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IL-6 treatment increases the survival of retinal ganglion cells *in vitro*: The role of adenosine A1 receptor

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ABSTRACT

IL-6 is a pleiotropic cytokine classically denominated pro-inflammatory. It has been already demonstrated that IL-6 can increase the survival of retinal ganglion cells (RGC) in culture. In this work, we show that the trophic effect of IL-6 is mediated by adenosine receptor (A1R) activation. The neutralization of extracellular BDNF abolished the IL-6 effect and the treatment with IL-6 and CHA (an agonist of A1R) modulated BDNF expression as well as pCREB and pTrkB levels.

1. Introduction

In 1986 Tadamitsu Kishimoto discovered interleukin-6 (IL-6) while searching for the molecule responsible for differentiation and growth of B lymphocytes [1,2]. IL-6 belongs to the neuropoietic cytokine family, which also includes ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin-M (OSM), interleukin-11 (IL-11), cardiotropin-1 (CT-1), neurotrophin-1 (NNT-1), interleukin-27 (IL-27), neuropoietin and B-cell-stimulating factor. These molecules share a common signaling pathway that involves a transmembrane glycoprotein of 130 kDa (GP130) [3–5].

The IL-6 effects occur through a specific binding protein, αIL-6R. The binding of IL-6 to the α-receptor subunit induces the homodimerization of gp130 subunits followed by the activation of cytoplasmic kinases of the Janus (JAK) family [4]. The activation of JAKs then leads to the stimulation of proteins from the STAT family (STAT3 and STAT1) – a family of signals transducers and activators of transcription [4]. STAT dimers translocate into the nucleus and induce transcription of immediate early genes, such as *junB*, and the rapid activation of a nuclear factor, termed acute-phase response factor (APRF) [4,6].

IL-6 is produced by Th1 and Th2 cells, 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 [6,8,9]. IL-6 synthesis can be induced by a variety of stimuli, but its major inducers are lipopolysaccharides (LPS), interleukin-1 (IL-1) and tumor necrosis factor (TNF) [10].

Much attention has been focused on the functional role of IL-6 in the CNS [11]. It promotes the differentiation of oligodendrocytes into mature oligodendrocytes and it induces the differentiation of cortical precursor cells into astrocytes. IL-6 also stimulates the activation of astrocytes and plays a role in the survival and differentiation of peripheral and central neurons [9,12]. In PC12, IL-6 induces the differentiation of these cells into a neuronal phenotype and supports their survival under adverse conditions in culture [13–15].

Previous work from our laboratory showed the trophic effect of IL-6 on axotomized rat retinal ganglion cells (RGC) kept in culture for 48 h [16]. Also it was shown that IL-6 protects RGC from an increase in intraocular pressure, a condition associated to glaucoma [17].

Adenosine regulates various physiological processes in several tissues. One good example is the cardiovascular system where adenosine is responsible for a decrease in blood pressure as a consequence of vasodilatation [18]. In the CNS, adenosine regulates neurotransmitter release, neuronal survival and differentiation [19]. The adenosine receptors can regulate cAMP production by the activation (A1 and A3 receptors) or the inhibition (A2a and A2b receptors) of the enzyme adenylyl cyclase [20].

The inhibition of neurotransmitter release by A1 adenosine receptor (A1R) activation appears to be mediated by the blockade of Ca^{2+} channels or activation of K^+ channels and hyperpolarization

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[21–23], while A2a adenosine receptor activity induces neuroprotection and facilitates the release of neurotransmitters [24,25].

Adenosine is a modulator of the inflammatory response due to the balance between the production of pro-inflammatory and anti-inflammatory cytokines [26]. The levels of extracellular adenosine depend either on the amount of adenosine synthesized in the extracellular compartment on the release of adenosine from the intracellular compartment [27].

It has been shown that adenosine stimulates the release of IL-6 in different tissues [28–30]. Besides, Biber and co-workers showed that IL-6 induces an increase in mRNA expression of A1R in the brain [31]. More recently, Biber and co-workers showed that IL-6 treatment enhances the A1R-mediated signaling in the brain under excitotoxic situations, contributing to an important decrease in neuronal degeneration [32].

The aim of this work was to analyze the participation of adenosine A1R on the trophic effect of IL-6 on RGC. Our results show that A1R are mediating the IL-6 effect on RGC survival. We also found that IL-6 and A1R stimulate a common downstream pathway involving BDNF and the transcription factor CREB.

2. Materials

IL-6 and antibodies anti-IL-6 and anti-BDNF were purchased from PeproTech (NJ, USA). Antibody anti-pCREB (Ser133) came from Cell Signaling (MA, USA). Antibody anti-A1R was bought from Millipore (MA, USA). Antibodies anti-actin and anti-p-TrkB and bovine serum albumin (BSA) came from Santa Cruz (CA, USA). The secondary antibody anti-rabbit came from Bio-Rad (CA, USA). Medium 199, trypsin and fetal calf serum were purchased from Gibco (MD, USA). Petri dishes were bought from TPP (Switzerland). Penicillin G, streptomycin sulfate, L-glutamine, poly-L-ornithine, horseradish peroxidase (HRP), tetramethylbenzidine, sodium nitroprusside, dimethylsulfoxide (DMSO), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), adenosine deaminase and *N*(6)-cyclohexyladenosine (CHA) were obtained from Sigma (MO, USA). K252a and NBI were purchased from Enzo Life Sciences (Switzerland). Materials used in Western blot experiments came from GE (CT-USA) and Ponceau red and H_2O_2 were purchased from VETEC (Brazil). Paraformaldehyde, glutaraldehyde, glycine and methanol were bought from J.T.Baker (Phillipsburg, NJ, USA). Entellan was supplied by Merck (Darmstadt, Germany).

3. Methods

3.1. Retrograde labeling 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. Afterwards, the animals survived for ~16 h before the procedures used for cell culture. This time lapse was necessary for the uptake and retrograde transport of HRP to the soma of RGC. Procedures using animals were approved by the local committee for animal care and experimentation (CEPA-projects #00196-10).

3.2. Cell culture

Primary cultures were prepared using procedures described elsewhere [17]. Briefly, rats at postnatal day 1 were killed by decapitation and their eyes were removed. The pigmented epithelium and attached tissues were separated from the neural retina 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 action of trypsin was stopped 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. After, 1 mL of the cell suspension was added to culture dishes, previously treated with poly-L-ornithine (50 μg/mL), at a plating density of 10^5cells/cm^2. After plating the cultures were incubated in 1 mL of complete medium for 4 h to allow for the attachment of cells to the substrate. Then, 1 mL of complete medium or 1 mL of complete medium containing the drugs to be tested was added to each Petri dish. Cultures were maintained 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).

3.3. Identification of RGC in culture

The presence of HRP in RGC was revealed according to the protocol of Mesulan [33]. The monolayers were fixed after 4 or 48 h *in vitro* with a mixture of 1% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (Karnovski solution) for 5 min, washed in phosphate buffer and then reacted with tetramethylbenzidine and H_2O_2. After reaction, the coverslips were washed in 0.2 M acetate buffer, dehydrated by air drying, immersed briefly in xylene and mounted in Entellan.

3.4. 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. These analyzes were performed using a double-blind process to minimize the influence of the researcher on the experimental results. As an internal control for the variable percentage of ganglion cells labeled with HRP in distinct experiments, the number of labeled cells at 4 h in culture was taken as 100% and the results were reported as percentage of 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.

3.5. Western blot

Cells were first 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 [34], 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 anti-A1R antibody (1:100), or anti-BDNF (1:3000), or anti-pCREB (1:5000) or anti-pTrkB (1:400), washed, incubated with peroxidase-conjugated anti-rabbit secondary antibody (1:10,000) and revealed by the Luminata (Millipore) chemiluminescence. To load control after revelation membranes were washed in TBS (pH 7.6), then incubated with glycine 0.2 M (pH 2.2) to remove the unbound primary and secondary antibodies. Quantitative analysis of blots was performed by scanning images and using the computer program Scion Image (Scion Corporation, MD, USA).

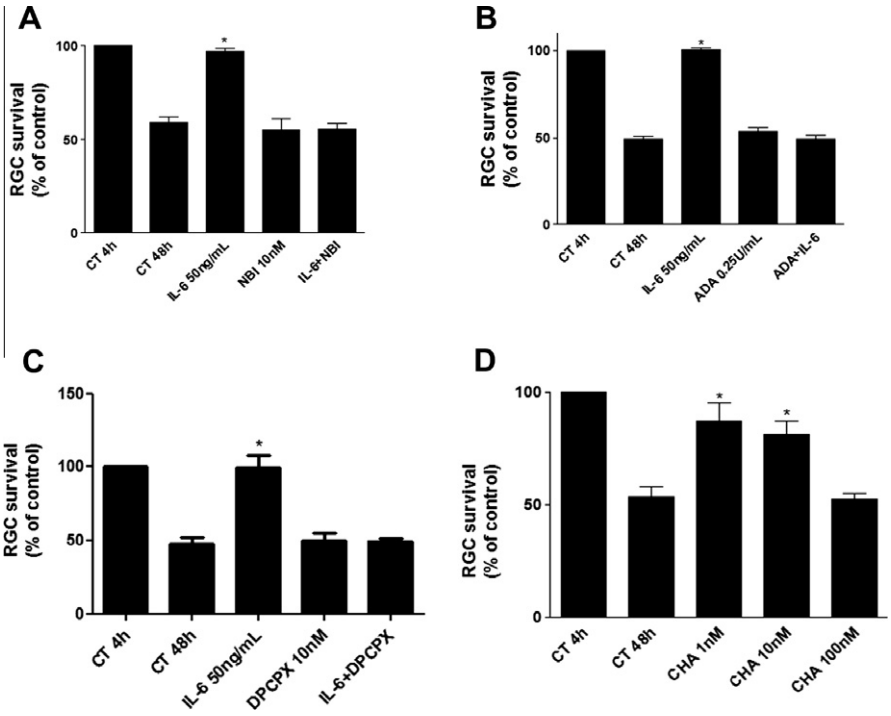

Fig. 1. (A) NBI blocks the effect of IL-6 on the survival of RGC. CT – Control; NBI (Nitrobenzyl ThioInosine) 10 nM – Inhibitor of Adenosine transporter), 50 ng/mL IL-6: interleukin 6, (n = 4–7). (B) Adenosine Deaminase inhibits the IL-6 effect on the survival of RGC. CT – Control; ADA (Adenosine Deaminase) 0.5 U/mL, 50 ng/mL IL-6; interleukin 6, (n = 7–10). (C) DPCPX blocks the effect of IL-6 on the survival of RGC. CT – Control; DPCPX (8-cyclopentyl-1,3-dipropylxanthine) 10 nM: Antagonist of Adenosine A1 Receptor), 50 ng/mL IL-6: interleukin 6, (n = 6–8). (D) CHA induces an increase in RGC survival. CT – Control; CHA (N6-cyclohexyl adenosine) 1–100 nM – Agonists of Adenosine A1 Receptor, (n = 6–8). All data are presented as mean ± SEM, P < 0.001 compared to control 48 h.

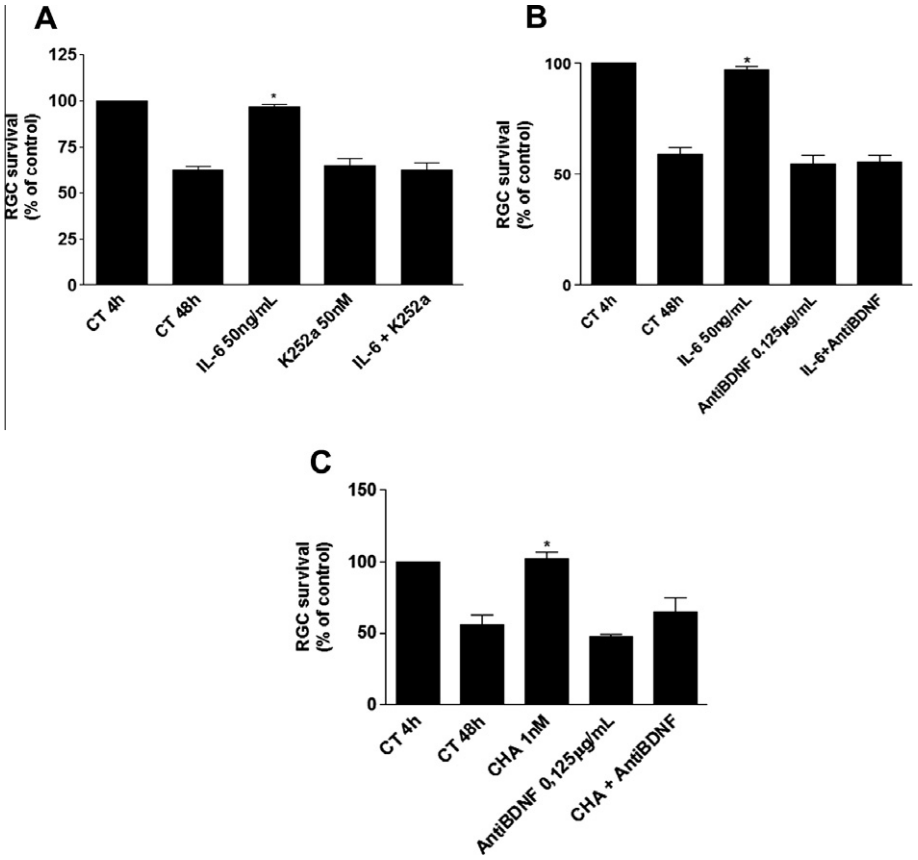

Fig. 2. (A) K252a blocks the effect of IL-6 on the survival of RGC. CT – Control. IL-6 50 ng/mL: IL-6: interleukin-6. K252a 50 nM: Antagonist of Trk receptors, (n = 6–9). (B) Anti-BDNF blocks the effect of IL-6 on the survival of RGC. CT – Control. IL-6 50 ng/mL: interleukin-6. Antibody anti-BDNF 0.125 µg/mL (n = 4–7). (C) Anti-BDNF blocks the effect of CHA on the survival of RGC. CT – Control. CHA 1 nM: Adenosine A1 Receptor Agonist. Antibody anti-BDNF 0.125 µg/mL (n = 5–11). All data are presented as mean ± SEM, P < 0.001 compared to control 48 h.

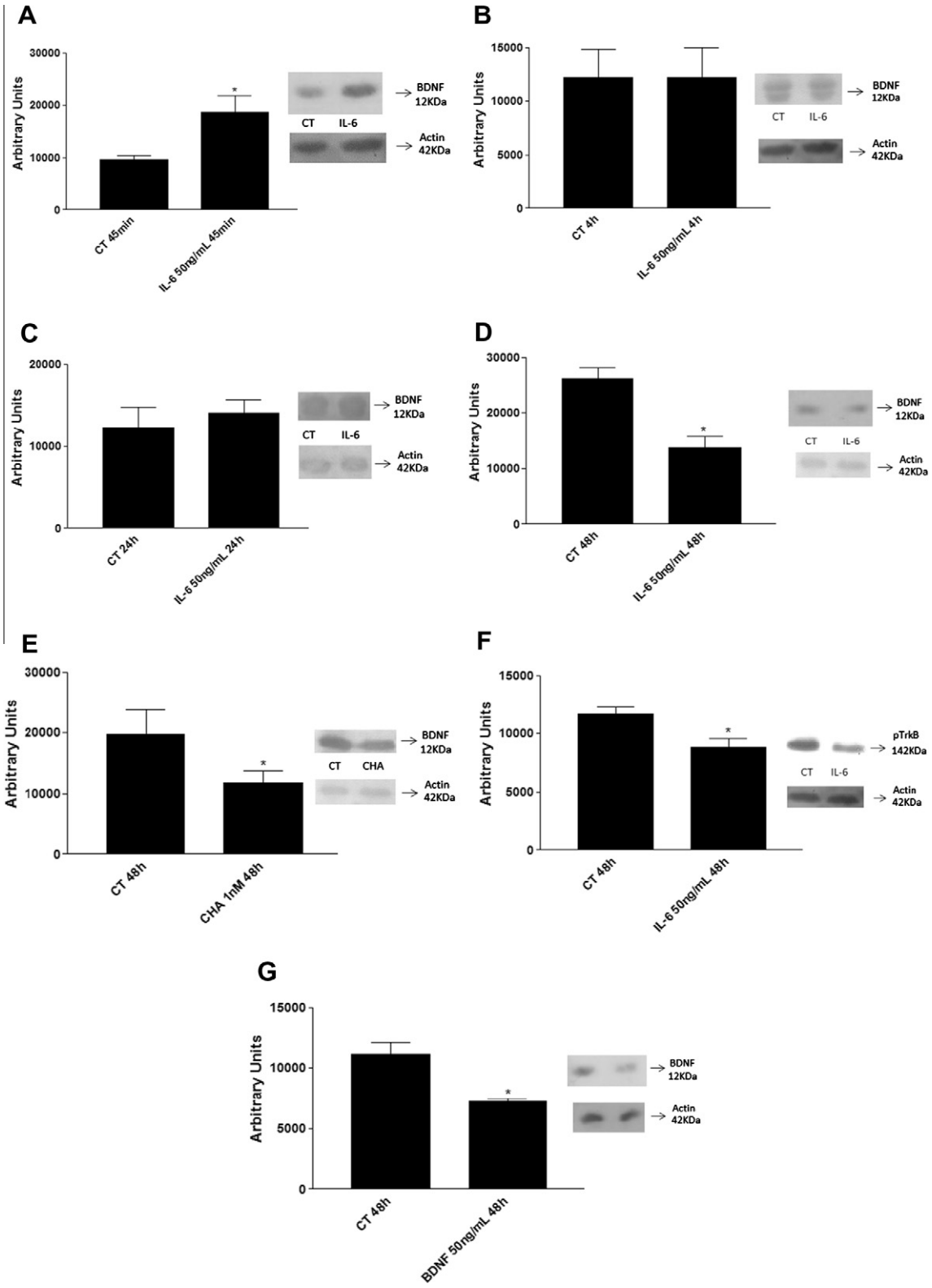

Fig. 3. (A) BDNF levels in cultures treated with IL-6 for 45 min. CT – Control. IL-6 50 ng/mL: interleukin-6. (B) BDNF levels in cultures treated with IL-6 for 4 h. CT – Control. IL-6 50 ng/mL: interleukin-6. (C) BDNF levels in cultures treated with IL-6 for 24 h. CT – Control. IL-6 50 ng/mL: interleukin-6. (D) BDNF levels in cultures treated with IL-6 for 48 h. CT – Control. IL-6 50 ng/mL: interleukin-6. (E) BDNF levels in cultures treated with CHA for 48 h. CT – Control. CHA 1 nM: A1 Adenosine Receptor Agonist. (F) Levels of pTrkB in cultures treated with IL-6 for 48 h. Control. IL-6 50 ng/mL: interleukin-6. (G) BDNF treatment for 48 h downregulates BDNF levels. CT – Control. BDNF 50 ng/mL. All data are presented as mean ± SEM ($n = 3$), $P < 0.001$ compared to CT.

4. Results

4.1. Adenosine is important to IL-6 effect

Fig. 1A shows that treatment with IL-6 (50 ng/mL) for 48 h induces a significant increase in the survival of RGC (≈65% when compared to the 48 h control cultures). However, when cultures were incubated with 10 nM NBI (an adenosine transporter inhibitor) the effect of IL-6 was abolished, suggesting that adenosine traffic is necessary for the trophic effect of IL-6 on RGC. Corroborating this idea, when we treated cells for 48 h with 0.25 U/mL adenosine deaminase (ADA) the IL-6 effect on RGC survival was inhibited (Fig. 1B). Together those results suggest that the presence of adenosine in the extracellular space is necessary for the trophic effect elicited by IL-6 on RGC.

4.2. Involvement of A1 adenosine receptors in the trophic effect of IL-6

Following we investigated the involvement of A1R in the IL-6 effect. Results presented in Fig. 1C show that treatment with an antagonist of A1R (10 nM DPCPX) abolished the trophic effect of IL-6. Accordingly, treating the cultures with the A1R agonist CHA (1 and 10 nM), showed an increase in RGC survival. The stronger effect (100% survival) was observed with 1 nM CHA and no effect was observed using 100 nM CHA (Fig. 1D).

4.3. BDNF is involved in the IL-6 effect

Fig. 2A shows that treatment with 50 nM K252a (a selective antagonist of Trk receptor) inhibited the IL-6 effect on RGC survival. Based on this result we evaluated whether BDNF was involved in IL-6 effect using an anti-BDNF antibody. Fig. 2B shows that the presence of anti-BDNF inhibited the IL-6 effect. To assess whether BDNF was also involved in the CHA effect we treated cells with anti-BDNF antibody in the presence of CHA. Fig. 2C demonstrates that CHA effect was also abolished in the presence of anti-BDNF.

4.4. Treatment with IL-6 or CHA modulates BDNF levels in retinal cell cultures

Fig. 3A shows that treatment with IL-6 for 45 min increased (94.6%) BDNF levels, although treatment of IL-6 for 4 or 24 h (Fig. 3B–D) did not change BDNF levels and 48 h of IL-6 stimulus downregulated (50% decrease) BDNF levels (Fig. 3D). Fig. 3E shows a similar effect (56% decrease) when cells were treated with CHA for 48 h. We then evaluated the levels of pTrkB receptor after treatment with IL-6 for 48 h. Fig. 3F shows that in the presence of IL-6 the pTrkB levels decreased. We also analyzed the BDNF capacity to downregulate its own levels. Fig. 3G shows that a 48 h BDNF treatment, similarly to IL-6 stimulus, decreased BDNF levels.

4.5. Treatment with IL-6 for 48 h decreases CREB phosphorylation

As CREB is an important transcriptional factor involved in BDNF synthesis [37] we evaluated the levels of pCREB after 4 and 48 h of IL-6 treatment. Fig. 4A shows that after 4 h of IL-6 treatment no changes were observed in the pCREB levels. On the other hand, when the cells were chronically exposed to IL-6 (48 h) we observed (Fig. 4B) a decrease of about 50% in pCREB levels.

5. Discussion

In the present work we show that adenosine and the activation of A1R mediate the trophic effect of IL-6 on RGC. It has been already demonstrated that IL-6 treatment decreases the excitability of post-synaptic transmission, due to an increase in the A1R activation, with a beneficial impact on neuronal survival [32]. Our present results are consistent with those findings since treatment with DPCPX (A1R antagonist) abolished the IL-6 effect while treatment with CHA (A1R agonist) induced an increase in RGC survival. Taken together, our results confirm the involvement of A1Rs in the IL-6 effect as previously demonstrated in other biological systems [31,32].

In 2001, we showed that the IL-6 effect depended on the release of vesicular polypeptides, since treatment with brefeldin A blocked the IL-6 trophic effect [16]. This evidence suggests that IL-6 trophic effect can be mediated by another molecule. It is already known that neurotrophins are involved in survival pathways. For this reason we investigated the participation of Trk receptors on the effect of IL-6 on RGC survival and observed the suppression of IL-6 effect when these receptors were blocked.

Studies show that in different experimental systems the effect of IL-6 are correlated to neurotrophins [35,36] and it has been demonstrated that IL-6 can modulate BDNF synthesis in different experimental models [35,37,38]. Accordingly, our data show that when BDNF action was blocked by neutralizing antibodies the effect of either IL-6 or CHA on the survival of RGC was abolished, demonstrating the involvement of BDNF in the IL-6 effect.

To investigate if IL-6 was regulating the synthesis of BDNF we analyzed by Western blot the levels of this neurotrophin after different time intervals of IL-6 treatment. The results show that IL-6 increases BDNF levels after 45 min of stimulus but not after 4 or 24 h. After 48 h of either IL-6 or CHA treatment we observed a decrease in BDNF levels. Interestingly, 48 h of BDNF treatment

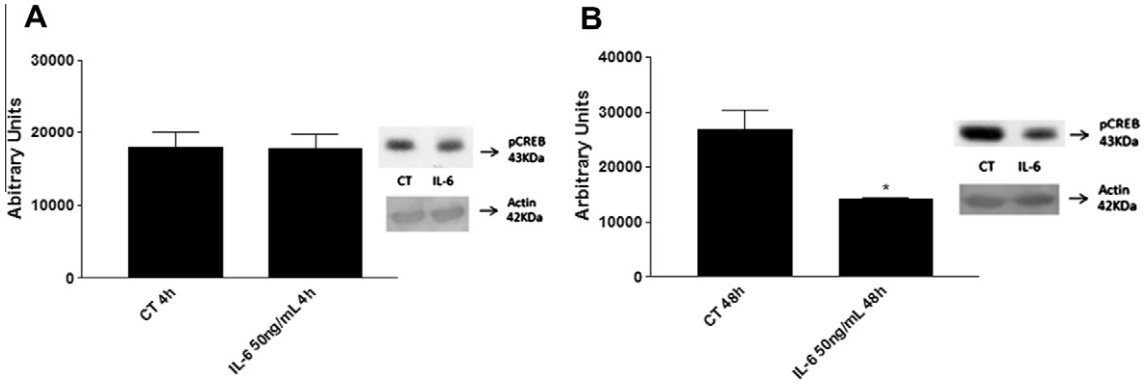

Fig. 4. (A) Levels of pCREB (pCREB) in cultures treated with IL-6 for 4 h. Control. IL-6 50 ng/mL: interleukin-6. Data are presented as mean ± SEM ($n = 3$). (B) Levels of pCREB in cultures treated with IL-6 for 48 h. Control. IL-6 50 ng/mL: interleukin-6. Data are presented as mean ± SEM ($n = 3$).

induced the same effect in BDNF levels, suggesting a capacity of BDNF to downregulate its own levels.

The relationship between TrkB receptor phosphorylation and the survival effects of BDNF in neuronal cells is well known [39]. To better understand the IL-6 effect on the control of BDNF levels, we investigated the levels of pTrkB in our cultures after IL-6 treatment. Our results show that the levels of pTrkB decreased after treatment with IL-6 for 48 h. These results are in accordance with our above mentioned results which show that BDNF levels are decreased after 48 h of IL-6 treatment.

The role of CREB (cAMP responsive element-binding protein) as a transcription factor mediating BDNF synthesis is well established [40]. We have found a decrease of pCREB levels after 48 h treatment with IL-6. This result is in disagreement with that observed by Murphy and co-workers after treatment of dorsal root ganglion neurons with IL-6 [38]. These contradictory results could be explained by the differences between retinal cells and the cells present in the dorsal root ganglia.

The death of RGC is observed in several important diseases such as glaucoma or diabetes and is responsible for the blindness observed in patients [17,41]. For this reason it is important to study mechanisms involved in RGC survival in order to establish appropriate pharmacological protocols capable of increasing the survival of these central neurons. Data from literature shows that IL-6 is able to rescue RGC from death induced by high pressure condition [17]. However, it is not easy to use IL-6 as a treatment since this molecule can act like a pro-inflammatory cytokine inducing side effects such as fever and even cell death [42–44]. In the present work, we demonstrate that the trophic effect of IL-6 on RGC is mediated by the activation of A1R. Given that A1R ligands are already widely used to treat different pathological conditions [45,46] the use of A1R agonists might lead to a new therapeutic strategy in diseases involving the death of RGC.

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