7-Methylxanthine Influences the Behavior of ADORA2A-DRD2 Heterodimers in Human Retinal Pigment Epithelial Cells

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Abstract

Introduction: The goal of this study was to investigate the presence of ADORA2A-DRD2 heterodimers in human retinal pigment epithelial (RPE) cells; determine if 7-methylxanthine (7-MX), a nonselective adenosine receptor antagonist which was used to control myopia progression, can influence the behavior of RPE cells through the ADORA2A-DRD2 receptor pathway; and assess the changes in the expression of signaling molecules during cellular signal transduction. ***Methods:*** Human RPE cells were cultured in vitro in the presence or absence of 7-MX. Cell proliferation was evaluated with the CCK-8 assay. Apoptosis and necrosis rates were determined by annexin V–FITC/propidium iodide staining and flow cytometry. Immunofluorescence and coimmunoprecipitation were used to examine the protein expression and colocalization of ADORA2A and DRD2 in RPE cells. ADORA2A and DRD2 were knocked down with small interfering RNAs (siRNAs). Changes in the protein expression of ERK1/2 and phospho-ERK1/2 (pERK 1/2), which are signaling molecules downstream of dopamine receptors, were evaluated by Western blot analysis. ***Results:*** Immunofluorescence and co-immunoprecipitation showed that ADORA2A and DRD2 were colocalized in RPE cells. The expression of ADORA2A in RPE cells was inhibited by treatment with 50 μmol/L 7-MX for 48 h, and the expression of DRD2, ERK1/2, and pERK1/2 was increased after treatment with 50 μmol/L 7-MX for 48 h. After siRNA-mediated knockdown of DRD2 in RPE cells and further treatment with 50 μmol/L 7-MX for 48 h, the expression of DRD2 was nearly restored to the level observed in the native control. At the experimental concentrations, 7-MX and siRNAs did not affect the proliferation or apoptosis of human RPE cells. ***Conclusions:*** ADORA2A and DRD2 heterodimers were present in RPE cells. 7-MX may affect the behaviors of RPE cells through the ADORA2A-DRD2 receptor pathway. 7-MX is an inhibitor of ADORA2A receptors that can prevent inhibition of the DRD2 receptor pathway and increase DRD2 receptor pathway activity. This phenomenon may explain the mechanism of action through which 7-MX can control myopia progression.

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Introduction

Myopia has become a major problem affecting global population health and national economic growth [1]. Once pathological myopia develops, the risk of blindness is greatly increased [2, 3]. Thus, it is important to control the rate of myopia progression in adolescents to reduce the rate of blindness and disability due to high myopia complications.

7-Methylxanthine (7-MX) is a metabolite of caffeine and theobromine, which has been shown potential to inhibit and prevent myopia progression. The results of several animal studies have demonstrated that 7-MX reduces the severity of myopia and axial length elongation caused by form deprivation in rabbits and guinea pigs [4–6]. A 36-month clinical trial showed that 7-MX reduces myopia progression in adolescents [7]. Although animal experiments and clinical trials have confirmed that 7-MX can control myopia to some degree, the underlying mechanisms are not known.

7-MX is a nonselective adenosine receptor antagonist that targets four adenosine receptor subtypes (ADORA1, ADORA2A, ADORA2B, and ADORA3), which are widely distributed in various tissues of the eye, including the retina, choroid, and sclera [8]. The RPE plays a critical role in relaying retinal growth signals to the choroids and sclera [9]. One of the most important mechanisms of myopia formation is the transfer of the visual signals affected by myopia from the neural retina to the RPE, where fluid exchange allows the transmission of molecular signals from the retinal and choroid layers to the sclera, followed by scleral remodeling [5]. Animal studies have shown that myopia is associated with changes in the retinal dopamine (DA) level [10]. DA is believed to be a retinal neurotransmitter involved in the signaling cascade that controls vision-guided eye growth. Mammalian and non-mammalian model studies have indicated that DA plays a critical role in the development of myopia [11]. 7-MX inhibits the expression of ADORA1 and ADORA2s in human RPE cells [12], which may change the expression of DA or other neurotransmitters that are associated with myopia. Adenosine and DA interact in the central nervous system, and the existence of ADORA2A-DRD2 heterodimers in the striatum has been suggested [13].

However, whether ADORA2A-DRD2 heterodimers are present in human eye tissues has not been demonstrated. Therefore, this study was conducted to investigate whether ADORA2A-DRD2 heterodimers are present in human RPE cells and whether 7-MX may the affect myopia progression through the ADORA2A-DRD2 pathway and the changes in the expression of signaling molecules during cellular signal transduction after 7-MX treatment to provide clues for the identification of effective drug targets and the development of drugs for controlling myopia.

Materials and Methods

Chemicals and Antibodies and siRNAs

7-MX (CAS: 552-62-5) was purchased from Aladdin (Shanghai, China), and the antibody against DRD2 (cat. sc-5303) was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against ADORA2A (cat. no. 51092-1-AP), ERK1/2 (cat. no. 112957-1-AP-1), phospho-ERK1/2 (pERK1/2) (cat. no. 28733-1-AP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were acquired from Proteintech (Wuhan, Hubei, China), and siRNAs specific for human ADORA2A and DRD2, which were used to suppress the expression of these molecules, were purchased from GenePharma Co., Ltd (Suzhou, China). The sequences of the siRNAs were as follows: ADORA2A sense and ADORA2A antisense – 5′-GGGAGCCAUGGAUAGUGCUTT-3′; 5′-AGCA-CUAUCCAUGGCUCCCTT-3′; DRD2 sense and DRD2 antisense – 5′-CCGUUAUCAUGAAGUCUAATT-3′; 5′-UUAGAC-UUCAUGAUAACGGTT-3′. Nonsilencing siRNA sense and antisense – 5′-GCGACGAUCUGCCUAAGAUdTdT-3′ and 5′-AUCUUAGGCAGAUCGUCGCdTdT-3′ – were used as a negative control.

Cell Culture and Transfection

ARPE-19 cells (human retinal pigment epithelial cells), HeLa cells (human cervical carcinoma cells), and SK-N-SH cells (human neuroblastoma cells) were grown in DMEM containing 10% fetal bovine serum (Analysis Quiz, Beijing) and 1% antibiotics. 7-MX (which has low solubility in water) was dissolved in dimethyl sulfoxide (DMSO) and further diluted with complete medium to the final concentration. All cells were purchased from Meisen Cell Technology Co., Ltd (Zhejiang, China). The cells were grown to 70% confluence before a variety of compounds were added. Treatment was carried out in complete medium containing 10% serum, and cells were collected at the indicated time points. For siRNA interference, cells were grown in the appropriate medium without antibiotics until they reached 50–60% confluence and transfected using GP-transfect-Mate (GenePharma, Suzhou, China) according to the manufacturer's protocol. Protein samples were collected for Western blot analysis 48 h after transfection.

Protein Extraction and Western Blotting

The harvested cells were homogenized and lysed in radioimmunoprecipitation assay buffer containing phosphatase inhibitors and protease inhibitors. The protein concentration was determined using BCA protein assay kits. After collection and denaturation, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA) by wet transfer. The membranes were blocked for 1 h with 5% skim milk and incubated overnight at 4°C with the following specific primary antibodies: DRD2 (Santa Cruz, sc-5303, 1:200 dilution) and ADORA2A (Proteintech, 51092-1-AP, 1:500 dilution). The next

day, the membranes were washed three times at 15-min intervals with Tris-buffered saline Tween and incubated with diluted secondary antibodies (1:2000, ZSGB-BIO, Beijing, China) for 1 h at room temperature. The membranes were washed with Tris-buffered saline Tween three times, developed with an enhanced chemiluminescent kit (New Cell & Molecular Biotech, Suzhou, China), and visualized with a multiplex fluorescent imaging system (BLT, GelView 6000Plus, Guangzhou, China).

Immunofluorescence

RPE cells were subcultured on cover glasses in 12-well plates until they reached 70–80% confluence and fixed with 4% paraformaldehyde for 40 min. Next, the cells were washed three times with PBS containing 0.1% Tween-20 for 5 min each, blocked for 30 min in quick blocking buffer (Beyotime Biotechnology, Shanghai, China), and incubated with anti-DRD2 (Proteintech, 22022-1-AP, 1:50 dilution) and anti-ADORA2a (Santa Cruz, sc-365235, 1:20 dilution) antibodies diluted in blocking buffer at 4°C overnight. Cells incubated in PBS without primary antibodies were used as a negative control. The next day, antibody-treated and negative control samples were washed three times with PBS containing 0.1% Tween-20 for 5 min each and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:200) (Abcam, Cambridge, UK) for 3 h at room temperature. Images were captured using a fluorescence microscope (Olympus, Lake Success, NY, USA).

Coimmunoprecipitation

Coimmunoprecipitation and RPE cell processing were performed according to the instructions of the Thermo Scientific Pierce Co-IP Kit (cat. no. 26149). Anti-DRD2 (Santa Cruz Biotechnology, mouse, sc-5303), and anti-ADORA2A (Proteintech, rabbit, 51092-1-AP) antibodies were used.

Analysis of Cell Viability by the CCK-8 Assay

Cell viability was measured by the CCK-8 assay (Dojindo, WTS, Japan). RPE cells were plated in 96-well plates at a density of 5×10^4 cells/well in 100 μL of complete culture medium. After overnight culture, the medium was replaced with drug-free complete medium or complete medium containing 7-MX or dimethyl sulfoxide. After 48 h of treatment, 10 μL CCK-8 solution was immediately added to each well. Subsequently, the cells were incubated for 1.5 h at 37°C. Cell viability was determined by measuring the optical density at 450 nm in a microplate spectrophotometer.

Flow Cytometry

RPE cells were treated with the indicated compounds, trypsinized, harvested (keeping all floating cells), washed with PBS buffer, incubated with fluorescein isothiocyanate-labeled annexin V and propidium iodide (PI) according to the instructions of the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences Inc., NJ, USA), and analyzed by flow cytometry (FACSAria, Becton Dickinson, Franklin Lakes, NJ, USA). Annexin V-positive and PI-negative cells were considered apoptotic cells, whereas PI-positive cells were considered necrotic cells.

Statistical Analysis

The distribution of the data was first assessed by the Kolmogorov-Smirnov test. Normally distributed data were analyzed using parametric tests (Student's *t* test for two groups or one-way ANOVA for three or more groups), and if the data were not normally distributed, a nonparametric test (the Mann-Whitney test for two groups or the Kruskal-Wallis test followed by Dunn's posttest for three or more groups) was used. GraphPad Prism version 9.00 for Windows (GraphPad Software, La Jolla, CA, USA) was used for analysis. All results were obtained from at least three independent experiments. A *p* value <0.05 was considered statistically significant.
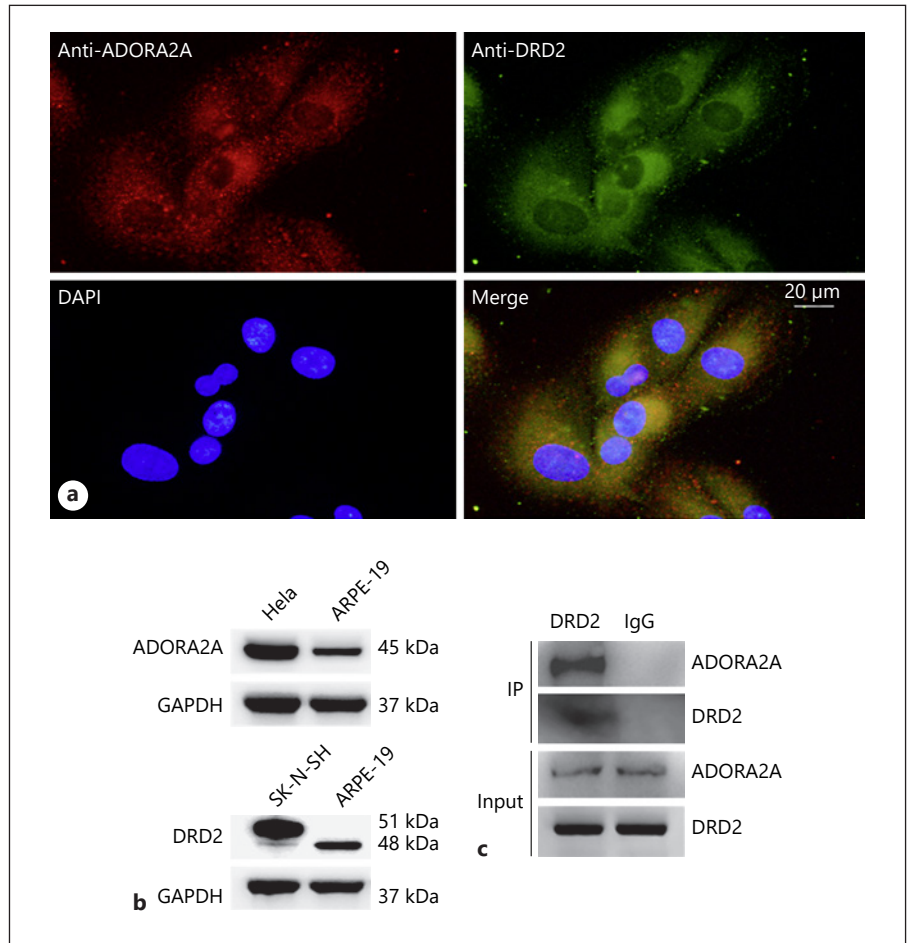
Results

The Presence of ADORA2A-DRD2 Heterodimers in Human RPE Cells

ADORA2A and DRD2 were expressed in ARPE-19 cells (Fig. 1a, b). There were two subtypes of DRD2 with molecular weights of 48 KDa and 51 KDa. The 51-KDa subtype of DRD2 was predominantly expressed in SK-N-SK cells (positive control), while the predominantly expressed subtype in RPE cells was the 48-KDa subtype (Fig. 1b). To gain insight into the relationship between adenosine signaling and DA function in terms of RPE cells, we examined the possible interaction between ADORA2A and DRD2. As indicated by immunofluorescence and coimmunoprecipitation assays, DRD2 and ADORA2A are expressed and colocalized in human RPE cells (Fig. 1a, c).

Changes in ADORA2AR and DRD2 Protein Levels after 7-MX Treatment

Immunoblotting showed that protein expression of DRD2 was increased after downregulation of ADORA2A protein expression by siRNA treatment for 48 h (Fig. 2a). Furthermore, as indicated by immunoblotting, DRD2 protein expression was elevated, and ADORA2A protein expression was decreased in RPE cells after treatment with 50 μmol/L 7-MX for 48 h (Fig. 2b). The selection of 7-MX drug concentrations for 50 μmol/L for this study was first based on previously reported safe concentrations in the literature [12, 14] and further tested for safety of this concentration by CCK-8 and flow cytometry assays as reported below (online suppl. Fig. S1; see www.karger.com/doi/10.1159/000525563 for all online suppl. material).

Additionally, immunofluorescence analysis showed that DRD2 protein expression was lower in the negative control RPE cells group (Fig. 2c). However, there was decreased ADORA2A protein expression and significantly increased DRD2 protein expression in RPE cells treated with 50 μmol/L 7-MX for 48 h (Fig. 2d) under the same

Fig. 1. DRD2 and ADORA2A can form heterodimers. **a** RPE cells were subjected to immunostaining using rabbit anti-D2R and mouse anti-ADORA2A antibodies and analyzed by fluorescence microscopy. The scale bar indicates 20 μm. **b** Expression of ADORA2A in RPE cells (positive control: HeLa cells). Expression of DRD2 in RPE cells (positive control: SK-N-SH cells). **c** Immunoprecipitation revealed the presence of ADORA2-DRD2 heterodimers in RPE cells.

cell volume, the same experimental time, and the same fluorescence microscopy (40X) conditions of observation as the negative control group.

7-MX Affects DRD2-Mediated Signal Transduction

The immunoblotting results showed that the expression of DRD2 was significantly reduced after administration of DRD2 siRNA alone for 48 h and significantly increased after treatment with 7-MX alone for 48 h (Fig. 3a). In addition, when cells were simultaneously treated with DRD2 siRNA and 7-MX for 48 h, the expression of DRD2 was restored to almost normal levels.

To further assess DRD2-mediated signal transduction, phosphorylated ERK (pERK) and ERK levels in RPE cells were analyzed. We further investigated the changes in the levels of pERK1/2 and ERK1/2, which are potential downstream markers of DA receptors used to assess D2R-mediated signal transduction. The anti-ERK antibody recognizes the region of the ERK protein around Thr202 and Tyr204, and the anti-phospho-ERK antibody recognizes

ERK protein phosphorylated at these amino acids. Western blot analyses of RPE cells revealed that both antibodies labeled bands at 44 KDa (ERK1/pERK1) and 42 KDa (ERK2/pERK2) and that pERK2 was more strongly phosphorylated than pERK1 (Fig. 3b). The protein expression levels of both ERK and pERK were increased after the treatment with 7-MX for 48 h (Fig. 3b).

Effects of 7-MX and siRNAs in RPE Cell Proliferation and Apoptosis

The effects of 7-MX on cell growth were assessed in human RPE cells incubated with 50 μmol/L 7-MX. The CCK-8 assay showed that there was no statistically significant change in growth at 24, 48, or 72 h (online suppl. Fig. S1a). The results of flow cytometry showed that the percentages of apoptotic and necrotic cells were not significantly different between the group incubated with 50 μmol/L 7-MX for 48 h and the negative control group (online suppl. Fig. S1b, c). In addition, the CCK-8 assay showed that at the assayed concentrations, the siRNAs
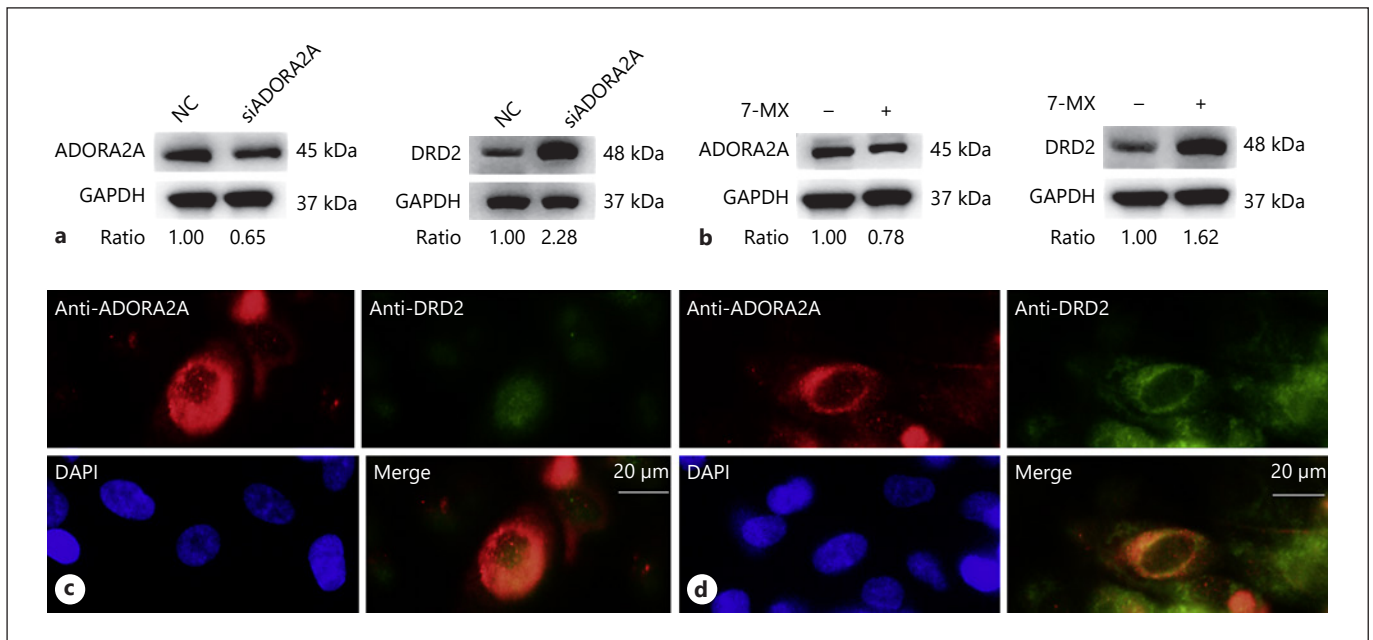
Fig. 2. a Cells were treated with siRNA of ADORA2A for 48 h, and then cell lysates were prepared and subjected to immunoblotting using the indicated antibodies. GAPDH was used as a loading control. **b** Cells were treated with 7-MX for 48 h, and then cell lysates were prepared and subjected to immunoblotting using the indicated antibodies. GAPDH was used as a loading control. **c** RPE cells were subjected to immunostaining using rabbit anti-D2R and mouse anti-A2aR antibodies and analyzed by fluorescence microscopy (×40). The scale bar indicates 20 μm. **d** RPE cells were treated with 50 μmol/L 7-MX for 48 h, subjected to immunostaining using rabbit anti-D2R and mouse anti-ADORA2A antibodies, and analyzed by fluorescence microscopy (×40). The scale bar indicates 20 μm.
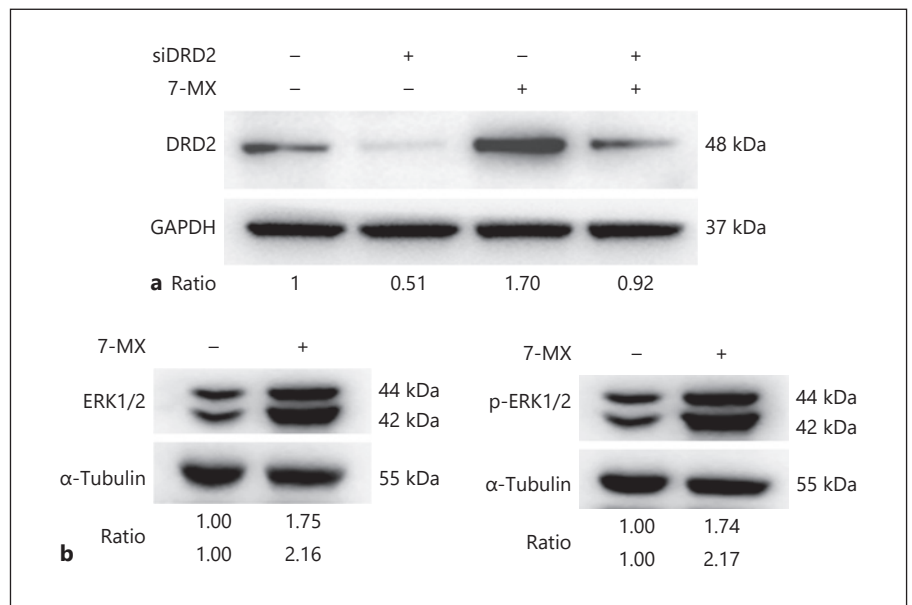

Fig. 3. a Human RPE cells were treated with or without siRNA targeting DRD2 and 50 μmol/L 7-MX for 48 h. DRD2 protein levels were measured by immunoblotting. **b** Changes in ERK1/2 and pERK1/2 protein levels upon 7-MX treatment.

did not have an effect on the proliferation of RPE cells ($p > 0.05$) (online suppl. Fig. S1d). The results of flow cytometry showed that the percentages of apoptotic and necrotic cells were not significantly different between the groups treated with siRNAs and the negative control group ($p > 0.05$) (online suppl. Fig. S1e, f).

Discussion/Conclusion

In the central nervous system, the DRD2 receptor and ADORA2A are colocalized in the globus pallidus and exist as heterodimers [13]. This receptor-receptor interaction plays an important role in the molecular regulation of the central nervous system. The evidence for striatal ADORA2A-DRD2 heterodimers has led to a new perspective on molecular mechanisms involved in schizophrenia and Parkinson's disease [15].

7-MX has been shown to be safe and effective in controlling the progression of myopia, but the mechanism is not yet clear. In the present study, it was demonstrated that ADORA2A-DRD2 heterodimers are present in human RPE cells. Activation of ADORA2A in the ADORA2A-DRD2 heterodimer inhibits the D2 receptor pathway [13].

The DRD2 receptor has been shown to be involved in metabolic and endocrine disorder-induced diseases such as diabetes and in an inflammatory model of depression [16]. In the pancreas, DA is reported to function through the DRD2 receptor as an inhibitory signal against insulin secretion [17]. In addition, DA is an important neurotransmitter in the retina that mediates diverse functions, including development, visual signaling, and refractive development. Retinal DA D2 receptor has been shown to be involved in myopia. Reduced retinal DA levels are observed in eyes deprived of sharp vision by either form deprivation myopia or lens-induced myopia [18]. Inducing high retinal DA levels by intravitreal application of a DA agonist can suppress the development of both FDM and LIM [19]. The RPE layer of the retina, which is located between the neuroretina and the choroid, plays an important role in mediating the transfer of molecular signals from the retina to downstream tissues, including the choroid and sclera. As RPE cells are tightly connected, signaling molecules involved in eye growth and development must be indirectly transmitted through certain receptors on the RPE surface to achieve downstream cascade reactions.

Previous studies have shown that 7-MX inhibits expression of ADORA1 and ADORA2s in human RPE cells [12], which may alter the expression of DA or other neurotransmitters associated with myopia. In this study, it was found that knockdown of ADOR2A by siRNA elevated D2 protein expression. The same phenomenon was observed in RPE cells treated with 7-MX as ADORA2A protein expression was reduced and DRD2 expression was increased in these cells. When the protein expression of DRD2 was knocked down by siRNA in RPE cells, the protein level of DRD2 was restored to the control level by incubation with 7-MX for 48 h. ADORA2A may therefore be an upstream regulator of DRD2 in RPE cells. In ADORA2A-DRD2 heterodimers, ADORA2A acts as an inhibitor of the D2 receptor. 7-MX is an inhibitor of ADORA2A; thus, inhibition of ADORA2A in RPE leads to increased protein expression of DRD2. This means that 7-MX may affect the behavior of RPE cells through the ADORA2A-DRD2 receptor pathway.

In retinal ganglia and photoreceptors, the ERK signaling pathway is one of the downstream cascades of DA receptors that control myopia progression [20, 21]. pERK levels can be used to assess D2R-mediated signal transduction [22]. DA activates the ERK signaling pathway via D2 receptors [23, 24]. A previous study indicated that as a downstream component of the DA signaling pathway, the ERK signaling pathway compiles the multiple effects of transduction cascades and D2 receptors expressed in chicken photoreceptors [20]. In this study, the protein expression of ERK1/2 and pERK1/2 was significantly increased, which indicates a change in the levels of upstream DA receptors. The enhancement of ERK1/2 phosphorylation and increase in ERK1/2 protein expression both suggest increased expression of upstream DA receptors.

Preclinical studies have assessed the cytotoxicity of 7-MX, which may be classified as a globally harmonized system category 5 drug with an LD50 > 2,000–5,000 mg/kg. Additionally, a 28-d repeated dose oral toxicity study demonstrated that 7-MX is nontoxic, and this conclusion is further supported by studies of its toxicity in cell lines [14]. When evaluating the cellular toxicity of a drug, % cell viability is a parameter of paramount importance in assessing the cell population's response to a drug. In this study, both the CCK-8 assay and flow cytometry demonstrated that at the experimental concentration of 50 μmol/L used in this study, 7-MX did not affect the proliferation or apoptosis of RPE cells and was safe. A potentiation limitation of this study is that we did not determine the dose-response relationship of 7-MX in RPE cells. However, our focus was identifying the possible mechanisms of action of 7-MX in controlling myopia progression while ensuring that the chosen concentration did not affect the viability of RPE cells.

In conclusion, 7-MX affects the behavior of RPE cells through the ADORA2A-DRD2 receptor pathway. In ADORA2A-DRD2 receptor heterodimers, ADORA2A inhibits the DRD2 receptor. 7-MX suppresses ADORA2A to prevent ADORA2A-mediated inhibition of the DRD2 receptor pathway and increase DRD2 receptor pathway activity, thus inhibiting inhibit myopia progression.

Statement of Ethics

This study was conducted in accordance with the tenets of the Declaration of Helsinki and approval from the Institutional Ethics Committee of the Peking University People's Hospital (No. 2018PHC059).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

Author Contributions

Yuzhuo Fan and Kai Wang wrote the manuscript. Yuzhuo Fan performed the experiments; Yuzhuo Fan and Jiarui Li conducted data processing and analysis. Kai Wang, Lvzhen Huang, and Mingwei Zhao provided the idea for this study and revised the manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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