Retinal Dopamine D2 Receptors Participate in the Development of Myopia in Mice

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Purpose. To learn more about the locations of dopamine D2 receptors (D2Rs) that regulate form-deprivation myopia (FDM), using different transgenic mouse models.

Methods. One eye of *D2R*-knockout (KO) mice and wild-type littermates was subjected to four weeks of monocular FDM, whereas the fellow eye served as control. Mice in both groups received daily intraperitoneal injections of either the D2R antagonist sulpiride (8 µg/g) or vehicle alone. FDM was also induced in retina- ($Six3^{cre}D2R^{fl/fl}$) or fibroblast-specific ($S100a4^{cre}D2R^{fl/fl}$) *D2R*-KO mice. A subset of retina-specific *D2R*-KO mice and $D2R^{fl/fl}$ littermates were also given sulpiride or vehicle injections. Refraction was measured with an eccentric infrared photorefractor, and other biometric parameters were measured by optical coherence tomography ($n \approx 20$ for each group).

Results. FDM development was attenuated in wild-type littermates treated with sulpiride. However, this inhibitory effect disappeared in the *D2R*-KO mice, suggesting that antagonizing D2Rs suppressed myopia development. Similarly, the development of myopia was partially inhibited by retina-specific (deletion efficiency: 94.7%) but not fibroblast-specific (66.9%) *D2R*-KO. The sulpiride-mediated inhibitory effects on FDM also disappeared with retinal *D2R*-KO, suggesting that antagonizing D2Rs outside the retina may not attenuate myopia. Changes in axial length were less marked than changes in refraction, but in general the two were correlated.

Conclusions. This study demonstrates that D2Rs located in the retina participate in dopaminergic regulation of FDM in mice. These findings provide an important and fundamental basis for further exploring the retinal mechanism(s) involved in dopamine signaling and myopia development.

Keywords: dopamine, myopia, D2 receptor, mouse

Myopia, the most common ametropia, has become a global health issue because of its rising prevalence.[1,2] In particular, the marked increase in incidence among schoolchildren is a concern, because high myopia can result in severe complications including cataract, macular degeneration, retinal detachment, glaucoma, and even blindness.[3] The etiology of myopia involves both genetic (e.g., parental myopia and myopia in twins)[4] and environmental (e.g., near work and education) factors.[5] Despite the global rise in the prevalence of myopia, the mechanism of myopia onset is still not fully understood.

A currently favored hypothesis is that a reduction in retinal dopamine levels, or inhibition of the dopamine pathway, results in myopia.[6,7] Consistent with this, treatments with exogenous dopamine, dopamine precursor L-dihydroxyphenylalanine, or the non-selective dopamine agonist apomorphine have a protective effect against myopia development in various animal models, including chicks,[8–18] guinea pigs,[19,20] rabbits,[21] tree shrews,[22] non-human primates,[23] and mice.[24,25] However, the underlying mechanism—including the specific type(s) of dopamine receptors, and the tissues and cells that express them—is largely undefined in these animal models. Studies carried out in chicks using various pharmacologic interventions, have shown that the inhibitory effects of exogenous dopamine,[16,17] dopamine agonists,[11,13] brief period of normal vision[8,12,26] or bright light exposure[27] against myopia development are mediated by dopamine D2 receptors (D2Rs). Similarly, a D2R agonist retards form-deprivation myopia (FDM) development in chicks[8] and tree shrews,[22] whereas a D2R antagonist enhances it.[28] The effects of D2Rs on the development of FDM appear to be species-specific.

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Table. Primers for Genotype Identification and qRT-PCR

Primer Name	Forward Primer	Reverse Primer
Primers for genotype identification of *D2R*-knockout mice		
D2R-WT	5′-TGATGACTGCGAATGTTGGTGTGC-3′	5′-CCGAGCCAAGCTAACACTGCAGAG-3′
D2R-KO	5′-TGATGACTGCGAATGTTGGTGTGC-3′	5′-AGGATTGGGAAGACAATAGCAG-3′
Primers for genotype identification of conditional *D2R*-KO (*D2R*CKO*) mice		
*D2R*CKO-WT	5′-CACTCCTGCTCACTCCTTG-3′	5′-CAGCACACTGTGAAGCACACAG-3′
*D2R*CKO-KO	5′-CACTCCTGCTCACTCCTTG-3′	5′-CTGAGCCCAGAAAGCGAAGGA-3′
Cre	5′-GCCTGCATTACCGGTCGATGC-3′	5′-CAGGGTGTTATAAGCAATCCC-3′
Primers for qRT-PCR		
18S	5′-CGGACACGGACAGGATTGAC-3′	5′-GTTCAAGCTGCCCGTCTCCTCATC-3′
D2R	5′-CCCTGGGTCGTCTATCTGGAG-3′	5′-GCGTGTGTTATACAACATAGGCA-3′
D1R	5′-CACGGCATCCATCCTTAACCT-3′	5′-TGCCTTCGGAGTCATCTTCCT-3′

* *D2R*CKO indicates retina- or fibroblast-specific *D2R*-KO.

In our previous studies, we found the inhibitory effect of apomorphine on FDM in mice was not abolished by *D2R* knockout (KO).[29] Furthermore, either systemic injection of a D2R antagonist or global genetic KO of *D2R*s inhibits FDM development.[30] The latter finding is supported by the observation that myopia development is inhibited by D2R antagonists in guinea pigs.[31,32] Thus this discrepancy in FDM development between chicks and mice may be due to a variety of factors including not only systematic species differences but also different dopamine concentrations reaching the ocular tissues, a difference in the site of action, and so on. Therefore, to determine the mechanism by which D2Rs modulate the development of myopia, the key step is to determine the action site of D2Rs.

D2Rs have been reported in various tissues. In the vertebrate retina, D2R proteins are found in photoreceptors and pigmented epithelium and as presynaptic autoreceptors on dopaminergic amacrine cells, in which their activation inhibits dopamine release.[33–36] Additionally, cells of the choroid layer express *D2R* mRNA in chicks.[37] Although dopamine receptors have not been consistently reported in the sclera, they have been found in corneal endothelial and epithelial cells.[38] Such wide distribution of D2Rs implies that many potential sites of action in the eye could mediate the effects of dopamine agonists on myopia.

The critical role of D2Rs in the modulation of myopia development has been primarily studied through pharmacological intervention.[6,7] However, intrinsic limitations of dopamine pharmacology, such as partial specificity and species-specific differences in dopamine receptors, might contribute to discrepancies in dopamine effects on myopia in different animal models. Furthermore, because D2Rs are expressed in various cell types and neural circuits in the retina and visual pathways,[35,39] dopaminergic action—even mediated by a single receptor type—might produce distinct effects on myopia development, by acting at different sites. Such limitations in the pharmacological approach impede any investigations into the site of action of D2Rs during myopia development. The use of transgenic models such as Cre-lox recombination to KO *D2R*s only in the neural retina/RPE (hereafter designated "retina" for convenience) or in specific cell types provides an attractive alternative for understanding the role of D2Rs at specific ocular sites in myopia development. Because no sclera-specific Cre mice are available, and because fibroblasts are the predominant cell population in the mammalian sclera, fibroblast-specific Cre mice are an appropriate alternative model for probing the possible roles of scleral *D2R*s in myopia.[40] Therefore, as an extension of our previous studies on the role of D2Rs in the myopia development,[29,30] this study will further clarify the site of action of D2Rs that regulate FDM—specifically, to distinguish between possible action via receptors in retina versus sclera. Using transgenic mouse models, we aimed (1) to determine whether the systemic inactivation of all D2Rs inhibits myopia development; (2) to identify the effects of either retina- or fibroblast-specific *D2R*-KO on normal refractive development and myopia progression; and (3) to determine whether the D2R-mediated pharmacological inhibition of FDM in mice is mediated by retinal D2Rs.

Materials and Methods

Animals

The protocols for using animals in this study were approved by the Animal Care and Ethics Committee at Wenzhou Medical University (Wenzhou, China). All experiments were conducted in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

***D2R*-Knockout (*D2R*-KO) Mice.** As previously described,[29,30,41] the systemic (whole-body) *D2R*-KO mouse model (*D2R*-KO) was generated by targeted mutagenesis of the *D2R* gene in embryonic stem cells. All of exon 7 and the 5′ half of exon 8 were deleted and replaced by a neomycin resistance cassette.[42] Heterozygous *D2R*-KO mice derived from the C57BL/6 background were bred to generate *D2R*-KO mice and their wild-type (*D2R*-WT) littermates.

Retina- or Fibroblast-Specific *D2R*-Knockout (*D2R*-KO) Mice. To determine the effects on myopia development of suppressing specifically retinal or scleral D2R signaling, $D2R^{fl/fl}$ mice (stock number 020631; The Jackson Laboratory, Bar Harbor, ME, USA) were crossed with Six3-Cre (a retinal cre, stock number 019755; The Jackson Laboratory) or S100a4-Cre mice (a fibroblast cre, stock number 012641; The Jackson Laboratory) to generate retina-specific ($Six3^{cre}D2R^{fl/fl}$)[43] or fibroblast-specific ($S100a4^{cre}D2R^{fl/fl}$)[44] *D2R*-KO mice. The Cre negative ($D2R^{fl/fl}$) littermate mice served as controls. In $D2R^{fl/fl}$ mice, two *loxP* sites were inserted flanking exon 2 of the *D2R* gene. These were bred to mice expressing the Cre recombinase enzyme, which resulted in offspring with exon 2 deleted in the Cre-expressing tissue. A frame-shift caused by the absence of exon 2 deletes an amino acid sequence, thus causing loss of D2R protein function. The identity (genotype) of the mice was confirmed by polymerase chain reaction (PCR) analysis of genomic DNA from tail-snips, using specific primers (Table).

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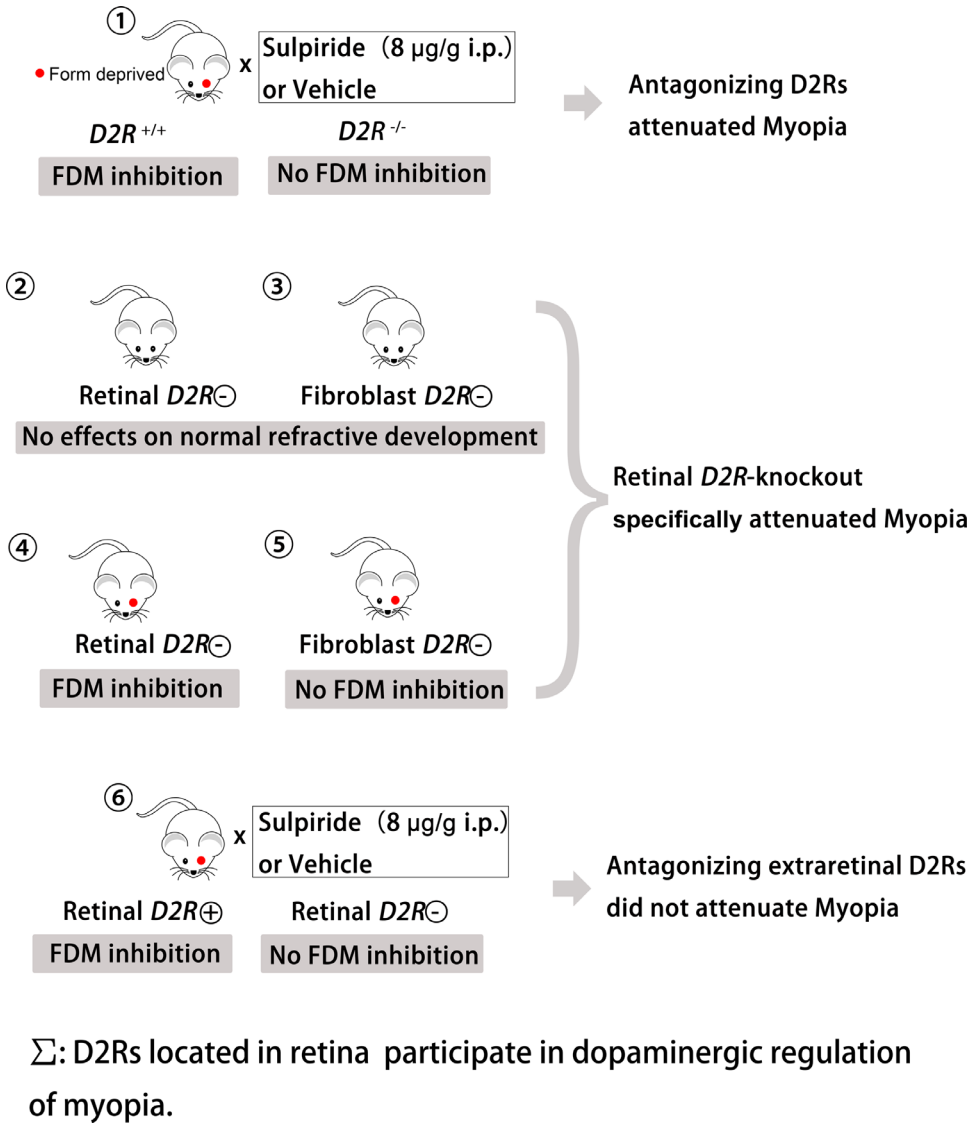

Figure 1. Pictorial representation of genetic and pharmacological manipulations used in this study. The experimental design consists of six parts: **(1)** to determine whether the inactivation of all D2Rs inhibits myopia development; **(2),(3)** to identify the effects of either retina- or fibroblast-specific *D2R*-KO on refractive development; **(4),(5)** to identify the effects of either retina- or fibroblast-specific *D2R*-KO on progression of monocular form-deprivation myopia (FDM); **(6)** to determine whether the D2R-mediated inhibition of FDM is mediated by D2Rs in the retina. Sulpiride 8 μg/g intraperitoneally: Mice received D2R antagonist sulpiride intraperitoneally; $D2R^{+/+}$ and $D2R^{-/-}$: WT and *D2R*-KO mice, respectively; Retinal *D2R*⊕ and Retinal *D2R*Θ: $D2R^{fl/fl}$ and retina-specific *D2R*-KO ($Six3^{cre}D2R^{fl/fl}$) mice, respectively; Fibroblast *D2R*Θ, fibroblast-specific *D2R*-KO ($S100a4^{cre}D2R^{fl/fl}$) mice.

Experimental Design

Pictorial representation of the genetic and pharmacological manipulations used in this study is provided in Figure 1. Gene expression was examined in the cornea, lens, neural retina, and sclera of both retina- and fibroblast-specific *D2R*-KO mice at 10 weeks of age. Retinal fundus images and electroretinograms were obtained in five-week-old and seven-week-old $Six3^{cre}D2R^{fl/fl}$ and $D2R^{fl/fl}$ mice. For FDM, mice were subjected to four weeks of form deprivation (FD), and ocular measurements were obtained before and after myopia induction. In mice that underwent normal refractive development, ocular measurements were tracked at two-week intervals from postnatal weeks four to 10.

Form Deprivation and Preparation for Drug Injection

Monocular FDM was induced by carefully gluing translucent diffusers over the right eye of each mouse. The diffusers, which degrade visual acuity, were worn for four weeks as previously described.[29,30,41] The contralateral fellow eye was untreated. A collar made from thin plastic was fitted around the neck to prevent the mice from removing the diffuser.

During the period of FD, daily intraperitoneal injections of all agents were made without anesthesia[45,46] in the lower right or left quadrant of the abdomen, using a microliter syringe attached to a 29-gauge needle. In rodents the ED50 value for the selective D2R antagonist sulpiride, applied

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systemically, is around 12.67∼18 µg/g,[47,48], with Ki being 15 nM for D2Rs compared to 45,000 nM for D1Rs.[49] Drug doses at 8 and 80 µg/g body weight, as used in this study, are within the range used in earlier studies.[30,50] Both doses of sulpiride were sufficient to inhibit the myopia development and showed no dose-dependent effects toward FDM in mice (Supplementary Fig. S1). Therefore we chose to use the lower dose, 8 µg/g—which is fully effective against FDM in mice, but less likely to have off-target effects than a higher dose—for the present study of the site of action of D2Rs. Sulpiride (Tocris Bioscience, Glasgow, UK) was injected at 8 µg/g body weight after dissolving it in the vehicle dimethyl sulfoxide (DMSO: 1.1 µg/g body weight; Sigma-Aldrich Corp., St. Louis, MO, USA). The injection volume in all groups was 1 µL/g body weight. In the following text, the notation either "-Veh" or "-Sul" indicates the injection of either vehicle or sulpiride (in the same vehicle) into mice of the genetically modified strains.

Gene Expression Analyses

The *D2R* mRNA expression levels in the cornea, lens, neural retina, and sclera of $Six3^{cre}D2R^{fl/fl}$, $S100a4^{cre}D2R^{fl/fl}$, and their $D2R^{fl/fl}$ littermates were determined by quantitative real-time PCR (qRT-PCR) analysis. The cornea, lens, and sclera were collected and homogenized separately using a ball mill, and total RNA was extracted using the RNeasy Fibrous Tissue Minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA from the retina was extracted using TRIZOL reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. After treatment with RQ1 RNase-Free DNase (Promega, Madison, WI, USA), the RNAs were reverse-transcribed, using random primers and Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) to synthesize the respective cDNAs.[40,44] The qRT-PCR was performed using the specific primers (Table 1) and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI ViiA 7 Real-Time PCR system (Applied Biosystems). The expression level of each target mRNA, relative to that of *18s rRNA* as reference, was determined with the $2^{-\Delta\Delta Ct}$ method.[51]

Fundus Imaging, Electroretinography (ERG), and Biometric Measurements

The retinal fundi of mice were viewed and photographed using the Phoenix MICRON IV imaging camera (Phoenix, Pleasanton, CA, USA). 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), as previously described.[29]

Non-cycloplegic refractive error was measured in a darkened room using an eccentric infrared photorefractor, as described in detail previously.[52] Briefly, each unanesthetized mouse was gently restrained, with its position adjusted until the first Purkinje image was clearly shown in the center of the pupil, indicating an on-axis measurement. The photorefractor rapidly acquired 10 individual values, and the average of those values was taken as the value for one measurement. This procedure was performed at least three times, and the mean of those values was reported as the refraction for that eye.

Ocular biometric measurements of anterior chamber depth, lens thickness, vitreous chamber depth (VCD), axial length (AL: from front of cornea to front of retina), and anterior corneal radius of curvature were performed with a custom-made spectral-domain optical coherence tomography instrument.[29,41] Each anesthetized mouse was placed in a cylindrical holder mounted on the positioning stage in front of the optical scanning probe. The optical axis of the eye was aligned with the axis of the probe by detecting the specular reflex on the corneal apex and posterior surface of the lens with an X-Y cross-scanning system. The raw data were exported and analyzed using custom-designed software to obtain the axial components and the anterior corneal radius of curvature. Each examiner was masked to the identity of the different groups while the measurements were made.

Statistics

Differential gene expression in two groups was assessed statistically by independent *t*-tests. Differences in fundus images were judged subjectively by an experienced ophthalmologist, who was blinded as to the treatment of each eye. Two-way repeated measures analysis of variance (ANOVA) was performed to assess the differences in ERG parameters at different flash intensities and the biometric measurements of refractive development at different times. Interocular differences in biometric parameters (FD eye minus fellow eye) were compared using a three-way repeated measures ANOVA, with genotype and pharmacologic/FD treatment as the two factors and time as the repeated measures. Bonferroni corrections were applied in post hoc analyses. Values of $P < 0.05$ were defined as significant (IBM SPSS, Version 19.0; IBM, Armonk, NY, USA).

Results

Antagonizing D2Rs Attenuated Myopia

First, to verify that whole-body (systemic) inactivation of all D2Rs inhibits myopia development, we tested the effects of sulpiride on FDM in *D2R*-KO mice. Baseline measurements of interocular differences of all biometric variables were similar in all treatment groups (all P values > 0.05). As previously reported,[29,30,41] the body weights of *D2R*-KO mice were less than those of *D2R*-WT mice (main effects of genetic KO: $F_{1,87} = 38.796$, $P < 0.001$, three-way repeated ANOVA; Fig. 2A).

After four weeks of FD, the myopic shift, measured as the difference between the deprived and fellow eyes, was greater than it was before treatment (main effects of time, $F_{1,87} = 170.547$, $P < 0.001$, three-way repeated measures ANOVA; Fig. 2B). A significant interaction was revealed for refraction, between *D2R*-KO and sulpiride treatment ($F_{1,87} = 8.336$, $P = 0.005$). After four weeks of FD, the myopic shift in the *D2R*-WT-Sul group (n = 18) was 46% lower than that in the *D2R*-WT-Veh group (n = 26) (interocular difference, -3.18 ± 0.30 diopter [D] in *D2R*-WT-Sul vs. -5.85 ± 0.58 D in *D2R*-WT-Veh, $P < 0.001$). The interocular difference in refractions between the deprived and fellow eyes was 69% lower in the *D2R*-KO-Veh group (n = 25) than in the *D2R*-WT-Veh group (n = 26) (-1.81 ± 0.33 D in *D2R*-KO-Veh, vs. -5.85 ± 0.58 D in *D2R*-WT-Veh; $P < 0.001$). Thus both systemic sulpiride treatment and systemic *D2R*-KO partially suppressed the FD-induced

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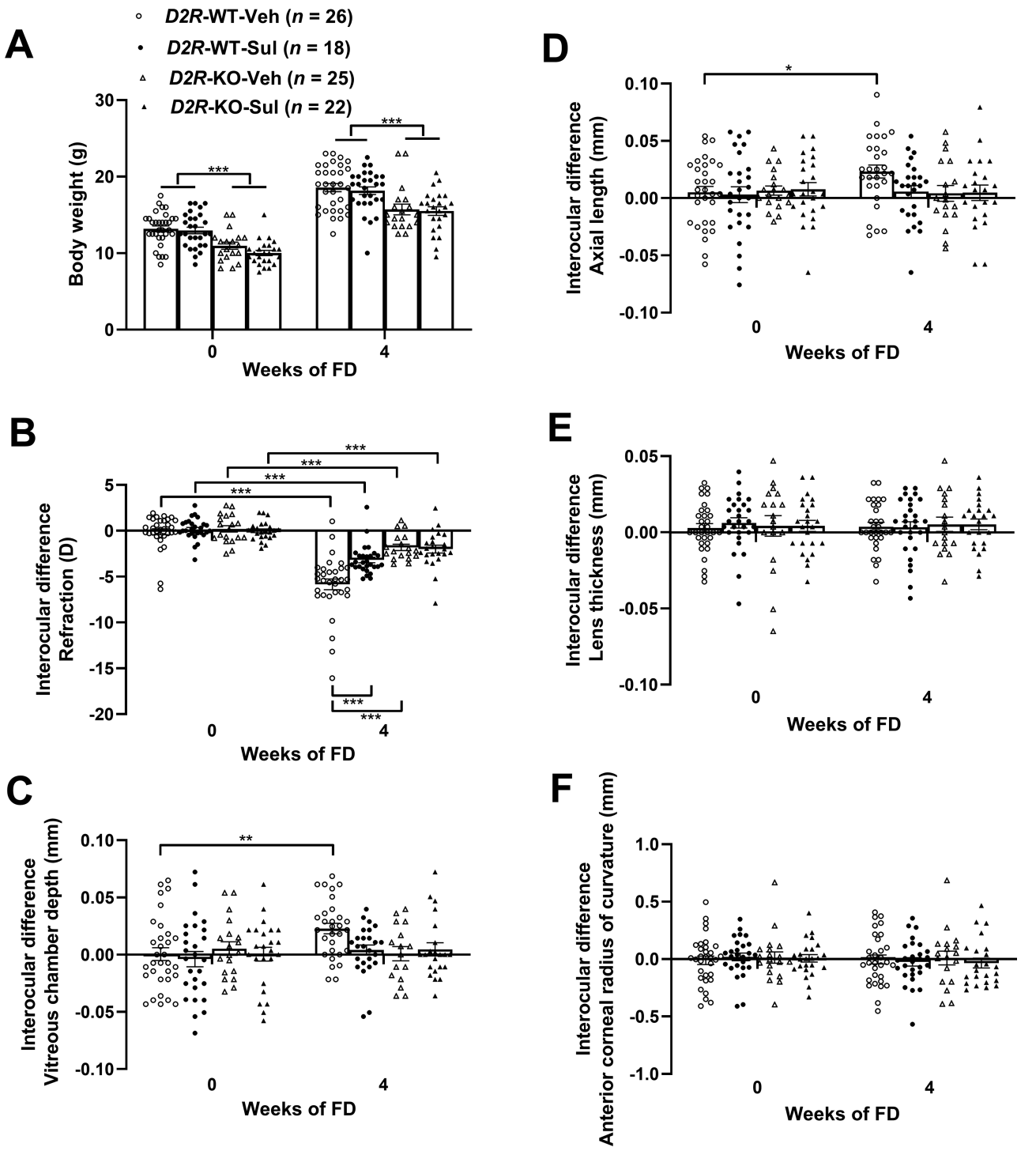

Figure 2. Effects of sulpiride and *D2R*-KO on body weight, refraction, and ocular dimensions in monocularly form-deprived (FD) mice. Biometric measurements in *D2R*-WT-Veh, *D2R*-WT-Sul, *D2R*-KO-Veh, and *D2R*-KO-Sul groups before and after four weeks of each treatment (four and eight weeks old, respectively). (**A**) Body weight. For panels B-F, interocular differences (deprived minus fellow eye) in: (**B**) refraction, (**C**) vitreous chamber depth, (**D**) axial length, (**E**) lens thickness, and (**F**) anterior corneal radius of curvature. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; three-way repeated measures ANOVA, post hoc simple effects analysis. Data are expressed as the mean ± standard error of the mean.

changes in refraction. As expected, there was no significant difference in refraction between the *D2R*-KO-Veh (n = 25) and *D2R*-KO-Sul (n = 22) groups (−2.00 ± 0.42 D in *D2R*-KO-Sul, vs. −1.81 ± 0.33 D in *D2R*-KO-Veh; $P > 0.05$). Therefore the inhibitory effect of sulpiride treatment on FD-induced changes in refraction disappeared in the absence of fully functional D2Rs. This dependence of sulpiride's antimyopia action on the presence of D2Rs further indicated that antagonism of D2Rs suppressed the myopic shift in refraction. However, no significant interaction between

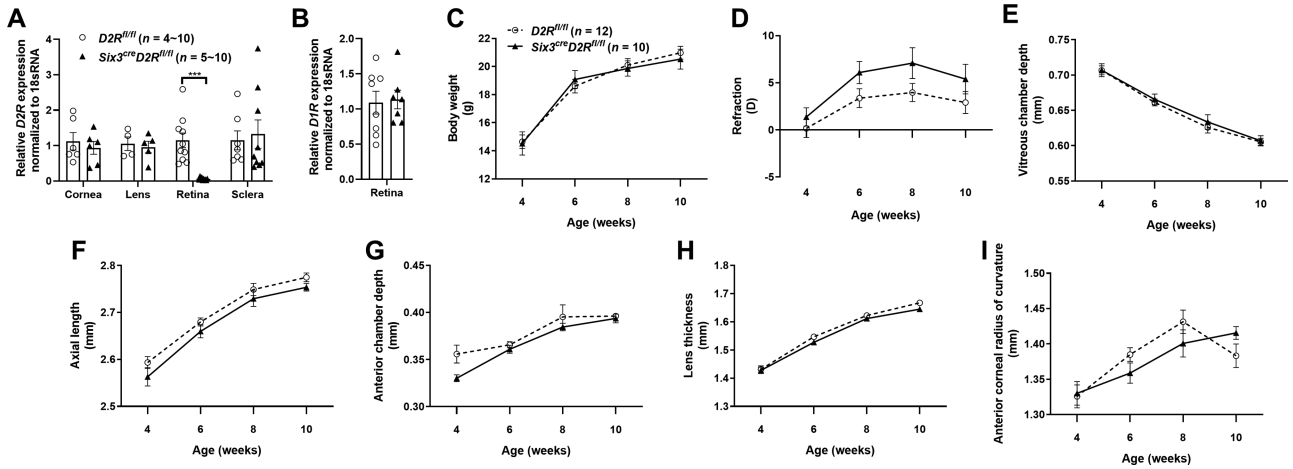

Figure 3. Effects of retina-specific *D2R*-KO on body weight, refraction, and ocular dimensions without form-deprivation. (**A**) Quantitative real-time PCR (qRT-PCR) assessed expression levels of *D2R* mRNA from cornea, lens, neural retina, and sclera of $Six3^{cre}D2R^{fl/fl}$ and littermate control mice ($D2R^{fl/fl}$). (**B**) Expression levels of *D1R* mRNA from retina of $Six3^{cre}D2R^{fl/fl}$ and $D2R^{fl/fl}$ mice. (**C-I**) Biometric measurements in $D2R^{fl/fl}$ and $Six3^{cre}D2R^{fl/fl}$ groups from four to 10 weeks old (sample sizes for all parameters are the same as those shown in **C**): (**C**) body weight, (**D**) refraction, (**E**) vitreous chamber depth, (**F**) axial length, (**G**) anterior chamber depth, (**H**) lens thickness, and (**I**) anterior corneal radius of curvature. ***$P < 0.001$. For comparing *D2R* and *D1R* expression between $D2R^{fl/fl}$ and $Six3^{cre}D2R^{fl/fl}$ groups, independent *t*-tests were used (**A** and **B**). For comparing the ocular parameters, No significant differences between $D2R^{fl/fl}$ and $Six3^{cre}D2R^{fl/fl}$ groups were found by two-way repeated measures ANOVA (**C–I**). Data are expressed as the mean ± standard error of the mean.

D2R-KO and sulpiride treatment was revealed for VCD or AL ($P > 0.05$; Figs. 2C, 2D). Neither *D2R*-KO nor sulpiride treatment, alone or in combination, had any effect on interocular differences of anterior chamber depth, lens thickness, or anterior corneal radius of curvature (all *P* values > 0.05, three-way repeated ANOVA; Figs. 2E, 2F and Supplementary Fig. S2A).

No Effects of Retina- or Fibroblast-Specific *D2R*-KO on Normal Refractive Development

Next, to determine whether D2Rs in retina or fibroblasts are involved in the regulation of normal refractive development, the refractive development of retina- or fibroblast-specific *D2R*-KO mice was monitored at different times. Retinal *D2R* gene expression in $Six3^{cre}D2R^{fl/fl}$ mice (n = 10) was nearly abolished and was highly significantly lower than that in $D2R^{fl/fl}$ littermates (n = 10) ($P < 0.001$, independent *t*-tests; Fig. 3A). In contrast, D1R gene expression was not significantly affected ($P > 0.05$, Fig. 3B). The specificity of *D2R*-silencing to the retina in $Six3^{cre}D2R^{fl/fl}$ mice was also apparent from the lack of difference in *D2R* expression levels in the cornea, lens, or sclera, between $Six3^{cre}D2R^{fl/fl}$ and $D2R^{fl/fl}$ mice ($P > 0.05$; Fig. 3A). Fundus images of $Six3^{cre}D2R^{fl/fl}$ mice did not reveal any obvious changes in retinal vasculature, pigmentation, nerve fiber arrangement, or optic nerve head (Supplementary Figs. S3A, S3B). The ERG a- and b-wave amplitudes in $Six3^{cre}D2R^{fl/fl}$ (n = 10) and $D2R^{fl/fl}$ (n = 9) mice were not significantly different from one another, under either scotopic or photopic conditions (main effects of genetic KO, $P > 0.05$ for each, two-way repeated ANOVA; Supplementary Figs. S3C, S3D).

In contrast with the systemic *D2R*-KO mice, which lacked D2Rs in all organs, tissues, and cell types and had reduced body weights, the retina-specific *D2R*-KO mice ($Six3^{cre}D2R^{fl/fl}$: n = 10) presented with body weights similar to those of control mice ($D2R^{fl/fl}$: n = 12) (main effects of genetic KO, $P > 0.05$, two-way repeated measures ANOVA; Fig. 3C). The refractions showed small but statistically insignificant trends toward more hyperopic refraction and shorter AL in retina-specific *D2R*-KO mice at each measurement time (main effects of genetic KO, $P > 0.05$; Figs. 3D, 3F). There were also no significant differences in VCD, anterior chamber depth, lens thickness, or anterior corneal radius of curvature during development from postnatal four to 10 weeks of age in the retinal *D2R*-KO mice (main effects of genetic KO, $P > 0.05$; Figs. 3E, 3G–I). Thus normal refractive development in mice was not affected by constitutive retinal *D2R*-KO.

Our qRT-PCR analysis showed that the level of *D2R* gene expression in sclera was 66.9% less in the fibroblast-specific *D2R*-KO mice ($S100a4^{cre}D2R^{fl/fl}$, n = 11) than in their WT littermates ($D2R^{fl/fl}$, *n* = 12) ($P = 0.039$, independent *t*-tests; Fig. 4A). However, the *D2R* expression level in lens of $S100a4^{cre}D2R^{fl/fl}$ mice was also lower by 39.5% than in $D2R^{fl/fl}$ mice ($P = 0.007$). There were no significant differences of *D2R* expression in cornea or retina, between $S100a4^{cre}D2R^{fl/fl}$ and $D2R^{fl/fl}$ mice ($P > 0.05$; Fig. 4A).

The fibroblast-specific *D2R*-KO mice ($S100a4^{cre}D2R^{fl/fl}$: *n* = 12) had normal body weights, not significantly different from those of littermate controls ($D2R^{fl/fl}$: n = 14) (main effects of genetic KO, $P > 0.05$, two-way repeated measures ANOVA; Fig. 4B). Refractive development was similar in fibroblast-specific *D2R*-KO and $D2R^{fl/fl}$ control mice (main effects of genetic KO, $P > 0.05$; Fig. 4C). However, in $S100a4^{cre}D2R^{fl/fl}$ compared to $D2R^{fl/fl}$ mice, AL increased (main effects of genetic KO, $F_{1,24} = 6.521$, $P = 0.017$; Fig. 4E), as did lens thickness (main effects of genetic KO, $F_{1,24} = 9.226$, $P = 0.006$; Fig. 4G). No significant differences were observed in VCD, anterior chamber depth, or anterior corneal radius of curvature, due to fibroblast-specific *D2R*-KO (main effects of genetic KO, $P > 0.05$; Figs. 4D, 4F, 4H).

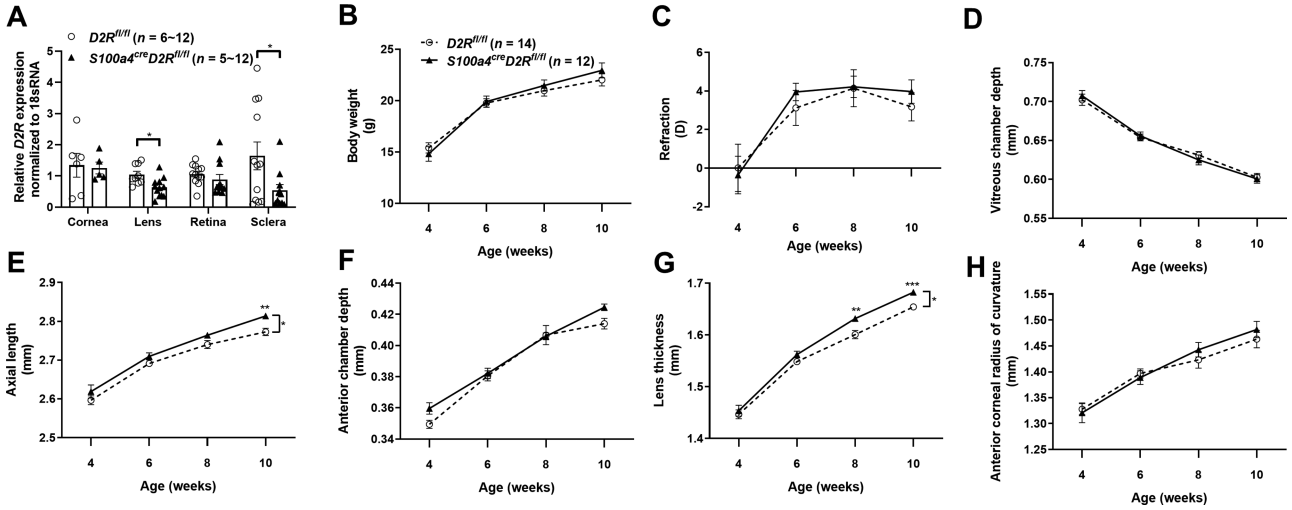

Figure 4. Effects of fibroblast-specific *D2R*-KO on body weight, refraction, and ocular dimensions without form-deprivation. (**A**) A qRT-PCR analysis of *D2R* mRNA expression levels in cornea, lens, retina, and sclera in $D2R^{fl/fl}$ and $S100a4^{cre}D2R^{fl/fl}$ mice. (**B–H**) Biometric measurements in $D2R^{fl/fl}$ and $S100a4^{cre}D2R^{fl/fl}$ groups from four to 10 weeks old: (**B**) body weight, (**C**) refraction, (**D**) vitreous chamber depth, (**E**) axial length, (**F**) anterior chamber depth, (**G**) lens thickness, and (**H**) anterior corneal radius of curvature. *$P < 0.05$. For comparing *D2R* expression between $D2R^{fl/fl}$ and $S100a4^{cre}D2R^{fl/fl}$ groups, independent *t*-tests were used (**A**). For comparing the ocular parameters between $D2R^{fl/fl}$ and $S100a4^{cre}D2R^{fl/fl}$ groups, two-way repeated measures ANOVA was used (**B–H**). Data are expressed as the mean ± standard error of the mean.

Thus a reduction of *D2R* expression in the sclera and lens contributed to the increases in AL and lens thickness.

Retina-Specific *D2R*-KO Lessens FDM

Then, to determine whether inactivation of retina- or fibroblast-specific D2Rs inhibits myopia, the development of FDM was monitored in retina- or fibroblast-specific *D2R*-KO mice. There was a significant interaction between retina-specific *D2R*-KO and FD treatment for body weights (interaction effects of genetic KO and FD, $F_{1,74} = 4.986$, $P = 0.029$, three-way repeated measures ANOVA; Fig. 5A). After four weeks of treatment, body weight was reduced by retina-specific *D2R*-KO combined with four weeks of FD ($Six3^{cre}D2R^{fl/fl}$-FD [n = 20] versus $Six3^{cre}D2R^{fl/fl}$-Control [n = 19], $P < 0.001$; $Six3^{cre}D2R^{fl/fl}$-FD vs. $D2R^{fl/fl}$-FD [n = 20], $P < 0.001$).

A significant interaction was found between treatment duration and FD effects for the interocular differences in refraction, VCD, and AL ($F_{1,74} = 200.080$, $P < 0.001$ for refraction; $F_{1,74} = 5.112$, $P = 0.027$ for VCD; $F_{1,74} = 10.728$, $P = 0.002$ for AL). Four weeks of FD resulted in significant myopia in the $D2R^{fl/fl}$-FD group compared to $D2R^{fl/fl}$-Controls (n = 19) (−7.30 ± 0.72 D vs. −0.17 ± 0.17 D; $P < 0.001$; Fig. 5B). In parallel, significant increases in VCD ($P = 0.007$; Fig. 5C) and AL ($P = 0.001$; Fig. 5D) were observed in the $D2R^{fl/fl}$-FD group compared to $D2R^{fl/fl}$-Control group. Thus FD induced significant ocular elongation and myopia in retina-specific $D2R^{fl/fl}$ mice.

The interocular differences in refraction in the $Six3^{cre}D2R^{fl/fl}$-FD group were also greater than those in the $Six3^{cre}D2R^{fl/fl}$-Control group (−5.41 ± 0.47 D in $Six3^{cre}D2R^{fl/fl}$-FD, vs. −0.11 ± 0.26 D in $Six3^{cre}D2R^{fl/fl}$-Control; $P < 0.001$; Fig.B). The increases in VCD, in the FD versus the control group, were not significantly different ($P > 0.05$; Fig. 5C). The increased AL, in the $Six3^{cre}D2R^{fl/fl}$-FD group after four weeks of FD (0.025 ± 0.008 mm in four weeks, vs. 0.001 ± 0.011 mm in 0 weeks; $P = 0.015$; Fig. 5D), was not significantly different from that in the $Six3^{cre}D2R^{fl/fl}$-Control group ($P > 0.05$). Thus FD in $Six3^{cre}D2R^{fl/fl}$ mice continued to induce significant myopia, with axial length following an increasing trend but nonsignificantly.

Interestingly, the interocular difference in refraction was approximately 26% lower in the $Six3^{cre}D2R^{fl/fl}$-FD group than in the $D2R^{fl/fl}$-FD group ($P = 0.005$; Fig. 5B). However, the differences between the increases in VCD (Fig. 5C) and AL (Fig. 5D), in the $Six3^{cre}D2R^{fl/fl}$-FD mice compared to the $D2R^{fl/fl}$-FD mice, were not statistically significant ($P > 0.05$). Therefore retina-specific *D2R*-KO only partially inhibited FD-induced changes in refraction in mice. Finally, the analysis showed no significant differences in the interocular differences of anterior chamber depth, lens thickness, or anterior corneal radius of curvature, between any two groups, after four weeks of either treatment (all *P* values > 0.05; Figs. 5E, 5F and Supplementary Fig. S2B).

Fibroblast-Specific *D2R*-KO has no Effect on FDM

Neither FD nor fibroblast-specific *D2R*-KO affected body weights after four weeks of treatment (main effects of FD or genetic KO: $P > 0.05$, three-way repeated measures ANOVA; Fig. 6A). A significant interaction was revealed for refraction, VCD and AL, between treatment duration and FD treatment ($F_{1,74} = 156.339$, $P < 0.001$ for refraction; $F_{1,74} = 4.937$, $P = 0.029$ for VCD; $F_{1,74} = 17.043$, $P < 0.001$ for AL). As expected, four weeks of FD successfully induced myopia (−4.96 ± 0.49 D in $D2R^{fl/fl}$-FD [n = 22], vs. −0.50 ± 0.29 D in $D2R^{fl/fl}$-Control [n = 22]; $P < 0.001$; Fig. 6B), with significant increases in VCD ($P = 0.038$; Fig. 6C) and AL ($P = 0.004$; Fig. 6D) in $D2R^{fl/fl}$ mice.

Similarly, the interocular difference in refraction in the $S100a4^{cre}D2R^{fl/fl}$-FD group (n = 17) was significantly greater than that in the $S100a4^{cre}D2R^{fl/fl}$-Control group (n = 17) (−5.93 ± 0.53 D, vs. −0.19 ± 0.27 D; $P < 0.001$). FD

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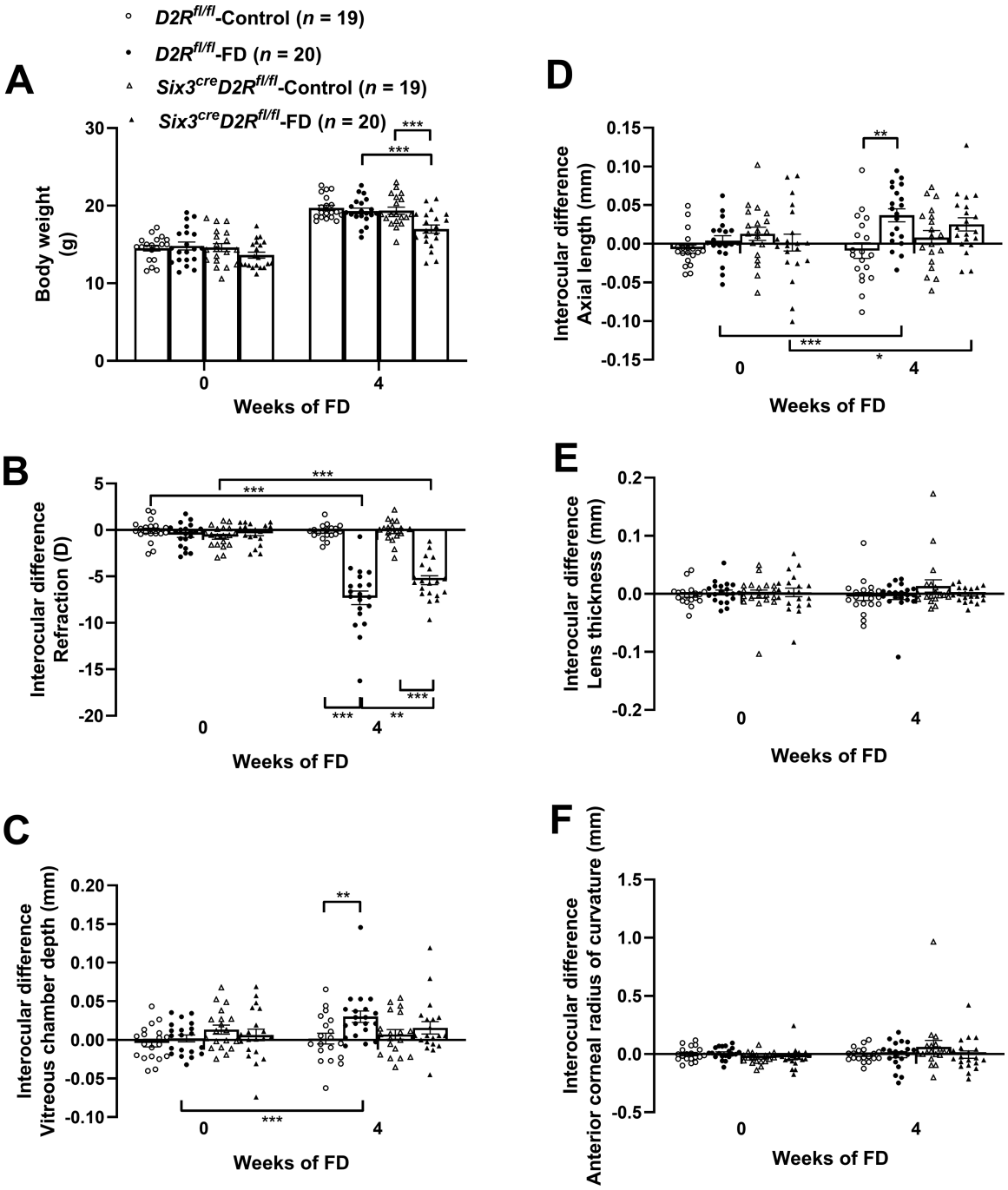

Figure 5. Effects of monocular form deprivation (FD) and retina-specific *D2R*-KO on body weight, refraction, and ocular dimensions. Biometric measurements in $D2R^{fl/fl}$-Control, $D2R^{fl/fl}$-FD, $Six3^{cre}D2R^{fl/fl}$-Control, and $Six3^{cre}D2R^{fl/fl}$-FD groups before and after four weeks of each treatment (four and eight weeks old, respectively). (**A**) Body weight. (**B-F**) Interocular differences (FD minus fellow eye) in: (**B**) refraction, (**C**) vitreous chamber depth, (**D**) axial length, (**E**) lens thickness, and (**F**) anterior corneal radius of curvature. $^{**}P < 0.01$, and $^{***}P < 0.001$; 3-way repeated measures ANOVA, post hoc simple effects analysis. Data are expressed as the mean ± standard error of the mean.

increased VCD by 0.022 ± 0.006 mm, and AL by 0.025 ± 0.011 mm, in the $S100a4^{cre}D2R^{fl/fl}$-FD group—compared to VCD by −0.003 ± 0.007 mm and AL by −0.003 ± 0.007 mm, respectively, in the $S100a4^{cre}D2R^{fl/fl}$-Control group ($P = 0.040$ for VCD; $P = 0.020$ for AL). Therefore FD-induced myopia was not significantly affected by fibroblast-specific *D2R*-KO.

Importantly, the differences in myopia and other biometric parameters between the deprived and fellow eyes, in the $S100a4^{cre}D2R^{fl/fl}$-FD group, did not differ from those in the $D2R^{fl/fl}$-FD group (all P values > 0.05, Figs. 6B–F, and Supplementary Fig. S2C). Even though it appears that the fibroblast-specific *D2R*-KO model did not affect the development of FDM, it must be interpreted with caution, because the deletion efficiency was only 66.9%. Thus it is difficult to completely exclude a possible role of scleral D2Rs in myopia. Additional experiments are clearly needed to further clarify this issue.

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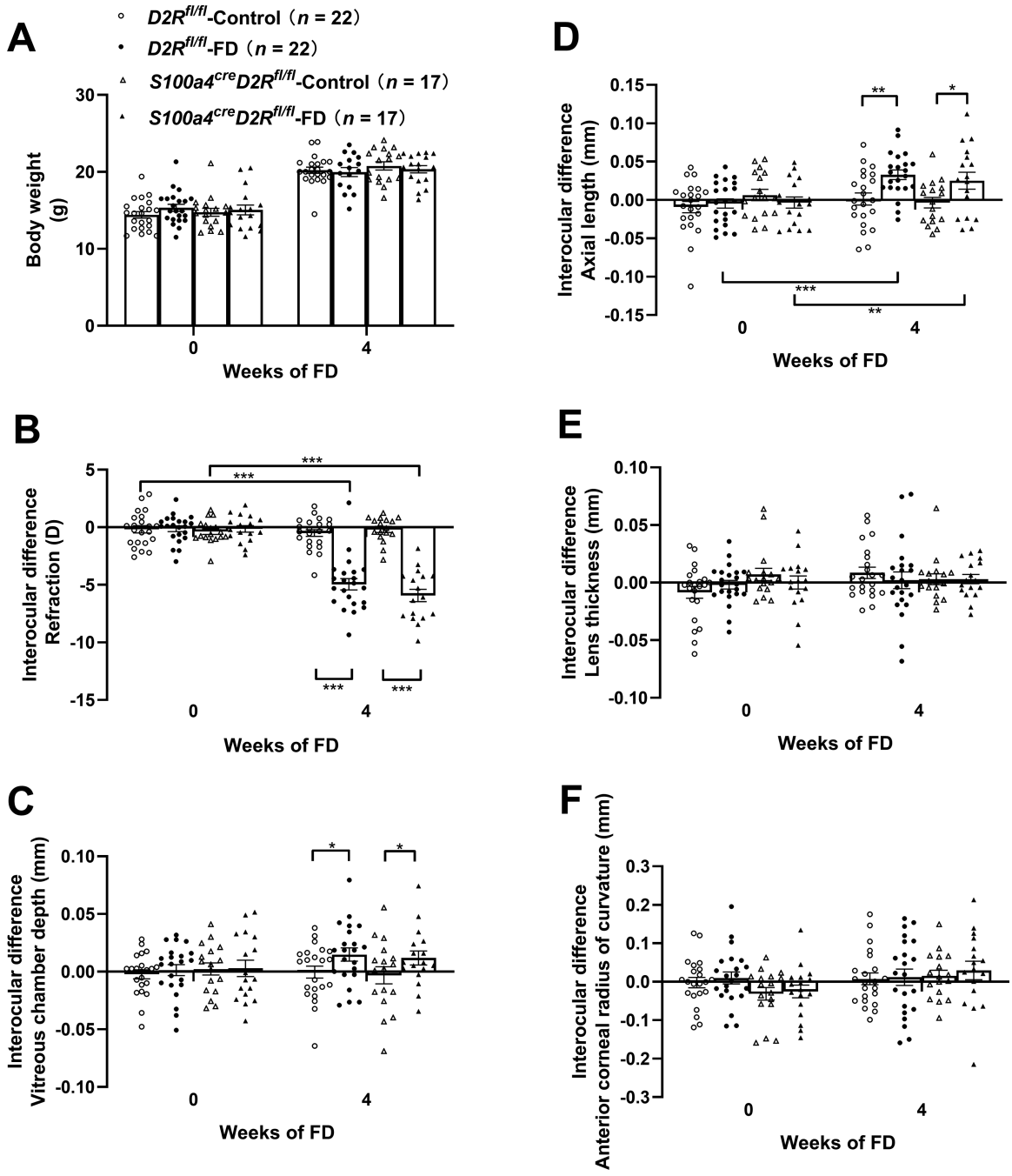

Figure 6. Effects of monocular form deprivation (FD) and fibroblast-specific *D2R*-KO on body weight, refraction, and ocular dimensions. Biometric measurements in $D2R^{fl/fl}$-Control, $D2R^{fl/fl}$-FD, $S100a4^{cre}D2R^{fl/fl}$-Control, and $S100a4^{cre}D2R^{fl/fl}$-FD groups before and after four weeks of each treatment (four and eight weeks old, respectively). (**A**) Body weight. (**B–F**) Interocular differences (FD minus fellow eyes) in: (**B**) refraction, (**C**) vitreous chamber depth, (**D**) axial length, (**E**) lens thickness, and (**F**) anterior corneal radius of curvature. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; three-way repeated measures ANOVA, post hoc simple effects analysis. Data are expressed as the mean ± standard error of the mean.

Antagonizing Extraretinal D2Rs Did Not Attenuate Myopia

Finally, although it has been clear that inactivation of retinal D2Rs inhibits myopia development, it is still unknown whether extraretinal D2Rs are involved in the regulation of myopia. Therefore, in order to further clarify whether inactivation of extraretinal D2Rs inhibits myopia development, we tested the effects of sulpiride on FDM in retina-specific *D2R*-KO mice. A significant interaction was revealed for body weights between retina-specific *D2R*-KO and treatment duration ($F_{1,65} = 31.364$, $P < 0.001$, three-way repeated measures ANOVA; Fig. 7A). After four weeks of FD treatment, retina-specific *D2R*-KO reduced body weights in both vehicle and sulpiride treatment groups ($Six3^{cre}D2R^{fl/fl}$-Veh [n = 15], versus $D2R^{fl/fl}$-Veh [n = 19], $P < 0.001$; and $Six3^{cre}D2R^{fl/fl}$-Sul [n = 16], vs. $D2R^{fl/fl}$-Sul [n = 19], $P < 0.001$).

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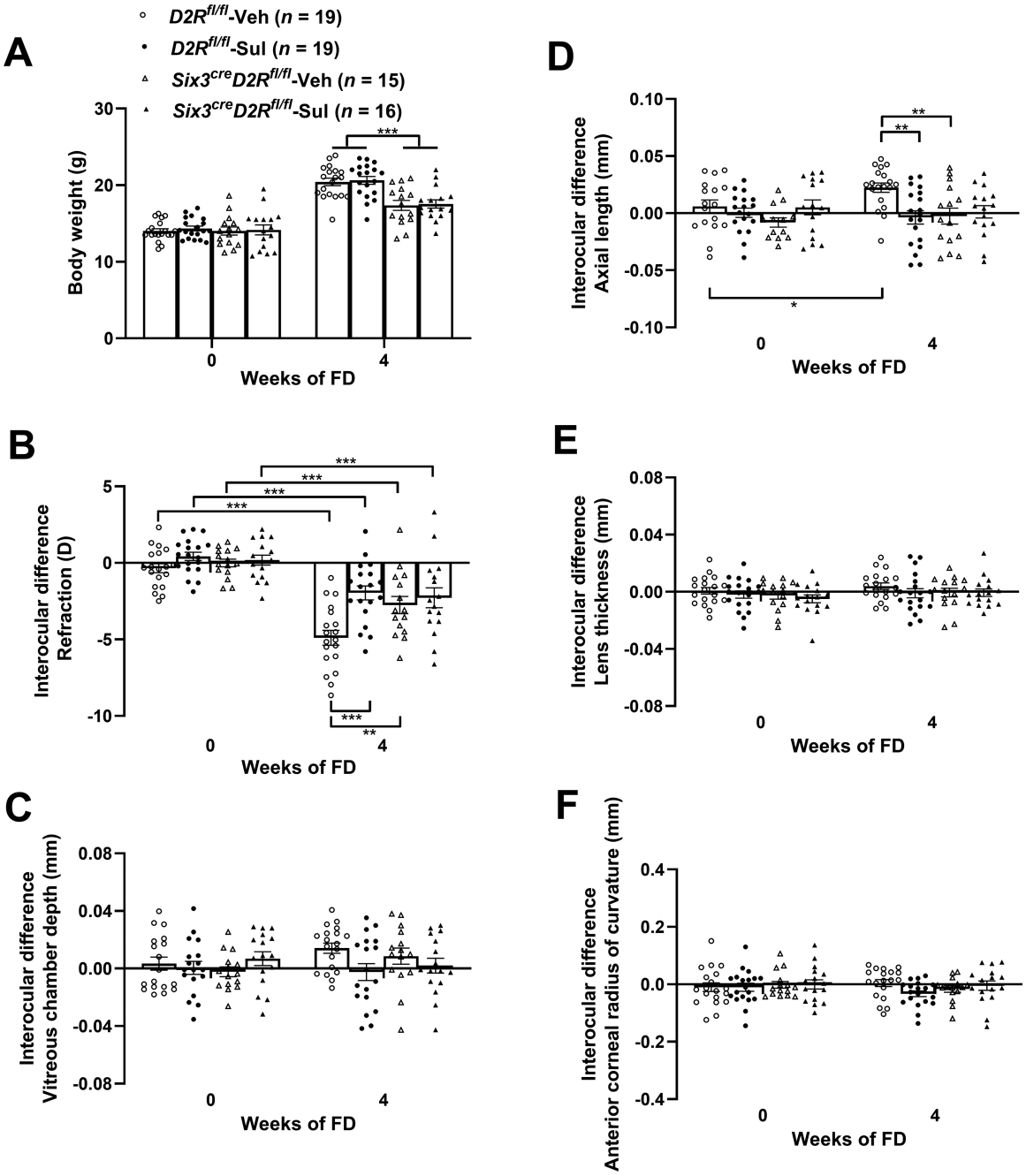

Figure 7. Effects of sulpiride and retinal *D2R*-KO on body weight, refraction, and ocular dimensions in monocularly form-deprived (FD) mice. Biometric measurements in $D2R^{fl/fl}$-Veh, $D2R^{fl/fl}$-Sul, $Six3^{cre}D2R^{fl/fl}$-Veh, and $Six3^{cre}D2R^{fl/fl}$-Sul groups before and after four weeks of each treatment (four and eight weeks old, respectively). (**A**) Body weight. (**B-F**) Interocular differences (deprived minus fellow eyes) in: (**B**) refraction, (**C**) vitreous chamber depth, (**D**) axial length, (**E**) lens thickness, and (**F**) anterior corneal radius of curvature. $^{**}P < 0.01$, and $^{***}P < 0.001$; three-way repeated measures ANOVA, post hoc simple effects analysis. Data are expressed as the mean ± standard error of the mean.

After four weeks of treatment, the refractive error was more myopic than before treatment (main effects of time, $F_{1,65} = 120.004$, $P < 0.001$; Fig. 7B). There was a significant interaction of refraction and AL, between retina-specific *D2R*-KO and sulpiride treatment ($F_{1,65} = 5.366$, $P = 0.024$ for refraction; and $F_{1,65} = 9.012$, $P = 0.004$ for AL). As expected, sulpiride inhibited FD-induced refractive changes in $D2R^{fl/fl}$ mice (by 60%) compared to those in Veh-treated mice (interocular difference: −1.96 ± 0.46 D in $D2R^{fl/fl}$-Sul, vs. −4.90 ± 0.48 D in $D2R^{fl/fl}$-Veh; $P < 0.001$; Fig. 7B); in parallel, the increase in AL was also reduced by sulpiride treatment ($P = 0.003$; Fig. 7D). Similarly, retina-specific *D2R*-KO reduced (by 44%) the FD-induced changes in refraction (interocular difference: −2.75 ± 0.56 D in $Six3^{cre}D2R^{fl/fl}$-Veh, vs. −4.90 ± 0.48 D in $D2R^{fl/fl}$-Veh; $P = 0.007$; Fig. 7B), and it suppressed the increase in AL ($P = 0.002$; Fig. 7D). Strikingly, this inhibitory effect of sulpiride on refraction or AL was no longer present in the $Six3^{cre}D2R^{fl/fl}$-Veh and

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Six3^{cre}*D2R*$^{fl/fl}$-Sul groups ($P > 0.05$; Figs. 7B–D), suggesting that the inhibitory effect of sulpiride on FDM depended on the existence of functional D2Rs in the retina. Therefore, because antagonizing D2Rs outside the retina did not attenuate myopia, D2Rs located in the retina are essential for dopaminergic regulation of emmetropization and myopia.

Interocular differences in anterior chamber depth, lens thickness, and anterior corneal radius of curvature in the different experimental groups were statistically indistinguishable (all P values > 0.05; Figs. 7E, 7F, and Supplementary Fig. S2D).

Discussion

There is an urgent need to develop more effective therapies for retarding the progression of myopia and its associated pathological complications. Although it is widely accepted that dopamine acts as a "stop" signal for myopic eye growth, it is imperative to investigate the sites of its action via D2Rs. In the present study, we showed that normal refractive development is unaffected whereas myopia development is attenuated in mice with retina-specific loss of function of the *D2R* gene, suggesting that myopia is characterized by changed D2R-mediated signaling in the retina. Furthermore, FDM development was not inhibited by sulpiride, a selective D2R antagonist, in these mice. Overall, the findings of our study support the hypothesis that the D2R-mediated inhibition of myopia is dependent on D2Rs in the retina. Thus our data have established which ocular tissues are the specific sites of action of D2Rs during the development of myopia in mice.

Although it has been previously shown that either genetic or pharmacological inactivation of D2Rs attenuates FDM development,[30] the results of the present study establish with high probability that the D2R antagonist sulpiride, in the dose and method of delivery used here, inhibits FDM in mice specifically via D2Rs in the retina. The reduction in myopic shift by sulpiride in the *D2R*-KO mice is opposite to the action of D2R agonists reported in chicks, wherein D2R activation was shown to be protective against myopia.[8,11-13,16,17] Underlying differences between retinal neural circuitry in these species could contribute to the variable effects of D2Rs on myopia control and ocular growth. Although in general the eyes of mammalian models are similar to those of humans, with respect to structure and biochemistry, mice are nocturnal animals with poorer visual acuity and lower sensitivity to defocus than the diurnal chicks,[53] However, discrepancies in the effects of dopamine on myopia are reported in other species as well. D2R antagonists have been shown to reverse apomorphine-induced suppression of myopia in chicks,[11,13] and both D2R agonist and antagonist were found to suppress myopia development in tree shrews.[22] Thus various factors such as partial specificity, as well as uncertainty of drug permeability and concentrations at the (unknown) site(s) of action, coupled with the use of only one or two fixed concentrations of drugs (rather than a wide range of concentrations that might distinguish between dose-dependent actions at different receptors) could also contribute to the differences in reported effects of D2Rs on myopia. In contrast, the variety of genetic manipulations available or possible in mice could be invaluable for overcoming the issues of partial specificity and uncertainty of the site of action, which are unavoidable with the pharmacologic approaches that are used in the study of other animal models of myopia. One could further refine this approach by engineering the transgenes to be conditional, so as to increase or decrease expression in just one eye and at a chosen age.

Given the widespread expression of D2Rs in eye, brain, and elsewhere in the body, it is not sufficient to demonstrate the role of the retinal site from the anti-myopia effect of systemic (whole-body) *D2R* KO. The phenotypic differences in body weights observed between systemic *D2R*-KO and retina-specific *D2R*-KO also highlight the importance of tissue- or cell-specific conditional mutant mouse models. These limitations of systemic *D2R*-KO mice would cause potential confounding effects to the inhibition of myopia observed in this study, although the effects of systemic *D2R*-KO, with respect to inhibiting FDM, were more obvious than those of retina-specific *D2R*-KO (Fig. 2 vs. Fig. 7). Our results agree with a previous study showing that retinal *D2R*s are a viable target to inhibit myopia development.[8] The partial inhibition of FDM by retina-specific *D2R*-KO suggests that either myopic development does not entirely depend on the role of retinal D2Rs or other signaling pathways are also involved in the control of axial growth of the eye. Specifically, in the present study FD induced axial elongation in retina-specific *D2R*-KO mice (Fig. 5) but not in their vehicle control group (Fig. 7), suggesting that even injection of vehicle alone inhibited axial growth in the retina-specific *D2R*-KO mice. Because the myopic shift in refraction of the *Six3*^{cre}*D2R*$^{fl/fl}$-Veh group was reduced by 44% compared with the *D2R*$^{fl/fl}$-Veh group, we cannot exclude the possibility that further reductions by treatment with sulpiride in the retina-specific *D2R*-KO mice would be too small to be detected.

For the fibroblast-specific *D2R*-KO (66.9% deletion efficiency) mice, the possibility that residual scleral *D2R*-mediated signaling contributed to the myopia development observed here could not be ruled out. One possibility for such a low efficiency could be limited expression of the S100a4 promoter driving Cre recombinase expression in scleral fibroblasts.[44] However, once the retinal *D2R*s were deleted, the inhibitory effects of sulpiride on FDM disappeared—that is, that D2Rs in the retina are the sole mediators of the anti-myopia effects of sulpiride in mice. This in turn suggests that D2Rs in the retina are the site of action for dopaminergic control of emmetropization and myopia.

Thus the dopaminergic mechanisms controlling refractive development appear to be localized chiefly in the retina, the major site of dopamine release in the eye. In the chick, dopamine content is far higher in the retina than in any other ocular tissue,[54] and during the development of FDM, retinal and vitreal levels of dopamine or its metabolite 3,4-dihydroxyphenylacetic acid become reduced,[9,19,55] whereas choroidal and scleral dopamine levels remain unaltered.[54] In much the same way, with partial-field FD, the decrease of retinal 3,4-dihydroxyphenylacetic acid is restricted to the specific retinal areas in which myopia is induced.[54,56] These results highlight the importance of local dopaminergic mechanisms in the regulation of ocular growth.

Identification of the specific site(s) of dopamine's anti-myopia action within the retina, including the neural retina and RPE, is a possible approach for future studies investigating D2R mechanisms. The inner retina is a likely candidate, because it is the site of dopamine secretion, and it houses presynaptic D2R autoreceptors on dopaminergic amacrine cells that, on activation, inhibit dopamine release. An increase in dopamine release, caused by lack of presynaptic autoinhibitory D2R autoreceptors in retina-specific

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D2R-KO mice,[57] could have contributed to the inhibition of myopia development in the present study. An equally likely candidate is the retinal pigment epithelium—a major source of several important cytokines and growth factors, which has been implicated in eye growth regulation.[58] Consistent with this view, intravitreal apomorphine (a nonspecific dopamine agonist) blocked FDM in chickens, probably by acting on D2-like receptors in the neural retina or pigmented epithelium.[11] Also, in vitro studies reported that the inhibitory effect of apomorphine on the proliferation of scleral chondrocytes was mediated by D2Rs in the pigmented epithelium.[59]

Some limitations of our current study include the measurement of refractive and biometric changes in mice, which have smaller eyes and greater individual differences than other animal models of myopia, as previously acknowledged.[60] The absence of significant changes in VCD and AL, especially in the FD-treated *D2R*-KO-Veh and $Six3^{cre}D2R^{fl/fl}$-Veh mice, is possibly attributable to the insufficient resolution of optical coherence tomography for accurately measuring micron-scale changes in VCD and AL in such small eyes. This is also the reason why large numbers of mice were involved in this study. Although Cre recombinase activity in $Six3^{cre}D2R^{fl/fl}$ mice extends to the optic nerve and the ventral forebrain,[43] these sites scarcely express *D2R*s. We can largely exclude the possibility that other, perhaps minor, cell types co-expressing *D2R*s might have contributed to the overall inhibition of myopia in the retina-specific *D2R*-KO mice.

In summary, our results highlight the importance of D2Rs in the retina in controlling emmetropization and myopia development in mice, and they establish that the retina is the site of action for dopaminergic control via D2Rs. With this knowledge, future studies using techniques such as optogenetics, conditional cell-specific transgenes, or designer receptors exclusively activated by designer drugs, will help to understand the retinal pathways involved in D2R-mediated myopia control.

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