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# IL-6, A1 and A2aR: A crosstalk that modulates BDNF and induces neuroprotection

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ABSTRACT

Several diseases are related to retinal ganglion cell death, such as glaucoma, diabetes and other retinopathies. Many studies have attempted to identify factors that could increase neuroprotection after axotomy of these cells. Interleukin-6 has been shown to be able to increase the survival and regeneration of retinal ganglion cells (RGC) in mixed culture as well as *in vivo*. In this work we show that the trophic effect of IL-6 is mediated by adenosine receptor (A2aR) activation and also by the presence of extracellular BDNF. We also show that there is a complex cross-talk between IL-6, BDNF, the Adenosine A1 and A2a receptors that results in neuroprotection of retinal ganglion cells.



## 1. Introduction

Retinal ganglion cell death is observed in diseases such as glaucoma, diabetes and ischemia [1–3] that cause blindness. It is therefore important to study the mechanisms involved in RGC survival to establish new appropriate pharmacological approaches to treat the different diseases related to RGC death.

In the past few years there has been considerable interest in the contribution of cytokines to neuroprotection. Although constitutive levels of cytokines in the brain are low, their production increases in response to neurotoxic and injury stimuli. One of cytokines that has been the subject of several studies is interleukin-6 (IL-6) [4–6]. Its effects in treatments are still subject to disagreement. Some authors relate IL-6 as a pro-inflammatory cytokine important in several brain pathologies, others have shown that IL-6 protects neurons in culture and is beneficial in several neurodegenerative diseases [4–6]. To date the effect of IL-6 in the brain and the events that could lead to cell death or to neuroprotection remain unclear.

IL-6 is produced by T-helper cells (Th1 and Th2), B cells, macrophages, astrocytes, fibroblasts, osteoblasts, keratinocytes, endothelial and mesangial cells [7]. In the central nervous system (CNS) it is synthesized mainly by microglial cells and astrocytes, but also by neurons [8,9]. IL-6 synthesis can be induced by a variety of stimuli, but its major inducers are lipopolysaccharides (LPS), interleukin-1 (IL-1) and tumour necrosis factor (TNF) [10].

The IL-6 effects occur through a specific binding protein, αIL-6R, that induces the homodimerization of gp130 subunits followed by the activation of cytoplasmic kinases of the Janus (JAK) family. The activation of JAKs then leads to the stimulation of proteins from the STAT family (STAT3 and STAT1) – a family of signal transducers and transcriptor activators. STAT dimers translocate into the nucleus and induce transcription of immediate early genes, such as *junB*, and the rapid activation of a nuclear factor called acute-phase response factor (APRF) [5,11].

Previous work from our laboratory demonstrated the trophic effect of IL-6 on axotomized rat retinal ganglion cells (RGC) kept in culture for 48 h [12]. More recently we showed that IL-6 treatment induced RGC survival depending on the adenosine A1 receptors (A1R) activation and that a transient levels of extracellular BDNF was also important for the IL-6 trophic effect [13]. Studies *in vivo* showed that IL-6 expression increases in retina after injury, suggesting its possible neuroprotective role [1]. Sappington and co-workers have shown that IL-6 also protects RGC from an increase in intraocular pressure, a condition associated to glaucoma [2].

Adenosine is a purine nucleoside that regulates processes such as blood pressure, vasodilatation, neurotransmitter release, neuronal survival and differentiation [14,15]. There are four identified

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receptors activated by adenosine: A1, A2a, A2b and A3. These receptors can regulate cAMP production by the activation or the inhibition of adenylyl cyclase. The activation of A1 and A3 adenosine receptors inhibits adenylyl cyclase activity, while A2a and A2b stimulate it [16].

Data from literature show that the inhibition of neurotransmitter release by A1 adenosine receptor activation appears to be mediated by the blockade of $Ca^{2+}$ channels or by the activation of $K^+$ channels [17–19]. Some authors also demonstrated that A2a adenosine receptor activity induces neuroprotection and facilitates the release of neurotransmitter [20,21].

Adenosine has also been described as a modulator of the inflammatory responses by regulating the balance between the production of pro-inflammatory and anti-inflammatory cytokines [22]. The extracellular presence of adenosine depends on either the amount of adenosine synthesized in the extracellular compartment or the release of adenosine from the intracellular compartment [23].

It has been shown that adenosine is able to stimulate IL-6 release in different tissues [24–26]. In astrocytes IL-6 can upregulate mRNA expression of A1R increasing accumulation of phosphatidylinositol, which also happened in a different study, when A1R was directly activated. The authors showed that adenosine can stimulate IL-6 release in hypoxic conditions increasing A1 receptor activity [27–29].

During the studies about the involvement of A1R on IL-6 effect [13] we found that cAMP production was important to IL-6 effect. Since A1R is classically involved in the adenylyl cyclase inhibition and therefore decreasing in cAMP levels, we considered the participation of other receptor that could mediate the production of cAMP. Here we show that adenosine A2a receptors (A2aR) are involved on IL-6 effect; additionally, we show the existence of a complex signalling pathway that involves modulation of IL-6, BDNF, A1R and A2aR levels resulting in the neuroprotection of axotomized RGC.

## 2. Materials and methods

### 2.1. Materials

IL-6, antibodies anti-IL-6 and anti-BDNF were purchased from PeproTech (USA). Zm241385, CHA and CGS21680 came from Tocris (UK). SQ22536 was bought from Enzo Life Science (Switzerland). H89 came from BIOMOL Research Laboratories (USA). Bovine serum albumin (BSA), anti p-CREB antibody and anti, anti actin antibody, A2aR antibody came from Santa Cruz (USA). Anti A1R antibody was bought from CHEMICON (USA). Anti rabbit and anti mouse HRP conjugated antibodies were purchased from Bio-Rad (USA). Medium 199 and trypsin were purchased from Gibco (USA). Petri dishes were bought from TPP (Switzerland). Penicillin G, streptomycin sulphate, L-glutamine, poly-L-ornithine, horseradish peroxidase (HRP), tetramethylbenzidine, sodium nitroprusside and dimethylsulphoxide (DMSO) were obtained from Sigma (USA). Paraformaldehyde, glutaraldehyde, glycine and methanol were bought from J.T. Baker (USA). Entellan was supplied by Merck (Germany).

### 2.2. Methods

#### 2.2.1. Retrograde labelling of retinal ganglion cells

Lister Hooded rats were anaesthetized by hypothermia within the first 24 h after birth and 1.0 μL of a 30% HRP solution in 2% DMSO was injected into each superior colliculus and the animal was kept for about 16 h. All procedures using animals were approved by the local committee for animal care and experimentation (CEPA-projects #00196-10).

#### 2.2.2. Cell culture

Primary cultures were prepared using procedures described elsewhere [12,13]. The animals were killed by decapitation and their eyes were removed. The retina was dissected in a calcium- and magnesium-free balanced salt solution (CMF) containing 100 μg/mL streptomycin + 100 U/mL penicillin. The retinae were incubated in CMF containing 0.1% trypsin for 16 min at 37 °C. The trypsin was inhibited by adding culture medium with 5% of fetal calf serum (FCS). Tissue was then resuspended in complete medium (culture medium supplemented with 2 mM glutamine, 100 μg/ml streptomycin, 100 U/mL penicillin and 5% FCS) and mechanically dissociated using a Pasteur pipette. Cultures were then incubated (density of $10^5$ cells/$cm^2$) in plates previously treated with poly-L-ornithine and kept in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C for different timeframes. We also tested the effect of the vehicles used for drug dilution and concluded that they did not influence the survival of RGC (data not shown).

#### 2.2.3. Identification of RGC in culture

According to the protocol of Mesulan [30] cells were fixed after 4 or 48 h *in vitro* with a mixture of 1% paraformaldehyde and 2% glutaraldehyde and 0.1 M Sodium phosphate buffer for 5 min, washed in phosphate buffer and then reacted with tetramethylbenzidine and $H_2O_2$. After the reaction, coverslips were washed in 0.2 M Acetate buffer, dehydrated, immersed briefly in xylene and mounted in Entellan.

#### 2.2.4. Presentation of data and statistical analysis

The number of RGC on each coverslip was evaluated by counting 1/20 of the total area using an Olympus microscope at a magnification of 400× under bright field. This quantification was performed using a double-blind process to minimise the influence of the researcher on the experimental results. As an internal control for the variable percentage of ganglion cells labelled with HRP in distinct experiments, the number of labelled cells at 4 h in culture was taken as 100% (i.e. control). The results were reported as percentages of the control. All data were expressed as mean ± standard error of the mean from experiments performed in duplicate or triplicate and each experiment was repeated at least three times. The overall statistical significance was first obtained by one-way analysis of variance (ANOVA). Statistical significance of all pairs of multiple groups of data was assessed by Newman–Keuls comparison test. A value of $P < 0.001$ was considered significant.

#### 2.2.5. Western blot

Cells were scraped off the dishes in sample buffer and the material boiled for 10 min. Total amount of protein in each sample was determined using the Bradford reagent [31], with BSA as standard. Samples containing 60 μg protein were submitted to 10% SDS–PAGE gel electrophoresis and the proteins transferred to PVDF membranes which were incubated overnight with the antibody, washed, incubated with peroxidase-conjugated anti-rabbit secondary antibody and revealed by Luminata (Millipore) chemiluminescence. Actin was used as a load control. The program ImageJ was used to quantificate all the blots.

#### 2.2.6. Elisa

Measurements of IL-6 were done on cell-free medium supernatants using ELISA kits, in accordance with the manufacturer's instructions (Duo Set, R&D Systems, Minneapolis, MN, USA).

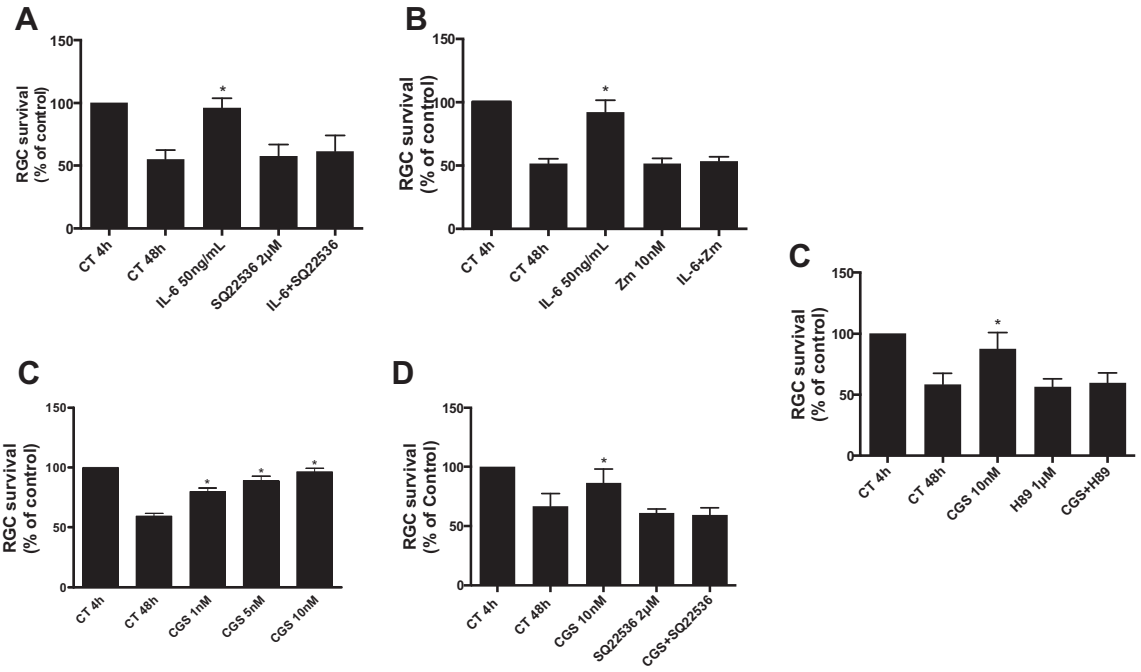


**Fig. 1. A – SQ22536 blocks the effect of IL-6 on the survival of RGC.** CT – Control; SQ22536 – 9-(tetrahydro-2-furyl)adenine = Adenylyl cyclase inhibitor; IL-6 – Interleukin-6. Data are presented as mean ± SEM ($n$ = 6–10), $P < 0.001$ compared to control 48 h. **B – Zm241385 blocks the effect of IL-6 on the survival of RGC.** CT – Control; Zm241385 – 4-(2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}-{1,3,5}triazin-5-yl-amino]ethyl)phenol) – A2aR antagonist; IL-6 = Interleukin 6. Data are presented as mean ± SEM ($n$ = 5–12), $P < 0.001$ compared to control 48 h. **C – CGS21680 induces an increase on the survival of RGC.**CT – Control; CGS21680 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Data are presented as mean ± SEM ($n$ = 5–6), $P < 0.001$ compared to control 48 h. **D – SQ22536 blocks the effect of CGS21680 on RGC.** CT – Control; SQ22536 – 9-(tetrahydro-2-furyl)adenine = Adenylcyclase inhibitor; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist, (6–12). **E – H89 blocked the effect of CGS21680 on RGC.** CT – Control; H89 – 5-isoquinolinesulfonamide, 2HCl = Protein Kinase A inhibitor; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Data are presented as mean ± SEM ($n$ = 6–10), $P < 0.001$ compared to control 48 h.

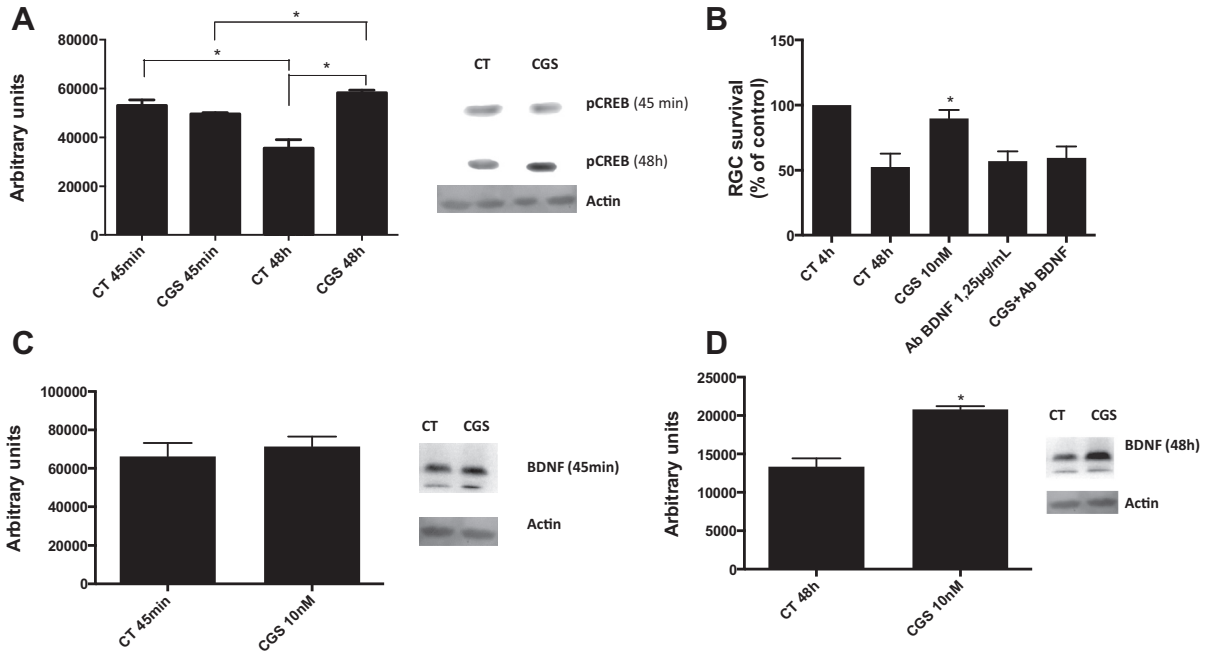


**Fig. 2. A – phospho-CREB levels.** CT – Control; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid – A2aR agonist. Data are presented as mean ± SEM ($n$ = 3), $P < 0.001$ compared to control 48h. **B – Antibody anti-BDNF blocked the effect of CGS21680 on the survival of RGC.** CT – Control; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Anti-BDNF 0.125 μg/mL. Data are presented as mean ± SEM ($n$ = 7–9), $P < 0.001$ compared to control 48 h. **C – BDNF levels after A2aR activation for 45 min.** CT = Control; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Data are presented as mean ± SEM ($n$ = 3), $P < 0.001$ compared to control 45 min. **D – BDNF levels after A2aR activation for 48 h.** CT = Control; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Data are presented as mean ± SEM ($n$ = 7–9), $P < 0.001$ compared to control 48 h.

## 3. Results

### 3.1. Adenosine A2aR are involved on IL-6 effect

During our effort to better understand the mechanism by which IL-6 increases the survival of RGC we investigated adenylyl cyclase activity. Fig. 1A shows that after treatment with SQ22536, a selective inhibitor of adenylyl cyclase activity, the IL-6 effect was abolished. This result leaded us to investigate the involvement of A2aR in the trophic effect of IL-6 on RGC. Therefore, we treated cultures with IL-6 for 48 h in the presence of A2aR antagonist, ZM241385 (ZM), and observed a complete blockade of IL-6 effect (Fig. 1B). Accordingly, when cultures were treated with an A2aR agonist (CGS21680) the RGC survival increased (Fig. 1C). Furthermore, cells were treated with CGS21680 in the presence of an adenyl cyclase inhibitor (Fig. 1D). As expected, the effect of CGS21680 was completely blocked. Also, following the inhibition of protein kinase A (PKA) by 1 μM H89 the effect of CGS21680 was abolished (Fig. 1E).

### 3.2. A2aR activation modulates BDNF

Initially cultures were treated with CGS21680 for 45 min or 48 h and the levels of phospho-CREB were analysed. Fig. 2A shows that treatment with CGS21680 for 48 h induced an increase (63%) in phospho-CREB levels. As phospho-CREB is a transcription factor related to BDNF production, we investigated the involvement of BDNF in the trophic effect of CGS21680 on RGC [34]. Fig. 2B demonstrates that the effect of CGS21680 was completely abolished by the presence of the antibody anti-BDNF. Following, we analysed the levels of BDNF after CGS21680 treatment. Our results show that treatment with CGS21680 for 45 min did not increase the levels of BDNF (Fig. 2C). However, after 48 h CGS21680 treatment the levels of BDNF increased 35% (Fig. 2D).

### 3.3. Interleukin-6 plays an important role on A1 and A2aR expression

To confirm that the effect of A1 and A2a receptors activation on RGC survival was mediated by IL-6 we neutralized it in the extracellular medium. Fig. 3A shows that in the absence of extracellular IL-6 both CHA and CGS21680 showed no trophic effect. In addition, the A1 and A2aR expressions were analysed after treatment with IL-6. Fig. 3B shows that after treatment with IL-6 for 24 h, the levels of A2aR significantly increased 85% and the levels of A1R decreased 48% (Fig. 3C). However, after IL-6 treatment for 48 h the levels of A2aR decreased 24% (Fig. 3D) and the levels of A1R increased 195% (Fig. 3E).

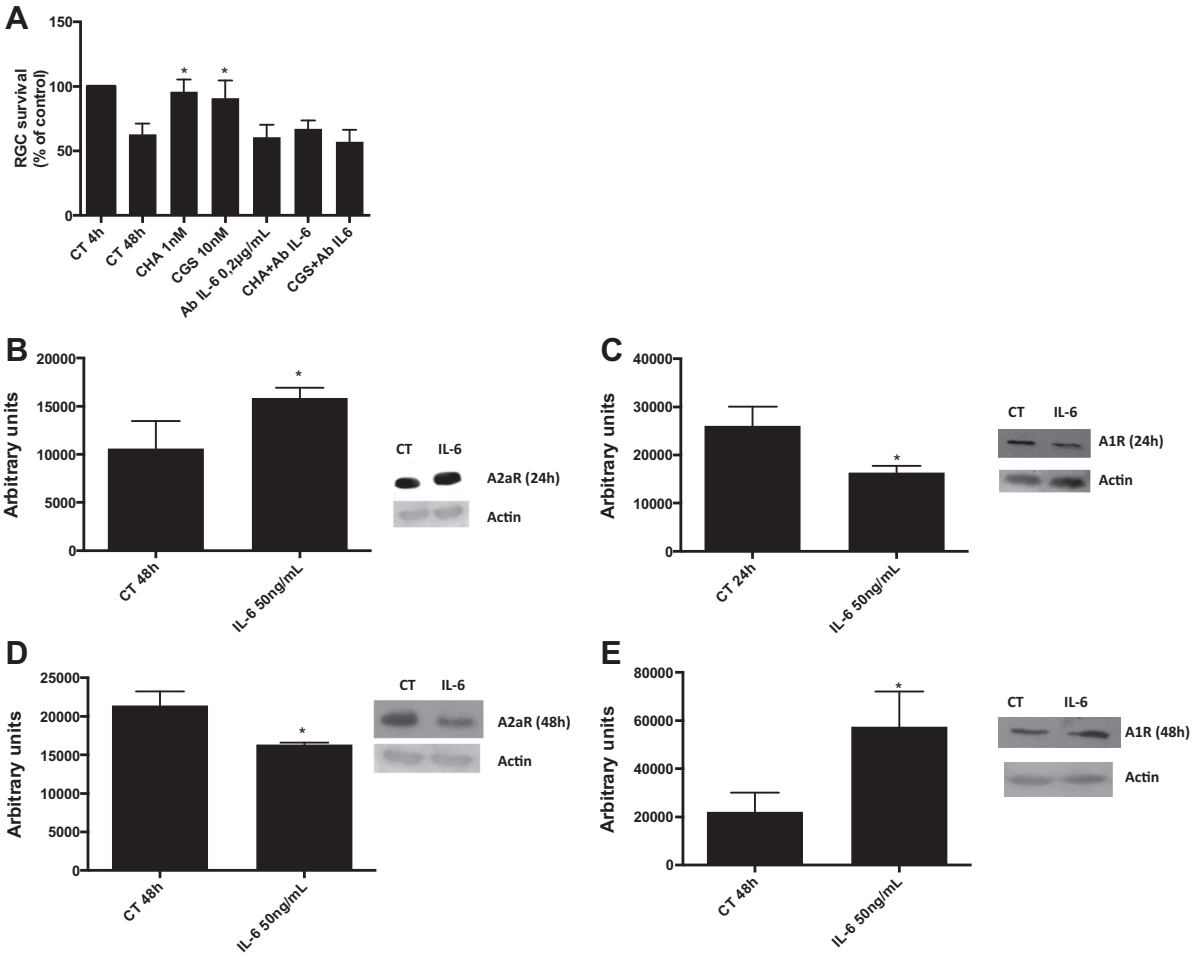


**Fig. 3. A – Anti-IL-6 antibody blocks the effect of both CHA and CGS21680.** CT = Control; CHA – N(6)-cyclohexyladenosine; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Anti-IL-6 0.2 μg/mL; Data are presented as mean ± SEM ($n$ = 6–9), $P$ < 0.001 compared to control 48 h. **B – IL-6 increases A2aR levels after 24 h of treatment.** CT = Control; IL-6 50 ng/mL = Interleukin-6. Data are presented as mean ± SEM ($n$ = 3), $P$ < 0.001 compared to control 24 h. **C – IL-6 increases A1R levels after 24 h of treatment.** CT = Control; IL-6 50 ng/mL = Interleukin-6. Data are presented as mean ± SEM ($n$ = 3), $P$ < 0.001 compared to control 24 h. **D – IL-6 increases A2aR levels after 48 h of treatment.** CT = Control; IL-6 50 ng/mL = Interleukin-6. Data are presented as mean ± SEM ($n$ = 3), $P$ < 0.001 compared to control 48 h. **D – IL-6 increases A1R levels after 48 h of treatment.** CT = Control; IL-6 50 ng/mL = Interleukin-6. Data are presented as mean ± SEM ($n$ = 3), $P$ < 0.001 compared to control 48 h.

### 3.4. The balance between A1 and A2aR activation mediates IL-6 levels intra and extracellular IL-6

The release of IL-6 was analysed after treatment with either CGS21680 or CHA for 48 h. Fig. 4A shows that A2aR activation increases 142% IL-6 release (291 μg/mL). On the contrary, A1R activation significantly decreased in 70% the release of the cytokine (26 μg/mL). The expression of IL-6 was also analysed after A1 and A2aR activation. Unexpectedly, as shown in Fig. 4B, CHA treatment for 48 h increased 94% IL-6 expression. As expected, Fig. 4C shows, that A2aR activation increased in 46% IL-6 expression. The IL-6 release was also analysed after treatment with BDNF for 48 h. Fig. 4D shows that BDNF also significantly increased in 83% the IL-6 expression (99 μg/mL).

## 4. Discussion

Earlier results from our group showed that IL-6 increase the survival of RGC [12,13] by inducing adenosine release and, as a consequence, A1R activation [13]. During our efforts to elucidate the signalling pathway triggered by L-6, the importance of adenylyl cyclase activation was observed. Since A1R is well known for its capacity to inhibit the adenyl cylcase [32], the participation of other adenosine receptors on IL-6 trophic effect was considered.

In culture of avian, long-term activation of A2aR blocked glutamate excitotoxicity on retinal neurons [21]. A2aR can also modulate STAT3 signal *in vitro* assuring neuroprotection after oxygen deprivation [29]. In agreement with those findings, our treatment of cultures with IL-6 in the presence of Zm241385 (A2a antagonist) did not increase the RGC survival. Furthermore, cultures that received treatment with A2a agonist CGS21680 presented an increase in RGC survival similar to that observed following IL-6 treatment. Additionally, cultures treated with CGS21680 showed similar morphologic characteristics to those treated with IL-6 (data not shown). In agreement with the literature, the inhibition of cAMP production completely inhibited CGS216801 trophic effect. This result indicates that the intracellular pathway involved in the trophic effect of CGS216801 is the classical pathway elicited by A2a receptors activation [16].

The cAMP-signalling cascade classically involves Protein Kinase A (PKA) activation [32]. In accordance to the literature CGS21680 effect was blocked after inhibition of PKA with H89, suggesting PKA involvement in A2aR signalling [16,32,33]. PKA is classically involved with CREB (cAMP responsive element-binding protein) phosphorilation [34]. Therefore the levels of phospho-CREB were analysed after treatment with CGS21680.

In the last few years, many authors have demonstrated the existence of a crosstalk between A2aR and BDNF. The literature shows that not only A2aR are involved in the mechanism of TrkB receptors transactivation, but they also modulate the effect of BDNF on synaptic transmition [35]. In agreement with the literature, the effect of CGS21680 was abolished in the presence of anti-BDNF antibody. Previous data from our group showed that after 45 min of IL-6 treatment BDNF levels increased and that treatment for 48 h with either IL-6 or the A1 receptor agonist (CHA) decreased

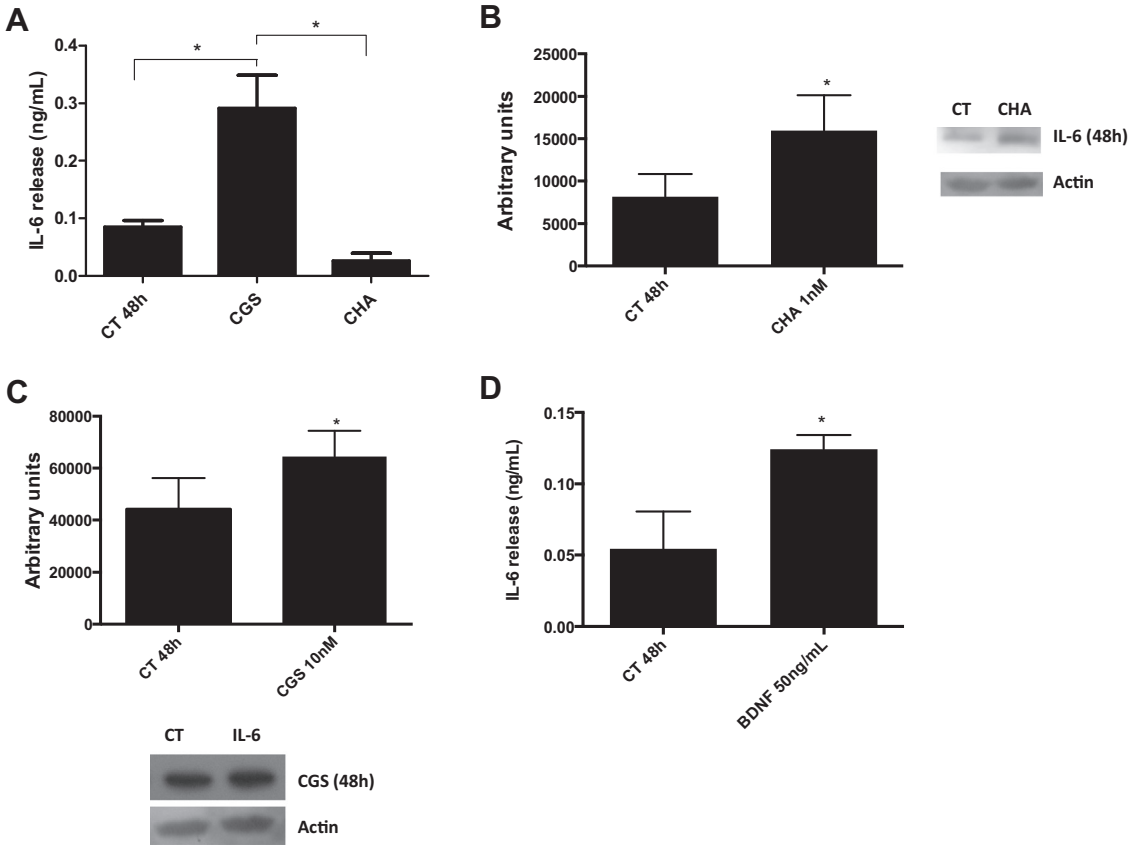


**Fig. 4. A – Adenosine A1 and A2aR modulates IL-6 release.** CT = Control; CHA – N(6)-cyclohexyladenosine = A1R agonist; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Data are presented as mean ± SEM ($n$ = 10–14), $P$ < 0.001 compared to control 48 h. **A – Adenosine A1 receptor activation increases IL-6 levels.** CT – Control; CHA – N(6)-cyclohexyladenosine = A1R agonist. Data are presented as mean ± SEM ($n$ = 3), $P$ < 0.001 compared to control 48 h. **B – Adenosine A2a receptor activation increases IL-6 levels.** CT = Control; CGS241385 – 3-[4-[2-[ [6-amino-9-[(2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxy-oxolan-2-yl]purin-2-yl]amino]ethyl]phenyl]propanoic acid = A2aR agonist. Data are presented as mean ± SEM ($n$ = 3), $P$ < 0.001 compared to control 48 h. **C – BDNF treatment increase the release of IL-6.** Data are presented as mean ± SEM ($n$ = 6–8), $P$ < 0.001 compared to control 48 h.

the levels of BDNF and phospho-CREB [13]. In this study we demonstrate that the activation of A2a receptor for 48 h, but not for 45 min, increased phospho-CREB and BDNF levels. This result suggests that in the signalling pathway triggered by IL-6 the A2aR activation comes first followed by A1R activation. This was confirmed by the treatment with IL-6 that decreased A1R and increased A2aR levels after 24 h. Additionally, after 48 h of treatment, the A1R levels increased whilst A2aR expression decreased. Together, these results suggest that both A2aR and A1R play an important role on BDNF levels and that IL-6 modulates BDNF levels through the regulation the adenosine A1 and A2aR expression.

Torres and Araujo (2001) showed that in order to increase the survival of RGC, IL-6 must be present in cultures for the full 48 h [12]. In agreement with the literature we showed that both CHA and CGS21680 effects were neutralized in cultures treated with anti-IL-6 antibody. Although the levels of IL-6 increased after both A1 and A2aR stimulation, after treatment with CHA cultures released low levels of IL-6 whereas after treatment with CGS21680 the cultures released higher levels of IL-6. While further studies are necessary, we suggest that the levels of IL-6 might be crucial to adenosine receptor modulation and that the balance between A1 and A2aR levels is related to the concentration of IL-6 in the extracellular medium.

Data from literature often relate IL-6 to BDNF [13,36,37]. Here we found that the increase in BDNF matches the extracellular presence of IL-6 and that BDNF releases IL-6. Based on this finding and given the fact the CGS effect depends on BDNF, we may conclude that the signalling pathways triggered by A2aR activation would be inducing IL-6 release through a BDNF stimulus.

IL-6 has multiple actions in different cell systems and its effect on RGC survival is not fully understood. Even if many questions remain unanswered, the signalling pathway triggered by IL-6 has shown to be promising in the treatment of diseases related to RGC death. A problem is that it is not easy to use IL-6 in clinical practice, since it can act as a pro-inflammatory cytokine inducing side effects such as fever or even cell death [38]. One possible strategy to tackle this problem is to use adenosine receptor agonists as downstream activators of the IL-6 pathway. Since there is an A2aR agonist already approved by the FDA for clinical practice [39], studies that relate adenosine receptors with IL-6 trophic effect might guide us to new pharmacological approaches that may benefit several patients who suffer diseases related to RGC death.

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