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## Mediation of Cannabidiol anti-inflammation in the Retina by Equilibrative Nucleoside Transporter and A<sub>2A</sub> Adenosine Receptor\*

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### Abstract

**Purpose**—Cannabidiol (CBD), a non-psychotropic, non-toxic compound has been shown to block diabetes- and endotoxin-induced retinal damage. However, the protective mechanism of this anti-inflammatory cannabinoid is not completely understood. The goal of this study is to determine the role of adenosine signaling in retinal inflammation and its potential modulation by CBD.

**Methods**—The adenosine receptor (AR) subtypes expressed in rat retinal microglial cells was assessed by quantitative real-time RT-PCR. AR function was determined via *in vitro* and *in vivo* inflammatory models. Microglial cells or rats were treated with or without lipopolysaccharide (LPS) in the presence or absence of adenosine, adenosine receptor agonists/antagonists, or CBD. Adenosine uptake and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) release in cells or in retinas were determined.

**Results**—Our results showed that A<sub>2A</sub>ARs are abundantly expressed in rat retinal microglial cells. Upon treating the cells or rats with LPS, activation of the A<sub>2A</sub>AR was the most efficient in mediating AR agonist- or CBD-induced TNF- $\alpha$  inhibition. CBD inhibited adenosine uptake via equilibrative nucleoside transporter 1 and synergistically enhanced adenosine's TNF- $\alpha$  suppression upon LPS treatment.

**Conclusions**—These results suggest that the activated A<sub>2A</sub>AR in the retinal microglial cells plays a major role in anti-inflammation in the retina, and that CBD anti-inflammatory effects are linked to adenosine uptake inhibition.

### Keywords

adenosine; cannabidiol; immunosuppression; lipopolysaccharide; microglia; retina

### INTRODUCTION

Retinal neuroinflammation is the common pathway in infectious and autoimmune retinitis, glaucoma, age-related macular degeneration, ischemic retinopathy in diabetic retinopathy and retinal vein occlusions.<sup>1-6</sup> Cytokine expression is induced via lipid mediators such as platelet activating factor and leukotrienes, oxygen-derived free radicals, complement components, and debris from dead or dying cells.<sup>7</sup> Proinflammatory cytokines, including tumor necrosis factor-

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$\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, and IL-6, are generated in tissues under infectious and ischemic insult, which serves to promote wound healing and clearance of necrotic tissue. However, excessive or chronic inflammation, especially in the context of neural tissue, leads to propagation of tissue injury and is the target for therapy in a variety of retinal diseases.<sup>4</sup>

Activated retinal microglia and macrophages are implicit in the pathogenesis of sight-threatening disease but are difficult to study *in vivo* due to their migratory behavior and morphological transformation.<sup>8</sup> Cultured retinal microglia activated by lipopolysaccharide (LPS) have been used to study microglial behavior as an *in vitro* model of neuroinflammation.<sup>9</sup> Studies have shown that following neuroinflammation through brain injury, activated microglia release proinflammatory cytokines such as TNF- $\alpha$  which instigates a cascade of inflammation and neurodegeneration.<sup>10</sup> We hypothesize that activated microglia in the diseased retina play a role similar to that of activated microglia in the brain.

Adenosine, a purine nucleoside, regulates a variety of physiological functions by stimulating specific extracellular receptors.<sup>11</sup> Under adverse conditions such as inflammation, adenosine production by damaged neurons is increased and helps to protect tissue against excessive damage.<sup>12</sup> Adenosine delivers potent suppressive effects on virtually all cells of the immune system by interacting with four subtypes of adenosine receptors (ARs), A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>.<sup>13</sup> The Gs protein-coupled A<sub>2A</sub>AR is most widely recognized to attenuate inflammation via a cAMP-mediated pathway, and its activation leads to inhibition of T cell expansion and differentiation, downregulation of neutrophil superoxide production and degranulation, and inhibition of proinflammatory cytokines expression including TNF- $\alpha$ .<sup>12</sup>

However, adenosine usually disappears very rapidly under physiological conditions, in part, due to rapid uptake into adjacent cells and subsequent intracellular metabolism,<sup>14</sup> preventing it from interacting with immune cell adenosine receptors and preventing sustained tissue protection. One way of retarding the disappearance of adenosine is by introducing nucleoside transporter inhibitors.<sup>15</sup>

It has recently been shown that nanomolar concentrations of cannabidiol (CBD), a non-psychoactive and non-toxic cannabinoid, inhibit uptake of adenosine by nucleoside transporters in both murine microglia and RAW264.7 macrophages. Moreover, *in vivo* treatment with a low dose of CBD was reported to decrease serum TNF- $\alpha$  levels in LPS-treated mice; this effect of CBD was reversed by treatment with the A<sub>2A</sub>AR antagonist ZM 241385 and completely absent in A<sub>2A</sub>AR<sup>-/-</sup> mice.<sup>16</sup> These studies demonstrated that CBD enhances adenosine signaling by inhibiting its extracellular removal revealing a non-cannabinoid receptor-mediated mechanism by which CBD decreases inflammation. Our previous work has shown that CBD inhibits glutamate-, LPS- and diabetes-induced retinal inflammation.<sup>5, 17, 18</sup> However, whether CBD inhibits these inflammatory diseases by blocking adenosine uptake has not been studied to our knowledge. The goal of this study was to determine whether adenosine mitigates the release of TNF- $\alpha$  in activated retinal microglial cells and to determine whether CBD curtails inflammation via enhancement of adenosine concentrations.

## METHODS

### Animal Treatment

All animal studies were carried out in accordance with the National Institutes of Health *Guide for the Use and Care of Laboratory Animals*. Male Sprague Dawley rats (200 – 225g) were pretreated for 1 h with CBD or vehicle, dimethylsulfoxide (DMSO), i.p. (CBD or DMSO : Cremophor : saline in a ratio of 1 : 1 : 18). After 30 min, rats were given 8-cyclopentyl-1,3-dipropylxanthine (CPX; Sigma, MO), 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-ylamino]ethyl}phenol (ZM 241385; Tocris Bioscience, Ellisville, MO), or vehicle

i.p. (CPX, ZM or DMSO : Cremophor : saline in a ratio of 1 : 1 : 8). After drug pretreatment, rats were anesthetized with isoflurane and given a single LPS (*Salmonella enterica*; Sigma, MO) injection at 0.35 mg/kg. After 24 hours, rats were killed by decapitation after anesthesia with isoflurane. Retinas and vitreous were collected for assay of total protein by the Bio-Rad DC protein assay, and for TNF- $\alpha$  levels by enzyme-linked immunosorbent assay (ELISA).

### Primary Rat Retinal Microglia Cultures

Microglial cells were isolated from retinas of newborn (within 24 h) rats as described previously with minor modifications.<sup>9, 18</sup> Briefly, retinas were collected and washed twice with ice-cold PBS and digested with 0.125% trypsin at 37° C for 3 min. Trypsin was inactivated by adding culture medium (DMEM/F12 (1:1) with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin). Tissues were triturated with a plastic pipette and washed twice. Cells were filtered through a Nitex nylon mesh (mesh opening 100 microns; Sefar America Inc.), collected by centrifugation, resuspended in culture medium and plated onto 100 cm<sup>2</sup> cell culture flasks at a density of  $2 \times 10^5$  cells/cm<sup>2</sup>. All cultures were maintained in a humidified incubator at 37° C and 5% CO<sub>2</sub> and fed on the third day, then once every 4 days. After 2 weeks, microglial cells were harvested in culture medium by shaking the flasks at 100 rpm for 1.5 h. The cell suspension was centrifuged and the detached cells re-plated in culture medium at designated densities for various experiments. The purity of the microglial cultures was ~98% as determined by immunocytochemical staining analysis for OX42, a macrophage/microglial marker. The morphology of microglia in culture was carefully examined by phase contrast and fluorescent microscopy.<sup>18</sup>

### Quantitative Real-Time RT-PCR

Total RNA was isolated from rat retinal microglial cells using RNAqueous –4PCR (Ambion). Subsequently, 1  $\mu$ g of total RNA was reverse transcribed using a mixture of random and poly-T primers according to the manufacturer's protocol (Invitrogen). Primers were designed for the rat A<sub>1</sub> (FWD, 5'- TCATTGCCTTGGTCTCTG-3'; REV, 5'- GTGGAAGTAGGTCTGTGG-3'), A<sub>2A</sub> (FWD 5'- TGCTTCCATCATGTCCTTGC-3'; REV, 5'- GCCCTTCGCCTTCATACC-3'), A<sub>2B</sub> (FWD, 5'-TTGGCATTGGATTGACTC-3'; REV, 5'- TATGAGCAGTGGAGGAAG-3'), and A<sub>3AR</sub> (FWD, 5'- TGCTTCCATCATGTCCTTGC-3'; REV, 5'- TGCTTCCATCATGTCCTTGC-3') using Beacon Design software (Bio-Rad; Kreckler et al 2006). PCR amplification (in SYBR Green Supermix) was performed using an iCycler iQ thermocycler (Bio-Rad) for 40 cycles of 25 s at 95° C followed by 45 s at an optimized annealing temperature for each AR. The cycle threshold, determined as the initial increase in fluorescence above background, was ascertained for each sample. Melt curves was performed upon completion of the cycles to ensure that nonspecific products were absent. For quantification of AR transcripts, a standard curve plotting cycle threshold versus copy number was constructed for each receptor subtype by analyzing 10-fold serial dilutions of plasmids containing the full-length rat AR clones.<sup>19</sup> AR transcript levels were expressed as copies/50 ng of total RNA.

### Cell Treatment

Microglial cells collected from culture flasks were seeded at a density of  $1 \times 10^5$  cells/well in 96-well plates. One day after seeding, the culture wells were washed with Cellgro Complete (Mediatech, Manassas, VA) and incubated in the same media with various treatments. Cells were pretreated with 1  $\mu$ M CBD (Cayman Chemical, Ann Arbor, MI) or vehicle dimethylsulfoxide (DMSO) for 30 min at 37° C, followed by adenosine (Sigma, MO), AR agonists (all from Sigma) or AR antagonists (all from Sigma except ZM 241385) at the indicated concentrations for 30 min at 37° C, finally treated with 30 ng/ml LPS (*Escherichia coli* OB4:1111; Sigma). After the indicated time course, culture media were collected and

assayed for TNF- $\alpha$  by ELISA. Subsequently, the cells were lysed with 0.4 N NaOH and assayed for total protein by the Bio-Rad DC protein assay.

### Adenosine Uptake Assays

Assays were carried out at 37°C in Earle's normal balanced salt solution (EBSS). Cells seeded at a density of  $5 \times 10^5$  cells/well in 24-well plates were washed once in EBSS and pre-incubated 30 min at 37°C with drug or DMSO vehicle. Uptake began after addition of 0.5  $\mu$ Ci [2- $^3$ H] adenosine (37 MBq; Amersham, UK). Nonspecific uptake was defined as uptake in the presence of 1 mM adenosine, which was added 1 min before [ $^3$ H]adenosine. After 1 min, buffer was rapidly aspirated, and cells were washed once with ice-cold PBS. Cells were solubilized in 0.2 M NaOH/1% SDS, and radioactivity was determined.

### Enzyme-Linked Immunosorbent Assay (ELISA) for TNF- $\alpha$

TNF- $\alpha$  levels in the supernatants of culture media were estimated with ELISA kits (R & D Systems, Minneapolis, MN) per the manufacturer's instructions. Briefly, diluted samples were added to a microplate pre-coated with a monoclonal antibody specific for rat TNF- $\alpha$ . After washing to remove unbound substances, an enzyme-linked polyclonal antibody specific for TNF- $\alpha$  was added. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added. The colored product yielded by the enzyme reaction was measured at 450 nm. The minimum detectable levels for TNF- $\alpha$  with this assay is 5 pg/ml. The sample levels were calculated from a standard curve. Subsequently, the cells were lysed with 0.4 N NaOH and assayed for total protein by the Bio-Rad DC protein assay. TNF- $\alpha$  released was expressed, after adjusted for protein, as a percentage of maximal TNF- $\alpha$  released from vehicle-treated cells. Retinal microglial cells were treated with LPS and culture media were collected at 5, 15, 20 and 30 min and 1, 2, 4, 6, 12 and 24 h after treatment and assayed for TNF- $\alpha$ . Levels of TNF- $\alpha$  began to increase significantly after 4 h of LPS treatment, peaking at the 6th h, then slowly leveled off within 24 h period. The levels of TNF- $\alpha$  in control culture media without LPS treatment did not change throughout the experimental period.<sup>18</sup>

Retinal and vitreous lysates from rat eyes used for TNF- $\alpha$  assay by ELISA was according to the manufacturer's instructions (R&D) with modifications. Pooled retinas and vitreous from each animal were placed in 150  $\mu$ l of lysis buffer [20 mM imidazole HCl, 100 mM KCl, 1 mM MgCl<sub>2</sub>, 1% Triton X-100, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EGTA, 1 mM EDTA, pH 6.8]<sup>20</sup> supplemented with a protease inhibitor cocktail (Sigma) followed by homogenization in a Mini-Bead beater with treated Ottawa sand.<sup>5</sup> The lysate was cleared of debris by centrifugation at 10,000  $\times$  g for 15 minutes (4°C), and 50  $\mu$ l of the supernatant was used for ELISA directly without further dilution. The sample TNF- $\alpha$  levels were calculated from a standard curve, corrected for protein concentration and expressed as a percentage of maximal TNF- $\alpha$  released from vehicle and LPS-treated animals.

### Data Analysis

The results are expressed as mean  $\pm$  SEM. Differences among experimental groups were evaluated by analysis of variance, and the significance of differences between groups was assessed by Fisher's PLSD post hoc test when indicated. Significance was defined as  $P < 0.05$ .

## RESULTS

### AR Expression in Retinal Microglial Cells

We first quantified mRNA expression of the four AR subtypes by real-time RT-PCR. The absolute copy numbers of the AR transcripts were calculated based on standard curves generated with AR cDNA clones.<sup>19</sup> As illustrated in Fig. 1, we detected abundant mRNA

expression of A<sub>2A</sub>AR (940±95 copies/50 ng of RNA) mRNA in rat retinal microglial cells. mRNA expression of A<sub>1</sub>AR, A<sub>2B</sub>AR or A<sub>3</sub>AR mRNA were barely detectable above background levels.

### A<sub>2A</sub>AR Regulation of TNF- $\alpha$ in Retinal Microglial Cells

To identify the AR subtype(s) involved in inhibiting TNF- $\alpha$  release in the retinal microglia in response to LPS stimulation, we examined the effect of the nonselective AR agonist adenosine-5'-*N*-ethylcarboxamide (NECA) in the presence of AR subtype-selective antagonists. The concentrations of each antagonist chosen for this study were based on the affinity and selectivity for the recombinant mouse AR subtypes determined by radioligand binding studies.<sup>19</sup> As shown in Fig 2A, cells pretreated with vehicle showed a 10-fold increase in LPS-induced TNF- $\alpha$  release compared to vehicle-treated control cells. Treatment with NECA at a concentration of 1  $\mu$ M potently inhibited LPS-stimulated TNF- $\alpha$  release (40%). When the cells were pretreated with the A<sub>1</sub>AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (CPX) (100 nM), the A<sub>2B</sub>AR antagonist 8-[4-[(4-Cyanophenyl) arbamoylmethyl] oxy] phenyl]-1,3-di(n-propyl) xanthine hydrate (MRS 1754) (1  $\mu$ M), or the A<sub>3</sub>AR antagonist 3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine-carboxylate (MRS 1523) (10  $\mu$ M), the inhibitory effect of NECA on TNF- $\alpha$  release was not affected. However, this effect was successfully blocked by 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5] triazin-5-ylamino]ethyl} phenol (ZM241385) at concentrations (100 and 500nM) capable of blocking A<sub>2A</sub>ARs. These results suggest that NECA inhibited LPS-induced TNF- $\alpha$  release from rat retinal microglia via the A<sub>2A</sub>AR.

To confirm the role of the A<sub>2A</sub>AR in regulating TNF- $\alpha$  release, we compared concentration-response curves generated with the A<sub>1</sub>AR agonist 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA), the A<sub>2A</sub>AR agonist 2-p-[2-Carboxyethyl]phenethylamino-5'-*N*-ethylcarboxamidoadenosine (CGS 21680), and the A<sub>3</sub>AR agonist N<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA). As revealed in Fig. 2B, treatment with CGS 21680 concentration-dependently inhibited LPS-stimulated TNF- $\alpha$  release. In contrast, IB-MECA and CCPA inhibited TNF- $\alpha$  release, but their potencies were low reflecting their low affinity for the A<sub>2A</sub>AR. Although, it is noticed that the potencies of IB-MECA and CGS 21680 were very similar. This is probably because the A<sub>3</sub>AR-selective IB-MECA can inhibit TNF- $\alpha$  release via the A<sub>2A</sub> and/or A<sub>2B</sub>AR in A<sub>2A</sub>AR-dominant systems.<sup>19</sup> These results suggest a TNF- $\alpha$  release regulatory effect mediated by the A<sub>2A</sub>AR.

### CBD Effects on Adenosine Uptake in Retinal Microglial Cells

CBD has the ability to enhance adenosine signaling through competitive inhibition of adenosine uptake by equilibrative nucleoside transporter 1 (ENT1) in murine microglia and macrophages.<sup>16</sup> To determine whether ENT1, which is *S*-(4-nitrobenzyl)-6-thioinosine (NBMPR) sensitive, is expressed in retinal microglial cells, we demonstrated NBMPR sensitivity in these cells. [<sup>3</sup>H]adenosine uptake assays were carried out in cells in the presence of 0.1  $\mu$ M NBMPR. To specifically examine inward [<sup>3</sup>H]adenosine transport, we examined uptake during the first minute after [<sup>3</sup>H]adenosine addition. As shown in Fig. 3, [<sup>3</sup>H]adenosine uptake was partially inhibited by NBMPR, suggesting the presence of ENT1 in these cells. To determine whether CBD inhibits adenosine uptake in retinal microglial cells via ENT1, we determined the effect of CBD on [<sup>3</sup>H] adenosine transport in the presence of 0, 0.02, 0.1, and 0.5  $\mu$ M NBMPR. NBMPR alone dose-dependently inhibited [<sup>3</sup>H] adenosine transport. CBD at 0.5  $\mu$ M also inhibited [<sup>3</sup>H] adenosine uptake. NBMPR at all levels did not enhance this inhibition, suggesting CBD is competing with NBMPR for ENT1 (Fig. 3). To determine if this inhibitory effect is dependent of known cannabinoid receptors, HU-210, a synthetic cannabinoid agonist which has an affinity for CB<sub>1</sub> and CB<sub>2</sub> receptors in the picomolar range<sup>21</sup> was tested for its ability to decrease [<sup>3</sup>H]adenosine uptake. HU-210 at 1  $\mu$ M did not

inhibit [<sup>3</sup>H]adenosine uptake (Fig. 3). These data suggest an adenosine uptake inhibitory effect of CBD independent of known cannabinoid receptors.

### Adenosine Mediation of CBD Effects on TNF- $\alpha$ in Retinal Microglial Cells

We tested the hypothesis that the TNF- $\alpha$ -inhibiting effect of CBD is mediated through enhancement of adenosine. This was done by comparing concentration-response curves generated with CBD or adenosine alone, then with the two reagents combined. Cells that were pretreated with adenosine alone from 1.5  $\mu$ M to 100  $\mu$ M followed by LPS showed a gradual decrease in TNF- $\alpha$  from 100% to 50% (Fig. 4). Cells that were pretreated with CBD alone from 0.03  $\mu$ M to 1  $\mu$ M followed by LPS showed a more drastic decrease in TNF- $\alpha$  from 100% to 20%. The drastic TNF- $\alpha$ -reducing effect by CBD at or near 1  $\mu$ M may be due to CBD's capability of being an anti-oxidant to scavenge oxygen radicals.<sup>22</sup> Interestingly, cells that were co-treated with adenosine from 6  $\mu$ M to 100  $\mu$ M and CBD from 0.06  $\mu$ M to 1  $\mu$ M showed a decrease in TNF- $\alpha$  that was much more drastic than the effect of either agent alone. This effect is particularly obvious at low concentrations of adenosine and CBD where TNF- $\alpha$ -inhibiting effect was not observed for either reagent alone (Fig. 4). Taken together, these results suggest that by inhibiting adenosine uptake, CBD synergistically enhances adenosine's TNF- $\alpha$  suppression upon LPS treatment.

### Adenosine Mediation of CBD Effects on TNF- $\alpha$ in the Retina

Adenosine uptake inhibitors decrease TNF- $\alpha$  in LPS-treated mice by increasing the amount of endogenous adenosine available to bind the A<sub>2A</sub>AR.<sup>15</sup> Because CBD inhibits adenosine uptake, we hypothesized that the A<sub>2A</sub>AR mediates TNF- $\alpha$  inhibition seen with CBD treatment.<sup>5, 18</sup> Rats were pretreated with CBD (1 mg/kg, i.p.) and with the A<sub>1</sub>AR antagonist CPX (3 mg/kg, i.p.) or the A<sub>2A</sub>AR antagonist ZM 241285 (10 mg/kg, i.p.) before LPS treatment (0.35 mg/kg, footpad). CBD-pretreated rats showed significantly decreased retinal TNF- $\alpha$  levels, compared with vehicle-pretreated rats (Fig. 5). Although this decrease remained unchanged upon co-treatment with CPX, pretreatment with ZM 241385 reversed the effects of CBD on TNF- $\alpha$  (Fig. 5). Neither antagonist alone significantly altered the effect of LPS to increase TNF- $\alpha$  levels (data not shown). These results correlate with the *in vitro* data obtained using microglial cells showing that CBD enhances the ability of adenosine to inhibit LPS-induced TNF- $\alpha$  release via the A<sub>2A</sub>AR.

## DISCUSSION

Inflammation-mediated neurodegeneration is of utmost clinical relevance. Inflammation in neural tissues involves production of reactive oxygen species that stimulate cellular release of proinflammatory cytokines. We studied the mechanisms for the activation of LPS-treated animals and retinal microglial cells as a model to simulate neuroinflammation because in uveitis or diabetes retinal microglia activated by reactive oxygen species release proinflammatory cytokines causing neurodegeneration and vascular permeability.<sup>8</sup> Adenosine has previously been shown to mitigate the proinflammatory cytokine release response in central neural tissue. A more complete understanding of adenosine receptor function in the retina should help develop novel therapeutic approaches to treat retinal disorders that are associated with inflammation.

CBD has been shown to block NMDA-, LPS-, or diabetes-induced retinal damage,<sup>5, 17, 18</sup> but the mechanism of protection is not completely understood. It has recently been demonstrated in macrophage/microglial cell lines and in LPS-treated mice that CBD may function by enhancing adenosine signaling.<sup>16</sup> In the present study, we demonstrate for the first time that CBD functions by a similar mechanism in the retina. In this study, we observed that the A<sub>2A</sub>AR is highly expressed in retinal microglial cells and that activation of this receptor

inhibits TNF- $\alpha$  production in response to LPS. CBD inhibited adenosine uptake in retinal microglial cells and synergistically enhanced adenosine's effect to suppress LPS-induced TNF- $\alpha$  release. CBD has been shown to competitively inhibit adenosine uptake by ENT1, which is NBMMPR-sensitive.<sup>16</sup> We determined that adenosine transport in retinal microglia is partially NBMMPR-sensitive and that the combined effect of adenosine uptake inhibition by NBMMPR and CBD is not additive. This suggests that CBD competitively inhibits adenosine transport via the ENT1 transporter in retinal microglial cells as well. *In vivo*, this inhibition increased the availability of endogenous adenosine to produce anti-inflammatory activity because the effects of CBD on TNF- $\alpha$  production induced by systemic administration of LPS were blocked by an A<sub>2A</sub> receptor antagonist. This mechanism unveils a likely explanation for the anti-inflammatory activity of CBD identified in other rat models.<sup>5, 18</sup>

CBD may mitigate inflammation through other pathways as well as enhancing adenosine signaling. For example, CBD is known to scavenge reactive oxygen species.<sup>22</sup> Therefore, the anti-inflammatory effect of CBD, especially at 0.5 – 1  $\mu$ M or higher concentrations, may be due to its ability to block oxidative stress that causes microglial activation and inflammation.

Adenosine binds to four different subtypes of G-protein coupled receptors (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub> receptors) that are variably expressed in immune cells. Previous studies have shown that AR subtypes except the A<sub>1</sub>AR are expressed in primary microglia and microglia cell lines.<sup>23, 24</sup> In this study, the real-time RT-PCR and pharmacological experiment using AR agonists as well as AR antagonists have shown that A<sub>2A</sub>AR is the most likely candidate for mediating the adenosine effect on TNF- $\alpha$  suppression in retinal microglial cells. The A<sub>2A</sub>AR mediates the suppressive effects of adenosine in macrophages as well as microglial cells.<sup>19</sup> LPS treatment induces macrophage infiltration in the uveitic retina.<sup>18, 25</sup> Therefore, the effects of CBD on TNF- $\alpha$  production in the eye may be due to enhanced A<sub>2A</sub>AR signaling in infiltrated macrophages in the retina. This mechanism highlights the role of macrophages or activated retinal microglia in retinal inflammation.

Drugs that enhance extracellular adenosine signaling have been of clinical interest in treatment of inflammation following myocardial or cerebral ischemia.<sup>26, 27</sup> CBD as an anti-inflammatory drug is an attractive alternative to smoked marijuana because of its lack of psychoactive effects.<sup>28</sup> CBD is known to be non-toxic in humans,<sup>29</sup> which has previously been a problem for other nucleoside inhibitor drugs.<sup>30, 31</sup> Another potential problem with chronic consumption of adenosine transporter inhibitor is the development of tolerance due to cell tolerance to adenosine through receptor desensitization.<sup>32</sup> It is important to determine the long-term effects of CBD use on adenosine receptors.

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## List of Nonstandard Abbreviations

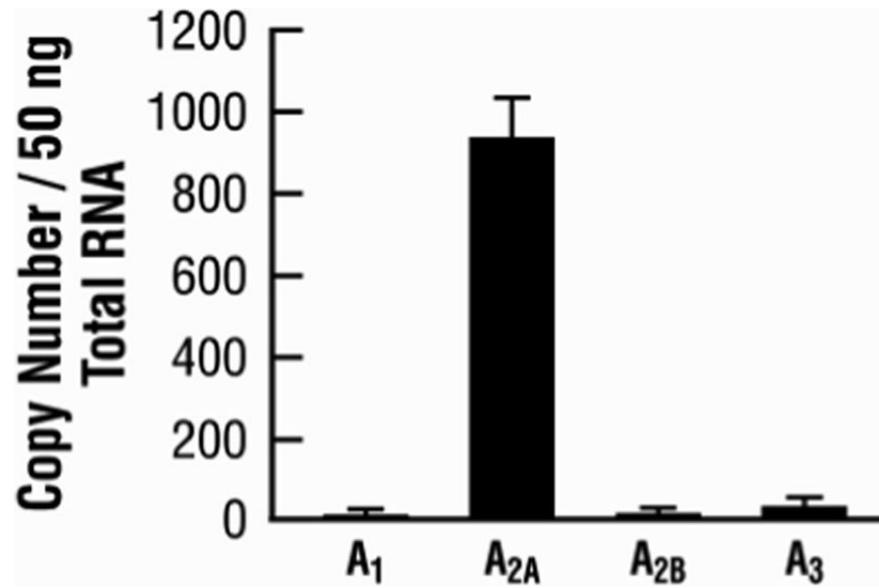
AR, adenosine receptor  
 ENT, equilibrative nucleoside transporter  
 CBD, cannabidiol  
 LPS, lipopolysaccharide  
 TNF- $\alpha$ , tumor necrosis factor- $\alpha$   
 RT-PCR, reverse transcription-polymerase chain reaction  
 FWD, forward  
 REV, reverse

NECA, adenosine-5'-*N*-ethylcarboxamide  
 CPX, 8-cyclopentyl-1,3-dipropylxanthine  
 ZM 241385, 4-{2-[7-amino-2-(2-furyl)[1,2,4]triazolo-[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl} phenol  
 MRS 1754, 8-[4-[(4-cyanophenyl) arbamoylmethyl]oxy]phenyl]-1,3-di(*n*-propyl) xanthine hydrate  
 MRS, 15233-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridine-carboxylate  
 CCPA, 2-chloro-*N*<sup>6</sup>-cyclopentyladenosine  
 CGS 21680, 2-*p*-[2-Carboxyethyl]phenethylamino-5'-*N*-ethylcarboxamidoadenosine  
 IB-MECA, *N*<sup>6</sup>-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide  
 NBMPR, *S*-(4-nitrobenzyl)-6-thioinosine

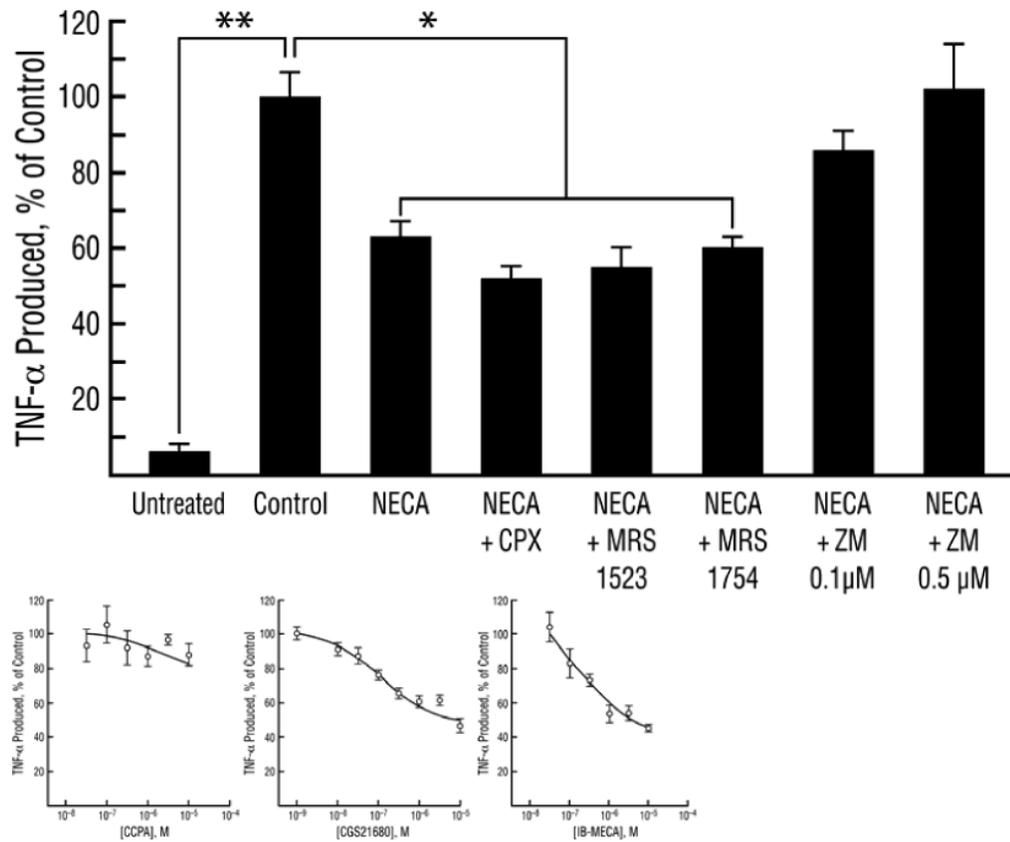
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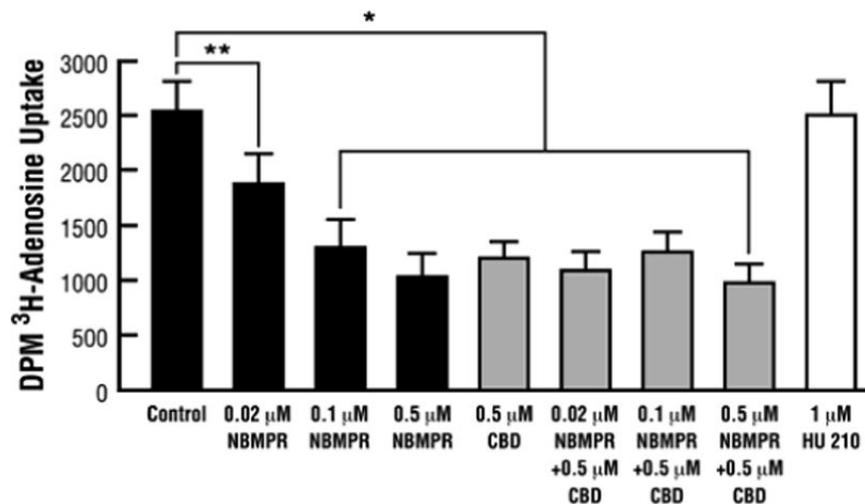


**Figure 1.** Expression of AR subtypes in retinal microglial cells. Expression of the four AR subtypes in rat retinal microglia in the form of mRNA levels as quantified by real-time RT-PCR ( $n=4 - 5$ ).

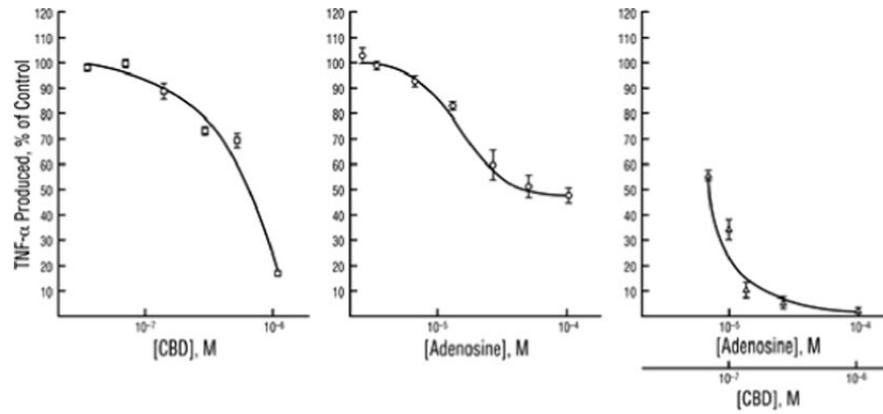


**Figure 2.**

Functional identification of AR subtypes in retinal microglial cells. TNF- $\alpha$  production was determined in LPS-treated (30 ng/ml, 12 hours) retinal microglial cells. **A.** In the presence of nonselective AR agonist NECA (1  $\mu$ M) and subtype-selective AR antagonists for A<sub>1</sub>AR (CPX, 100 nM), A<sub>2A</sub>AR (ZM241385, 100 and 500 nM), A<sub>2B</sub>AR (MRS 1754, 1  $\mu$ M) and A<sub>3</sub>AR (MRS 1523, 10  $\mu$ M). TNF- $\alpha$  levels were determined by ELISA ( $n=4-8$ ). **B.** In the presence of varying concentrations of subtype-selective AR agonists: A<sub>1</sub>AR, CCPA, A<sub>2A</sub>AR, CGS 21680 and A<sub>3</sub>AR, IB-MECA.

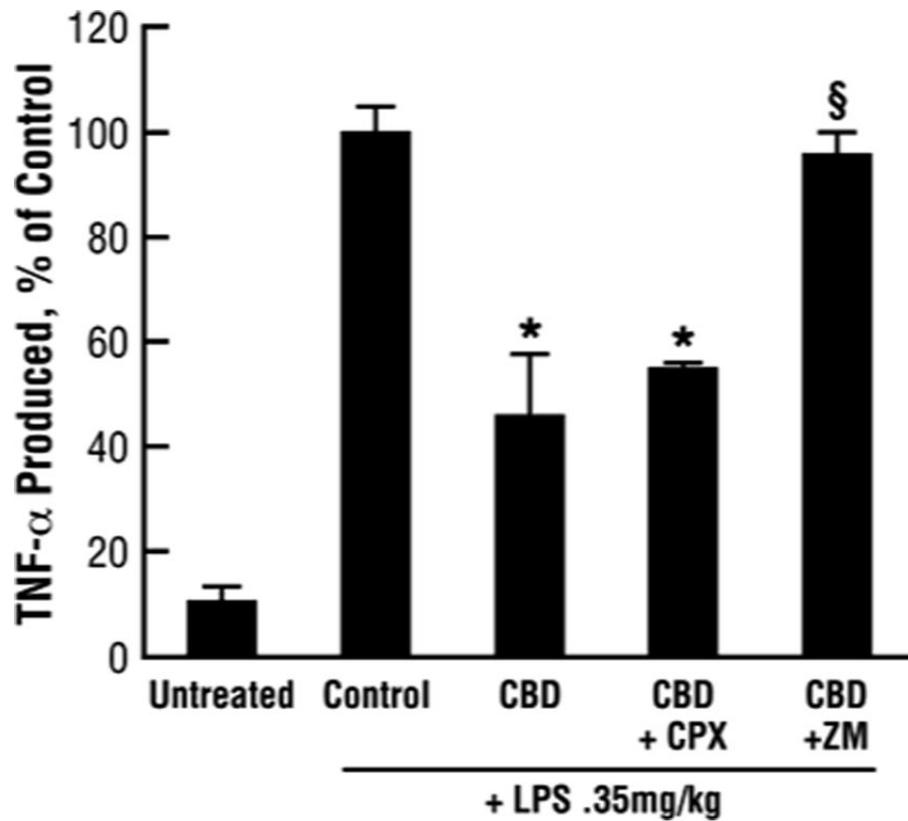


**Figure 3.** CBD or NBMPR, but not HU210, inhibits [<sup>3</sup>H]adenosine uptake in retinal microglial cells. Cells were pretreated with NBMPR (0, 0.02, 0.1, and 0.5 μM), CBD (0.5 μM), NBMPR combined with CBD, or HU210 (1 μM) for 30 min at 37°C, and uptake of 0.5 μCi [<sup>3</sup>H]adenosine over a period of 1 min was assayed. Nonspecific uptake, determined in the presence of 1 mM adenosine, was subtracted from each data point ( $n \geq 5$ ). \*,  $P < 0.005$ , \*\*,  $P < 0.05$ , compared with the vehicle control (one-way ANOVA followed by the post hoc test (Fisher's PLSD)).



**Figure 4.**

CBD enhances adenosine's ability for TNF- $\alpha$  inhibition. Retinal microglial cells were pretreated with CBD (0.03 – 1  $\mu$ M) or vehicle for 0.5 hr. Cells were then treated with vehicle or adenosine (1.5 – 100  $\mu$ M) for another 0.5 hr. Lastly, cells were treated with LPS (30 ng/ml). Twelve hours after LPS treatment, culture media were collected. TNF- $\alpha$  levels were determined by ELISA ( $n=4 - 8$ ).



**Figure 5.** CBD decreases TNF- $\alpha$  via activation of  $A_{2A}$ AR in the retina. Male SD rats were pretreated before LPS injection with a single dose of CBD (1 mg/kg, i.p.) or vehicle. Thirty minutes later, rats were given vehicle, CPX (3 mg/kg, i.p.), or ZM 241385 (ZM, 10 mg/kg, i.p.). Rats were treated with LPS (0.35 mg/kg, footpad) 1 h after CBD injection. Twenty-four hours after LPS treatment, rats were killed, and retina and vitreous were collected. TNF- $\alpha$  levels were determined by ELISA ( $n = 6$ ). \*,  $P < 0.05$  compared with vehicle control; §,  $P > 0.05$  compared with control and  $P < 0.05$  compared with CBD alone (one-way ANOVA followed by the post hoc test (Fisher's PLSD)).