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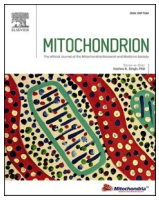

Original Research Article

Neuroprotective compounds alter the expression of genes coding for proteins related to mitochondrial function in activated microglia

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ABSTRACT

A hallmark of neuroinflammatory disorders is mitochondrial dysfunction. Nevertheless, the transcriptional changes underlying this alteration are not well-defined. Microglia activation, a decrease in mitochondrion biogenesis and a subsequent alteration of the redox are common factors in diseases coursing with neuroinflammation. In the last two decades, components of the adenosinergic system have been proposed as potential therapeutic targets to combat neuroinflammation. In this research, we analyzed by RNAseq the gene expression in activated microglia treated with an adenosine A_{2A} receptor antagonist, SCH 582561, and/or an A_3 receptor agonist, 2-Cl-IB-MECA, since these receptors are deeply related to neurodegeneration and inflammation. The analysis was focused on genes related to inflammation and REDOX homeostasis. It was detected that in the three conditions (microglia treated with 2-Cl-IB-MECA, SCH 582561, and their combination) more than 40 % of the detected genes codified by the mitochondrial genome were differentially expressed (FDR < 0.05) (14/34, 16/34, and 13/34) respectively, being almost all of them (>85 %) upregulated in the microglia treated with adenosinergic compounds. Also, we analyzed the differential expression of genes related to mitochondrial function and oxidative stress codified by the nuclear genome. Additionally, we evaluated the oxygen consumption rate (OCR) of mitochondria in microglia treated with LPS and IFN-γ, both alone and in combination with adenosinergic compounds. The data showed an improvement in mitochondrial function with the antagonist of the adenosine A_{2A} receptor, compared to the effects of pro-inflammatory stimulus, confirming a functional effect consistent with the RNAseq data.

1. Introduction

A substantial proportion of the most prevalent metabolic alterations, such as diabetes, metabolic syndrome, and obesity co-occur with cognitive and neuronal symptoms and increase the chances of developing neurodegenerative diseases, which are characterized by increased reactive oxygen species (ROS) levels and by inflammation due to the activation of microglia (Franco et al., 2019; Wright, 2022). Clinical features can be divided into systemic symptoms (SS), and those that are neurological (NS). Nevertheless, due to the priority in addressing SS to prevent early death, NS are underdiagnosed and, therefore, underexplored, and undertreated. This has led to the consideration of a previously underestimated axis that is known as neuroimmunometabolism (Larabee et al., 2019) and focuses on comprehending the implications that metabolic alterations can have on the nervous system through the activity of immune cells, including the peripheral macrophages and their equivalent in the CNS: microglia.

Metabolic alterations are associated with impaired energy production caused by abnormal processes in various phases of nutrient absorption, cellular uptake, and catabolism. Specifically, these

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abnormalities have in common mitochondrial dysfunction, which leads to a decreased rate of energy production within the electron respiratory chain. This dysfunction is one of the primary factors contributing to the excessive production of ROS (Quincozes-Santos et al., 2017), and to the skewing of macrophages/microglia toward a pro-inflammatory phenotype (Wang et al., 2018).

Clear examples of the link between systemic metabolic alterations and nervous system impairment are the increased chances of suffering from Alzheimer's disease (Arnold et al., 2018) and Parkinson's disease (Han et al., 2023) of diabetic patients. The pro-inflammatory effects of the metabolic alterations are not only due to activation of peripheral macrophages but of microglia (Dhananjayan et al., 2017)(Spiteri et al., 2022). Hence, it is paramount to address how activation of macrophages and microglia impact mitochondrial function. There is evidence of neuroinflammation in both animal models (Nagayach et al., 2022) and patients (Vargas-Soria et al., 2023). In fact, the inflammatory processes observed systemically in patients have a correlate in the central nervous system, where microglia are key to orchestrating both pro-inflammatory and neuroprotective molecules and controlling ROS production.

The approval in Japan and the U.S. of a first-in-class adenosinergic drug, an adenosine A_{2A} receptor ($A_{2A}R$) antagonist, has fueled hopes for more approvals of therapeutic agents acting on adenosine receptors. The approved drug is istradefylline, a synthetic methylxanthine. Consumption of natural methylxanthines, caffeine, and theophylline, which are non-selective antagonists of adenosine receptors, decreases the risk of suffering from Parkinson's and Alzheimer's disease (Bibbiani et al., 2003; Cunha, 2016, 2008; Eskelinen et al., 2009; Eskelinen and Kivipelto, 2010; Espinosa et al., 2013; Franco, 2009; Gelber et al., 2011; Ikeda et al., 2002; Kachroo and Schwarzschild, 2012; Kalda et al., 2006; Lindsay et al., 2002; Nehlig, 2016; Serrano-Marín et al., 2020; Silva et al., 2018), thus suggesting its effect in hindering ROS production and/or inflammation. In cell and animal models it has been shown that istradefylline and other $A_{2A}R$ antagonists are anti-inflammatory. A_{2A} ($A_{2A}R$) and A_3 (A_3R) receptors, as other members of the G protein-coupled receptor (GPCR) family, interact to form a functional unit in which the A_3R functionality is impaired (Lillo et al., 2020). Blockade of the A_3R is reverted in the presence of $A_{2A}R$ antagonists. Consequently, it is hypothesized that the combination of $A_{2A}R$ antagonists and A_3R agonists could provide greater hindrance to ROS production than that provided by each compound alone.

Upon activation, microglia can polarize in two extreme phenotypes that, for instrumental purposes, are known as pro-inflammatory (M1) and neuroprotective (M2) (Franco and Fernández-Suárez, 2015). There is interest in knowing whether $A_{2A}R$ antagonists and/or A_3R agonists may shift microglia from the M1 to the M2 phenotype. Activation of the $A_{2A}R$ in activated microglia leads to oxidative stress revealed by an increase in nitric oxide production and release (Saura et al., 2005). Thus, the potential of adenosine receptor ligands goes from the polarization of microglia to the regulation of oxidative stress. In fact, tools are needed to reduce the oxidative stress associated with various diseases that sooner or later affect mitochondria and/or the expression and activity of factors necessary for REDOX homeostasis (Fan et al., 2017).

Several gene ontologies (GOs) that become affected upon pharmacological treatment cannot be detected by comparing the control and the pathological condition using models of neurological diseases. There are many confounder issues, being important the difficulty in the separation of the different cell types. Proposed solutions for this problem are the single-cell RNASeq (scRNASeq), and the single-nucleus RNA-Seq (snRNASeq). snRNASeq provides background noise and excess data, making difficult real progress in knowledge. snRNASeq is unsuitable for the study of microglia, which is one of the most important cell types in terms of the pathophysiology of neurological symptoms related to metabolic disorders. This study aimed to analyze the impact of adenosine receptor ligands on the expression profile of REDOX and mitochondrial-related genes in activated microglia. Specifically, 2-Cl-IB-MECA, an A_3R agonist, and SCH 58261, an $A_{2A}R$ antagonist, were administered both individually and in combination. The identification of genes associated with mitochondrial function led to subsequent assays of mitochondrial respiration, revealing results that corroborated the findings from RNAseq data analysis.

2. Materials and Methods

2.1. Reagents

Chloroform (C2432-500ML) was obtained from Sigma Aldrich (St. Louis, MO, US), Trizol (15696026) was obtained from Ambion Life Technologies (Waltham, MA, US), isopropanol (131090.1211) was obtained from PanReac AppliChem (Chicago, IL, US), lipopolysaccharide (L4391-1MG) and human interferon-γ (I3265-1MG) were obtained from Sigma_Aldrich (St. Louis, MO. US), 2-Cl-IB-MECA (1104) and SCH 58261 (2270) were obtained from Tocris Bioscience (Bristol, UK).

2.2. Cells

The HMC3 cell line derives from human fetal brain-derived primary microglial cells (ATCC Cat# CRL-3304); it was cultured in DMEM medium (Sigma_Aldrich) supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine (Sigma_Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C and 5 % CO_2 in a humidified incubator.

Primary microglial cells were obtained from the brain of 2 to 4 days-old CD-1 mouse pups. Cells obtained as described elsewhere (Navarro et al., 2018) were plated at a density of 40,000 cells/0.32 cm^2. Repeated pipetting and passage through a 100 μm-pore mesh was followed by centrifuging at 200 x g for 7 min. The cell pellet was placed in Dulbecco's modified Eagle medium (DMEM) containing 2 mM L-glutamine (DMEM-g) and cells were seeded in 6-well plates (3.5 x 10^5 cells/ml density). 24 h later, cells were placed in DMEM-g supplemented with MEM Non-Essential Amino Acid Solution (1/100) and 10 % (v/v) heat inactivated Fetal Bovine Serum (FBS) and containing 100 U/mL penicillin/streptomycin. All cell culture reagents were from Invitrogen, Paisley, Scotland, United Kingdom. Cells were cultured for 15 days in a 5 % CO_2 humid atmosphere (37 °C) prior activation using 0.01 % (v/v) lipopolysaccharide (LPS) and 0.002 % (v/v) interferon-γ (IFN-γ). 24, 32 and 40 h after LPS/IFN-γ treatment, vehicle or adenosine receptor ligands were added to the culture. At 48 h counted since the beginning of activation with LPS and IFN-γ, RNA was extracted using Trizol (details in (Lillo et al., 2023)). Purification was made using isopropanol and chloroform. Purity and integrity were assessed by, respectively, the 280/260 absorption ratio and the RNA Integrity Number (RIN). Further quality control for each sample was performed at German facilities of Novogene where sequencing was performed. Following mRNA purification using poly-T oligo-attached magnetic beads, first-strand cDNA synthesis was achieved using random hexamer primers; for second-strand cDNA synthesis dTTP (non-directional library) or dUTP for (directional library). For the non-directional library, it was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. For the directional library, it was ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification, and purification. Qubit and real-time PCR for quantification was used for checking and bioanalyzer for size distribution. Sequences were obtained using Novogene NovaSeq 6000 (pair end 150), being 151 + 8 + 8 + 151 the sequencing cycles. Quality control of sequencing data (raw reads) was first achieved via in-house "perl" scripts. "Clean reads" were obtained upon removal of reads containing adapter or ploy-N; low-quality reads were also removed prior final analysis. Paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5.

2.3. RNAseq data processing

Feature Counts v1.5.0-p3 software was used for counting the reads to

each gene-derived mRNA. Fragments per Kb was calculated considering the length of a given gene and reads count for the gene. Differential expression analysis was done using millions base pairs sequenced (FPKM) and considering the effect of sequencing depth and gene length for the reads.

2.4. Database retrieval and differential expression analysis

Data were retrieved from the Gene Set Experiments uploaded to Gene Expression Omnibus with the accession numbers GSE214330 for the control samples and the treated with A_3R agonist, and GSE222696 for the samples treated with the A_{2A}R antagonist and with the combination of A_3R agonist and A_{2A}R antagonist.

Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR package. Differential expression analysis was performed using the DESeq2R package (1.20.0). The software uses the negative binomial distribution for analysis and determination of P-values; the Benjamini-Hochberg approach was employed for assessing the false discovery rate. The "edgeR" R package was used for comparing the expression in two different experimental conditions. Differentially expressed genes were selected by having a false discovery rate (FDR) < 0.05 and a fold change (FC) of less than 0.67 or greater than 1.5.

2.5. Gene ontology analyses

For the gene ontology analysis, the g:Profiler and the EnrichR online software were used (https://biit.cs.ut.ee/gprofiler/gost, and https://maayanlab.cloud/enrichr-kg, respectively; both accessed on February 14, 2023). The gene ontology analysis of the mitochondrial genes encoded by the mitochondrial chromosome that were differentially expressed was performed with g:Profiler. The association of the genes with the biological processes was not only done using empirical information but of various kinds of bioinformatical prediction. To assess the mitochondrial genes encoded by the nuclear genome, Enrichr tool "interactive and collaborative HTML5 gene list enrichment analysis tool" was used, selecting as curated database the database of GO related to biological processes. Afterwards, hits were clustered using the REVIGO online tool (https://revigo.irb.hr/); which allows a 2D-plot for grouping GOs by similarity. Cytoscape software (v.3.9.1) (https://cytoscape.org/) was used to improve the comparison analysis.

STRING is defined as a "*database of known and predicted protein–-protein interactions*" https://string-db.org/ accessed on March 29, 2023). STRING was used for obtaining the GOs of the differentially expressed genes with the following settings: full network, i.e., considering indirect (functional) and direct (physical) interactions, no additional shells (only the products of the genes provided to STRING were considered) and a confidence of 0.4.

2.6. Methods for finding altered oxidative cellular pathways

Based on empirical and bioinformatic prediction tools, differentially expressed genes (DEGs) related to oxidative cellular pathways were obtained and a subsequent gene ontology analysis was performed. For this analysis, EnrichR online software was used. GO terms were considered enriched at a statistical significance of < 0.05; they were later used and clustered using the REVIGO online tool, which allows grouping GOs in other GOs of a higher hierarchical order. Cytoscape software (v.3.9.1) was used to improve the better revealing comparisons of the results.

2.7. Oxygen consumption rate determination

Mitochondrial oxygen consumption rate (OCR) was evaluated in the human HMC3 microglial cell line using a Seahorse Bioscience XF Extracellular Flux Analyzer (Seahorse Bioscience, North Billerica, MA). HMC3 cells (10.000 cells/well) were seeded in 96-well plates and grown for 24 h. Then, cells were treated with 0.01 % (v/v) LPS and 0.002 % (v/v) IFN-γ for 24 h for inducing the pro-inflammatory microglial phenotype with or without an adenosine A_{2A}R antagonist, SCH 582561, and/or an A_3R agonist, 2-Cl-IB-MECA (24, 32 and 40 h after LPS/IFN-γ treatment). After treatments, cells were washed and incubated for 1 h at 37 °C in a non-CO_2 incubator with assay medium (XF DMEM base medium supplemented with 2 mM glutamine, 10 mM glucose, and 1 mM sodium pyruvate, pH 7.4). Basal respiration, maximal respiration, and the spare respiratory capacity were calculated from the OCR measurements with the sequential addition of Oligomycin (1 μM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) at 1 μM, and antimycin A/ rotenone (0.5 μM) using Wave Desktop 2.6.1. Data were normalized with total μg of protein calculated with Pierce BCA Protein Assay Kit (Thermo Scientific).

3. Results

3.1. Analysis of nuclear gene expression of products related to the management of oxidative stress

3.1.1. Differentially expressed genes related to REDOX homeostasis

The expression of genes involved in the oxidative response was checked in the three conditions (LPS/IFN-γ-activated microglia treated with 2-Cl-IB-MECA, activated microglia treated with SCH 582561 and activated microglia treated with the two adenosine receptor ligands. Several genes were significantly up/downregulated in all three treatments (Fig. 1 and Tables 1 and 2). Genes that were significantly upregulated in all three conditions code for C-reactive protein (CRP), glycosylation end product receptor (RAGE), proline-rich 5 protein and lipocalin 2. Several genes directly and/or indirectly related to REDOX homeostasis in the CNS were significantly downregulated, up to 27 detected in all three treatments (Fig. 1 and Table 2).

3.1.2. Mitochondrial products coded by nuclear genes

The mitochondrion is key in handling oxidative stress, by helping to produce both radical scavengers and the reducing power needed manage oxidative stress. We checked the transcripts encoded by nuclear DNA and coding for mitochondrial products. Data from four conditions were considered: activated microglia, activated microglia treated with 2-Cl-IB-MECA, activated microglia treated with SCH 582561 and activated microglia treated with the two adenosine receptor ligands. Compared with the control (activated microglia), a significant number of differentially expressed genes (DEGs) were detected. Some of them were coding for proteins involved in both metabolism and detox processes. Genes that were significantly upregulated (versus activated microglia) in all three conditions code for: alcohol dehydrogenase 1 −iron containing-, branched chain ketoacid dehydrogenase E1, −ß polypeptide-, 7A1 subunit of cytochrome *c* oxidase, family 24 member of cytochrome P450 monooxygenases (Cyp24a1), dehydrogenase E1 and transketolase domain containing 1, dual specificity phosphatase 2 and mono-ADP ribosyl hydrolase 1. Except for the latter, all these proteins participate directly or indirectly in the regulation of REDOX events. When analyzing both up and downregulated DEGs, gene ontologies (GOs) related to oxidative stress mediators and oxidative stress management were identified (Fig. 2). Genes that were significantly downregulated (versus activated microglia) in all three conditions code for: member 9 of ATP-binding cassette, sub-family A (ABCA9), FK506 binding protein 10, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2, phorbol-12-myristate-13-acetate-induced protein 1, serine protease 35, riboflavin kinase, solute carrier family 25, member 48 and Tubb3 class III member of beta tubulins (Table 1 and 2). These proteins are less related to oxidative stress than those coded by upregulated genes; however, they are related to events that are relevant to the immunological functionality of microglia. A bar graph of the fold change (FC) for each differentially expressed gene (DEG) in each treatment (A_3R agonist and/or

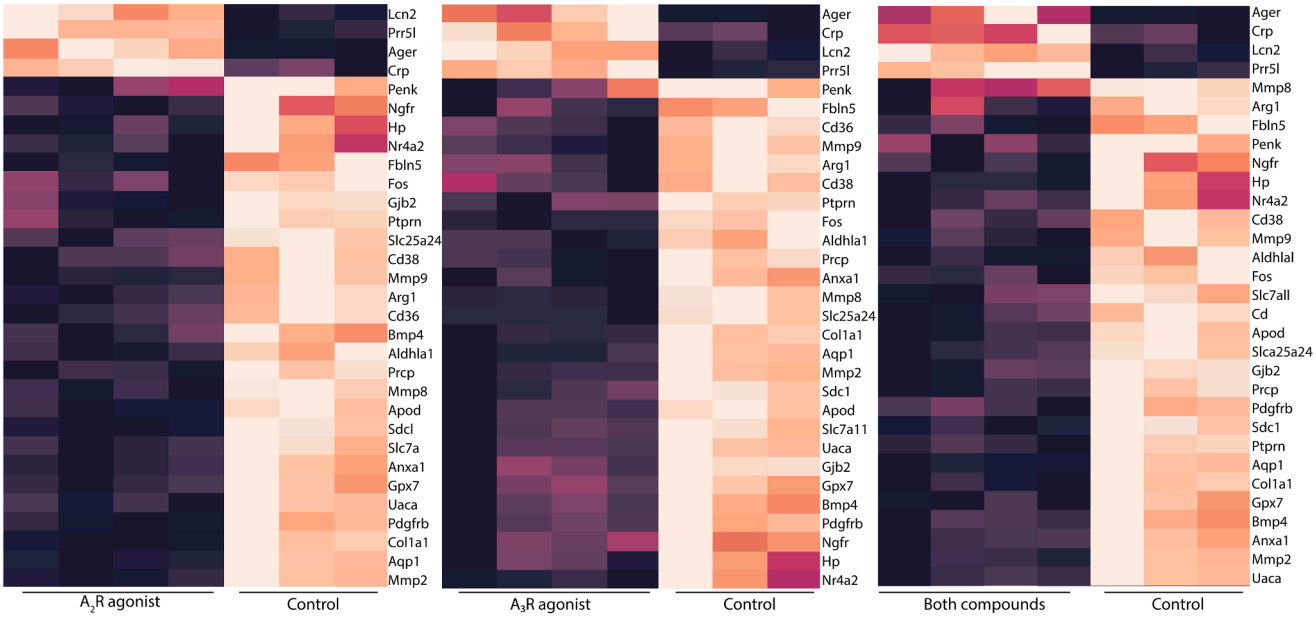

Fig. 1. Heatmap of expression of nuclear genes encoding for proteins related to mitochondrial function and REDOX homeostasis. Genes that are differentially expressed (versus activated microglia: "control") and that meet the criteria false discovery rate (FDR) < 0.05 and ii) fold change (FC) > 1.5 for upregulated genes or fold change (FC) < 0.67 for downregulated genes. Conditions treatment with the $A_{2A}R$ antagonist (SCH 58261, left), treatment with the A_3R agonist (2-Cl-IB-MECA, middle) and both (right). In the columns presenting data from treatments with adenosine receptor ligands, the darker the color, the more downregulated the expression of the gene.

$A_{2A}R$ antagonist) is provided in Fig. 3. Downregulation can go as low as 5-fold less compared with control (activated microglia), as in the case of serine protease 35 and upregulation goes up to 3-fold in the case of dual specificity phosphatase 2; also noticeable is the 2.7-fold increase of Cyp24a1 in the treatment with the $A_{2A}R$ antagonist. The connection between REDOX homeostasis and immunological regulation events in activated microglia is analyzed below.

For the gene ontology analysis of the nuclear DEGs that code for mitochondrial proteins, we used the procedure described in *Methods* but restricting the search to the genes included in MitoCarta3.0, an inventory of genes encoding mammalian mitochondrial proteins provided by the Broad Institute. When performing the gene ontology analysis of significantly upregulated/downregulated genes in each treatment (SCH 58261, 2-Cl-IB-MECA and both), we detected several ontologies some of which are related to REDOX homeostasis, to protection against neurodegeneration and/or to neuroinflammation. Subsequent analysis was performed only using genes that were found up/downregulated in all three treatments. Upon in-deep analysis using the downregulated genes, several of the detected GOs were related to alterations in the energy production and cell survival processes; examples are apoptosis (GO:0006915), regulation of NAD(P)H oxidase activity (GO:0033860), ATP transport (GO:0015867) and mitochondrial outer membrane permeability (GO:0097345) (Fig. 3). The GOs arising when considering upregulated genes were, among others, fat-soluble vitamin catabolism (GO:0042363), cellular response to vitamin D and its metabolism (GO:0071305, GO:0033273, GO:0042359 and GO:0033280), steroid catabolic process (GO:0006706) and fatty acid omega-oxidation (GO:0010430). Additionally, two GOs related to glutamate, a neurotransmitter, a source of carbons for the mitochondrial tricarboxylic acid cycle and a molecule actively involved in CNS homeostasis were found: glutamate catabolic process (GO:0006538), and 2-oxoglutarate metabolic process (GO:0006103) (Fig. 3).

3.2. *Differential expression of genes in the mitochondrial DNA*

Analysis on the expression of genes encoded by the mitochondrial DNA was done using data obtained in activated microglia, and in activated microglia treated with an A_3R agonist, 2-Cl-IB-MECA, and/or an $A_{2A}R$ antagonist, SCH 582561. Compared with the control (activated microglia), a significant number of transcripts were differentially expressed. The number of differentially expressed genes was 16, 14 and 13 in cells treated, respectively, with the A_3R agonist, the $A_{2A}R$ antagonist, and the combination. Most of DEGs (>85 %) were significantly upregulated in the microglia treated with adenosine receptor ligands. First, it should be noted that no gene for a mitochondrial protein was significantly downregulated. Significant downregulation was only occurring for genes coding for one transfer and two and ribosomal RNAs (Table 2). Significantly upregulated genes coded for transfer RNAs, but also for the mitochondrial proteins (Table 1). Proteins coded by genes upregulated in all three conditions were: mitochondrial ATP synthase 6 (mt-Atp6), mitochondrial cytochrome *b*, mitochondrial encoded cytochrome *c* oxidase II, mitochondrial NADH dehydrogenase 1 and mitochondrial NADH dehydrogenase 4L. The FC for each DEG in each of the treatments is shown in Fig. 4. The values indicate that individual and combined treatments led to minor changes in the FC of DEGs thus suggesting that adenosine actions via $A_{2A}R$ or via A_3R are only slightly affecting the expression and function of mitochondrial RNAs or of proteins encoded by the genes in the mitochondrial chromosome. Hence, the results concerning the mitochondrial genome were not further considered.

3.2.1. *STRING analysis*

To look for networks connecting the DEGs and to assess how adenosine receptor ligands may affect REDOX homeostasis in activated microglia, the STRING tool was used (See Methods). Nuclear DEGs were considered in the analysis that is, excluding; for the reasons given section 3.2, DEGs of the mitochondrial genome. Genes that were upregulated in all three conditions or downregulated in all three conditions led to the connections displayed in Fig. 5 and Fig. 6, respectively. Six of the differentially expressed genes do not interact according to standard STRING criteria. Adding to the analysis other genes that were upregulated led to the connections displayed in Supplementary Figure S1. The main feature in Fig. 5 is the cluster of genes coding for mitochondrial proteins that, via mitochondrial-encoded cytochrome *c* oxidase II, is

Table 1
Significantly upregulated genes in nuclear or mitochondrial chromosomes that code for mitochondrial products and/or for proteins involved in REDOX homeostasis. Genes in the list are related to oxidative stress production regulation, and to mitochondrial function. In red: genes that do not code for proteins but for transfer or ribosomal RNAs. FC: Fold change. Mt: mitochondrial. Chr: Chromosomal.

UPREGULATED																	
Oxidative stress						Mitochondrial products encoded by mitochondrial chr						Mitochondrial products encoded by nuclear chr					
$A_{2A}R$ antagonist		A_3R agonist		Combined treatment		$A_{2A}R$ antagonist		A_3R agonist		Combined treatment		$A_{2A}R$ antagonist		A_3R agonist		Combined treatment	
Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC
Crp	3.56	Crp	3.66	Crp	3.06	mt-Atp6	1.17	mt-Atp6	1.15	mt-Atp6	1.13	Adhfe1	2.01	Adhfe1	2.04	Adhfe1	1.88
Ager	2.07	Ager	2.10	Ager	2.04	mt-Co2	1.22	mt-Co2	1.2	mt-Co2	1.16	Bckdhb	1.8072	Bckdhb	1.79	Bckdhb	1.63
Prr5l	1.68	Prr5l	1.72	Prr5l	1.65	mt-Cytb	1.10	mt-Cytb	1.14	mt-Cytb	1.09	Cox7a1	1.56	Cox7a1	1.67	Cox7a1	1.68
Lcn2	1.63	Lcn2	1.54	Lcn2	1.51	mt-Nd1	1.19	mt-Nd1	1.2	mt-Nd1	1.18	Cyp24a1	2.73	Cyp24a1	2.40	Cyp24a1	2.25
Lpo	1.53	Lpo	1.51			mt-Nd4l	1.37	mt-Nd4l	1.8	mt-Nd4l	1.43	Dhtkd1	1.61	Dhtkd1	1.62	Dhtkd1	1.64
Nos3	1.64			Nos3	1.69	mt-Tc	1.35	mt-Tc	1.3	mt-Tc	1.25	Macrod1	1.7400	Macrod1	1.77	Macrod1	1.61
Cxcl1	1.59					mt-Tn	1.58	mt-Tn	1.28	mt-Tn	1.26	Dusp26	2.95	Dusp26	2.55	Dusp26	2.11
Gstk1	1.56					mt-Tp	1.14	mt-Tp	1.14	mt-Tp	1.19	Aldh1l1	1.5436	Aldh1l1	1.57		
		Mt3	1.50			mt-Nd6	1.13	mt-Nd2	1.07			Gatb	1.55	Gatb	1.52		
		Epas1	1.55			mt-Co3	1.18	mt-Co3	1.15			Me3	1.63	Me3	1.67		
								mt-Nd4	1.14	mt-Nd4	1.09	Nudt6	1.79	Nudt6	1.65		
												Sdsl	1.59	Sdsl	1.60		
												Slc25a18	1.56	Slc25a18	1.50		
														Mterf1b	1.69	Mterf1b	1.76
														Acss1	1.63	Acss1	1.53
														Maob	1.64	Maob	1.51
												Slc25a45	1.59				
												Efhd1	2.1465				
												Gatm	1.53				
												Gstk1	1.56				
														Slc25a21	1.54		
														Slc25a48	1.98		
														Cstad	1.53		
														Slc25a43	1.52		

connected to Dhtkd1-coded dehydrogenase and, via mitochondrial-encoded NADH:ubiquinone oxidoreductase core subunit 1, to C reactive protein and lipocalin 2. The mesh in Supplementary Figure S1 is similar but more populated and with one more gene, nitric oxide 3 (NOS3), that is differentially expressed in the treatment with the antagonist and in the dual treatment (Fig. 7B). NOS3 enhances the connectivity as it is connected to both mitochondrial-encoded cytochrome *c* oxidase II and NADH:ubiquinone oxidoreductase core Subunit 1; in addition, NOS3 allows further connectivity to C reactive protein and to glycine amidinotransferase, a mitochondrial enzyme, and EPAS1 transcription factor.

Genes that were downregulated in all three conditions led to the interaction networks displayed in Fig. 6. Comparing the genes in the figure with those in the list of DEGs in Table 2, it is noticed that, according to standard STRING criteria, up to 15 of them were non interacting. A main cluster of interactions included three metalloproteinases (MMPs), the nerve growth factor receptor (ß-polypeptide), Col1a1-coded collagen subunit, bone morphogenetic protein 4 and *fos* transcription factor. Linked to some of the nodes in the main interaction cluster are proteins relevant for cell fate and/or for regulation of

Table 2
Significantly downregulated genes in nuclear or mitochondrial chromosomes that code for mitochondrial products and/or for proteins involved in REDOX homeostasis. In red: genes that do not code for proteins but for transfer or ribosomal RNAs. FC: Fold change. Mt: mitochondrial. Chr: Chromosome.

DOWNREGULATED																	
Oxidative stress						Mitochondrial products encoded by mt Chr						Mitochondrial products encoded by nuclear Chrs					
$A_{2A}R$ antagonist		A_3R agonist		Combined treatment		$A_{2A}R$ antagonist		A_3R agonist		Combined treatment		$A_{2A}R$ antagonist		A_3R agonist		Combined treatment	
Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC	Gene	FC
Aldh1a1	0.40	Aldh1a1	0.46	Aldh1a1	0.46	mt-Ti	0.174					Abca9	0.53	Abca9	0.53	Abca9	0.55
Anxa1	0.53	Anxa1	0.59	Anxa1	0.55			mt-Rnr1	0.77	mt-Rnr1	0.82	Fkbp10	0.61	Fkbp10	0.62	Fkbp10	0.61
Apod	0.32	Apod	0.36	Apod	0.34			mt-Rnr2	0.87	mt-Rnr2	0.83	Hmgcs2	0.21	Hmgcs2	0.35	Hmgcs2	0.26
Aqp1	0.19	Aqp1	0.27	Aqp1	0.20							Pmaip1	0.60	Pmaip1	0.56	Pmaip1	0.66
Arg1	0.25	Arg1	0.42	Arg1	0.45							Prss35	0.19	Prss35	0.24	Prss35	0.21
Bmp4	0.52	Bmp4	0.58	Bmp4	0.52							Rfk	0.60	Rfk	0.62	Rfk	0.60
Cd36	0.44	Cd36	0.44	Cd36	0.45							Slc25a24	0.52	Slc25a24	0.55	Slc25a24	0.54
Cd38	0.60	Cd38	0.66	Cd38	0.66							Tubb3	0.62	Tubb3	0.58	Tubb3	0.52
Col1a1	0.22	Col1a1	0.25	Col1a1	0.23							Alas2	0.43	Alas2	0.42		
Fbln5	0.55	Fbln5	0.61	Fbln5	0.59							Acaca	0.62				
Fos	0.53	Fos	0.55	Fos	0.60							Bcat1	0.63				
Gjb2	0.41	Gjb2	0.50	Gjb2	0.44							Chdh	0.64				
Gpx7	0.53	Gpx7	0.56	Gpx7	0.51							Mrps10	0.64				
Hp	0.53	Hp	0.65	Hp	0.57							Hspb7	0.31				
Mmp2	0.32	Mmp2	0.37	Mmp2	0.32									Recql4	0.56		
Mmp8	0.43	Mmp8	0.62	Mmp8	0.57											Clic4	0.66
Mmp9	0.30	Mmp9	0.34	Mmp9	0.38												
Ngfr	0.41	Ngfr	0.41	Ngfr	0.40												
Nr4a2	0.43	Nr4a2	0.46	Nr4a2	0.44												
Pdgfrb	0.59	Pdgfrb	0.63	Pdgfrb	0.59												
Penk	0.52	Penk	0.49	Penk	0.47												
Prcp	0.63	Prcp	0.65	Prcp	0.62												
Ptprn	0.58	Ptprn	0.52	Ptprn	0.53												
Sdc1	0.50	Sdc1	0.56	Sdc1	0.51												
Slc25a24	0.52	Slc25a24	0.55	Slc25a24	0.54												
Slc7a11	0.54	Slc7a11	0.53	Slc7a11	0.62												
Uaca	0.57	Uaca	0.59	Uaca	0.59												
Ptgs2	0.63	Ptgs2	0.59														
S100a8	0.60	S100a8	0.64														
Ednra	0.62			Ednra	0.60												
Mmp3	0.60			Mmp3	0.64												
Mycn	0.54			Mycn	0.44												
Ppargc1b	0.50			Ppargc1b	0.64												
Wnt16	0.28			Wnt16	0.47												
Alox5	0.58																
Cryaa	0.22																
Nox4	0.61																
Pla2r1	0.65																
Xdh	0.64																
		Areg	0.43														
				Cygb	0.34												

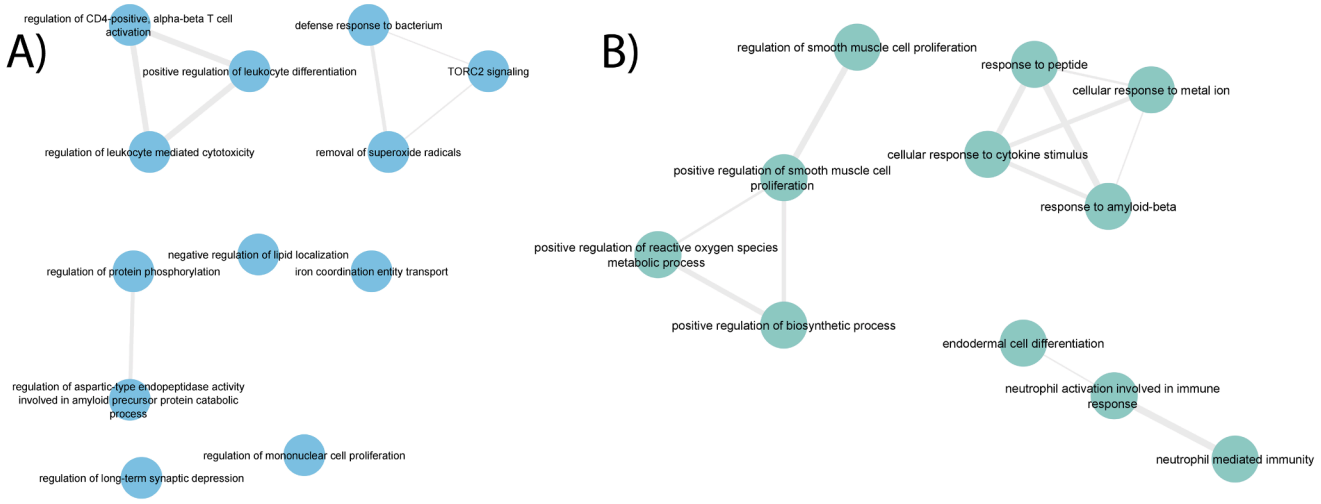

Fig. 2. Graphs of overrepresented GOs when considering DEG involved in mitochondrial metabolism and REDOX homeostasis. A) GOs shared among genes that were significantly upregulated in the three treatments. B) GOs shared among genes that were significantly downregulated in the three treatments.

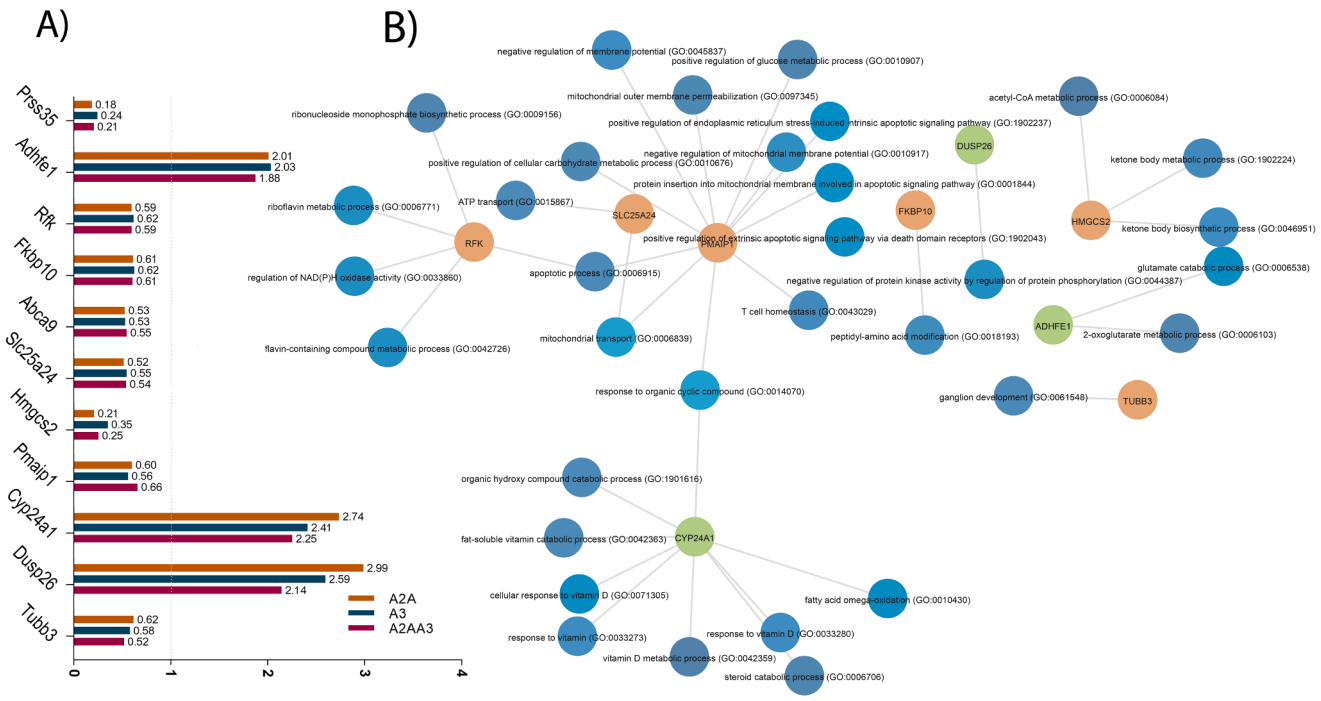

Fig. 3. Differential expression of nuclear gene-encoded mitochondrial products. Panel A: Fold change (FC) of the DEGs (encoding mitochondrial proteins) that were identified in both individual and combined treatments: FCs related to $A_{2A}R$ antagonist treatment are in orange, FCs related to A_3R agonist treatment are in blue and FCs related to the combined treatment are in red (all versus data obtained using activated microglia incubated with vehicle instead of with adenosine receptor ligands). **Panel B:** Interaction graph showing the overrepresented GOs when considering genes displayed in A. Significantly upregulated genes in green and significantly downregulated genes are in orange. The associated GOs are in blue. The lighter the blue, the smaller the false discovery rate (FDR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

microglial activation. An independent set of interactions is guided by haptoglobin that interacts with CD36 and arginase 1. Adding other genes that were downregulated in only one or two conditions led to a more populated map of interactions but without gross qualitative changes, that is, MMPs being the main nodes connecting to inflammation-related proteins and to the nerve growth factor receptor and *fos* (Supplementary Figure S2).

The connection map regarding DEGs common to the three treatments, irrespective of whether they were up or downregulated, is shown in Fig. 7. Remarkably, the image seems as resulting from the combination of the image in Fig. 5 and the image in Fig. 6 meaning that the combined treatment with the adenosine receptors ligands leads to upregulation of mitochondrial products and/or proteins contributing to the REDOX homeostasis and to downregulation of proteins related to the regulation of microglial activation. We consider relevant to include the gene coding for NOS3 that, as previously indicated, was differentially expressed in the treatment with the antagonist and in the dual treatment (in the treatment with the agonist the FC was 1.48, i.e., did not reach the threshold level of 1.5). When incorporated (Fig. 7 panel B), NOS3 is the main node that connects the two types of clusters. Two other nodes that

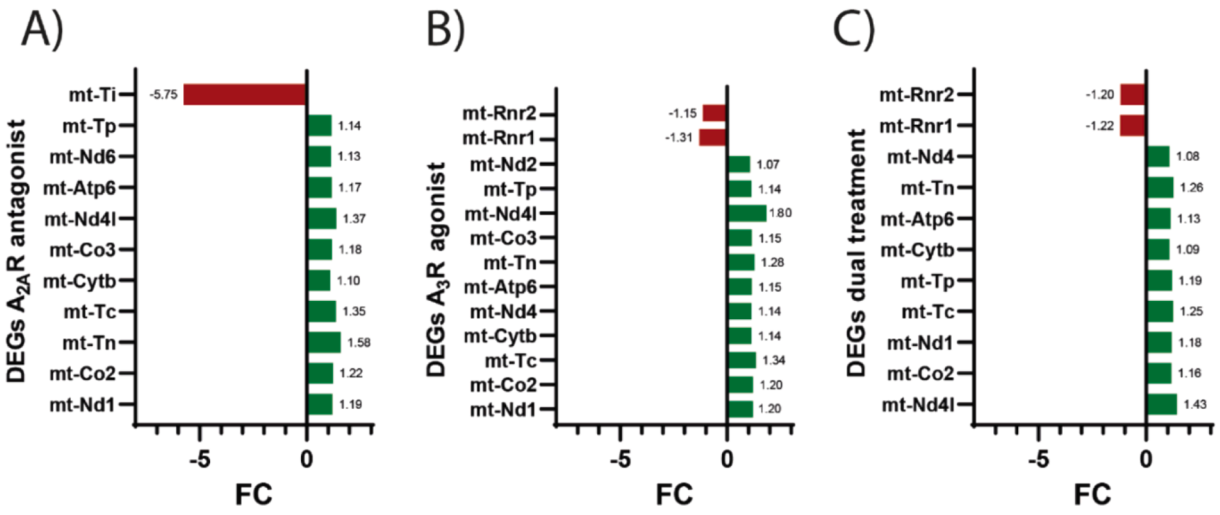

Fig. 4. Histograms showing the fold change (FC) of mitochondrial gene expression comparing data obtained in the absence and presence of the adenosine receptor ligands. Panel A: activated microglia treated with A_{2A}R antagonist (versus data obtained using activated microglia). **Panel B:** microglia treated with A_3R agonist (versus data obtained using activated microglia). **Panel C:** microglia treated with A_{2A}R antagonist and A_3R agonist (versus data obtained using activated microglia). Only genes with significantly different expression (a fold change $0.67 > FC > 1.5$) and FDR < 0.05 are shown.

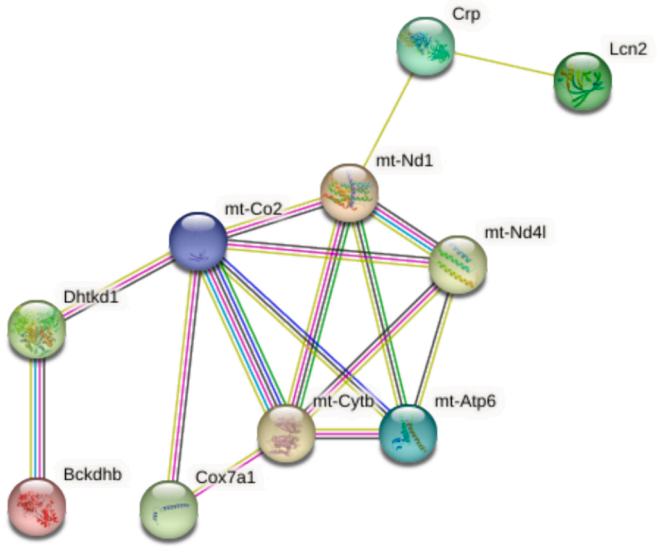

Fig. 5. STRING analysis of the protein products of the genes that were upregulated in the three treatments. Six proteins for which no interaction was found with standard STRING parameters are not displayed.

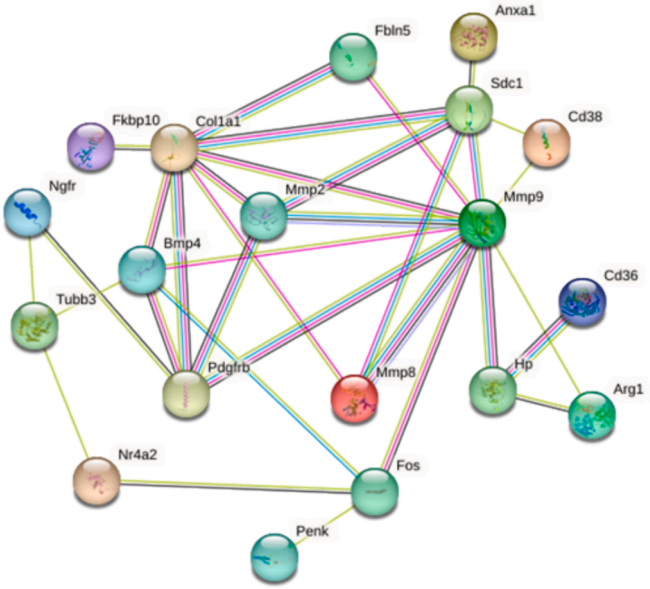

Fig. 6. STRING analysis of the protein products of the genes that were downregulated in the three treatments. 15 proteins for which no interaction was found with standard STRING parameters are not displayed.

connect the two types of clusters are *fos* and C reactive protein. The bottom part of Fig. 7 is mainly constituted by proteins related to mitochondrial function coded by upregulated genes, whereas the upper part is mainly constituted by proteins participating in the control of oxidative stress coded by downregulated genes.

3.3. Mitochondrial respiration in the HMC3 human microglial cell line

To investigate whether RNAseq findings correlate with changes in mitochondrial function, we compared respiration in LPS/IFN-γ activated HMC3 cells treated or not with the A_{2A}R antagonist, SCH 582561, with the A_3R agonist, 2-Cl-IB-MECA, or both (Fig. 8). Compared to resting microglia, activation of cells with LPS/IFN-γ activated microglia resulted in a significant decrease in basal (rate of OCR under basal conditions), maximal (maximum respiratory rate that the cell can achieve), and compensatory (capability to compensate energy demands of the cell with mitochondrial inhibition) respiration, confirming the metabolic reprograming in response to an inflammatory stimulus. However, co-treatment of the activated microglial cells with the A_{2A}R antagonist, SCH 582561, alone, or combined with the A_3R agonist, 2-Cl-IB-MECA, protects mitochondria from the maximal and compensatory respiratory pro-inflammatory effects. Neither single nor combined cotreatments with adenosine ligands led to changes in the basal respiration rate of activated cells (Fig. 8 A-C).

4. Discussion

Neuroprotection is crucial in managing metabolic diseases accompanied by neurological alterations. Traditionally, strategies to delay or prevent neuronal death focused directly on affected neurons. A newer, promising approach consists of targeting microglia, as neurodegeneration often triggers microglial activation, the release of pro-

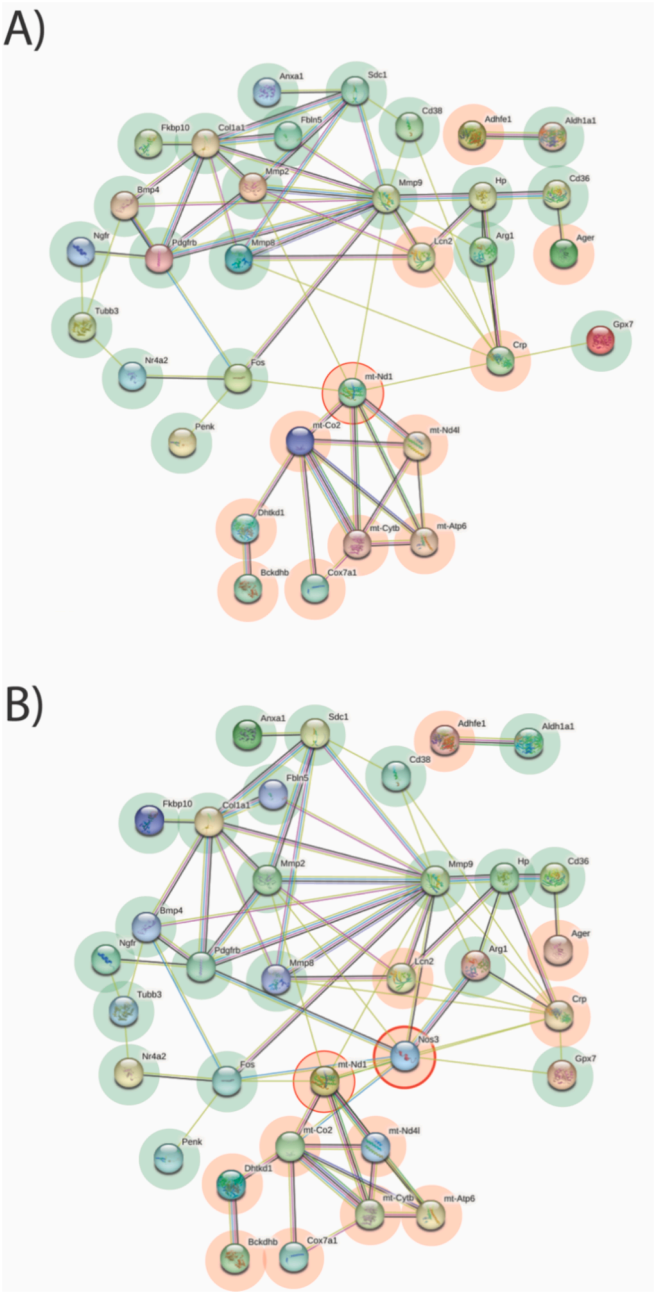

Fig. 7. STRING analysis of the protein products of the genes that were both upregulated and downregulated in the three treatments. Panel A. Without considering NOS3. **Panel B.** Considering NOS3 (see text). Those genes that were upregulated are highlighted in red, whilst those downregulated are highlighted in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

inflammatory cytokines, and increased oxidative stress. The current understanding is that neuroprotection can be achieved by shifting pro-inflammatory (M1) microglia to the neuroprotective (M2) phenotype. This phenotype is expected to produce neuroprotective factors and reduce oxidative stress. We are particularly interested in determining whether antagonists of the adenosine $A_{2A}R$ and agonists of the adenosine A_3R induce such a shift in microglial polarization. Additionally, we aimed to determine whether targeting cell surface receptors upregulated in activated microglia affects the expression of mitochondrial proteins. Using an RNAseq approach we have addressed, in activated microglia, the effect of $A_{2A}R$ antagonists and/or A_3R agonists on the expression of genes that are related to REDOX homeostasis and that code for mitochondrial proteins. The results provide insights into how adenosine ligands could afford neuroprotection by decreasing the expression of genes such as the protein tyrosine phosphatase receptor type N (PTPRN) and the nuclear receptor subfamily 4 group A member 2 (NR4A2), both of which have been related to neurologic processes directly linked with metabolic alterations. PTPRN is essential for the physiological production of norepinephrine, dopamine, and serotonin in the brain; it acts as a receptor for the PTP peptide, an autoantigen recognized by immunoglobulins present in the serum of insulin-dependent diabetes mellitus patients. Additionally, increasing the expression of genes such as Nos3 and Lcn2 appears to lead to anti-inflammatory effects (Lim et al., 2021; Yuste et al., 2015).

The adenosine receptor ligands used in this study exhibit anti-inflammatory properties and offer neuroprotection in both in vitro and in vivo models. GO analyses suggest that treating neuroinflammatory conditions with $A_{2A}R$ antagonists and/or A_3R agonists may exert their effects by reversing mitochondrial alterations induced by inflammation and enhancing metabolic processes that facilitate energy production, such as fatty acid oxidation and the Krebs cycle. These effects could promote neuronal survival in a neuroinflammatory context (Franco and Serrano-Marín, 2023; Wright, 2022; Yin et al., 2016).

Strategies aimed at enhancing mitochondrial antioxidant capacity, reducing ROS production, or repairing damaged mitochondria hold promise for potentially mitigating oxidative stress-related diseases. The STRING analysis of products of genes that are significantly upregulated in all conditions reveals a network containing proteins that are essential for redox reactions taking place in the mitochondria, for electron chain transport and ATP synthesis by oxidative phosphorylation. The main cluster is connected by C reactive protein and lipocalin 2, two “acute phase” inflammation biomarkers. Lipocalin 2 has been directly implicated in oxidative stress and plays a role in inflammation derived from ischemic stroke and traumatic brain injury (Kim et al., 2022). Although still considered a pro-inflammatory molecule, results reported in 2007 suggested that microglia secrete lipocalin 2 which, acting in an autocrine fashion, endows microglia with increased susceptibility to apoptosis, raising the question of whether it is helping to stop neuroinflammation (Lee et al., 2007). Its upregulation in response to oxidative stress suggests that it may act as a protective mechanism to mitigate the damaging effects of ROS (Parmar et al., 2018). Our results support an anti-inflammatory/neuroprotective role in microglia rather than a damaging role.

When both significant upregulated and significant downregulated genes are considered, the STRING analysis leads to almost independent networks. Remarkably, the two networks are only connected by the product of mtND1 gene, i.e., by a component of mitochondrial NADH: ubiquinone oxidoreductase, which is key for mitochondrial function in general and for managing oxidative stress in particular.

The effect of adenosinergic ligands on mitochondrial function aligned with the transcriptomics findings. Activated microglia showed a significant decrease in basal and maximal respiratory rates, likely resulting from a less aerobic metabolism. Activated microglia may experience oxidative stress due to their less aerobic metabolism. Additionally, the increased production of pro-inflammatory agents in activated microglia can further contribute to oxidative stress. Our results suggest $A_{2A}R$ antagonists and A_3R agonists act in coordination to increase aerobic respiration leading to reduced oxidative stress and to decrease production of inflammatory cytokines. These effects highlight the crucial role of mitochondria and mitochondrial genes in mediating the benefits of adenosinergic ligands. Consequently, the therapeutic potential of adenosinergic ligands for inflammatory conditions lies in their ability to target mitochondria, thereby providing sufficient energy and avoiding oxidative stress thus protecting mitochondria from the consequences of the expression of the M1 microglial phenotype.

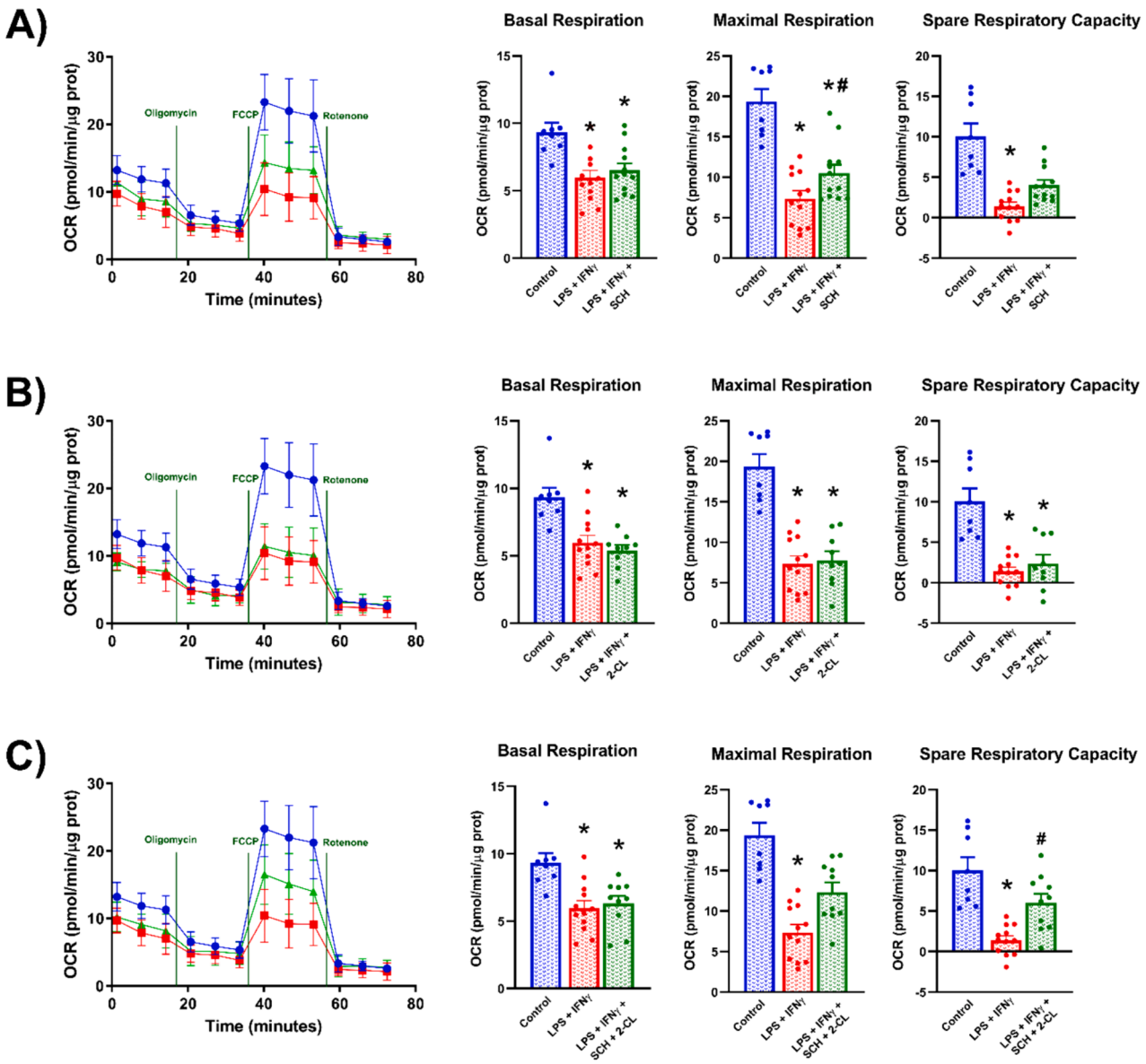

Fig. 8. Mitochondrial respiration parameters in HMC3 cells. The mitochondrial respiration (OCR levels) was detected in HMC3 cells treated with LPS + IFNγ (red line/bars), and with adenosine ligands (green lines/bars), or in untreated cells (control; blue). OCR was estimated under basal conditions or following the addition of oligomycin (O; 1 µM), the uncoupler FCCP (F; 1 µM) or the inhibitors rotenone + antimycin A (R/A; 0.5 µM), and rates of basal respiration, maximal respiration and spare respiratory capacity are shown. Bars represent the mean ± standard error of the mean (SEM) for resting (blue bars) and LPS/IFN-γ activated cells treated with saline (red bars) or with adenosine receptor ligands (green bars). Panel **A.** Treatment with SCH 582561 (**SCH**), the $A_{2A}R$, antagonist. Panel **B.** Treatment with 2-Cl-IB-MECA (**2-Cl**), the A_3R, agonist. Panel **C.** Combined (**SCH + 2-Cl**), treatment. *$p < 0.05$ compared to the control, #$p < 0.05$ compared to LPS/IFNγ. One Way ANOVA with Student-Newman-Keuls Method post hoc test or Kruskal-Wallis One Way Analysis of Variance on Ranks with Dunn's post hoc test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5. Conclusions

Transcriptomic data and asessment of mitochodrial function indicate that targeting activated (reactive) microglia with an A_{2A} receptor antagonist (SCH 58261), an A_3 receptor agonist (2-Cl-IB-MECA), and their combination can effectively reduce neuroinflammation. SCH 58261 modulates the microglial transcriptome by downregulating proinflammatory genes and enhancing mitochondrial function. 2-Cl-IB-MECA also decreases proinflammatory gene expression, but does not affect mitochodrial respiration. Importantly, the combination treatment of 2-Cl-IB-MECA and SCH 58261 shows a potentially synergistic effect, evidenced by a unique trascriptomic profile and superior improvements in mitochondrial respiration compared to either treatment alone. These findings suggest that dual targeting of A_{2A} and A_3 receptors in activated microglia offer an effective therapeutic strategy for neuroinflammation.

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CRediT authorship contribution statement

Joan Serrano-Marín: Conceptualization, Formal analysis,

Supervision, Validation, Writing – original draft, Writing – review & editing. **Rita Valenzuela:** Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Cristina Delgado:** Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Aloia Quijano:** Investigation, Methodology, Writing – review & editing. **Gemma Navarro:** Investigation, Supervision, Writing – review & editing. **José Luis Labandeira –García:** Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing. **Rafael Franco:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mito.2024.101934.

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