (APO) can inhibit FDM development in animal models, including chickens,5,17,18 rabbits,19 monkeys,20 and guinea pigs.8,21 Although the stability of retinal dopamine during FDM development in mice is different from the decreased levels in other species,12–14 daily systemic APO injection in mice nevertheless offers protection against FDM development by at least 75%, in agreement with other species.22 Therefore, the mouse is not an outlier compared with other animal models with respect to the development of FDM and responses to APO. Collectively, the inhibitory effect of APO on FDM suggests that activation of dopamine receptors can prevent myopia development. APO, as well as dopamine, also produces more consistent and effective inhibition of
myopia development than other dopamine agents for all species studied so far. These results also support the idea that decreased dopamine activity contributes to the development of FDM.

As a nonselective dopamine agonist, APO binds to all types of dopamine receptors, and although it can bind to serotonergic and adrenergic receptors, it has much lower affinities for them compared with dopamine receptors. The dopamine receptors consist of two subfamilies, D1-like (D1, D5) and D2-like (D2, D3, and D4) receptors, which are positively and negatively linked to adenyl cyclase, respectively. Studies based on autoradiography and immunohistochemistry found that horizontal, bipolar, amacrine, and ganglion cells express mainly D1-like receptors, whereas photoreceptors express D2 receptors. Similar to dopamine, the affinity of APO for D2 receptors (D2Rs) is up to 22-fold greater than for D1 receptors (D1Rs).

An important step in evaluating the involvement of dopamine signaling in refractive development and eye growth is to determine the specific type of dopamine receptors involved. Based on previous results of combined pharmacologic intervention, D2R activation may have a major role in the APO-mediated inhibition of axial eye growth. The inhibitory effect of APO on FDM in chickens was abolished by coadministration of the dopamine antagonist haloperidol, which shows somewhat greater affinity for D2Rs compared with D1Rs. Furthermore, Rohrer et al. showed that in chickens the APO-induced protection against FDM was almost completely abolished by the D2R antagonist spiperone but not by the D1R antagonist SCH 23390. Although pharmacologic intervention is an important and useful approach, it has its intrinsic limitation of partial specificity. It is difficult to totally block the effect of APO by D2R antagonists due to the uncertainty over local drug concentration and distribution. These limitations make it difficult to draw definitive conclusions about the exact role of dopamine receptor subtypes in the APO-mediated inhibition of myopia.

If APO attenuates FDM mainly through a D2R mechanism, then activation of D2Rs would be expected to prevent myopia. However, our recent studies show that either D2R KO or systemic administration of the D2R antagonist sulpiride attenuates FDM development in mice by approximately 50%. The latter finding is also supported by the observation that myopia development is inhibited by D2R antagonists in albino guinea pigs. The partial inhibition of FDM in C57BL/6 mice by APO may reflect opposing effects arising from activation of different subtypes of dopamine receptors. In contrast to D2Rs, inactivation of D1Rs by the D1R antagonist SCH59166 inhibited the development of myopia in mice. Because of the complexity and inherent limitations of dopamine receptor pharmacology, a more direct approach to dissecting the roles of D2Rs and D1Rs in myopia development would be helpful. Thus, the use of genetic KO mice may provide complementary and more definitive insight regarding the roles of D2Rs and D1Rs.

In this study, we used mouse D2R-KO and D1R-KO models as a potentially more direct approach for evaluating the roles of D2Rs and D1Rs in myopia development. First, we determined whether retinal structure and function were altered in the KO models compared with WT mice treated with selective antagonists for D2Rs and D1Rs. Thus, we analyzed retinal layer thickness and electroretinograms (ERGs) in genetic D2R-KO and D1R-KO mice and in wild-type (WT) mice treated with D2R or D1R antagonists. If the transient pharmacologic blockade of D2Rs and D1Rs produces similar effects to D2R KO and D1R KO, this would largely exclude these phenotypes being attributed to developmental confounding effects of the genetic KO. We then utilized the D2R-KO and D1R-KO mice to study the involvement of the dopamine receptors in the inhibitory effect of APO on FDM development. By combining pharmacologic and genetic approaches in the current study, we sought to overcome the limitations of each approach to better understand the roles of D2Rs and D1Rs in myopia development. We found that APO attenuates myopia development by a D1R- but not by a D2R-dependent mechanism.

**Materials and Methods**

**Animals**

The study was approved by the Animal Care and Ethics Committee at Wenzhou Medical University (Wenzhou, China), and all treatment and care of animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**D2R-KO Mice.** The D2R-KO mice were generated by targeted mutagenesis of the D2R gene in embryonic stem cells, in which all of exon 7 and the 5' half of exon 8 were deleted and replaced by a neomycin resistance cassette. Heterozygous D2R-KO mice (+/-) derived from the C57BL/6 background were bred to generate D2R KO (-/-) and their WT littermates (+/+). PCR genotyping procedure was briefly reported previously and is described in detail at the website for the Jackson Laboratory (available in the public domain, https://www.jax.org/strain/003190).

**D1R-KO Mice.** The D1R-KO mice were obtained from The Knockout Mouse Project Repository (KOMP; The Knockout Mouse Project, Mouse Biology Program, University of California, Davis, CA, USA) and backcrossed for up to 10 generations with C57BL/6J to maintain a C57BL/6J genetic background. Briefly, to generate a germline KO allele, a neomycin resistance cassette was inserted into the critical exon 7 of the target gene. This leads to the disruption of the gene's translational frame, and premature termination occurs at the neo-polycistronic site. Homozygous D1R KO (-/-) and WT (+/+ ) littermates used in these studies were generated from inbred heterozygote mice (+/–). The genotypes were determined by PCR analysis of genomic DNA extracted from the toenail of each mouse, as described in detail at the KOMP's website for this line (available in the public domain, https://www.komp.org/Products/php?cloneID=558080).

**Experimental Design**

**Retinal Structure and Function Analysis.** Retinal structure and function were evaluated in 5-week-old D2R-KO (n = 11), D1R-KO (n = 13), and WT control mice (D2R-WT, n = 10; D1R-WT, n = 17). In parallel with KO mice, 35 C57BL/6 mice (4 weeks old) were randomly assigned to four different groups. One group received daily intraperitoneal injections of the D2R antagonist sulpiride (D2R-WT-sulpiride, n = 8), while the second group (controls) received the vehicle dimethylsulfoxide (D2R-WT-DMSO, n = 9). The third group received daily intraperitoneal injections of the D1R antagonist SCH39166 (D1R-WT-sCH39166, n = 9), along with the fourth group (controls) that received the vehicle DMSO (D1R-WT-DMSO, n = 9). Retinal layer thickness and ERG responses were measured at the end of 1 week of each treatment (5 weeks old).

**APO-Treated D2R-KO Mice.** Forty-seven D2R-KO mice (4 weeks old) were randomly divided into two groups: D2R-WT-Veh (n = 24; Veh, vehicle composed of ascorbic acid solvent for injection of APO) and D2R-KO-APO (n = 25). Fifty-six WT littermates were randomly divided into two control groups: D2R-WT-Veh (n = 25) and D2R-WT-APO (n = 31).
APO-Treated D1R-KO Mice. Thirty-two D1R-KO mice (4 weeks old) were randomly assigned to two groups: D1R-KO-Veh (n = 16) and D1R-KO-APO (n = 16). Thirty-six WT littersmates were randomly divided into two control groups: D1R-WT-Veh (n = 16) and D1R-WT-APO (n = 20).

Form Deprivation

Form deprivation was produced by covering the right eye of each animal with a handmade white translucent occluder.53 The occluder was attached carefully to the fur around the eye with expanded polystyrene glue and remained in place for 4 weeks. A collar made from thin plastic was fitted around the neck to prevent the mouse from removing the occluder. Body weight, refraction, axial components, and corneal radius of curvature of the occluded and nonoccluded fellow eyes were measured prior to and at the end of the 4 weeks of each treatment (4 and 8 weeks old, respectively) in all groups.

Preparation for Drug Injection

Daily intraperitoneal injections of all agents were made in the lower right or left quadrant of the abdomen without anesthesia using a microliter syringe attached to a 29-gauge needle. Sulpiride (80 μg/g body weight; Tocris Bioscience, Glasgow, UK) or SCH91966 (0.4 μg/g body weight; Tocris Bioscience) were injected after dissolving in DMSO (1.1 μg/g body weight; Sigma-Aldrich, Buchs, Switzerland). APO (5 μg/g body weight; Tocris Bioscience) was injected after dissolving in distilled H2O containing 1 μg/μL ascorbic acid (ICN Biomedicals, Inc., Irvine, CA, USA) to retard oxidation. Solutions of 1 μg/μL ascorbic acid were used as the vehicle control for APO treatment. The injection volume in all groups was 1 μL/g body weight. The pharmacologic doses were chosen based on our34,35 and other studies.34,35

Retinal Layer Thickness Measurements

Thicknesses of the retinal layers were quantified from optical coherence tomography (OCT) images using an ophthalmic imaging platform (Heidelberg Retina Angiograph Spectralis system; Heidelberg Engineering, Heidelberg, Germany). A 30-diopter (D) lens was added in front of the OCT camera, and OCT images centered on the optic nerve head (ONH) were obtained from each eye. The thickness of individual retinal layers was quantified using a standard Early Treatment Diabetic Retinopathy Study grid, which was centered manually on the ONH.36 Mean values of the outer ring (corresponding to a distance of 600 μm from the center of the ONH, approximately 17°) were analyzed by an experienced annotator using the Heidelberg system. The measurements included thicknesses of the retinal nerve fiber layer (RNFL), ganglion cell layer to inner plexiform layer (GCL-IPL), inner nuclear layer to outer plexiform layer (INL-OPL), outer nuclear layer (ONL), photoreceptor layer (PR), and total retinal thickness.

ERG Recording

To assess overall retinal function, scotopic and photopic flash ERGs were recorded with a custom-built Ganzfeld dome connected to a computer-based system (Q450SC UV; Roland Consult, Wiesbaden, Germany).35,36 All mice were reared under cyclic light/dark conditions and dark-adapted overnight before the experiments. In the pharmacologic experiments, record- ings were started 45 minutes after injection of pharmacologic antagonists or vehicle for each mouse. General anesthesia was achieved with an intraperitoneal injection of ketamine (70 mg/ kg) and xylazine (7 mg/kg), and the pupils were dilated with 1% tropicamide and 2.5% phenylephrine hydrochloride. A small amount of 2.5% methylcellulose gel was applied to the eye, and a custom gold wire loop electrode was placed over the cornea as the active electrode. Needle reference and ground electrodes were inserted into the cheek and tail, respectively. Body temperature was maintained by placing the animals on a 37°C warming pad during the experiment.

For scotopic flash ERGs, white LED stimuli of five intensities (−3.699, −2.201, −0.699, 0.301, and 0.799 log cd/s/m²) were used without background lighting to generate and record rod-dominant responses and mixed rod- and cone-driven responses as the intensities increased. Recordings were started from the lower light intensity and progressed to higher levels. For each of the intensities between −3.699 and −0.699 log cd/s/m², ERGs were averaged from five single flashes. The interstimulus interval was 15 seconds. For intensities between 0.301 and 0.799 log cd/s/m², ERGs were averaged from three single flashes. The interstimulus interval was 40 seconds. Signals were band-pass filtered between 1 and 300 Hz for a- and b-waves and between 50 and 300 Hz for oscillatory potentials (OPs). The amplitude of each ERG a-wave was measured from the baseline to the trough, and the implicit time was measured from the stimulus onset to the trough of the a-wave. The ERG a-wave amplitudes and implicit times were examined only at the three highest luminances. The amplitude of each b-wave was measured from the trough of the a-wave to the peak of the b-wave, and the implicit time was measured from the stimulus onset to the peak of the b-wave. OPs were consistently detectable from only the four highest flash intensities, and therefore only these flash intensities were used in OP analysis. The sum of OPs (OP1+OP2+OP3+OP4) was used for analysis.

Following 10 minutes of light adaptation with a rod-saturating background (1.398 log cd/m²), photopic stimuli were presented at 1 Hz, with −0.699, −0.201, 0.301, 0.799, and 1.301 log cd/s/m² stimuli against the same background white lighting used for light adaptation. The responses to 50 flashes were averaged and filtered through a band-pass of 1 to 300 Hz.

Biometric Measurements

Refraction was measured at the vertical pupil meridian in darkness using an eccentric infrared photorefactor designed by Schaeffel.35,37 Briefly, each unanesthetized mouse was placed on a platform that was slowly adjusted until a clear first Purkinje image occurred in the center of the pupil, indicating an on-axis measurement. The data were then automatically recorded by the program designed by Schaeffel33 and reported as the mean of at least three measurements, each of which was averaged from 10 individual values to create that measurement.

A custom-made spectral-domain OCT (SD-OCT) system was used to measure the anterior chamber depth (ACD, from the posterior corneal surface to the anterior lens surface), lens thickness (LT), vitreous chamber depth (VCD, from the posterior lens surface to the vitreous-retina interface), axial length (AL, from the anterior corneal surface to the vitreous-retina interface), and corneal radius of curvature.38,39 Each anesthetized mouse was placed in a cylindrical holder and mounted on the positioning stage in front of a modified slit lamp. The SD-OCT scanning position was aligned along the optical axis of the eye during the measurement with an X-Y cross-scanning system. The raw SD-OCT data were exported and analyzed using custom-designed software to obtain the axial components and corneal radius of curvature.38,39 The mean of three repeated SD-OCT measurements for each eye was used for analysis.
Statistics

The data, which were all verified to be normally distributed, are presented as means ± SEMs. Independent t-tests were used to analyze the changes in retinal layer thicknesses, and repeated measures ANOVA was performed to assess the changes in ERG parameters with changes in flash intensity. Intergroup differences of the biometric parameters were compared by 2-way repeated measures ANOVA with genotype (WT versus KO) and pharmacologic treatment (vehicle versus APO) as two factors and time (baseline versus 4 weeks) as repeated measures. Bonferroni corrections were applied in post hoc analysis. Values of $P < 0.05$ were considered to be significant. All statistical analyses were performed with statistical software (IBM SPSS, Version 19.0; IBM, Armonk, NY, USA).

RESULTS

Specificity of Changes in Retinal Structure and Function

No significant differences in RNFL, GCL+IPL, INL+OPL, ONL, PR, or total retinal thickness between D2R-WT and D2R-KO mice (all $P$ values > 0.05, independent t-tests; Fig. 1A–C) were observed. Similarly, there were no significant differences in RNFL, GCL+IPL, INL+OPL, ONL, PR, or total retinal thickness between D2R-WT-DMSO and D2R-WT-sulpiride mice (all $P$ values > 0.05, Fig. 1D–F) after 1 week of treatment. All retinal layer thicknesses were similar between D1R-WT and D1R-KO mice (all $P$ values > 0.05; Fig. 1G–I). None of the retinal layer thicknesses were affected after 1 week of SCH39166 treatment (all $P$ values > 0.05; Fig. 1J–L).

Implicit times of the a- and b-waves for both scotopic and photopic conditions were not affected by either genetic KO or pharmacologic antagonist treatment (all $P$ values > 0.05, repeated measures ANOVA; see Supplementary Fig. S1 for details). The a-wave, b-wave, and summed OP amplitudes were not significantly different between D2R-WT and D2R-KO mice under scotopic conditions ($P > 0.05$ for each, repeated measures ANOVA; Fig. 1A, B). However, the a- and b-wave amplitudes were significantly higher under photopic conditions in D2R-KO mice compared with D2R-WT mice (a-wave amplitude, $F_{1,19} = 13.618$, $P = 0.002$; b-wave amplitude, $F_{1,19} = 7.195$, $P = 0.015$; Fig. 1C). The sulpiride treatment had no significant effect on a-wave, b-wave, and summed OP amplitudes in scotopic conditions ($P > 0.05$ for each; Fig. 1D, E) or a- and b-wave amplitudes in photopic conditions ($P > 0.05$ for each; Fig. 1F). In scotopic conditions, D1R-KO mice had significantly decreased b-wave amplitudes compared with D1R-WT mice ($F_{1,28} = 6.335$, $P = 0.017$; Fig. 1G), but there were no differences in the summed OP amplitudes ($P > 0.05$; Fig. 1H). In photopic conditions, D1R-KO mice had decreased b-wave amplitudes compared with D1R-WT mice ($F_{1,28} = 4.830$, $P = 0.036$; Fig. 1I). Consistent with these findings, 1 week of SCH39166 treatment significantly decreased both b-wave ($F_{1,16} = 6.623$, $P = 0.020$; Fig. 1J) and summed OP amplitudes ($F_{1,16} = 5.186$, $P = 0.059$; Fig. 1K) in scotopic conditions and b-wave amplitudes in photopic conditions ($F_{1,16} = 6.212$, $P = 0.025$; Fig. 1L).
D2R-Independent Inhibitory Effect of APO on FDM

The D2R-KO mice had a smaller body weight in comparison with the D2R-WT mice both prior to and after 4 weeks of pharmacologic treatment (D2R-KO-Veh and D2R-KO-APO versus D2R-WT-Veh and D2R-WT-APO, $F_{1,99} = 42.353$, $P < 0.001$, main effect of genotype, 2-way repeated ANOVA; Fig. 3A, Table 1). Some ocular measurements also revealed slower growth rate in the D2R-KO mice. For D2R-KO mice compared with D2R-WT mice, the fellow nondeprived eyes had shorter ACDs ($F_{1,99} = 13.351$, $P < 0.001$; Table 1) and shorter ALs ($F_{1,99} = 7.532$, $P = 0.007$). Therefore, a comparison of raw data between the D2R-WT and D2R-KO eyes would provide potentially misleading results due to the different rates of

![Graphs and images showing scotopic and photopic ERG a-wave, b-wave, and summed OP amplitudes in treated mice: D2R-KO (A–C), D2R antagonist sulpiride (D–F), D1R-KO (G–I), and D1R antagonist SCH39166 (J–L). *$P < 0.05$ and **$P < 0.01$; 1-way repeated measures ANOVA, post hoc simple effects analysis. SE, standard error of the mean.](http://www.arvojournals.org/2018/05/2627)

D2R-Independent Mechanism of Apomorphine in Myopia

**FIGURE 2.** Scotopic and photopic ERG a-wave, b-wave, and summed OP amplitudes in treated mice: D2R-KO (A–C), D2R antagonist sulpiride (D–F), D1R-KO (G–I), and D1R antagonist SCH39166 (J–L). *$P < 0.05$ and **$P < 0.01$; 1-way repeated measures ANOVA, post hoc simple effects analysis. SE, standard error of the mean.
development of these two groups. Therefore, we expressed all treatment effects as interocular differences, that is, the differences between form-deprived eyes and fellow eyes (Fig. 3).

A significant interaction between measurement time and APO treatment in the interocular differences of refraction and AL was revealed ($F_{1,99} = 24.565$, $P < 0.001$ for refraction; $F_{1,99} = 4.175$, $P = 0.044$ for AL, interaction effect, 2-way repeated measures ANOVA, post hoc simple effects analysis. SE, standard error of the mean.)
TABLE 1. Body Weight, Refraction, and Ocular Biometry for APO Treatment in D2R-KO Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Eye Refraction, D</th>
<th>ACD, mm</th>
<th>VCD, mm</th>
<th>LT, mm</th>
<th>VCD, mm</th>
<th>AL, mm</th>
<th>CRC, mm</th>
<th>VCD, mm</th>
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<td>Baseline</td>
<td>25</td>
<td>12.35</td>
<td>0.35</td>
<td>2.35</td>
<td>1.00</td>
<td>0.329</td>
<td>6</td>
<td>0.004</td>
<td>1.440</td>
</tr>
<tr>
<td>Deprived</td>
<td>6</td>
<td>0.005</td>
<td>1.03</td>
<td>0.318</td>
<td>0.86</td>
<td>0.335</td>
<td>6</td>
<td>0.006</td>
<td>1.447</td>
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<tr>
<td>Fellow</td>
<td>6</td>
<td>0.005</td>
<td>1.03</td>
<td>0.318</td>
<td>0.86</td>
<td>0.335</td>
<td>6</td>
<td>0.006</td>
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<tr>
<td>D2R-WT-APO, n = 25</td>
<td>31</td>
<td>11.94</td>
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<td>1.00</td>
<td>0.329</td>
<td>6</td>
<td>0.004</td>
<td>1.440</td>
</tr>
<tr>
<td>Deprived</td>
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<td>D2R-KO-Veh, n = 24</td>
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<td>6</td>
<td>0.007</td>
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<tr>
<td>Deprived</td>
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<td>1.443</td>
<td>0.005</td>
<td>0.002</td>
<td>0.004</td>
<td>6</td>
<td>0.006</td>
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<tr>
<td>Fellow</td>
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<td>D2R-KO-APO, n = 23</td>
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<td>Deprived</td>
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Data are expressed as the mean ± SEM. CRC, corneal radius of curvature.

5.47 ± 0.001 for AL; Fig. 3C, D). Thus, D2R KO inhibited the form deprivation–induced changes in refraction, VCD, and AL in D2R-WT mice. The interocular refraction difference for the D2R-KO-APO group was also 56% lower than in the D2R-KO-Veh group (−1.30 ± 0.39 D in D2R-KO-APO versus −2.97 ± 0.42 D in D2R-KO-Veh, P = 0.031; Fig. 3B). Therefore, the inhibitory effect of APO on FDM remained even in the absence of D2Rs. However, differences between D2R-KO-Veh and D2R-KO-APO groups, in both interocular differences in VCD and AL, were not significant (P > 0.05 for each; Fig. 3C, D).

The difference in myopia between the deprived and fellow eyes induced in the D2R-KO-Veh group was 46% lower than in the D2R-WT-Veh group (−2.97 ± 0.42 D in D2R-KO-Veh versus −5.47 ± 0.96 D in D2R-WT-Veh, P = 0.003, 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 3B). The D2R-KO-Veh group also presented with smaller interocular differences in VCD, 0.006 ± 0.006 mm, and AL, 0.007 ± 0.004 mm, compared with that of 0.22 ± 0.003 mm and 0.28 ± 0.006 mm, respectively, in D2R-WT-Veh mice (P = 0.001 for VCD and P = 0.001 for AL; Fig. 3C, D). Thus, D2R KO inhibited the form deprivation–induced changes in refraction, VCD, and AL in vehicle-treated mice. No significant differences with respect to interocular differences in refraction or other biometric parameters between D2R-WT-APO and D2R-KO-APO after 4 weeks of each treatment (all P values > 0.05, Fig. 3) were revealed. Similarly, the analysis showed no significant differences in the interocular differences of ACD, LT, and corneal radius of curvature between any two groups after 4 weeks of each treatment (all P values > 0.05, Fig. 3E-G). Therefore, D2R KO had no effect on FDM in APO-treated mice.

**D1R-Dependent Inhibitory Effect of APO on FDM**

Similar to the D2R-KO studies, the D1R-KO mice had a smaller body weight in comparison with the D1R-WT mice both prior to and after 4 weeks of pharmacologic treatment (D1R-KO-Veh and D1R-KO-APO versus D1R-WT-Veh and D1R-WT-APO). There were no significant differences between the APO and vehicle groups for any of the baseline measurements (all P values > 0.05, 2-way repeated ANOVA, post hoc simple effects analysis).

After 4 weeks of treatment, the myopic shift, measured as the difference between the deprived and fellow eyes, in the D2R-WT-APO group was 83% lower than in the D2R-WT-Veh group (−0.92 ± 0.27 D in D2R-WT-APO versus −5.47 ± 0.96 D in D2R-WT-Veh, P < 0.001, 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 3B). In parallel with the refraction changes, in the D2R-WT-APO group the interocular difference in VCD, −0.001 ± 0.005 mm, was completely suppressed compared with a difference of 0.022 ± 0.003 mm in the D2R-WT-Veh group (Fig. 3C, P = 0.001). Similarly, the interocular difference in AL for the D2R-WT-APO group, −0.002 ± 0.005 mm, was also completely suppressed in comparison with a difference of 0.028 ± 0.006 mm in the D2R-WT-Veh group (Fig. 3D, P = 0.001). Thus, APO treatment attenuated the form deprivation–induced changes in refraction, VCD, and AL in D2R-WT mice. The interocular refraction difference for the D2R-KO-APO group was also 56% lower than in the D2R-KO-Veh group (−1.30 ± 0.39 D in D2R-KO-APO versus −2.97 ± 0.42 D in D2R-KO-Veh, P = 0.031; Fig. 3B). Therefore, the inhibitory effect of APO on FDM remained even in the absence of D2Rs. However, differences between D2R-KO-Veh and D2R-KO-APO groups, in both interocular differences in VCD and AL, were not significant (P > 0.05 for each; Fig. 3C, D).

The difference in myopia between the deprived and fellow eyes induced in the D2R-KO-Veh group was 46% lower than in the D2R-WT-Veh group (−2.97 ± 0.42 D in D2R-KO-Veh versus −5.47 ± 0.96 D in D2R-WT-Veh, P = 0.003, 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 3B). The D2R-KO-Veh group also presented with smaller interocular differences in VCD, 0.006 ± 0.006 mm, and AL, 0.007 ± 0.004 mm, compared with that of 0.22 ± 0.003 mm and 0.28 ± 0.006 mm, respectively, in D2R-WT-Veh mice (P = 0.001 for VCD and P = 0.001 for AL; Fig. 3C, D). Thus, D2R KO inhibited the form deprivation–induced changes in refraction, VCD, and AL in vehicle-treated mice. No significant differences with respect to interocular differences in refraction or other biometric parameters between D2R-WT-APO and D2R-KO-APO after 4 weeks of each treatment (all P values > 0.05, Fig. 3) were revealed. Similarly, the analysis showed no significant differences in the interocular differences of ACD, LT, and corneal radius of curvature between any two groups after 4 weeks of each treatment (all P values > 0.05, Fig. 3E-G). Therefore, D2R KO had no effect on FDM in APO-treated mice.
FIGURE 4. Effects of D1R KO and APO treatment on body weight, refraction, and ocular dimensions. Biometric measurements in D1R-WT-Veh, D1R-WT-APO, D1R-KO-Veh, and D1R-KO-APO groups before and after 4 weeks of each treatment. (A) Body weight. For panels B–G, interocular differences for deprived and fellow eyes: (B) Refraction, (C) VCD, (D) AL, (E) ACD, (F) LT, (G) corneal radius of curvature. *P < 0.05, **P < 0.01, and ***P < 0.001; 2-way repeated measures ANOVA, post hoc simple effects analysis. SE, standard error of the mean.
Table 2. Body Weight, Refraction, and Ocular Biometry for APO Treatment in D1R-KO Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Refraction, D</th>
<th>ACD, mm</th>
<th>VCD, mm</th>
<th>LT, mm</th>
<th>BCVA</th>
<th>Eye</th>
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<tbody>
<tr>
<td>Baseline</td>
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<tr>
<td>D1R-WT-Veh, n = 16</td>
<td>12.72 ± 0.53</td>
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<tr>
<td>D1R-WT-APO, n = 16</td>
<td>12.75 ± 0.58</td>
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<tr>
<td>D1R-KO-APO, n = 20</td>
<td>10.51 ± 0.42</td>
<td></td>
<td>1.00</td>
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</table>

Data are expressed as the mean ± SD.

After 4 weeks of treatment, the interocular refraction difference for the D1R-WT-APO group was 81% lower than in the D1R-WT-Veh group (\(-1.00 \pm 0.87\) D in D1R-WT-APO versus \(-5.26 \pm 0.75\) D in D1R-WT-Veh, \(P < 0.001\), 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 4B). In parallel with the refraction changes, in the D1R-WT-APO group, the interocular difference in VCD, 0.011 ± 0.006 mm, was significantly less than in the D1R-WT-Veh group, 0.033 ± 0.007 mm (Fig. 4C; \(P = 0.023\)). Similarly, the interocular difference in AL for the D1R-WT-APO group, −0.003 ± 0.008 mm, was negligible in comparison with a difference of 0.028 ± 0.006 mm in the D1R-WT-Veh group (Fig. 4D; \(P = 0.005\)). Thus, APO attenuated the form deprivation–induced changes in refraction, VCD, and AL in D1R-WT mice (Fig. 4). Remarkably, interocular differences in refraction, VCD, and AL for the D1R-KO-APO group were not significantly different from those of the D1R-KO-Veh group (\(P > 0.05\) for all; Fig. 4B-D). Therefore, the inhibitory effect of APO treatment on FDM disappeared in the absence of D1Rs.

The interocular difference in refraction for the D1R-KO-APO group was approximately four-fold greater than in the D1R-WT-APO group (−4.44 ± 0.64 D in D1R-KO-APO versus −1.00 ± 0.87 D in D1R-WT-APO, \(P = 0.001\), 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 4B). The interocular difference in VCD for the D1R-KO-APO group, 0.030 ± 0.004 mm, was approximately three times that of the D1R-WT-APO group, 0.011 ± 0.006 mm (\(P = 0.055\); Fig. 4C). For the interocular difference of AL in the D1R-KO-APO group, the increase was 0.027 ± 0.007 mm, much more than the change in the D1R-WT-APO group, −0.003 ± 0.008 mm (\(P = 0.005\); Fig. 4D). Thus, D1R KO prevented the APO-induced changes in refraction, VCD, and AL (Fig. 4). However, the interocular differences for refraction, VCD, or AL between D1R-WT-Veh and D1R-KO-Veh groups were not significant (\(P > 0.05\) for each; Fig. 4). Therefore, D1R KO had no effect on FDM in vehicle-treated mice.

Neither D1R KO nor APO treatment, applied alone or in combination, had any effect at any time point, on interocular differences of ACD, LT, or corneal radius of curvature (all \(P\) values \(> 0.05\), 2-way repeated ANOVA; Fig. 4E-G).

**Discussion**

Consistent with our previous studies, 22,28 FDM was largely inhibited (approximately 80%) by the nonselective dopamine agonist APO and partially inhibited (approximately 50%) in D2R-KO mice. In this study, we further determined that the APO-mediated myopia inhibition could be completely abolished in D1R-KO mice but not in D2R-KO mice. This indicates the specificity of D1Rs for the APO pharmacologic effect on form-deprivation myopia and the nonessential role of D2Rs in this process in mice. However, the development of myopia was not affected by D1R KO in this study. This suggests that two different mechanisms are involved in FDM development versus APO-mediated effect on ocular growth. It also suggests a possible developmental compensation effect of D1R KO.

In this study, neither D2R KO nor D1R KO seriously altered retinal structure and function as revealed by the unchanged thicknesses of the retinal layers and only minor changes in ERG recordings. However, it is worth noting that the visual signal processes must be altered in KO mice as dopamine plays an important and extensive modulatory role in the vertebrate visual system. 36,40 Reported and observed effects of D1Rs and D2Rs on the ERGs seem to be contradictory. Pharmacologic blockade or genetic mutation of D2Rs in this study slightly increased b-wave and summed OP amplitudes, which are similar to the effects mediated by D2R antagonists in goldfish. 41

**Investigative Ophthalmology & Visual Science**

**D1R-Dependent Mechanism of Apomorphine in Myopia**

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and cats, and by genetic KO in mice, but is opposite to that seen in rabbits. We also found that suppression of the b-wave and summed OP amplitudes by the D1R antagonist SCH39166 resembled the ERG phenotype of the D1R-KO mice. Similar results showing a reduction of the b-wave amplitude have been obtained by other authors working with D1R-KO mice, and D1R antagonist-treated goldfish. However, in frogs, pharmacologic blockage of D1Rs increased the amplitudes of b-waves of both dark- and light-adapted ERGs. While the reasons for these contradictory results are unknown, adaptation and stimulus intensities, age, and species differences are likely contributing factors. The transient pharmacologic blockade of D2Rs and D1Rs produced effects similar to those in D2R-KO and D1R-KO mice. Thus, the results of comparative analyses of the effects of genetic and pharmacologic inactivation of D2Rs and D1Rs suggest that the KO phenotypes cannot be simply attributed to developmental confounding effects in these mice. Therefore, it seems likely that altered effects of APO in D2R-KO and D1R-KO mice reflect the lack of D2Rs and D1Rs, respectively, though other mechanisms, for example, the noradrenergic system, cannot be completely excluded.

The baseline refractions for D1R-WT and D1R-KO mice were more myopic and had increased ACDs and ALs compared with D2R-WT and D2R-KO mice, respectively. The differences in refraction and biometric measurements between these groups are not attributable to different genetic backgrounds for the two sets of WT mice and different feeding conditions, although they were developed from a C57BL/6j background. Additionally, the experimental time was relatively long, and therefore several factors (e.g., different batches of mice and subtle changes in the environment) might also have affected the absolute values of refraction and biometric measurements. However, these discrepancies cannot account for the main results of our study, as the D2R-KO and D1R-KO mice were compared only with their WT littermates. This approach limited and excluded potential confounding effects of development and long experimental times.

The inhibitory effect of APO on FDM in D2R-KO mice was evident in refraction but not in VCD or AL. It is possible that the small APO-induced changes measured in VCD and AL were not statistically significant because of the relatively low resolution (6 μm, theoretically) of the SD-OCT used in this study. Also, as the myopia induced in the D2R-KO-Veh group was reduced by 5% compared with the D2R-WT-Veh group, further reductions by APO treatment in the KO group may not have been large enough to be detected. The inhibitory effect of APO on FDM in the D2R-KO-APO group was 56% of that in the D2R-KO-Veh group. Furthermore, the myopia induced in the D2R-KO-APO group was similar to the D2R-WT-APO group, suggesting that the inhibitory effect of APO on myopia does not depend on the presence of D2Rs. Other dopamine receptors, such as D1Rs, may be involved in this inhibitory effect of APO on FDM. In agreement with this hypothesis, the myopia shift induced in D1RKO-APO mice was not different from that in D1RKO-Veh mice, but it was much greater than that in the D1R-WT-APO mice. Changes in the corresponding VCD and AL were consistent with the refractions for all groups. Therefore, the inhibitory effect of APO on the development of myopia is lost in the absence of D1Rs. This suggests that activation of D1Rs is responsible for APO-mediated inhibition of myopia and that other receptor subtypes including D2Rs, have minimal roles. However, our earlier study showed that inactivation of D2R by either D2R KO or the D2R antagonist sulpiride partially attenuated FDM development in mice. The latter result also implies that the changes induced by form deprivation are not totally dependent on the presence of D2Rs. This interpretation is supported by our finding that D1Rs are involved in the APO-mediated inhibition of ocular growth and myopia.

The role of D1Rs in the control of ocular growth and myopia development has not been well studied and remains unclear. In this study, the myopia induced in the D1R-KO group was similar to that in the D1R-WT-Veh group. In contrast, the inhibition of myopia development by APO treatment in the D1R-WT group was largely lost in the D1R-KO-APO group. These results suggest that D1Rs are not required for the development of FDM but are required for the inhibitory effect of APO on the latter. The signal pathways underlying these complicated response patterns remain to be elucidated. Previous pharmacologic manipulation showed that D1Rs play a role in axial eye growth, but the nature of that role is not clear. Intravitreal injection of the D1R antagonist SCH 23390 enhanced FDM development in chickens. Similarly, preliminary studies indicated the D1R agonist SKF 38393 inhibited FDM, whereas the D1R antagonist SCH 39166 enhanced FDM in mice (Zhou X, et al. IOVS 2014:55:ARVO E-Abstract 3038). However, other studies in chickens and tree shrews found that the development of FDM was not affected by intravitreal injection of the D1R antagonist SCH 23390. In the present study, the mouse D1R gene was deleted throughout the body during early development, and the D1R-KO mice exhibited growth retardation and small body weight compared with WT mice due to reduced food and water intake, as reported previously. Further studies are needed to determine the exact role of the D1R signaling cascades in the control of ocular growth and myopia development.

In chickens, the inhibitory effect of APO on myopia appears to be D2R dependent, as it was blocked by local administration of a D2R antagonist but not by a D1R antagonist. In contrast, in the present study, prevention of myopia development by APO in mice appeared to be dependent on D1Rs because systemic administration of APO did not inhibit FDM in D1R-KO mice. A species difference, that is, between chickens and mice, probably contributes to the different receptor mechanisms of APO in control of ocular growth and myopia development. Mice are nocturnal animals, less dependent on vision behaviorally than chickens, which are diurnal. The ocular growth of chickens is also highly sensitive to visual manipulation. Nonetheless, the conclusion from results in mice that myopia development is promoted by D2R activation and inhibited by D1R activation is largely supported by findings from guinea pigs (Zhou X, et al. IOVS 2013:54:ARVO E-Abstract 3678). The action of APO, which binds efficiently to D1- and D2-like receptors, depends on the local concentration, the endogenous retinal dopamine level (especially the extracellular dopamine level), and the active status of the dopamine receptors. Obviously, differences in routes of drug administration (intravitreal injection in chickens and intraperitoneal injection in our study) lead to different drug distributions within the retina. During FDM development, the unchanged dopamine level in mice is also different from the reduced dopamine level in chickens. However, with respect to the active status of dopamine receptors, which is the most important point while considering the receptor mechanism of APO activity, genetic KO provides an alternative approach that is much more complete and persistent than pharmacologic intervention. Various studies have shown that dopamine receptors are downregulated or upregulated in response to the increased or reduced dopamine concentration. Such changes cannot be excluded using pharmacologic intervention. Therefore, the partial specificity of dopaminergic drugs and uncertainty of local dopamine and applied drug concentrations all likely contribute to differences in experimental...
outcomes between the chicken and mouse. Even though the genetic mouse models are somewhat limited by the presence of developmental effects, as models for investigating myopia they avoid the above limitations. More studies are needed to clarify the mechanism of dopamine signaling in refractive development and eye growth.

Although dopamine receptors are widely distributed in the retina, brain, and other tissues throughout the body, it is reasonable to assume that the site of action of APO on ocular growth is in the retina. Systemic administration of APO with the same dose used in this study (5 μg/g body weight) decreased the ERG a- and b-wave amplitudes in our previous study.22 This indicates that the route and dose of systemic APO administration here was adequate for stimulating retinal dopamine receptors. The D2R-KO and D1R-KO mice used in this study were generated by targeted mutagenesis of the respective genes in embryonic stem cells. Therefore, the D2Rs or D1Rs were deleted throughout the body during early development.28,31,32,52 Systemic administration of APO would probably have stimulated both extra- and intraretinal dopamine receptors. Therefore, it is possible that the APO effects are exerted on extraretinal sites, including nonretinal ocular and extraocular sites (e.g., sclera, anterior pituitary, central nervous system); thus, the actual role of retinal dopamine receptors in inhibition of FDM remains to be resolved. The possibility that there are both retinal and nonretinal ocular sites of action mediating the ocular growth effects of APO and that they are differently affected, based on the route of APO administration, cannot be excluded. Additional studies using focal deletion of retinal and extraretinal dopamine receptors are required to clarify its sites of action.

Mice are nocturnal animals with poor vision acuity and little sensitivity to defocus compared with the chickens.57 However, mammalian models in general have eyes similar to humans with respect to structure and biochemistry, and a great deal is known about the biology and genetics, particularly of mice. Most important of all, the various available genetic KO models, which permit specific and complete inactivation of the target gene to overcome partial specificity of pharmacologic approach, are irreplaceable by other animal models for exploring the mechanisms underlying myopia development. Therefore, the genetic KO mouse models are expected to be novel resources and provide new insights into the signaling mechanisms that regulate eye growth.

In conclusion, we found that in mice the protective effect of APO against the development of myopia was dependent on the presence of D1Rs but not D2Rs. This suggests a D1R specificity for the pharmacologic inhibitory effect of APO on FDM and a nonessential role of D2Rs for this effect in mice. These findings now provide additional new insights into the roles of dopamine receptors in the development and inhibition of myopia.

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References


