

# The Role of the Dopamine D2 Receptor in Form-Deprivation Myopia in Mice: Studies With Full and Partial D2 Receptor Agonists and Knockouts

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**PURPOSE.** The purpose of this study was to explore the role and mechanism of D2 receptor (D2R) involvement in myopia development and the effects of the full D2R agonist quinpirole and partial D2R agonist aripiprazole on postnatal refractive development and form-deprivation myopia (FDM).

**METHODS.** C57BL/6 ("B6") mice, raised either in a visually normal or unilateral form-deprivation environment, were divided into three subgroups, including an intraperitoneally injected (IP) vehicle group and two quinpirole (1 and 10 µg/g body weight) treatment groups. The effects of quinpirole on FDM were further verified in D2R-knockout (KO) mice and corresponding wild-type littermates. Then, the modulation of normal vision development and FDM by aripiprazole (1 and 10 µg/g body weight, IP) was assessed in C57BL/6 mice. All biometric parameters were measured before and after treatments, and retinal cyclic adenosine phosphate (cAMP) and phosphorylated ERK (pERK) levels were analyzed to assess D2R-mediated signal transduction.

**RESULTS.** Neither quinpirole nor aripiprazole affected normal refractive development. FDM development was inhibited by quinpirole at low dose but enhanced at high dose, and these bidirectional effects were validated by D2R-specificity. FDM development was attenuated by the partial D2R agonist aripiprazole, at high dose but not at low dose. Quinpirole caused a dose-dependent reduction in cAMP levels, but had no effect on pERK. Aripiprazole reduced cAMP levels at both doses, but caused a dose-dependent increase of pERK in the form-deprived eyes.

**CONCLUSIONS.** Reduction of D2R-mediated signaling contributes to myopia development, which can be selectively attenuated by partial D2R agonists that activate D2Rs under the low dopamine levels that occur with FDM.

**Keywords:** myopia, dopamine D2 receptor, quinpirole, bidirectional effect, aripiprazole

The prevalence of myopia is markedly increasing worldwide, and, in some Asian populations, it is present in nearly 90% of the surveyed children.<sup>1,2</sup> The rising prevalence is associated with an increase in the severity of myopia, which, in turn, increases the risks of many sight-threatening complications exponentially.<sup>3</sup>

Among the numerous studies investigating the signal cascade of myopia, dopamine released from retinal amacrine cells has long been proposed as an important messenger linking postnatal eye growth and myopia.<sup>4,5</sup> Studies from multiple experiments across different species, including primates,<sup>6</sup> chickens,<sup>7</sup> and guinea pigs,<sup>8</sup> have shown that the synthesis and release of retinal dopamine, and/or the content of its main metabolite 3,4-dihydroxyphenylacetic acid, are reduced in response to form-deprivation myopia (FDM). However, in wild-type (WT) mice, retinal dopamine levels remain unaltered after induction of FDM.<sup>9–11</sup> This

suggests subtle, but as yet undetected, changes of the total retinal dopamine levels, and/or changes in other components of the dopaminergic system, such as rates of release and re-uptake (for instance, extracellular dopamine levels could be changed by FDM in mice). This hypothesis is supported by the observations that myopia development in mice is inhibited by the exogenous applications of either apomorphine, a nonselective dopamine agonist, or L-dihydroxyphenylalanine (L-DOPA),<sup>12–14</sup> and it is enhanced by removing the cellular sources of retinal dopamine with 6-hydroxydopamine (6-OHDA).<sup>15</sup> Additionally, dopaminergic drugs, including exogenous apomorphine and L-DOPA, have no effect on the axial growth of eyes with normal vision, but they selectively modulate FDM.<sup>8,12,13,16</sup> Therefore, myopia that is induced by form-deprivation (FD) may be different from that which arises with normal visual input. Together, these studies suggest that increasing dopamine

levels in the eyes can prevent myopic growth signals, and dopamine receptor activation is needed for normal refractive eye growth under FD visual conditions.

Dopamine receptors are coupled to G-proteins and are divided into two families, the D1- and D2-like receptors (D1Rs and D2Rs, respectively), which are positively and negatively linked to the synthesis of the intracellular second messenger cyclic adenosine phosphate (cAMP), respectively.<sup>17</sup> Stimulation of D2Rs also modulates other pathways (e.g. activating the two isozymes of extracellular signal-regulated kinase (ERK)).<sup>18,19</sup> D2R activation may have a major role in the dopamine-mediated inhibition of axial growth.<sup>7,20</sup> The D2R antagonist spiperone, but not the D1R antagonist SCH 23390, completely abolished the protective effects of apomorphine against FDM in chickens.<sup>20</sup> In contrast, our previous studies that showed the development of FDM in mice was attenuated by either D2R genetic knock-out (KO) or by the D2R antagonist sulpiride.<sup>21</sup> Similarly, myopia development was enhanced by the D2R agonist quinpirole and inhibited by sulpiride in both pigmented and albino guinea pigs.<sup>22,23</sup> Thus, this apparent discrepancy between mice and chickens likely reflects the complexity of D2R-mediated actions, and could result from species differences in retinal circuitry and the regulation of D2Rs during myopia development.

The exact role and intrinsic mechanism of D2R involvement in myopia development still remains to be critically evaluated. Application of the partial D2R agonist aripiprazole (also referred to as a “dopamine stabilizer” that increases dopaminergic neurotransmission when it is too low and decreases it when it is too high)<sup>24,25</sup> provided an alternative approach to solving this problem, because the mechanism of action of aripiprazole is different from that of other agents having an affinity for dopamine receptors. As a partial agonist, aripiprazole has a high affinity for the receptors; but it possesses limited activity at D2Rs, compared to that of dopamine.<sup>26</sup> As confirmed in multiple studies, aripiprazole could act as either agonist or antagonist, depending on endogenous dopamine levels and signaling status.<sup>26,27</sup> In the presence of a low-dopaminergic environment, aripiprazole acts as a D2R agonist, resulting in an increase in dopaminergic neurotransmission; in high-dopaminergic conditions, however, aripiprazole blocks D2Rs, leading to a decrease in dopaminergic neurotransmission.<sup>28</sup>

In this study, we first used the full and selective D2R agonist quinpirole at low and high doses to assess the role of D2Rs in the modulation of normal vision development and FDM. We then validated the role of D2Rs in D2R-KO mice. We found that during the development of FDM, quinpirole exerted a bidirectional effect on the development of myopia such that activation of D2Rs with a low dose of quinpirole attenuated it, whereas a high dose exacerbated it. These findings provide additional evidence that the development of myopia is characterized by reduced D2R-mediated signaling. Furthermore, normalization (but not overactivation) of the reduced D2R-mediated signaling in FDM may have a protective effect. This is consistent with the previous findings that exogenous apomorphine and L-DOPA selectively inhibit FDM, but have no effect on normal vision development.<sup>8,12,13,16</sup>

Based on these observations, our strategy was to selectively revert the D2R-mediated signaling in FDM to the normal levels, without overstimulation. The search for such a strategy led us to propose the use of partial D2R agonists, albeit the multiple, nonspecific pharmacological

targets other than D2Rs,<sup>28,29</sup> reverse myopia development for its dopamine stabilizer property. Specifically, partial D2R agonists, such as aripiprazole, can selectively increase D2R-mediated signaling in FDM, in which the activity of dopamine-dependent pathways is reduced; however, it will not produce overstimulation as would full D2R agonists. Our results show that the complementary actions of quinpirole and aripiprazole provide the most accurate and balanced evidence to date for the roles of D2Rs in myopia development in a mammalian model.

## MATERIALS AND METHODS

### Animals

**Ethics.** This study was approved by the Animal Care and Ethics Committee at Wenzhou Medical University (Wenzhou, China), and all treatment and care of animals adhered to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

**D2R-KO Mice.** As described previously,<sup>21,30,31</sup> the D2R-KO mice were generated by deleting the entire exon 7 and the 5' half of exon 8, replacing both 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 (+/+). The genotype of the mice was determined by polymerase chain reaction analysis of toenail DNA, as reported previously,<sup>30,32</sup> and as described in detail at the website for The Jackson Laboratory (<https://www.jax.org/strain/003190>).

### Experimental Design

**Animal Holding and Lighting Conditions.** All mice were reared under a daily 12-hour light/12-hour dark cycle (incandescent lights on at 8:00 AM and off at 8:00 PM) in the animal facilities, with illuminance at the cage floor approximately 500 lux. The room temperature was maintained at 25 deg Celsius (°C) and mice received food and water ad libitum.

**Quinpirole-Treated C57BL/6 Mice.** For the normal visual environment, 46 C57BL/6 mice (4 weeks old) were randomly divided into 3 subgroups. Vehicle-treated mice, designated as Veh ( $n = 15$ ), received only the solvent, 0.1% ascorbic acid (Sigma-Aldrich Corp.) used for injection of quinpirole (Tocris Bioscience, Glasgow, UK). Mice receiving a low dose of quinpirole, 1 µg/g body weight, were designated 1QNP ( $n = 15$ ), and mice receiving a high dose, 10 µg/g body weight, were designated 10QNP ( $n = 16$ ). For the FD environment, 79 C57BL/6 mice (4 weeks old) were randomly assigned to three subgroups: FD-Veh ( $n = 24$ ), FD-1QNP ( $n = 27$ ), and FD-10QNP ( $n = 28$ ).

**Quinpirole-Treated D2R-KO Mice and WT Littermates.** D2R-KO mice ( $n = 71$ , 4 weeks old) were randomly divided into three groups: D2R-KO-Veh ( $n = 21$ ), D2R-KO-1QNP ( $n = 27$ ), and D2R-KO-10QNP ( $n = 23$ ). WT littermates ( $n = 66$ ) were randomly divided into three control groups: D2R-WT-Veh ( $n = 28$ ), D2R-WT-1QNP ( $n = 19$ ), and D2R-WT-10QNP ( $n = 19$ ). All of these mice were raised in the FD environment.

**Aripiprazole-Treated Mice.** For the normal visual environment, 44 C57BL/6 mice (4 weeks old) were randomly divided into 3 subgroups. Vehicle-treated mice, designated as Veh ( $n = 13$ ), received only the solvent, 10% *N,N*-dimethylformamide (Sigma-Aldrich Corp., St. Louis, MO,

USA) used for injection of aripiprazole (Sigma-Aldrich Corp.). Mice receiving a low dose of aripiprazole, 1 µg/g body weight, were designated 1APZ ( $n = 13$ ), and mice receiving a high dose, 10 µg/g body weight, were designated 10APZ ( $n = 18$ ). For the FD environment, 48 C57BL/6 mice (4 weeks old) were randomly assigned to three subgroups: FD-Veh ( $n = 17$ ), FD-1APZ ( $n = 16$ ), and FD-10APZ ( $n = 15$ ).

### Form Deprivation

FD was achieved by carefully gluing a hand-made translucent occluder to the fur around the right eye of each mouse and leaving it attached for 4 weeks in the relevant groups. A collar made from thin plastic was fitted around the neck to prevent the mouse from removing the occluder. The daily injections of vehicle or drug were performed at approximately 9 to 10 AM during the FD treatments. Body weight, refraction, axial components, and corneal radius of curvature were measured prior to and at the end of the 4 weeks of each treatment (4 and 8 weeks old, respectively) in all groups. To eliminate any effect of anesthesia, the mice were euthanized by cervical dislocation 48 hours after the final biometric measurements. The retinas of the occluded and nonoccluded fellow eyes in the three FD groups were collected between 9:30 AM and 10:30 AM, 30 minutes after the last drug injection. This interval was chosen to match the onset of the pharmacological effect of aripiprazole at these doses.<sup>33,34</sup> In order to assess D2R-mediated signal transduction, the retinas were analyzed for cAMP ( $n = 10$ –17 for each group) and pERK levels ( $n = 5$ –6 for each group).

### Preparation for Drug Injection

All drugs were administered without anesthesia by daily intraperitoneal injections in the lower right or left quadrant of the abdomen using a 1-ml syringe cannula (Shanghai Kindly Medical Devices Co., Ltd, Shanghai, China) attached to a 29-gauge needle. Quinpirole was injected after dissolving in distilled H<sub>2</sub>O containing 0.1% ascorbic acid to retard oxidation. Aripiprazole was injected after dissolving in 10% *N,N*-dimethylformamide, as previously described.<sup>35,36</sup> The injection volume in all groups was 1 µl/g body weight. The pharmacological doses were chosen to achieve effective drug concentration in the central nervous system, on the basis of previous studies.<sup>36,37</sup>

### Biometric Measurements

A detailed description of the recording apparatus has been reported.<sup>12,21</sup> Refraction was measured in a darkened room using an eccentric infrared photorefractor designed by Schaeffel.<sup>31,38,39</sup> Briefly, each unanesthetized mouse was gently restrained, with its position adjusted until a clear first Purkinje image occurred in the center of the pupil, indicating an on-axis measurement. The data were reported as the means of at least 3 measurements, each of which was averaged from 10 individual values to create that measurement.

The anterior chamber depth, lens thickness, vitreous chamber depth (VCD), axial length (AL, from the anterior corneal surface to the vitreous-retina interface), and anterior corneal radius of curvature were measured with a custom-made spectral-domain optical coherence tomography (SD-OCT) system.<sup>12,21,31</sup> General anesthesia was achieved with an intraperitoneal injection of ketamine (70 mg/kg) and

xylazine (7 mg/kg). 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 during the measurement with an X-Y cross-scanning system. The raw SD-OCT data were exported and analyzed by custom-designed software to obtain the axial components and the corneal radius of curvature. The mean of three repeated SD-OCT measurements for each eye was used for analysis.

### Retinal cAMP Assay

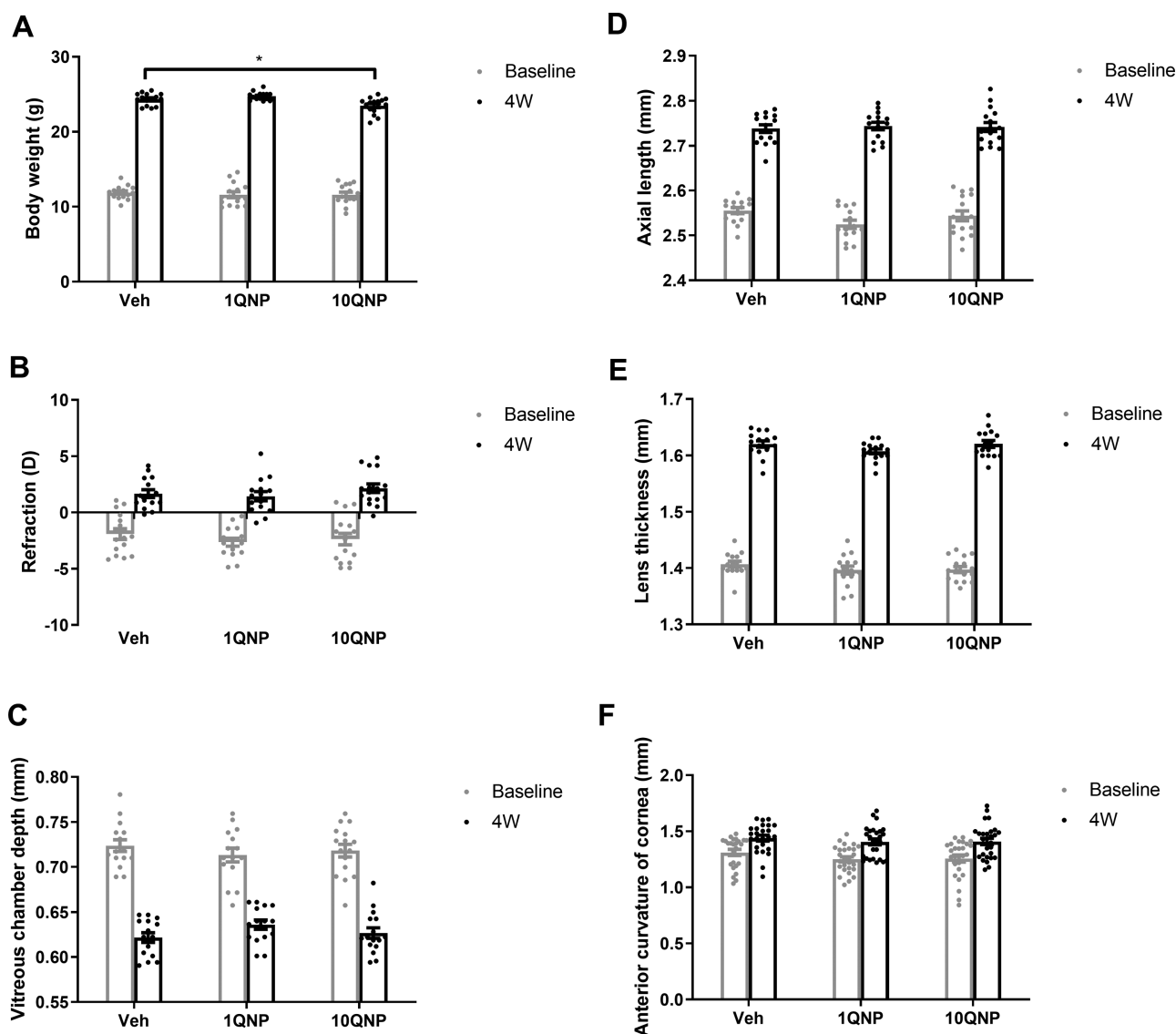
The retinal cAMP levels were measured using a <sup>125</sup>I-cAMP radioimmunoassay kit (Institute of Isotopes Ltd., Budapest, Hungary), as previously reported.<sup>40</sup> After removal from the posterior eye cup, the retina was weighed and then homogenized in 1 ml of acetate buffer. Then 2 ml of dehydrated ethanol was added, and the supernatants were collected after centrifugation (1,200 × g, 4°C, 15 minutes). The remaining pellets were re-suspended in another 2 ml of 75% ethanol and the suspension was centrifuged; supernatants were collected again, pooled with the supernatants from the first spin, and dried overnight at 60°C. The residual powder was dissolved in 1 ml acetate buffer, and 0.1 ml was assayed, according to the manufacturer's instructions. Radioactivity in the re-suspended residue was determined by a gamma scintillation counter (xh6080; Xi'an Nuclear Instrument Factory, Xi'an, Shaanxi, China) for at least 60 seconds, and the amount of cAMP was expressed as fmol cAMP/mg retinal wet-weight.

### Western Blot Analysis

As previously described in detail,<sup>41</sup> protein extracts from each sample were loaded onto 10% sodium dodecyl sulfate polyacrylamide gels and electrotransferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk for 2 hours at room temperature and then incubated with primary rabbit monoclonal antibodies against phospho-ERK1/2 (pERK; dilution 1:1000; 4370S; Cell Signaling Technology, Danvers, MA, USA) or ERK1/2 (dilution 1:1000; 4695S; Cell Signaling Technology), overnight at 4°C. The blots were also probed with mouse monoclonal antibodies to  $\alpha$ -tubulin (dilution 1:1000; ab7291; Abcam, Cambridge, MA, USA) antibody as loading controls. After washing three times with tris-buffered saline containing 0.1% Tween-20 for 10 minutes each, the membranes were incubated with IRDye 800CW goat anti-mouse IgG (dilution 1:2000; 926-32210; Odyssey, Lincoln, NE, USA) or IRDye 800CW goat anti-rabbit IgG (dilution 1:2000; 926-32211; Odyssey) antibodies for 2 hours at room temperature. Densitometric analysis of the protein bands was conducted using Image J version 1.48 software (National Institutes of Health, Bethesda, MD, USA), and the pERK values were normalized to the corresponding loading total ERK.

### Statistical Analysis

Data, which were all verified to be normally distributed, were presented as mean  $\pm$  standard error of the means. The effects induced by FD were shown as interocular differences (FD eye minus fellow eye) instead of the directly measured absolute values, to avoid the potential confounding variable of different growth rates due to the drug or genetic KO



**FIGURE 1.** Effects of quinpirole treatment on body weight, refraction, and ocular dimensions in C57BL/6 mice under a normal visual environment. Biometric measurements in Veh, 1QNP, and 10QNP groups before and after 4 weeks of treatment: (A) body weight, (B) refraction, (C) vitreous chamber depth, (D) axial length, (E) lens thickness, and (F) anterior corneal radius of curvature. \* $P < 0.05$ ; 2-way repeated measures ANOVA, post hoc simple effects analysis. Veh, vehicle; 1QNP or 10QNP, 1 or 10  $\mu$ g quinpirole/g body weight, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

treatments. Statistical significance was determined by 2-way or 3-way repeated measures ANOVA, with measured time (baseline versus 4 weeks for the analysis of biometric parameters) or eyes (FD versus fellow eyes for the cAMP and pERK analyses) as repeated measures. Bonferroni corrections were applied in post hoc analyses. Values of  $P < 0.05$  were taken to be significant. All statistical analyses were performed with SPSS (IBM, Version 19.0; IBM, Armonk, NY, USA).

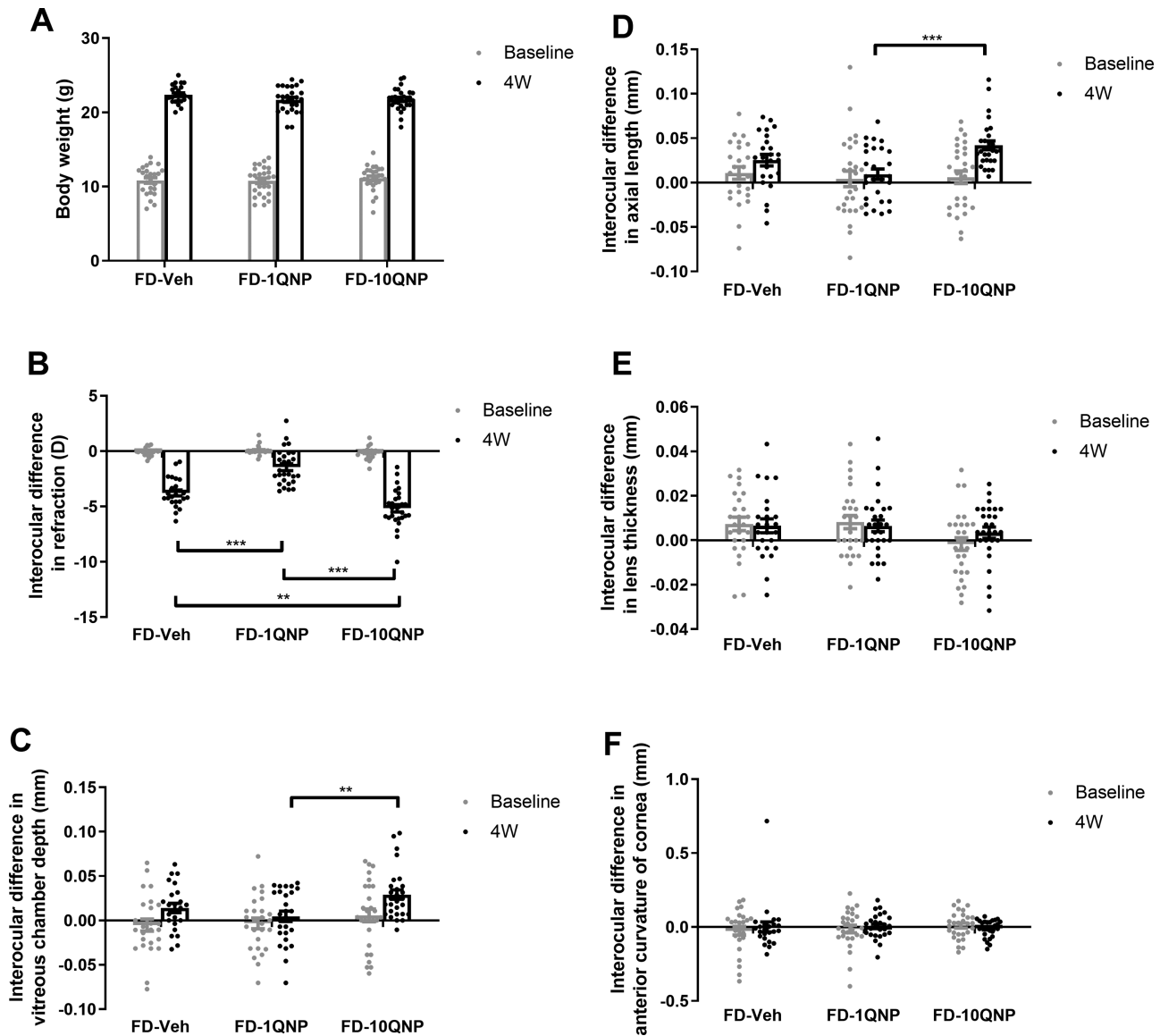
## RESULTS

### The Bidirectional Effect of Quinpirole on FDM Development in C57BL/6 Mice

With unobstructed vision in the normal visual environment (no form-deprivation), mice treated with the high dose of quinpirole (10QNP) had slightly but significantly lower

body weight after 4 weeks of treatment than did Veh mice (Fig. 1A;  $P = 0.019$ , 2-way repeated ANOVA). There were no differences in any of the measured ocular biometric parameters, among the Veh, 1QNP, and 10QNP groups, either before or after 4 weeks of treatments ( $P > 0.05$ ; Figs. 1B–F, Supplementary Table S1). Therefore, normal postnatal refractive development in mice was not affected by the daily IP injection of quinpirole.

Refraction and ocular biometry absolute data of C57BL/6 mice FD and fellow eyes treated with quinpirole are presented in Supplementary Table S2. Under the FD environment, treatment with quinpirole and the elapsed 4 weeks of the study period had main and interaction effects on interocular differences of refraction, VCD, and AL, as determined by 2-way repeated ANOVA (Supplementary Table S1). For all baseline results, none of the interocular differences in biometric measurements among the three vehicle and



**FIGURE 2.** Effects of quinpirole treatment on body weight, refraction, and ocular dimensions in form-deprived (FD) C57BL/6 mice. Biometric measurements in FD-Veh, FD-1QNP, and FD-10QNP groups before and after 4 weeks of treatment. (A) Body weight. For panels B–F, interocular differences for deprived and fellow eyes: (B) refraction, (C) vitreous chamber depth, (D) axial length, (E) lens thickness, and (F) anterior corneal radius of curvature. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ; 2-way repeated measures ANOVA, post hoc simple effects analysis. FD-Veh, form-deprived mice treated with vehicle; FD-1QNP or FD-10QNP, form-deprived mice treated with 1 or 10  $\mu\text{g}$  quinpirole/g body weight, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

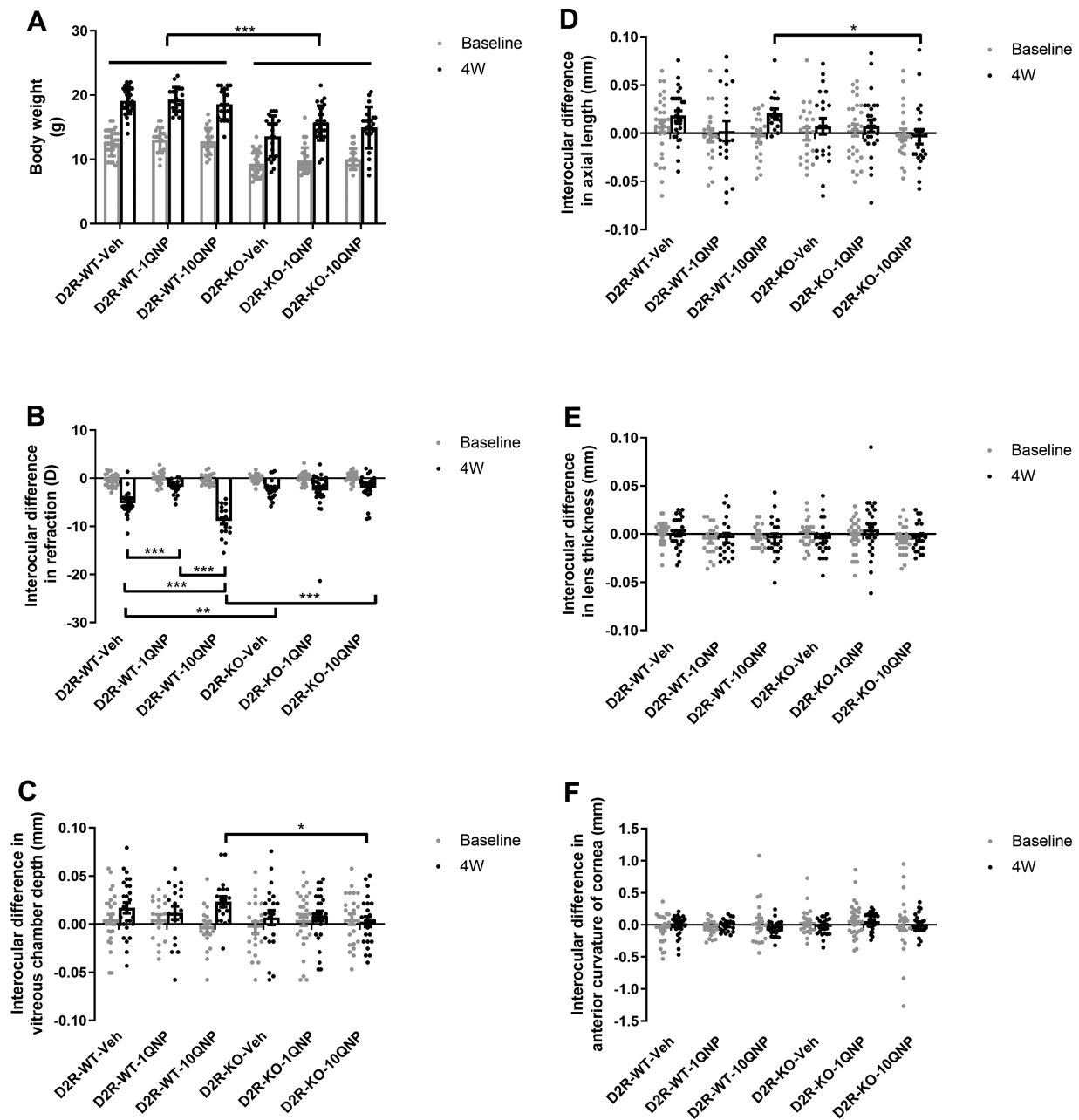
quinpirole FD groups were significant (all  $P$  values  $> 0.05$ , 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 2). After 4 weeks of treatment, the high and low doses of quinpirole had opposite effects on the development of FDM: the low dose (1QNP) inhibited myopia development ( $-3.77 \pm 0.26$  diopter (D) in FD-Veh versus  $-1.45 \pm 0.31$  D in FD-1QNP,  $P < 0.001$ ), whereas the high dose (10QNP) promoted it ( $-3.77 \pm 0.26$  D in FD-Veh versus  $-5.16 \pm 0.33$  D in FD-10QNP,  $P = 0.006$ ). In parallel with the refraction changes, the interocular differences of VCD and AL in FD-1QNP and FD-10QNP also were affected in opposite ways, relative to FD-Veh ( $P = 0.006$  for VCD;  $P < 0.001$  for AL; Figs. 2C, 2D). Thus, the low and high doses of quinpirole treatment had opposite effects on FDM in C57BL/6 mice.

There were no interocular differences in body weight, anterior chamber depth, lens thickness, or anterior corneal

radius of curvature among the different FD groups ( $P > 0.05$ ; Figs. 2A, 2E, 2F, and Supplementary Figure S1) at week 4 of the experiment.

### The Opposite Effects of Quinpirole on FDM Development Were Mediated by D2Rs

To determine whether D2Rs alone could mediate these opposite effects of QNP on FDM in mice, the effects of different doses on myopia development were examined using D2R-KO mice. Consistent with our previous reports,<sup>21,31</sup> the physical growth in the D2R-KO mice was slower, as revealed by a lower body weight in comparison with the D2R-WT mice of the same age (main effects,  $F_{1,131} = 105.231$ ,  $P < 0.001$ , 3-way repeated ANOVA; Fig. 3A). Refraction and

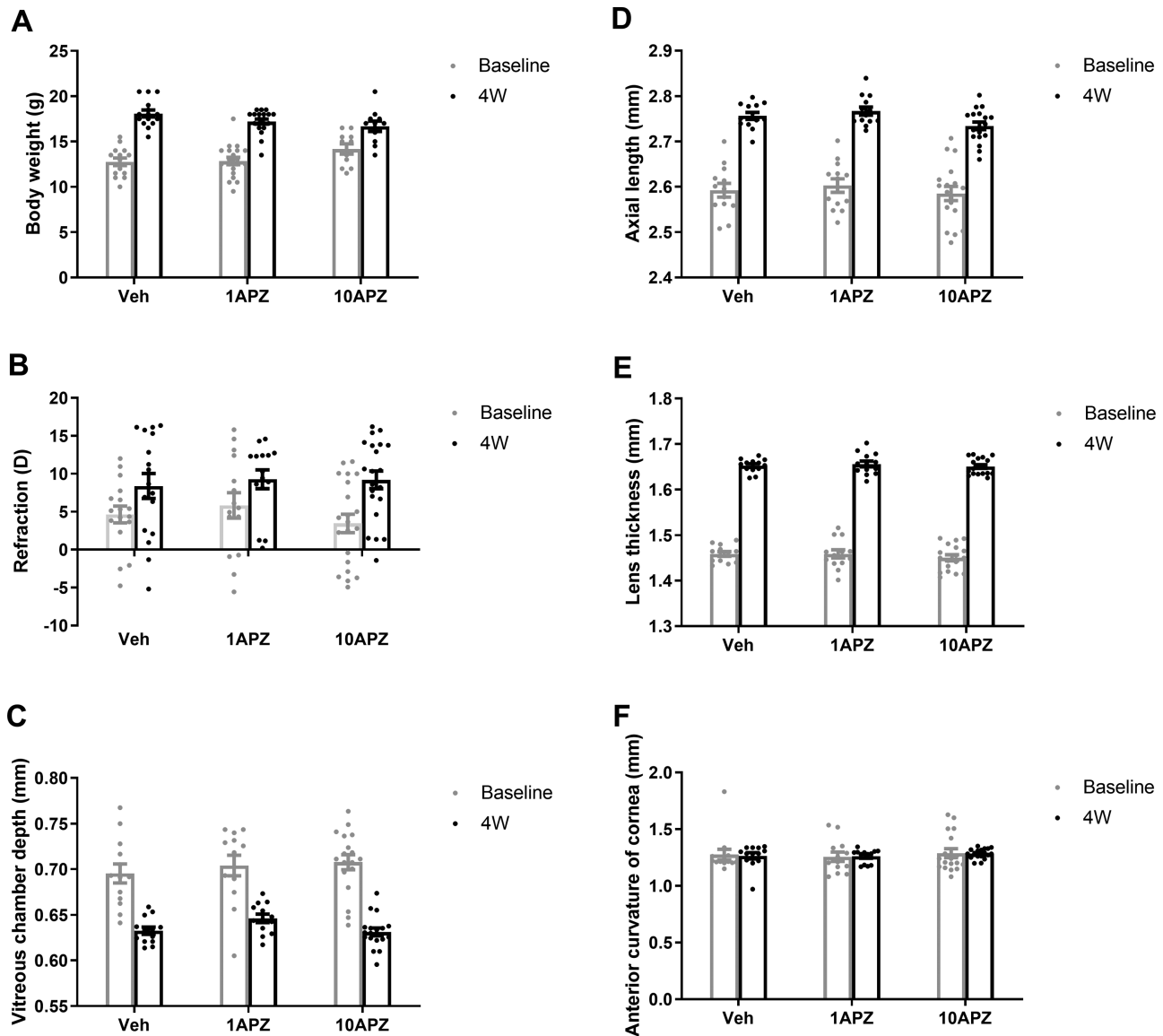


**FIGURE 3.** Effects of quinpirole and D2R-KO treatment on body weight, refraction, and ocular dimensions in form-deprived mice. Biometric measurements in D2R-WT-Veh, D2R-WT-1QNP, D2R-WT-10QNP, D2R-KO-Veh, D2R-KO-1QNP, and D2R-KO-10QNP groups before and after 4 weeks of each treatment. (A) Body weight. For panels B–F, interocular differences for deprived and fellow eyes: (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 \*\*\* $P < 0.001$ ; 3-way repeated measures ANOVA, post hoc simple effects analysis. D2R-WT-Veh, dopamine D2 receptor-wild-type mice treated with vehicle; D2R-WT-1QNP or D2R-WT-10QNP, dopamine D2 receptor-wild-type mice treated with 1 or 10  $\mu\text{g/g}$  body weight quinpirole respectively; D2R-KO-Veh, dopamine D2 receptor-knock out mice treated with vehicle; D2R-KO-1QNP or D2R-KO-10QNP, dopamine D2 receptor-knock out mice treated with 1 or 10  $\mu\text{g/g}$  body weight quinpirole, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

ocular biometry absolute data of D2R-KO mice FD and fellow eyes treated with quinpirole are presented in Supplementary Table S3.

The main and interaction effects of quinpirole treatment and D2R-KO on interocular differences of refraction, VCD, and AL were assessed by 3-way repeated ANOVA (Supplementary Table S4). For all baseline results, there were no significant differences between the interocular differences

in biometric parameters of any two groups ( $P > 0.05$ , 3-way repeated ANOVA, post hoc simple effects analysis; Fig. 3). After 4 weeks of treatment, as described above for C57BL/6 mice, the high and low doses of quinpirole had opposite effects on the development of FDM in the D2R-WT mice ( $F_{2,131} = 27.860$ ,  $P < 0.001$ ; Fig. 3B). As in our previous studies,<sup>21,31</sup> the myopia induced in the D2R-WT-Veh group was 2.31 times greater than in the D2R-KO-Veh group ( $F_{1,131}$



**FIGURE 4.** Effects of aripiprazole treatment on body weight, refraction, and ocular dimensions in mice under normal visual environment. Biometric measurements in Veh, 1APZ, and 10APZ groups before and after 4 weeks of treatment: (A) body weight, (B) refraction, (C) vitreous chamber depth, (D) axial length, (E) lens thickness, and (F) anterior corneal radius of curvature.  $P > 0.05$  for all comparisons; 2-way repeated measures ANOVA, post hoc simple effects analysis. Veh, vehicle; 1APZ or 10APZ, 1 or 10  $\mu\text{g}$  aripiprazole/g body weight, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

= 12.503,  $P < 0.001$ ; see Fig. 3B). Importantly, the opposing effects of quinpirole on FDM in WT mice were not significant in D2R-KO mice ( $P > 0.05$ ; see Fig. 3B). Because D2R-KO partially attenuated FDM development, further suppression by the low dose of quinpirole treatment in the KO group might not have been large enough to be detected. However, D2R-KO indeed prevented the 10QNP-induced effect of myopia enhancement ( $F_{1,151} = 57.656$ ,  $P < 0.001$ ; see Fig. 3B).

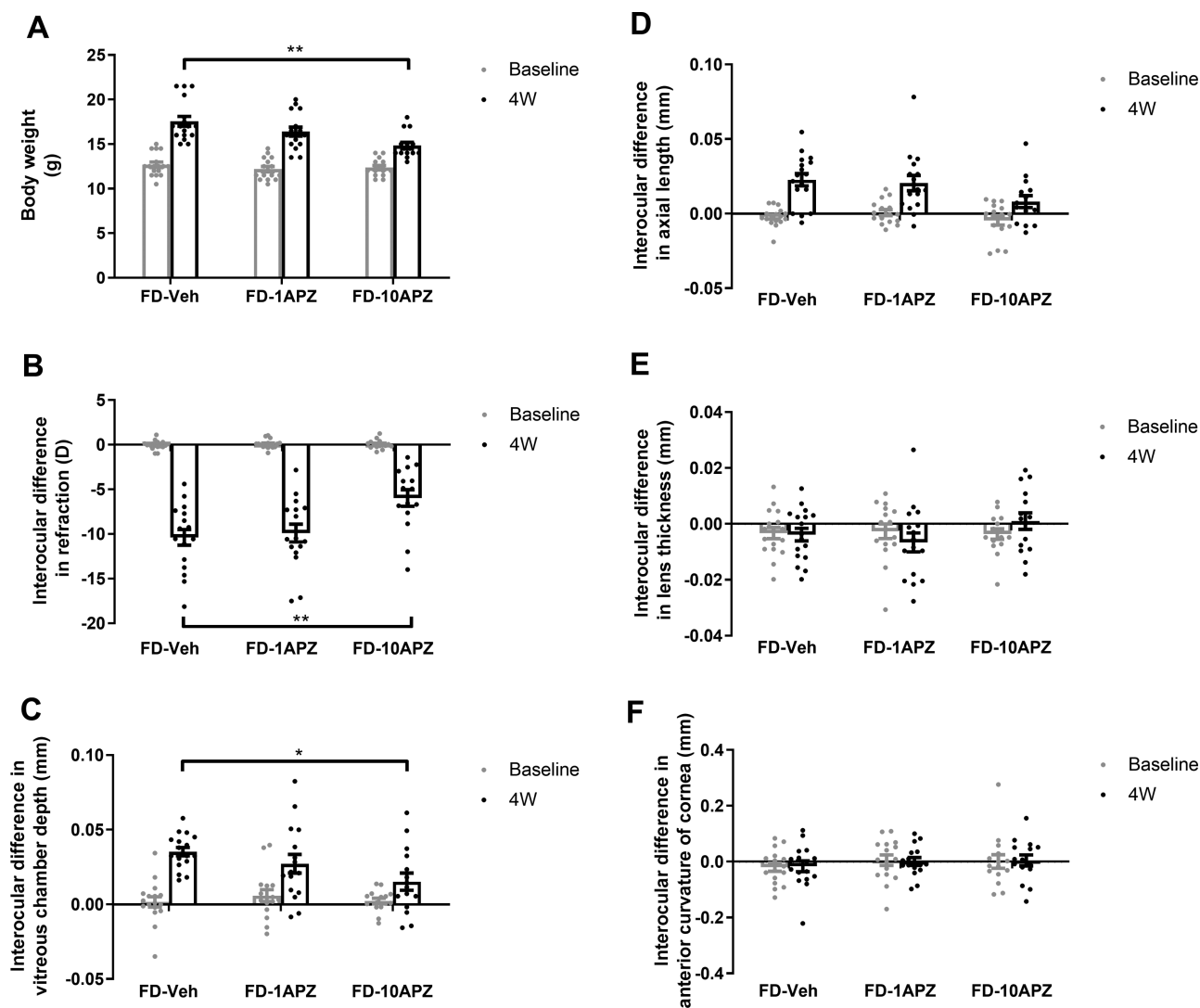
The interocular differences of VCD and AL in D2R-WT-1QNP and D2R-WT-10QNP were affected only slightly, and not significantly ( $P > 0.05$ ), relative to D2R-WT-Veh (Figs. 2C, 2D). Consistent with the refraction changes, there were no differences in the interocular differences of VCD and AL among the three D2R-KO groups ( $P > 0.05$  for

each; Figs. 3C, 3D). Therefore, the opposing effects of quinpirole treatment on FDM diminished in the absence of D2Rs.

Neither quinpirole nor D2R-KO treatment, applied alone or in combination, had any effect at any time on interocular differences in anterior chamber depth, lens thickness, or anterior corneal radius of curvature (all  $P$  values  $> 0.05$ , 3-way repeated ANOVA; Figs. 3E, 3F, and Supplementary Figure S1).

### Aripiprazole Attenuated FDM

In mice reared in the normal visual environment, there were no differences in body weight or in any of the measured ocular biometric parameters among the Veh, 1APZ, and 10APZ groups, either before or after 4 weeks of treatments



**FIGURE 5.** Effects of aripiprazole treatment on body weight, refraction, and ocular dimensions in form-deprived mice. Biometric measurements in FD-Veh, FD-1APZ, and FD-10APZ groups before and after 4 weeks of treatment: (A) body weight. For panels B–F, interocular differences for deprived and fellow eyes: (B) refraction, (C) vitreous chamber depth, (D) axial length, (E) lens thickness, and (F) anterior corneal radius of curvature. \* $P < 0.05$  and \*\* $P < 0.01$ ; 2-way repeated measures ANOVA, post hoc simple effects analysis. FD-Veh, form-deprived mice treated with vehicle; FD-1APZ or FD-10APZ, form-deprived mice treated with 1 or 10  $\mu\text{g}$  aripiprazole/g body weight, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

( $P > 0.05$ , 2-way repeated ANOVA; Fig. 4, Supplementary Table S5). Therefore, normal postnatal refractive development in mice was not affected by the daily injection of aripiprazole.

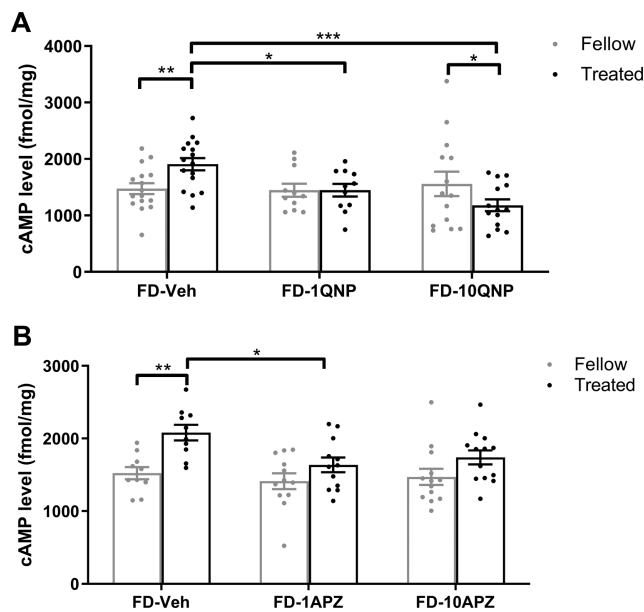
Under FD, vehicle-treated mice had greater body weight after 4 weeks of treatment than did mice treated with the high dose of aripiprazole ( $P = 0.001$ ; Fig. 5A). Thus, the physical growth of the FD-10APZ mice was slower than FD-Veh mice. Refraction and ocular biometry absolute data of FD and fellow eyes treated with aripiprazole are presented in Supplementary Table S6.

Treatment with aripiprazole and the elapsed 4 weeks of the study period had main and interaction effects on interocular differences of refraction, VCD, and AL, as determined by 2-way repeated ANOVA (Supplementary Table S5). For each of the baseline measurements, there were no differences between the aripiprazole and vehicle groups (all  $P$  values  $> 0.05$ , 2-way repeated ANOVA, post hoc simple effects anal-

ysis; see Fig. 5). After 4 weeks of treatment, the myopic shift, measured as the difference between the deprived and fellow eyes, was 1.74 times greater in the FD-Veh group ( $-10.38 \pm 0.88$  D) than in the FD-10APZ group ( $-5.98 \pm 0.92$  D;  $P = 0.005$ , 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 5B). In parallel with the refraction changes, the elongation of VCD in the FD-Veh group was enhanced, as reflected in the greater interocular differences of VCD than in the FD-10APZ group ( $P = 0.023$ , Fig. 5C). There was a tendency for interocular differences in AL to be greater in the FD-Veh group than in the FD-10APZ group, but this difference was not significant ( $P = 0.076$ ; Fig. 5D). Thus, the high dose of aripiprazole treatment attenuated the FD-induced changes in refraction and VCD. However, the inhibitory effect of aripiprazole on FDM development at the low dose was not significant ( $P > 0.05$ ).

There were no interocular differences in anterior chamber depth (Supplementary Figure S1), lens thickness





**FIGURE 6.** Effects of quinpirole and aripiprazole treatments on retinal cAMP levels in mice after 4 weeks of form deprivation. **(A)** Quinpirole. **(B)** Aripiprazole. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ; 2-way repeated measures ANOVA, post hoc simple effects analysis. FD-Veh, form-deprived mice treated with vehicle; FD-1QNP or FD-10QNP, form-deprived mice treated with 1 or 10  $\mu\text{g}$  quinpirole/g body weight; FD-1APZ or FD-10APZ, form-deprived mice treated with 1 or 10  $\mu\text{g}$  aripiprazole/g body weight, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

(Fig. 5E), or corneal radius of curvature (Fig. 5F) among the different FD groups ( $P > 0.05$ ) at week 4 of the experiment.

### Effect of Quinpirole and Aripiprazole on Retinal cAMP and pERK Levels

As reported above, FDM, but not normal refractive development, was affected by quinpirole and aripiprazole. This suggests that D2R-linked signaling was altered during the development of FDM. However, it is unknown how the underlying D2R-linked signaling was changed during this process. Therefore, we further investigated the changes in cAMP and pERK levels, both of which were potential markers to assess D2R-mediated signal transduction.

Retinal cAMP levels were measured by radioimmunoassay, and the main and interaction effects of drug treatment and FD on cAMP and pERK were determined by 2-way repeated ANOVA (Supplementary Table S7). In the FD-Veh group, retinal cAMP levels were lower in the fellow eyes than in the FD eyes after 4 weeks of FD ( $P = 0.005$ , 2-way repeated ANOVA, post hoc simple effects analysis; Fig. 6A). This interocular difference disappeared in the FD-1QNP group ( $P > 0.05$ ), and was reversed in the FD-10QNP group ( $P = 0.021$ ). Quinpirole treatment caused a dose-dependent inhibition of cAMP levels in the FD eyes ( $F_{2,39} = 9.770$ ,  $P < 0.001$ ).

In either the FD-1APZ or the FD-10APZ group, the retinal cAMP levels in the fellow eyes were not significantly different from those in the FD eyes ( $P > 0.05$ , Fig. 6B). The cAMP level in the retinas of deprived eyes was greater in the FD-Veh group than in the FD-1APZ group ( $P = 0.015$ , see

Fig. 6), but the difference between the FD-Veh group and the FD-10APZ group was not significant ( $P = 0.076$ ).

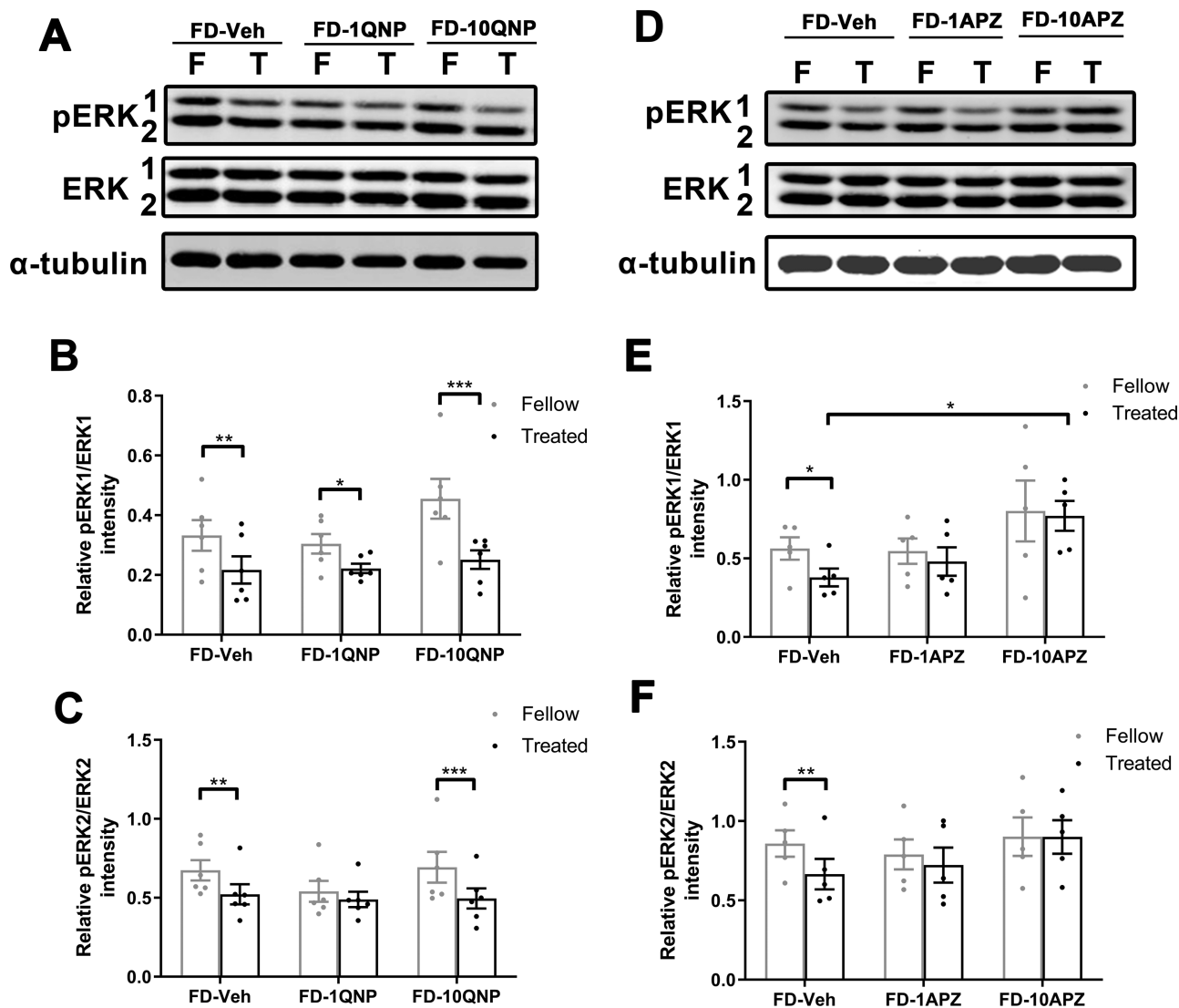
The anti-ERK antibody recognizes the region of ERK proteins around Thr202 and Tyr204, and the anti-phospho-ERK antibody recognizes ERK that is phosphorylated at those amino acids. In Western blots of mouse retinas, both antibodies labeled corresponding bands at 44 kDa (ERK/pERK1) and 42 kDa (ERK/pERK2) (Figs. 7A, 7D). Consistent with results of other studies,<sup>42,43</sup> pERK2 was more strongly phosphorylated than pERK1 (see Fig. 7A). Quantitatively, the pERK results, normalized to  $\alpha$ -tubulin, were consistent with those normalized to total ERK (data not shown), thus only those data normalized to total ERK were reported in detail here. In the FD-Veh group, the levels of retinal pERK1/ERK1 and pERK2/ERK2 in the fellow eyes after 4 weeks of FD were greater than in the FD eyes ( $P = 0.008$  for pERK1/ERK1;  $P = 0.001$  for pERK2/ERK2; 2-way repeated ANOVA, post hoc simple effects analysis, Figs. 7B, 7C). However, neither low nor high doses of quinpirole treatment had effects on pERK1/ERK1 and pERK2/ERK2 (relative levels of phosphorylation), as there were no differences in the FD eyes among the FD-Veh, FD-1QNP, and FD-10QNP groups ( $P > 0.05$ , see Figs. 7B, 7C).

Notably, these differences between either pERK1/ERK1 or pERK2/ERK2, in FD and fellow eyes in both the FD-1APZ and FD-10APZ groups, were not statistically significant ( $P > 0.05$ , Figs. 7E, 7F). Furthermore, the pERK1/ERK1 levels of the deprived eyes were lower in the FD-Veh group than in the FD-10APZ group ( $P = 0.017$ , see Fig. 7E). However, the retinal pERK1/ERK1 levels in the deprived eyes, in the FD-1APZ and FD-Veh groups, were not statistically significant ( $P > 0.05$ ).

## DISCUSSION

In this study, neither quinpirole nor aripiprazole affected normal vision development in mice. In contrast, FDM development was inhibited by the full D2R agonist quinpirole at low dose but enhanced at high dose, and the D2R-specificity of these bidirectional effects was validated in the D2R-KO mice. These results suggest that myopia is characterized by reduced D2R-mediated signaling. Further, it suggests that normalization (but not overactivation) of the reduced D2R-mediated signaling in FDM may have a protective effect. This view is consistent with the finding that FDM development was selectively attenuated by the partial D2R agonist aripiprazole, which activates the D2Rs under low dopamine levels, such as occurs in FDM. Together, these results suggest that the onset of vision-dependent myopia is a fundamentally different process from the normal development of eye growth and vision, and that downregulation of signaling via D2Rs contributes to myopia development.

Quinpirole, at the low dose (1  $\mu\text{g}/\text{g}$  body weight), inhibited myopia development; but at the high dose (10  $\mu\text{g}/\text{g}$  body weight), it promoted it. These bidirectional dose-dependent effects were not present in D2R-KO mice, strongly indicating they required D2Rs. Previous long-held and mixed views stated that D2R activation in chickens,<sup>7,20</sup> but inactivation in mice,<sup>21</sup> contributes to the inhibition of myopia development. However, we have now demonstrated for the first time that D2R activation exerts bidirectional control of FDM. This suggests that normalization of D2R activation, but not overstimulation, is important for protection against myopia. As D2Rs expressed on different neurons exert opposing functions toward dopamine transmission, it would be reasonable



**FIGURE 7.** Effects of quinpirole and aripiprazole treatments on retinal ERK phosphorylation in mice after 4 weeks of form deprivation as measured by Western blots. Two bands were typically observed: (A, D) Upper bands: pERK1 and ERK1, both 44 kDa. Lower bands: pERK2 and ERK2, both 42 kDa. Quantitation of the phosphorylation levels of ERK1 (B, E) and ERK2 (C, F) were normalized to the corresponding total ERK1 and ERK2. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ ; 2-way repeated measures ANOVA, post hoc simple effects analysis. FD-Veh, form-deprived mice treated with vehicle; FD-1QNP or FD-10QNP, form-deprived mice treated with 1 or 10  $\mu$ g quinpirole/g body weight, respectively; FD-1APZ or FD-10APZ, form-deprived mice treated with 1 or 10  $\mu$ g aripiprazole/g body weight, respectively. Data are expressed as the mean  $\pm$  standard error of the mean.

to propose that D2 autoreceptors (D2S isoform, expressed presynaptically in dopaminergic neurons) and heteroreceptors (D2L isoform, expressed in post-synaptically in non-dopaminergic neurons) are responsible for the bidirectional responses to activation of D2Rs.<sup>44</sup> D2R autoreceptors are 3 to 10 times more sensitive to dopamine agonists, including quinpirole, than are D2R heteroreceptors.<sup>45,46</sup> However, this possibility is likely ruled out, because it is unconvincing that the reduced extracellular dopamine levels, resulting from activation of D2 autoreceptors by low-dose quinpirole, would lead to FDM inhibition.

As far as we know, this is the first evidence that partial agonists can attenuate FDM development without affecting refractive development under the normal visual environment. Based on the pharmacological profile of partial D2R agonist,<sup>27</sup> the selective inhibitory effect of aripiprazole on

FDM is attributed to activation of D2Rs under low levels of dopamine. This result is also consistent with the finding that quinpirole might produce physiological effects at low dose, and super-physiological effects at high dose (as in FDM). Therefore, both the full D2R agonist quinpirole at low dose (but not at the high dose), and the partial D2R agonist aripiprazole, could inhibit myopia by activation of D2Rs under low-dopamine conditions.

The molecular basis for these effects of quinpirole and aripiprazole is not clear. Retinal cAMP content was increased, whereas the phosphorylation of ERK was decreased, during the development of FDM. This finding was similar to that of our previous study,<sup>40</sup> in which scleral cAMP levels were increased in FD eyes of guinea pigs (whereas the increase in the retina was minor). The present results suggest that D2R-linked signaling was reduced

during the development of FDM, thus implying a reduction in dopamine levels in FDM in mice, as in various other species.<sup>6–8</sup> In FD eyes, quinpirole caused a dose-dependent reduction in cAMP levels, but no effect on pERK. In contrast, both the low and high doses of aripiprazole reduced cAMP levels, but the level of pERK increased in FD eyes. These findings suggest three important points. First, the reduced cAMP level (shared by quinpirole and aripiprazole) could contribute to the D2R-mediated modulation of FDM. Second, the changes in cAMP levels and pERK showed similar patterns of inhibition (of cAMP) or no effect (on pERK) for both the low and high doses of quinpirole, thus not accounting for the bidirectional effects of quinpirole. The mechanism underlying the bidirectional effects of quinpirole at the low and high doses is not clear. We suggest that the higher dose of quinpirole might act at D2Rs combined with other receptors, or might act via separate signaling pathways (viz., biased signaling) to produce distinct effects,<sup>47</sup> but only when administered and acting concurrently. These possibilities need to be clarified by additional experiments. The third important point is that quinpirole (both at low and high doses) did not affect pERK, whereas aripiprazole increased pERK, in FD eyes. This suggests that quinpirole and aripiprazole might exert control of myopia through distinct signaling pathways. The difference in pERK signaling between the two drugs might be due to functional selectivity (i.e. biased signaling for D2Rs by aripiprazole),<sup>48</sup> or to action at receptors other than D2R, such as the 5-hydroxytryptamine receptors.<sup>28,29</sup> Either or both of these might account for the myopia control exerted by aripiprazole. Additional experiments using D2R-KO mice are clearly needed to further clarify the D2R specificity of aripiprazole. Aripiprazole can also affect metabolism<sup>49,50</sup> in a way that might contribute to the reduced bodily growth of the mice in this study. Nonetheless, we have not shown any direct link between altered metabolism and myopia development.

How, exactly, full or partial D2R agonists specifically control myopia inhibition remains to be clarified by future studies? Myopia associated with relatively low dopamine levels can be pharmacologically targeted by partial D2R agonists, such as aripiprazole, which has a dopamine stabilizing property, and, therefore, acts as a D2R agonist in the low dopamine environment. Aripiprazole does not affect eye growth under normal conditions with physiological levels of dopamine. This is a critical concern in developing dopamine-based therapy for myopia treatment. Aripiprazole is effective and well tolerated in short- (4–8 weeks), intermediate- (26 weeks), and long-term (52 weeks) clinical trials for the treatment of schizophrenia and schizoaffective disorder.<sup>51</sup> However, the broad spectrum of dopamine actions on development, and on the central nervous system (CNS), cardiovascular, and endocrine functions, raises serious concerns and poses difficult challenges. Therefore, additional studies are required to demonstrate the effectiveness and safety of aripiprazole and similar drugs for the prevention and treatment of myopia.

It is likely that both systemic administration of the drugs and D2R gene deletion act at both retinal and extra-retinal sites to exert control of myopia development. Therefore, the possible contribution of extra-retinal action in the myopia development observed here could not be ruled out. Thus, this study focused only on the aggregate roles that D2Rs play in FDM. Additional studies using focal deletion of retinal and extra-retinal D2R receptors are required to clarify this issue. Because D2Rs are widely expressed by neurons

of many types in the retina, it is also likely that the growth-modulating effects of these drugs are not due to action at a single postsynaptic site. Therefore, further studies aimed at identifying the roles of specific retinal cell types will also be necessary to more fully understand how dopamine influences ocular growth and refractive development.

In summary, from the perspective of visual development, the novel findings of this study are the bidirectional control of ocular growth and refraction by full D2R agonists and the attenuation of FDM by partial D2R agonists with the ability to activate the D2Rs under low dopamine levels, such as what occurs in FDM. Our results suggest that a reduction of D2R-mediated signaling contributes to myopia development. These findings provide complementary and fresh insights into the role of dopamine receptors during the development of myopia.

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