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Adenosine Receptor Distribution in Rhesus Monkey Ocular Tissue

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Abstract

Adenosine receptor (ADOR) antagonists, such as 7-methylxanthine (7-MX), have been shown to slow myopia progression in humans and animal models. Adenosine receptors are found throughout the body, and regulate the release of neurotransmitters such as dopamine and glutamate. However, the role of adenosine in eye growth is unclear. Evidence suggests that 7-MX increases scleral collagen fibril diameter, hence preventing axial elongation. This study used immunohistochemistry (IHC) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) to examine the distribution of the four ADORs in the normal monkey eye to help elucidate potential mechanisms of action. Eyes were enucleated from six Rhesus monkeys. Anterior segments and eyecups were separated into components and flash-frozen for RNA extraction or fixed in 4% paraformaldehyde and processed for immunohistochemistry against ADORA1, ADORA2a, ADORA2b, and ADORA3. RNA was reverse-transcribed, and qPCR was performed using custom primers. Relative gene expression was calculated using the C_t method normalizing to liver expression, and statistical analysis was performed using Relative Expression Software Tool. ADORA1 immunostaining was highest in the iris sphincter muscle, trabecular meshwork, ciliary epithelium, and retinal nerve fiber layer. ADORA2a immunostaining was highest in the corneal epithelium, trabecular meshwork, ciliary epithelium, retinal nerve fiber layer, and scleral fibroblasts. ADORA2b immunostaining was highest in corneal basal epithelium, limbal stem cells, iris sphincter, ciliary muscle, ciliary epithelium, choroid, isolated retinal ganglion cells and scattered scleral fibroblasts. ADORA3 immunostaining was highest in the iris sphincter, ciliary muscle, ciliary epithelium, choroid, isolated retinal ganglion cells, and scleral fibroblasts. Compared to liver mRNA, ADORA1 mRNA was significantly higher in the brain, retina and choroid, and significantly lower in the iris/ciliary body. ADORA2a expression was higher in brain and retina, ADORA2b expression was higher in retina, and ADORA3 was higher in the choroid. In conclusion, immunohistochemistry and RT-qPCR indicated differential patterns of expression of the four adenosine receptors in the ocular tissues of the normal non-human primate. The presence of ADORs in scleral fibroblasts and the choroid may support mechanisms by which ADOR antagonists may prevent myopia. The potential effects of ADOR inhibition on both anterior and posterior ocular structures warrant investigation.

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Abstract

Keywords

Adenosine receptors; ocular distribution; Rhesus monkey; myopia control; immunohistochemistry

1.0 Introduction

Myopia, especially high myopia, is a growing problem in many areas of the world (Bar Dayan et al., 2005; Holden et al., 2016; Lin et al., 2004; Vitale et al., 2008; Williams et al., 2015; Woo et al., 2004). Myopia is a risk factor for sight-threatening conditions such as glaucoma, retinal detachments, and macular choroidal neovascularization (Koh et al., 2016; Mitchell et al., 1999; Saw et al., 2005; Shen et al., 2016; Wong et al., 2014). Maculopathy secondary to high myopia is a leading cause of irreversible legal blindness in many Asian countries (Hsu et al., 2004; Iwase et al., 2006; Wong et al., 2014). In the United States alone, the annual direct cost of correcting distance vision is over \$3.8 billion, and the total financial burden to the U.S. economy is over \$35 billion yearly (Frick, 2012; Rein et al., 2006; Vitale et al., 2006). Thus strategies to prevent myopia or to slow its progression would be beneficial for both individual patients and national health care systems.

Myopia is most commonly attributed to increased axial length of the eye. Axial growth ultimately occurs through tissue remodeling that increases the creep rate of the sclera, allowing the tough, fibrous connective tissue to stretch and axially elongate the eye (Phillips et al., 2000; Siegwart and Norton, 1999). Myopic axial elongation results in a reduction in scleral collagen content and a decrease in collagen fibril diameter (McBrien et al., 2000). The discovery that 7-methylxanthine (7-MX), a metabolite of caffeine (1,3,7-trimethylxanthine), increased scleral collagen fibril diameter (Trier et al., 1999) suggested that methylxanthines could protect against myopia progression. Subsequently, 7-MX was shown to significantly reduce myopic progression in rabbits, guinea pigs, macaques, and children (Cui et al., 2011; Hung et al., 2018; Nie et al., 2012; Trier et al., 2008). Though the precise mechanism by which 7-MX alters scleral collagen and slows myopia progression is unknown, caffeine and its metabolites are known to block adenosine receptors non-selectively at low or physiological concentrations (10–50 μM) (Daly et al., 1983; Fredholm, 1985). At much higher concentrations, caffeine inhibits phosphodiesterases (100–10,000 μM) and activates ryanodine receptor channels (300–2,000 μM) (Fredholm, 2011). Since it is debatable whether such high concentrations are physiologically relevant, adenosine receptor blockade is the most likely mechanism of action.

Adenosine is a ubiquitous neuromodulator present in various forms in all cells of the body, and adenosine receptors (ADORs) are membrane-bound G-protein coupled receptors. Four adenosine receptors have been identified, A1, A2a, A2b, and A3, each of which is coupled to different constellations of G-proteins (Fredholm et al., 2001). As a general rule, ADORA1 and ADORA3 are coupled to Gi/o proteins to initiate inhibitory intracellular signaling, leading to decreases in intracellular cyclic adenosine monophosphate (cAMP) and inhibition of protein kinase A. ADORA2a and ADORA2b are coupled to G_{s/olf} to initiate excitatory or facilitative intracellular signaling (Alfinito et al., 2002; Epperson et al., 2009; Freund et al., 1994; Gao et al., 1999; Gerwins and Fredholm, 1995; Jockers et al., 1994; Kull et al., 2000; Olah, 1997; Oliveira and Correia-de-Sa, 2005; Palmer et al., 1995). ADORA1, ADORA2a, and ADORA3 are activated at physiologic concentrations of adenosine, but ADORA2b is only activated at higher concentrations, such as those resulting from inflammation, ischemia, or tissue damage (Fredholm, 2007). ADORs form homo- and hetero-dimers with other ADORs, P2 adenine receptors, dopamine receptors, and adrenergic receptors (Chandrasekera et al., 2013; Ciruela et al., 1995; Ferré et al., 1992; Fredholm et al., 2011; Ginés et al., 2000; Gracia et al., 2013; Komatsu et al., 2012; Orru et al., 2011; Sattin et al., 1975).

ADORs accomplish a variety of physiological effects in different tissues. In neurons, ADORs regulate the release of neurotransmitters such as dopamine and glutamate (Ferré et al., 1992; Fredholm and Dunwiddie, 1988; Ginsborg and Hirst, 1972; Gonçalves et al., 2015; Quarta et al., 2004; Stella et al., 2003). In smooth muscle, ADORA1 and ADORA2b regulate proliferation and differentiation (Dubey et al., 1998b; Gerwins and Fredholm, 1995). In cardiac fibroblasts, ADORA2b inhibits collagen formation (Dubey et al., 1998a). However, ADORA2a stimulates collagen production in other tissues, such as skin and liver (Chan et al., 2006a; Chan et al., 2006b). Treatment with caffeine generally reduces fibrosis, for example, in the skin, liver and lung (Donejko et al., 2014; Tatler et al., 2016; Wang et al., 2014). In the eye, adenosine increases blood flow in the choroid and optic nerve head, suggesting that ADORs on vascular cells promote vasodilation (Polska et al., 2003). Adenosine signaling via ADORs plays a role in the pathologic vascularization seen in retinopathy of prematurity and diabetic retinopathy (Chen et al., 2017; Vindeirinho et al., 2016). ADORA1 and ADORA2b in the corneal endothelium regulate hydration and promote deturgescence (Tan-Allen et al., 2005), and ADORA1 and ADORA2 receptors regulate fluid transport across the retinal pigment epithelium (Kawahara et al., 2005). The functions of ADORs in scleral fibroblasts have not been fully elucidated; however, knockout of ADOR2a reduces collagen fibril diameter in mouse sclera, and activation of ADORA2a in cultured human scleral fibroblasts stimulates expression of collagen type I, III, and V mRNA (Zhou et al., 2010).

Though studies have investigated the localization of different adenosine receptors in discrete parts of the eye in different species, systematic studies encompassing the entire eye are lacking in species used in myopia research. A summary of previous studies reporting ADOR distribution can be seen in Table 1. One study examined the expression of all four ADORs in the entire rat eye (Kvanta et al., 1997), but species-specific differences between the rat and larger species have been noted: specifically, ADORA3 is expressed in human and guinea pig sclera, but not in rat sclera (Cui et al., 2008; Cui et al., 2010; Kvanta et al., 1997); and

ADORA1 is expressed in human and monkey photoreceptors, but not in rat photoreceptors (Braas et al., 1987). The Rhesus monkey (*Macaca mulatta*) has been used extensively in myopia research (Bradley et al., 1999; Fernandes et al., 2003; Hung et al., 2018; Qiao-Grider et al., 2007, 2010; Smith, 2013; Smith and Hung, 1999; Tigges et al., 1990), but to date no study has examined the distribution of ADORs in the Rhesus eye. The retina of a related species, the Cynomolgus monkey (*Macaca fascicularis*) was found to contain ADORA1 in the retinal nerve fiber layer, retinal ganglion cells, the inner plexiform layer, the inner nuclear layer, and in photoreceptors (Braas et al., 1987); but no other ocular tissues were examined, and no other ADORs were investigated. This study sought to examine the distribution of the ADORs in the anterior and posterior segments of normal Rhesus monkeys using immunohistochemistry (IHC) and reverse-transcription quantitative polymerase chain reaction (RT-qPCR) to begin to understand potential mechanisms by which adenosine receptor blockers slow the progression of myopia and to identify the structures most likely to be affected by ADOR inhibition.

2.0 Materials and methods

2.1 Tissue dissection

Ocular tissue from six normal Rhesus monkeys, ages 150 days (n = 2), 4 yrs (n = 2), 6 yrs (n = 2), was utilized for these experiments. Animals were sacrificed with injection of a pentobarbital sodium/phenytoin sodium (390mg/50mg) cocktail, and eyes were enucleated immediately. Eyes were dissected into anterior chambers and eyecups. The anterior chamber of one eye per animal was separated into components and each flash-frozen separately for RNA extraction. The anterior chamber of the fellow eye was fixed whole in 4% paraformaldehyde (Sigma, St. Louis, MO) in 0.1 M phosphate buffer (J.T. Baker, Phillipsburg, NJ) and was processed for IHC. Eyecups were dissected into 5 petals. Two petals were separated into layers and flash-frozen separately for RNA extraction, and the remaining petals were fixed in paraformaldehyde and processed for immunohistochemistry, as described below. Tissue samples were kept at -80°C until use.

Additionally, liver and brain (striatum) of four Rhesus monkeys were harvested, and flash-frozen in liquid nitrogen for RNA extraction. Brain tissue was used as a positive control tissue in which all four ADORs are known to be expressed abundantly. Since ADORs are ubiquitously expressed, no true negative control tissue exists; however, liver is known to express ADORs at very low levels; therefore, liver was chosen as a quasi-negative control tissue.

2.2 Immunohistochemistry

For ADOR immunohistochemistry, ocular tissue (cornea, iris, ciliary body, lens, retina, choroid, and sclera) was fixed in 4% paraformaldehyde overnight, and infiltrated with sucrose (Sigma, St. Louis, MO) in sodium phosphate buffer (J.T. Baker, Phillipsburg, NJ) 5%, 10%, and 15% solutions for 1 hour each, 20% overnight, then a 1:1 solution of 20% sucrose:optimum cutting temperature (OCT) freezing compound (Sakura Finetek, Torrance, CA) for 1 hour.

Tissue was positioned in molds (Sakura Finetek, Torrance, CA) with 1:1 20% sucrose:OCT compound with orientation marked, and flash-frozen in liquid nitrogen. Sections (10µm thick) were cut on a cryostat (Leica Biosystems, Buffalo Grove, IL) onto coated slides (Hydrophilic Plus, Bio SB, Santa Barbara, CA) and stored at -20°C. Slides were post-fixed with 4% paraformaldehyde for 10 minutes, rinsed with deionized water (dH₂O) for 5 minutes, treated with 1% NaBH₄ (Sigma, St. Louis, MO) for 2 minutes, washed with dH₂O x3, washed with Hank's Balanced Salt Solution (HBSS without phenol red, Sigma, St. Louis, MO) x3, and blocked (10% goat serum, 0.5% Triton X-100, 1% fish gelatin, 5% BSA in HBSS, all from Sigma, St. Louis, MO) for 1 hour. Primary antibodies were ADORA1, ADORA2a, ADORA3, (Sigma, St. Louis, MO) and ADORA2b (Thermo Fisher Scientific, Waltham, MA), each used at 1:100 and applied for 2 nights at 4°C. See Table 2 for antibody information. For each run, one slide was incubated in blocking buffer instead of primary antibody as a no-primary control to assess for non-specific staining of the secondary antibody. Slides were washed in HBSS x3, and secondary antibody (goat anti-rabbit Alexa fluor 488, 1:200, Molecular Probes, Life Technologies, Eugene, OR) was applied for 1 hour at room temp. Slides were washed in HBSS x3 and dH₂O x3, and coverslipped with Prolong Diamond (Life Technologies, Eugene, OR) mounting medium. Slides were allowed to dry overnight, then coverslips were sealed with clear nail polish. Slides were imaged on a fluorescent inverted microscope (Olympus IX71 with Q Capture Pro software, Olympus, Waltham, MA).

2.3 Primer design

Rhesus macaque genomic and mRNA sequences were obtained from the University of California at Santa Cruz (UCSC) Genome Browser for validated and provisional genes and from Ensembl for predicted genes. Alternative splicing information was obtained from Ensembl. To maximize detection, each sequence was examined to find regions contained in all transcripts of a single gene. RT-qPCR Primer information is provided in Table 3. ADORA2a had three non-overlapping transcripts; therefore, primer pairs had to be designed for each transcript separately. Discontinuous mega-BLAST (National Center for Biotechnology Information, National Institute of Health) was used to find undesirable regions of homology with other genes. All ADOR sequences had high homology with each other in all regions spanning introns, thus primers were designed in the non-homologous 3' portion of the final exon for each gene. This necessitated DNase digestion of RNA samples prior to reverse-transcription, although DNase digestion causes significant degradation of RNA integrity. Primers for reference genes were designed to span an intron. Primers were designed using Primer3 (UT, UMassMed) and tested *in silico*: primer pairs were excluded if In-Silico PCR (UCSC) showed no products or more than one product or if OligoAnalyzer (Integrated DNA Technologies, Skokie, IL) showed homodimers or heterodimers with a $\Delta G < -7$ kcal/mol or hairpins with melting temperatures $>40^{\circ}\text{C}$. Primers were ordered from Integrated DNA Technologies (Skokie, IL). The efficiency of each primer pair was determined by generating standard curves using serial dilutions of Rhesus brain cDNA. Amplification specificity was determined by generating dissociation curves and by running PCR products on 1.5% agarose gels (Phenix, Candler, NC). For each gene, the primer pair that showed the best combination of efficiency, amplification consistency, tight dissociation curves, and tight bands at the expected size was chosen for use. The primers selected each

had a dynamic range of four orders of magnitude. Reference genes had the additional requirement that they had to amplify at approximately equal levels in all tissues used in this study. Of five reference genes tested (*B2m*, *HMBS*, *HPRT1*, *HSP90AB1*, and *YWAZ*), only two (*B2m*, *HSP90AB1*) met all requirements. See Table 3 for primer information.

2.4 RT-qPCR

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed to detect RNA expression. Frozen tissue was thawed in activated RLT buffer (Qiagen, Hilden, Germany) and homogenized (LabGen 125, Cole-Parmer, Vernon Hills, IL). RNA was extracted (RNeasy kit, Qiagen, Hilden, Germany) with on-column DNase digestion, and concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA quality was analyzed with a Bioanalyzer (Agilent, Cedar Creek, TX), and samples with an RNA Integrity Number less than 7.0 were excluded from analysis. RNA was reverse-transcribed (500 ng/reaction) with oligo-dT primers (AffinityScript, Agilent, Cedar Creek, TX), and quantitative PCR was performed in triplicate 25 µl reactions containing 2% of each RT reaction and 300 nM primers with SYBR Green chemistry (Brilliant II, Agilent, 95°C 30 s, 60°C 30 s, 72°C 30 s × 40 cycles) with dissociation curves on an Mx3005P with MxPro software (Agilent, Cedar Creek, TX). PCR products were run on 1.5% agarose gels to verify specificity. RT-minus controls for each sample (RT reactions without reverse transcriptase) showed no amplification.

2.5 Quantitative Analysis

Traditional methods of RT-qPCR analysis (Ct and standard curve methods) assume an experimental design in which gene expression in experimental conditions is compared to gene expression in control conditions. In this study, gene expression was measured in various tissues all from the normal monkey, therefore all samples would be considered “control” samples. This necessitates either use of a non-traditional analysis method, or unconventional use of a traditional method with the caveat that said method is not necessarily appropriate in this instance. Both approaches were utilized in this study.

Non-traditional analysis was performed using Excel (Microsoft) by taking the expression ratio of each target gene to the geometric mean of the reference genes, according to equation 1,

$$\text{Expression Ratio} = \frac{(E_{\text{target}})^{Ct_{\text{target}}}}{\text{Geomean}[(E_{\text{ref}})^{Ct_{\text{ref}}}]}$$
 Equation 1:

where E is primer efficiency ($E = 10[-1/\text{slope}]$) as determined from standard curves, and Ct (Crossing threshold) is the number of amplification cycles required for fluorescence to reach threshold. Because the expression of ADORs was always less than the expression of the reference genes, expression ratios were always between 0 and 1, and most were between 0 and 0.1. Though this method is the most appropriate because it makes no assumptions about control versus experimental samples, this yielded numbers not conducive to statistical

analysis and graphs that were difficult to interpret. For this reason, a modified traditional analysis was also performed.

Modified traditional analysis was performed using Relative Expression Software Tool (REST, Multiple Condition Solver Version 2). REST calculates the relative expression ratios of target genes by dividing the expression of the target gene by the expression of the reference gene using equation 2,

$$\text{Normalized Expression Ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}} (\text{control mean} - \text{sample mean})}}{\text{Geomean}[(E_{\text{ref}})^{\Delta CP_{\text{ref}} (\text{control mean} - \text{sample mean})}]}$$

Equation 2:

where CP (Crossing point) is the difference of the control sample mean Ct minus the test sample mean Ct. P-values are determined by REST using Pair Wise Fixed Reallocation Randomization Tests. For this analysis, liver tissue was designated as the control tissue because liver is known to express ADORs at very low levels (Dixon et al., 1996), therefore ADOR expression in other tissues should be higher than their expression in liver and most expression ratios should be positive. It should be noted that designation of liver as control is somewhat arbitrary, however it does allow for comparison of gene expression across tissues.

3.0 Results

3.1 Immunolocalization

Immunostaining of the four adenosine receptor subtypes in Rhesus anterior segment ocular tissues is shown in Figure 1, with relative staining presented in Table 4. In the anterior chamber, the corneal epithelium and limbal rete pegs, which contain corneal stem cells, showed moderate immunoreactivity of ADORA2a and ADORA2b (A2a > A2b), and low to no immunoreactivity to ADORA1 and ADORA3 (A3 > A1). ADORA2b staining was highest in the basal epithelium, which contains mitotically active cells, whereas ADORA2a staining was even across the epithelial layers. ADORA2a staining was also high in stromal keratocytes. All four ADORs showed high immunoreactivity in corneal endothelium, ciliary epithelium, and the ciliary muscle. In the iris sphincter muscle, ADORA1, ADORA2b, and ADORA3 immunostaining were high, and ADORA2a staining was low. No ADOR staining was noted in the iris dilator muscle. In the iris stroma, ADORA1, ADORA2a, and ADORA2b staining was high. In the trabecular meshwork, ADORA1 and ADORA2a staining were high, ADORA2b staining was moderate, and ADORA3 staining was low.

Immunostaining of ADORs in Rhesus posterior segment ocular tissues is shown in Figure 2 and Table 4. In the posterior chamber, all four anti-ADORA antibodies showed high immunoreactivity in nuclei in the ganglion cell layer and in the retinal nerve fiber layer. The outer plexiform layer contained moderate ADORA1, ADORA2b, and ADORA3 staining, but low ADORA2a staining. The outer and inner nuclear layers showed very low to no

staining of all four ADORs. The photoreceptor inner segments showed moderate immunoreactivity of all four ADORs. The retinal pigment epithelium exhibited moderate ADORA2b and ADORA3 staining and low ADORA1 and ADORA2a staining. The choroid showed moderate ADORA2b and ADORA3 staining, low ADORA2a staining, and no ADORA1 staining. A posterior ciliary nerve coursing through the choroid showed high ADORA2a staining. Scleral fibroblasts exhibited high ADORA2a immunostaining, moderate ADORA2b staining, low ADORA3 staining, and no ADORA1 staining. Additionally, scattered scleral fibroblasts contained higher levels of ADORA2b immunoreactivity than adjacent fibroblasts.

3.2 Gene Expression

Mean Crossing thresholds (Cts) of each gene in each tissue are shown in Table 5. The Ct is the number of amplification cycles required for fluorescence to reach a threshold, therefore the lower the Ct, the more abundantly the gene is expressed. Cts above 35 are equivalent to noise from primer dimers and are considered not expressed. All genes tested had Cts lower than 35 and were therefore considered to be expressed in all tissues tested. There were no differences between the three age groups (150 days, 4 years, 6 years). Expression ratios (ERs) of ADOR expression to reference gene expression were calculated for each ADOR for each tissue. Because ADOR expression was consistently much less than reference gene expression, all ERs were less than one, and most less than 0.1. In the liver and iris/ciliary body, all ERs were less than 0.1. In the brain, ADORA2a transcripts one and three (ADORA2aT1, T3) achieved ERs higher than 0.5, transcript two (T2) had an ER of 0.1, and all other ADORs had ERs much less than 0.1. In the retina, ADORA2aT1 and T3 had ERs above 0.1 and all other ADORs had ERs less than 0.1. In the choroid, ADORA2b was the only ADOR to have an ER above 0.1.

Because the above expression ratios are mostly between 0 and 0.1 and are thus difficult to interpret, an alternative analysis was performed to generate normalized expression ratios (NERs), normalizing expression of each gene in each tissue to its expression in rhesus liver. As explained above, choosing liver as a control tissue is semi-arbitrary; therefore, this method is less appropriate than the above ER method. However, because the methods most commonly used to analyze RT-qPCR results generate NERs, these results are in a more familiar format and are thus more easily interpreted. This method also allows statistical analysis to determine significant differences.

In the brain, normalized expression ratios of ADORA1 and all three ADORA2a transcripts were significantly higher than in liver, and ADORA2b and ADORA3 were not significantly different from liver (Table 6). In the retina, ADORA1, all three ADORA2a transcripts, and ADORA2b were all significantly higher than in liver, and only ADORA3 was not significantly different from liver. In the choroid, ADORA1 and ADORA3 were higher than in liver, but ADORA2aT1 was significantly lower than in liver. ADORA2aT2 and T3 and ADORA2b were not significantly different between choroid and liver. In the iris and ciliary body, ADORA1 was significantly lower than in liver, and all other ADORs were not significantly different from liver.

4.0 Discussion

In this study, adenosine receptor distribution was characterized in the Rhesus monkey anterior and posterior ocular tissue. We found that ADORA1 was localized to the iris sphincter muscle, trabecular meshwork, ciliary epithelium, and retinal nerve fiber layer. ADORA2a was localized to the corneal epithelium, trabecular meshwork, ciliary epithelium, and retinal nerve fiber layer, and also present in scleral fibroblasts. ADORA2b was localized to the corneal basal epithelium, limbal stem cells, iris sphincter, ciliary muscle, ciliary epithelium, the choroid, isolated retinal ganglion cells and scattered scleral fibroblasts. ADORA3 was localized to the iris sphincter, ciliary muscle, ciliary epithelium, the choroid, isolated retinal ganglion cells, and scleral fibroblasts.

Expression of ADORs in the retinal ganglion cell layer is consistent with previous studies, though their function is not entirely understood. ADORs in nervous tissue are known to modulate neurotransmitter release. In the central nervous system, ADORA1 activation has been shown to modulate potassium and calcium currents, thereby resulting in presynaptic inhibition (Dunwiddie and Masino, 2001). Adenosine applied to purified retinal ganglion cell cultures or to intact rat retina preparations decreases glutamate receptor-induced calcium influx (Hartwick et al., 2004). Stimulation of ADORA3 may play a neuroprotective role by reducing Ca^{+2} overload in retinal ganglion cells (Zhang et al., 2006b).

In another study, authors found that ADORA1 were present on the intrinsically photosensitive retinal ganglion cells (ipRGCs), and application of adenosine to rat retinas reduced light-evoked spiking of ipRGCs through suppression of a cAMP-mediated pathway (Sodhi and Hartwick, 2014). The ipRGCs are a small subset of mammalian retinal ganglion cells that are directly sensitive to light through the activation of melanopsin (see review by Lucas, 2013). The ipRGCs are primarily involved in non-image forming functions, including regulation of pupil size and circadian rhythm entrainment. The ipRGCs have synaptic connections to bipolar cells, as well as amacrine cells, and have been shown to reciprocally modulate dopamine, which has known roles in myopic eye growth (Hannibal et al., 2017; Ostergaard et al., 2007). Localization of ADORs to the ganglion cell layer in Rhesus monkeys, along with previous evidence for a role of adenosine in ipRGC signaling, may implicate this pathway as a potential modulator for myopic eye growth. Further investigation of ipRGC function and eye growth in animals treated with adenosine receptor agonists and antagonists may help to clarify these interactions.

ADORs modulate blood flow, most likely by stimulating vasodilation (Polska et al., 2003). Adenosine infusion increases choroidal blood flow in cats (Portellos et al., 1995), though which ADOR is responsible for this effect is not fully established. A recent study in humans showed that acute oral administration of caffeine causes thinning of the choroid from 30 minutes to 4 hours after intake (Altinkaynak et al., 2016). Conversely, a recent study in Rhesus monkeys showed that long-term (150 day) oral administration of 7-MX resulted in chronic thickening of the choroid, accompanied by a decrease in experimental myopia (Hung et al., 2018). In the CNS, acute and chronic effects of caffeine differ significantly, presumably because chronic administration of caffeine causes differential regulation of ADOR1 and ADORA2a (Brito et al., 2016; Karcz-Kubicha et al., 2003). The choroid is a

critical component in the vision-dependent cascade of structural changes in myopic eye growth (Nickla, 2006). However, the mechanism by which the thickness of the choroid is modulated has not been elucidated. With immunostaining, ADORA2a, A2b and A3 were localized to the choroid. The observation of ADORs in the choroid itself leaves open the possibility that methylxanthines may target the choroid directly.

Here, ADORA2a, ADORA2b, and ADORA3 expression was localized to the sclera. In rabbits, chronic treatment with 7-MX resulted in an increase in scleral collagen content and increased collagen fibril diameter in the posterior sclera (Trier et al., 1999). A previous study found all four types of ADORs in human scleral fibroblasts (Cui et al., 2008). These findings may suggest a direct role of methylxanthines in preventing scleral remodeling in response to myopia-inducing visual stimuli in experimental animals.

In this study, ADOR immunoreactivity was noted in all three layers of the cornea. ADORA2b expression was especially interesting, as it was localized in the basal layer of the corneal epithelium and in the limbal rete pegs, the source of corneal stem cells. Adenosine and ADORA2a play a critical role in the differentiation of stem cells (Kang et al., 2018; Katebi et al., 2009), but the role of ADORA2b in stem cells is unknown. The cornea and sclera are fibrous connective tissues composed of collagen fibers and specialized fibroblasts ("keratocytes" in the cornea). The primary structural difference between the cornea and sclera consists in the arrangement of the collagen fibers: regularly-arranged collagen lamellae confer transparency, whereas irregularly arranged fibers confer opacity. Because of this similar composition, it is possible that the changes previously noted in sclera following methylxanthine treatment may occur in the cornea as well. Thus it would be relevant to evaluate whether methylxanthine treatment causes changes in corneal thickness, corneal stem cell viability, and corneal collagen fibril diameter. In Rhesus monkeys, oral 7-MX treatment did not alter corneal power or curvature (Hung et al., 2018), indicating that any changes to the cornea are optically unimportant. However, if methylxanthines are shown to increase corneal thickness or corneal collagen fibril diameter, they may also prove useful in non-surgical treatment of corneal ectasias such as keratoconus.

ADOR immunoreactivity was high in parasympathetically-innervated smooth muscle, namely the iris sphincter muscle and the ciliary muscle. No ADOR immunoreactivity was noted in the sympathetically-innervated iris dilator muscle. Immunoreactivity against all four ADORs was noted in the ciliary epithelium, which produces aqueous humor. These findings suggest that ADOR antagonists could have effects on pupil size, amplitude of accommodation, and/or intraocular pressure (IOP). ADORA1 agonists decrease IOP by increasing the activity of collagen-degrading metalloproteinases, increasing outflow facility through the trabecular meshwork (Li et al., 2018). ADORA1 inhibition by A1-selective antagonists raises IOP. Conversely, ADORA2a/ADORA3 inhibition lowers IOP by modulating aqueous humor production (Avila et al., 2002; Crosson, 1995; Crosson and Gray, 1994, 1996; Wang et al., 2010). Since methylxanthines are non-selective ADOR inhibitors, the pro- and anti-hypertensive effects of ADOR inhibition appear to balance out. No differences in IOP or pupil size were noted between Rhesus monkeys given oral 7-MX versus age-matched controls given a normal diet (Hung et al., 2018). Also, in a small study, topical caffeine had no effect on IOP in patients with primary open angle glaucoma or ocular

hypertension (Chandra et al., 2011). This indicates that though IOP can be affected by ADOR inhibition, methylxanthines appear to have a better safety profile than non-methylxanthines. Interestingly, the matrix metalloprotease/tissue inhibitor of metalloproteinase (MMP/TIMP) system, known to be modulated by ADORA1, is implicated in the scleral remodeling seen in form deprivation myopia (Liu et al., 2017), which may suggest a link between IOP control and myopia. The effect of methylxanthines on amplitude of accommodation has not been assessed.

For a therapy to be beneficial, its therapeutic effects should outweigh its non-therapeutic effects; therefore it is important to be mindful of potential unwanted side effects. Because ADORs are found throughout the body, modulation of ADOR function as an anti-myopia therapy could have effects in systemic tissues. However, no adverse systemic effects of oral 7-MX administration in Rhesus monkeys (Hung et al., 2018) or children (Trier et al., 2008), have been reported. Caffeine is well known to cause systemic effects such as increased heart rate, blood pressure and wakefulness (Huang et al., 2005; Turnbull et al., 2017). The different effects of 7-MX and caffeine indicate that the adverse effects of each methylxanthine should be evaluated individually. Systemic side effects may be reduced by topical application. In the small intraocular pressure study mentioned above, instillation of 1% (52.5 mM) caffeine eye drops three times a day for 6 days had no effect on either blood pressure or subjective assessment of well-being (Chandra et al., 2011).

One identified side effect of caffeine has the potential to become a therapy for cataract, the leading cause of blindness world-wide (Pascolini and Mariotti, 2012). The observation that caffeine added to culture medium prevents opacification of rat lenses *in vitro* after UVB irradiation (Varma et al., 2008) led to several *in vivo* studies demonstrating that pre-treatment with caffeine significantly reduced or eliminated opacification from multiple cataractogenic stimuli, primarily via caffeine's ability to scavenge reactive oxygen species (Kronschlager et al., 2013; Varma and Hegde, 2010; Varma et al., 2010a; Varma et al., 2010b). Because this is unrelated to ADOR inhibition, the crystalline lens was not evaluated in this study.

Limitations of this study include lack of data on scleral gene expression. Grinding techniques strong enough to homogenize the tough tissue of the monkey sclera typically destroyed its delicate RNA content, therefore we were unable to analyze scleral ADORA mRNA expression. Another limitation is the small number of subjects (n = 6), since the expense of primate research renders large studies unfeasible. Another limitation was that our RT-qPCR analysis used only two reference genes instead of the recommended four (Bustin et al., 2009). Only two of the five reference genes tested satisfied our criteria of specificity, consistent amplification efficiency, and equal expression in the target tissues. Few studies have identified reference genes in *Macaca mulatta*, (Ahn et al., 2008; Noriega et al., 2010), and none have assessed their expression in the eye, indicating a need for further studies identifying ocular reference genes in the Rhesus monkey.

5.0 Conclusions

Adenosine receptors were localized to all tissues of the Rhesus monkey eye, though the expression patterns vary between the four receptor subtypes. The presence of ADORs in scleral fibroblasts suggests a mechanism by which ADOR antagonists may increase scleral stiffness to prevent myopia. However, the presence of ADORs in such locations as the cornea, limbal rete pegs, the trabecular meshwork, the iris sphincter, and the ciliary muscle suggests that inhibition of adenosine receptors may affect more than just the sclera. Further studies should focus on characterizing the anti-myopiagenic effects of methylxanthines, as well as potential non-therapeutic effects of long-term methylxanthine treatment.

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Abbreviations

ADOR	adenosine receptor
cAMP	cyclic adenosine monophosphate
CBE	ciliary body epithelium
cDNA	complimentary DNA
dH₂O	deionized water
GCL	ganglion cell layer
HBSS	Hank's balanced salt solution
IHC	immunohistochemistry
INL	inner nuclear layer
IPL	inner plexiform layer
ONL	outer nuclear layer
OPL	outer plexiform layer
PR-IS	photoreceptor inner segments
RGC	retinal ganglion cell
RNFL	retinal nerve fiber layer
RPE	retinal pigment epithelium

RT-qPCR reverse-transcriptase quantitative polymerase chain reaction

TM trabecular meshwork

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Highlights

- All adenosine receptors (ADOR) subtypes were found in Rhesus monkey ocular tissue
- ADORs were found in cornea, iris, ciliary body, retina, choroid and sclera
- ADOR antagonists may prevent myopia through binding on sclera and choroid

Figure 1:

Immunohistochemistry showing ADOR immunoreactivity in anterior segment structures. A) Corneal epithelium and stroma: ADORA1 was only in layer of sloughed tissue, not in living cells; ADORA2a was present in both epithelium and stromal keratocytes; ADORA2b and ADORA3 were present in epithelium and not in stroma. B) Limbal rete pegs: ADORA1 was present only in sloughing layer; ADORA2a, ADORA2b and ADORA3 were present throughout the thickness of the peg; ADORA2b was highest in the basal epithelium, where corneal stem cells reside. C) Corneal endothelium (arrow), iris sphincter (arrowhead), and iris dilator (double arrowhead): ADORA1 was high in corneal endothelium, the iris stroma, and the iris sphincter muscle, but was not found in the iris dilator muscle; ADORA2a was high in the corneal endothelium (which in this specimen abuts the anterior iris), and the iris stroma, but moderate in the iris sphincter muscle and not found in the iris dilator muscle; ADORA2b was high in the corneal endothelium, the iris stroma, and the iris sphincter muscle, but was not found in the iris dilator muscle; ADORA3 was high in the corneal endothelium, and the iris sphincter muscle, and low in the iris stroma, and was not found in the iris dilator muscle. D) Trabecular meshwork and ciliary body muscle: ADORA1 was high in the trabecular meshwork and ciliary muscle; ADORA2a was high in the trabecular meshwork and moderate in the ciliary muscle; ADORA2b and ADORA3 were high in the ciliary muscle and low in the trabecular meshwork. E) Ciliary processes and epithelium: of

all four ADORs was high in the ciliary epithelium; Immunoreactivity in the ciliary process cores was largely from non-specific binding of the secondary antibody to red blood cells.

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Figure 2:

Immunohistochemistry showing ADOR distribution in posterior segment structures. A) Retina: ADORA1 immunoreactivity was mainly in the ganglion cell layer (GCL), outer plexiform layer (OPL), and photoreceptor inner segments (PR-IS); ADORA2a immunoreactivity was mainly in the retinal nerve fiber layer surrounding the ganglion cells, the outer nuclear layer (ONL), and PR-IS; ADORA2b and ADORA3 immunoreactivity were highest in isolated retinal ganglion cells and the retinal nerve fiber layer, followed by the OPL and PR-IS. B) Retinal pigment epithelium (RPE) and choroid: ADORA1 immunoreactivity was nearly absent in the RPE and choroid; ADORA2a immunoreactivity was nearly absent in the RPE but was moderate in the choroid and high in posterior ciliary nerves (asterisk); ADORA2b immunoreactivity was high in the RPE and choroid; ADORA3 immunoreactivity was high in the RPE but mostly absent in the choroid. C) Sclera and orbital tissue: ADORA1 immunoreactivity was absent in the sclera and high in orbital tissue; ADORA2a immunoreactivity was moderate in scleral fibroblasts and high in orbital tissue; ADORA2b and ADORA3 immunoreactivity were high in scattered scleral fibroblasts and moderate in remaining scleral fibroblasts and orbital tissue. * Posterior ciliary nerve.

Figure 3:
Non-traditional expression ratio analysis of gene expression of ADORs relative to reference genes arranged by tissue.

Figure 4:
Traditional Ct analysis of ADOR gene expression in target tissue compared to their expression in liver tissue.

Table 1:

Previous investigations on ocular ADOR distribution

Author, Year	Species	Technique(s)	ADOR Distribution
(Goodman et al., 1983)	Mice, rats	Radio-labeled adenosine binding	Enucleation of one eye depleted ADORs in contralateral superior colliculus, suggesting ADORs in RGC axons.
(Braas et al., 1987)	Cynomolgus monkey, rat, Guinea pig, human	Radio-labeled agonist binding	All species: A1 in RNFL, RGCs, IPL Monkey, human: A1 also in INL and photoreceptors Studied only A1 only in retina
(Friedman et al., 1989)	Human	RPE cultures; agonist/antagonist binding	RPE has A2a but not A1 Studied only RPE
(Blazynski, 1990)	Rabbit, mouse	Radio-labeled agonist binding	A1: inner retina, IPL A2: RPE, PR Studied only retina and RPE
(Blazynski, 1993; Blazynski and McIntosh, 1993)	Bovine	Radio-labeled agonist binding	RPE has A2a and/or A2b Studied only RPE
(McIntosh and Blazynski, 1994)	Bovine	Radio-labeled agonist binding	A2a and A2b on rod outer segment plasma membranes Studied only photoreceptors
(Kvanta et al., 1997)	Rat	In situ hybridization	A1: CBE, GCL>INL A2a: CBE, INL > GCL, ONL > RPE A2b: CBE A3: none Looked at entire rat eye
(Tan-Allen et al., 2005)	Bovine	Cultured corneal endothelium, RT-PCR, Western blot, cAMP Ca^{+2} Cl^{-} assays, membrane potentials	Corneal endothelium has A1 and A2b Studied only corneal endothelium
(Zhang et al., 2006a)	Rat	Retrograde-labeling of RGCs, laser capture microdissection, immunopanning, RT-PCR	A3 is expressed in RGCs Studied only A3 only in RGCs
(Dong et al., 2007)	Human	Primary cultures of RPE, RT-PCR, immunocytochemistry	A2a is co-expressed with dopamine D2 receptors in RPE Studied only A2a and D2 only in RPE
(Cui et al., 2008)	Human	Primary cultures of scleral fibroblasts, Western blot, Immunohistochemistry of frozen scleral sections	A1: in nucleus A2a: on side of cytoplasm A2b: in nucleus and cytoplasm A3: weak in cytoplasm Studied only scleral fibroblasts
(Cui et al., 2010)	Guinea pig	Immunohistochemistry, Western blot in form-deprived versus normal eyes	A1: retina >> choroid, sclera A2a: retina > choroid, sclera A2b: choroid > retina > sclera A3: barely detectable in sclera > retina, choroid Form deprivation myopia decreased A1, increased A2b Studied only posterior segment
(Wan et al., 2011)	Human	D407 RPE cell line, RT-PCR, Western blot, confocal fluorescence microscopy	A1: in nucleus, perinucleus, cytoplasm A2a: in one side of perinucleus and cytoplasm A2b: strong in nucleus, perinucleus, cytoplasm A3: weak in cytoplasm Studied only RPE cell line
(Brito et al., 2016)	Chick	In ovo caffeine; WB, IHC	Caffeine exposure in ovo increased retinal A1, decreased A2a Studied only retina

Table 2:

Antibody information

Antibodies	Cat. No.	Source	Species	Dilution
α -ADORA1	A268	Sigma	Rabbit	1:100
α -ADORA2a	A269	Sigma	Rabbit	1:100
α -ADORA2b	AB1589P	Sigma	Rabbit	1:100
α -ADORA3	PA5-36350	ThermoFisher	Rabbit	1:100
α -Rabbit-AlexaFluor488	A11008	Molecular Probes	Goat	1:200

Table 3:

RT-qPCR Primer information

Gene	Gene status	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)	Efficiency	Slope, Y intercept	R ²
ADORA1	Provisional	GGAGAGGCAG AAAGGGTAGG	ACCTCATGGCT GTTTGAAGC	116	107.0%	–3.164, +23.69	0.993
ADORA2aT1	Predicted	AGGCAGCAAG AACCTTTCAA	GGTACTCCCTC CACTCACCA	189	98.0%	–3.370, +21.49	0.992
ADORA2aT2	Predicted	ATGATGCTGC TGCCAGAAC	TCCTCACAGGA GCCAAGTG	114	99.8%	–3.326, +23.80	0.974
ADORA2aT3	Predicted	CAGTGATGGA GAGCAGGTCA	TCCATGGATCA TCAGGACAC	187	91.9%	–3.533, +22.08	0.991
ADORA2b	Predicted	GACACGGCTG GCTTATTCTC	TTCAAGCTGTC AGCGTCATC	196	96.7%	–3.404, +25.52	0.968
ADORA3	Validated	TGTCATGGCT GCCTTTATCC	TGTGTCCAAAG AATCAGAGGG	212	102.2%	–3.271, +26.74	0.963
B2m	Provisional	CACCCCAAT GAAAAAGATG	CCTCCATGATG CTGCTTACA	106	99.3%	–3.339, +21.36	0.999
HSP90AB1	Validated	TCTTCAGGCTT TTCCCTTGA	GGTTCTTCTGCT GCCACTTC	107	96.5%	–3.409, +20.62	0.998

Table 4:

Relative immunostaining intensity for antibodies against ADORA1, ADORA2a, ADORA2b, ADORA3, and no-primary control for Rhesus monkey ocular tissue

Antibody	Cornea	Limbic rete pegs	Iris	Trabecular meshwork	Ciliary muscle	Ciliary epithelium	Retina	RPE	Choroid	Sclera
ADORA1	-	-	+++	++	+++	+++	+++	-	-	-
ADORA2a	++	+	++	++	++	+++	+++	-	++	++
ADORA2b	++	++	++	+	+++	+++	+++	++	++	++
ADORA3	+	+	+	+	+++	+++	+++	++	-	++
Control	-	-	-	-	-	-	-	-	-	-

Table 5:

Mean Crossing Threshold (Ct) for ADORs and reference genes in Liver, Brain, Retina, Choroid, and Iris/ciliary body.

Gene	Liver	Brain	Retina	Choroid	Iris/CB
ADORA1	27.326	23.576	25.027	21.784	28.666
ADORA2aT1	27.318	21.278	22.934	27.963	25.761
ADORA2aT2	28.283	23.408	24.805	26.084	27.981
ADORA2aT3	29.182	22.145	24.435	28.884	27.504
ADORA2b	27.945	26.347	24.626	27.589	25.545
ADORA3	28.248	28.037	29.269	24.044	28.577
B2m (ref)	20.329	21.35	22.479	19.842	19.564
HSP90AB1 (ref)	21.512	20.164	19.938	20.638	19.883

Table 6:

Normalized 2-log expression ratio of Rhesus monkey ocular tissues compared to liver, * and ** indicate significance at $p < 0.05$ and < 0.001 , respectively

Gene	Brain	Retina	Choroid	Iris/CB
ADORA1	3.79 ± 1.17 $p = 0.02^*$	2.72 ± 0.99 0.02^*	5.15 ± 2.31 0.01^*	-2.58 ± 0.04 0.03^*
ADORA2a T1	5.72 ± 2.58 0.02^*	4.56 ± 1.73 0.01^*	-1.29 ± 0.10 0.13	0.34 ± 0.14 0.91
ADORA2a T2	4.73 ± 2.08 0.02^*	3.78 ± 1.60 0.001^{**}	1.53 ± 0.73 0.19	-0.87 ± 0.14 0.70
ADORA2a T3	6.48 ± 2.93 0.03^*	4.77 ± 2.25 0.004^{**}	-0.39 ± 0.12 0.71	0.42 ± 0.18 0.82
ADORA2b	1.42 ± 0.55 0.06	3.55 ± 1.47 0.001^{**}	-0.32 ± 0.11 0.75	1.18 ± 0.62 0.54
ADORA3	0.066 ± 0.029 0.90	-0.73 ± 0.13 0.35	3.60 ± 1.85 0.04^*	-1.50 ± 0.09 0.36