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DOTTORATO DI RICERCA IN
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CICLO XXX
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Glial cells and neuroinflammation: the adenosinergic contribution

Settore Scientifico Disciplinare BIO/14

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to Matteo and my family

TABLE OF CONTENTS

ABBREVIATIONS

AC, adenylyl cyclase

ActD, actinomycin D

AD, Alzheimer's disease

ADA, adenosine deaminase

Ado, adenosine

ANOVA, analysis of variance

ARs, adenosine receptors

ATP, adenosine triphosphate

BAY 60-6583, 2-[[6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]-acetamide

BBB, blood-brain barrier

BNDF, brain-derived neurotrophic factor

CGS 21680, 2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate

CHA, N(6)-cyclohexyladenosine

Cl-IB-MECA,1-[2-Chloro-6-[[(3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N* methyl-β-D-ribofuranuronamide

CNS, central nervous system

DAPI, 4′,6′-diamino-2-phenyl-indole

DMEM, Dulbecco's modified Eagle medium

DMSO, dimethyl sulfoxide solution

ENHA, erythro-9-(2-hydroxy-3-nonyl) adenine

ERK, extracellular signal-regulated kinase

GFAP, antiglial fibrillary acidic protein

GLUT, Glucose transporter

HIF-1, Hypoxia-inducible factor-1

IL, interleukin

iNOS, inducible nitric oxide synthase

JNK, c-Jun N-terminal kinase

LPS, lipopolysaccharide

MAPK, mitogen-activated protein kinase

MRS 1523, 3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate

MS, multiple sclerosis

NF-kB, nuclear factor-kB

NGF, nerve growth factor

NO, nitric oxide

PBS, phosphate-buffered saline

PD, Parkinson's disease

PGE_2, prostaglandin E2

PI3K, phosphoinositide 3-kinases

PKC, protein kinase C

PLC, phospholipase C

PSB 36, 1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3hydroxypropyl)-1*H*-purine-2,6-dione

PSB 603, 8-[4-[4-(4-Chlorophenzyl)piperazide-1-sulfonyl)phenyl]]-1-propylxanthine

ROS, reactive oxygen species

Rottlerin,1-[6-[(3-Acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2dimethyl-2H-1- benzopyran-8-yl]-3-phenyl-2-propen-1-one

SB202190, 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole

SCH 442416, 2-(2-Furanyl)-7-[3-(4-methoxyphenyl)propyl]-7 H-pyrazolo[4,3 e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SH5, D-3-Deoxy-2-O-methyl-myo-inositol 1-[(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate]

siRNA, small interfering RNA

SP600125, 1,9-Pyrazoloanthrone

SQ22,536, 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine

TLR, toll-like receptor

TNF, tumour necrosis factor

U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene

U73122, 1-[6-[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione

VEGF, vascular endothelial growth factor

ABSTRACT

The hallmark of neuroinflammation is the activation of microglia, the immunocompetent cells of the CNS, releasing a number of pro-inflammatory mediators implicated in the pathogenesis of several neurological diseases such as AD, PD, MS and ischemic stroke. The innate immune TLR4, localized on the surface of microglia, is a first-line host defense receptor against invading microorganisms and LPS, a component of the cell wall of gram-negative bacteria, was first identified as the TLR ligand. ARs interacting with TLR4 influence on many immune properties of microglia and are implicated in numerous physiological and pathophysiological states in CNS. Indeed, Ado, a ubiquitous nucleoside with important immunomodulatory functions, has been found to take part in the principal microglial activation processes spanning from proliferation, process extension, retraction, migration and cytokine production.

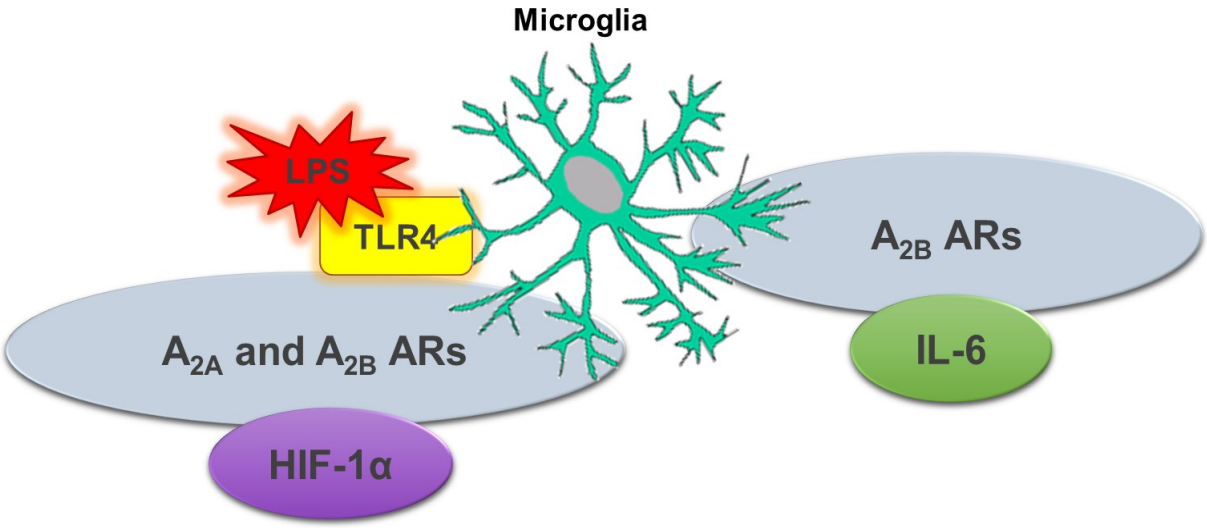
On this background, the aim of this study was to investigate the adenosinergic contribution to glial cell mediated-neuroinflammation by analyzing the expression of ARs and the signalling pathways, transcription factors and cytokines activated by them in different pathophysiological conditions linked to hypoxic and inflammatory conditions.

The aim of studies reported in **chapter 1** was to investigate whether Ado may affect microglia functions by acting on HIF-1α modulation, the main transcription factor of hypoxia-inducible genes, involved in the immune response, being regulated in normoxia by inflammatory mediators. Primary murine microglia were activated with LPS with or without Ado, Ado receptor agonists and antagonists and HIF-1α accumulation and downstream genes regulation were determined. Ado increased LPS-induced HIF-1α accumulation leading to an increase in HIF-1α target genes involved in cell metabolism and pathogens killing but did not induce HIF-1α dependent genes related to angiogenesis and inflammation. The stimulatory effect of Ado on HIF-1α and its target genes was essentially exerted by activation of A_{2A} through ERK1/2 (or p44/42) and A_{2B} subtypes via p38 MAPKs and Akt phosphorylation. Furthermore, the nucleoside raised VEGF and decreased TNF-α levels, by activating A_{2B} subtypes. Our results show that Ado increases GLUT-1 and iNOS gene expression in a HIF-1α-dependent way, through A_{2A} and A_{2B} ARs, suggesting their role in the regulation of microglial cells function following injury. However, inhibition of TNF-α adds an important anti-inflammatory effect only for the A_{2B} subtype.

The aim of studies reported in **chapter 2** was to indagate the potential role of ARs in the modulation of IL-6 secretion and cell proliferation in primary microglial cells. The A_{2B} AR agonist BAY 60-6583 stimulated IL-6 increase under normoxia and hypoxia, in a dose- and time-dependent way. In cells incubated with the blockers of PLC, PKC-ε and PKC-δ the IL-6 increase due to A_{2B} AR activation was strongly reduced, whilst it was not affected by the

inhibitor of AC. Investigation of cellular signalling involved in the A_{2B} AR effect revealed that only the inhibitor of p38 MAPK was able to block the agonist effect on IL-6 secretion, whilst inhibitors of ERK1/2, JNK1/2 MAPKs and Akt were not. Stimulation of p38 by BAY 60-6583 was A_{2B} AR dependent, through PLC, PKC-ε and PKC-δ but not AC pathway, in both normoxia and hypoxia. Finally, BAY 60-6583 increased microglial cell proliferation involving A_{2B} AR, PLC, PKC-ε PKC-δ and p38 signalling. Our results show for the first time that Ado by activating A_{2B} ARs increases IL-6 protein levels and cell proliferation through a pathway dependent on PLC, PKC-ε, PKC-δ, and p38 signalling.

These findings add new molecular pathways activated by Ado in microglia to give a reduction of genes and cytokines involved in inflammation and hypoxic injury that may cohexist in stroke, ischemia and other CNS disorders.



Schematic representation of the topics of the thesis

GENERAL INTRODUCTION

Microglia

Microglia — from micro (small) and glia (glue) — are the immunocompetent cells of the CNS and continuously screen the cerebral microenvironment to respond to pathogens and injury and to maintain the brain homeostasis in both health and disease [1]. They were discovered, for the first time, in 1899 by the German neurologist Franz Nissl, who called them "Staebchen zellen" or "rod cells" for their nucleus form [2]. However, the most complete characterization of microglia was in 1919 with Pio Del Rìo-Hortega, a Spanish neuroscientist, who introduced the modern terminology of how we describe glial cells today, i.e. by differentiating between “macroglia” and “microglia”. In particular, Rìo-Hortega refined his description of “microglia” as the nonneuronal, nonastrocytic element of the CNS, distinct from neurectodermal oligodendroglia and oligodendrocytes, which constitute the “macroglia” [3]. For years, microglia were no longer the focus of neuroscience research until the revolutionary work of Georg Kreutzberg’s group that generated a new preparation, the facial nerve lesion, and examined the resultant response in the facial nucleus. This allowed Kreutzberg’s group to analize the process of microglial activation, which in turn stimulated the evolution of research in the field of neuroscience [4], [5].

In the healthy CNS, microglial cells are well integrated in the neuronal glial network and are present in all brain areas with different density between 5% in the *corpus callosum* and 12% in the *substantia nigra*. They are found in analogous amounts to neurons ranging from 100 to 200 billion cells depending on the condition (i.e., healthy, infected, diseased). In contrast to neurons, microglia can proliferate, mainly during infection and damage and in the presence of endogenously released toxic proteins [6].

These cells belong to the monocyte/macrophage lineage. They share functional features and functions with macrophages including the expression of TLRs [7], surface molecules such as the glycoprotein F4/80 and the complement receptor 3 (CD3 also known as CD11b or as Mac-1) [8]–[10], major histocompatibility complex (MHC) class I and II, the capacity to present antigens to $CD4^+$ T cells to exert an effect on the functional T cells phenotype [11], the ability to polarize their phenotype, which can be influenced by inflammatory T cells and the lymphocyte regulatory T cells [12], [13] (Table. 1). Nevertheless, they are dissimilar from other tissue macrophages due to their low expression levels of the macrophage-antigen CD45 and their relatively inactive phenotype [14], [15]. A “resting” inactive microglial cell is characterized by a little soma with extended ramified processes [16]. Under healthy conditions, microglial cell processes do not overlap with processes of adjacent cells and each cell seems to have a scavenger function for its own close region. The position of the cell soma remains stable, while the processes of the “resting” microglia are continuously extending and dretracting to patrol the

tissue environment. An interesting observation is that this morphology occurs only *in vivo* and is relatively absent in cultured microglia cells. Nimmerjahn et al., have demonstrated, through *in vivo* imaging of microglia, that in intact brain tissue these cells had dynamic processes which continuously scan the neighboring microenvironment [17].

Upon tissue damage, inflammation, viral or bacterial insult, or external materials, microglia can quickly retract their processes, turn into a “ameboid” active phenotype and become efficient mobile effector cells [18]. The activation of these cells is extremely different and depends on the context and type of the stressor or pathology, for example, during chronic immune challenges, microglia that are close to blood vessels seem to lose their ramifications becoming more “amoeboid”. It is not clear whether this process is a step involved during the differentiation of blood-derived cells or whether it depends on phenotypic variations of the resident cells [19]. The status of activation of microglia can be subdivided in two “extreme states”: a “classically” M1 state and an “alternatively” M2 polarization [20], [21] (Fig. 1). Specifically, M1 microglia are often associated with acute infection and have a crucial role in the protection against microorganisms, while, M2 cells partecipate in tissue remodelling, repair, and healing [21]. This functional classification depends on the microenvironment of chemokines or cytokines as a result of microbial products or injured cells [21]. IFN-γ and LPS polarize microglia towards the M1 state and induce the release or expression of IL-1, IL-6, IL-12, IL-23, and iNOS, while, IL-4, IL-10, and IL-13 polarize microglia towards the M2 state, which produce IL-10 and express arginase 1 [21], [22]. However, during disease both of these “extremes” as well as intermediate states may be present. Recent works suggest that microglia do not only take part in immunological processes but also play a role in non-pathological conditions by, for example, eliminating or remodelling synapses and support of myelin turnover [23].

TAB. 1: Phenotypes of microglia [13].

Phenotype	Identification Markers
Resting State	Iba-1 $CD45^{int}$ $CD11b^{+}$ F4/80 Isolectin (IB4)
Classical Activation (M1)	MHCII CD16 (FcγR III) CD 32 (FcγR II) CD80 (B7-1) CD86 (B7-2) CD40 (TNF)
Alternative Activation (M2)	Arg-1 CD68 (ED1) Fizz1 (Relmα) Ym-1 CD206 (MR) Dectin-1

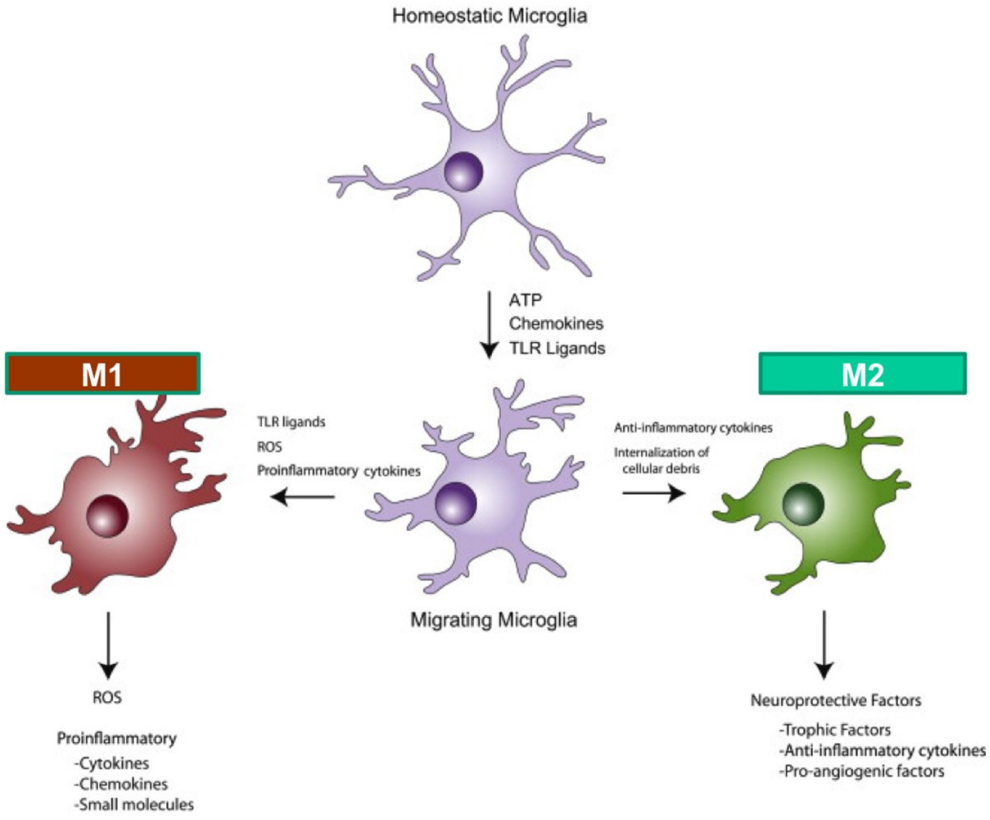


FIG. 1: Activation and phenotypic shift of microglia from the resting state to the M1/M2 [24].

Origin of microglia

Macrophages, perivascular and parenchymal microglia seem to originate from myeloid progenitors but the precise origin of these cells still remains to be completely established. It is supposed that parenchymal microglia derive from neuroectodermal matrix cells and that, macrophages or mesenchymal progenitors originate from the yolk sac, establishing themselves in the brain during the embryonic stage [1]. In mice, microglial precursors can be detected in the fourth ventricle at embryonic day 13, in humans microglial-like cells can already be detected at 13 weeks of gestation, while ramified microglia are found at week 21. Well-differentiated microglial cells are present close to term of gestation and most of them are generated after birth and after the formation of the BBB [2], [25].

Nevertheless, new data propose the existence of other subpopulations of microglial cells i.e. those deriving from the primitive pial macrophages, from the yolk sac and those newly differentiated from monocytes or their progenitors [26], [27].

Furthermore, there are different hypothesis about the maintenance of the microglial population in the adult CNS: one theory is that adult microglia are maintained by the division of progenitor cells already present in the brain or via self-replication, another supposition suggests that circulating precursors are capable to infiltrate the CNS and differentiate into microglia [28]. However, new studies demonstrate that the intrinsic adult microglial population does not originate from blood cells but only from an intrinsic source. For example, a recent work shows that adult mouse brains depleted of microglia by specific inhibitors completely repopulated with novel microglia through the proliferation of nestin-positive cells that then differentiate into microglia [4].

Microglial "on" and "off" signalling

Several exogenous and endogenous signals converge on microglia to actively maintain or modify their functional state and to coordinate the specific repertoire of microglial functions. Conversions between "resting" and "activated" states are triggered when microglial cells identify the presence, anomalous concentration, or unusual molecular size of certain factors [6].

Microglia are governed, in particular, by two mechanisms: the "on" and the "off" receptor-mediated signalling. The "on" signalling is represented by a new signalling molecule that appears in the brain, is recognized by microglia and generates activation, while the "off signal" maintains a continuing signalling to restrain microglial cells [29], [30].

The "on" signals are represented not only by infectious agents (e.g. structures associated with bacterial cell walls, viral envelopes, or their respective DNAs and/or RNAs) but also by molecules delivered following tissue injury such as the calcium-binding S100 proteins, the heat shock proteins (HSP60, HSP70, HSP90, and gp96) and the chromosomal protein high-mobility group B1 (HMGB1) [31]. The biochemical nature and the fonts of these signals are diverse, as the variety of receptors present on microglia. Indeed, microglia can be alerted and activated by both pathogen- and damage/danger associated molecular patterns (PAMPs/DAMPs), that include the TLR family, and by modifications of the physiological concentration of some neuro- and cotransmitters that could indicate alterations of neuronal activity [32], [33].

For example, purinergic receptors are important for the maintenance of injured neurons and facilitate tissue homeostasis by microglia (Fig. 2) [34]. In particular, release of nucleotides such as ATP in the CNS, especially after nerve injury, mimics an inflammatory injury in the parenchyma and could immediately activate microglia for the release of neurotrophic factors [35]–[38].

Moreover, microglial activation and maturation are also closely controlled by endogenous transcription factors like Runt-related transcription factor 1 (Runx1), ETS (E-twentysix) family transcription factor Pu.1, and interferon regulatory factor 8 (Irf8) that are crucial regulators of the differentiation process during embryonic development [28], [39] (Table. 2).

The "off" signalling is due to a loss of constitutive signalling. In the healthy brain, microglia are in close contact with neurons, for which they serve important developmental support and maintenance functions, such as clearance of aberrant proteins [5], [17], [35], [40]–[42]. Healthy neurons, that are in close contact with microglia, preserve the inactive state of these cells *via* secreted and membrane-bound signals, including CD200, CX_3CL1 (fractalkine or neurotactin), CD47, the receptor expressed on myeloid cells 2 (TREM2), neurotransmitters and neurotrophins [30], [43], [44] (Fig. 2).

CD200 is a surface molecule usually expressed on neurons, astrocytes and oligodendrocytes [45] but its receptor (CD200R) is exclusively expressed on macrophages in the CNS, including microglial cells. The interaction of neuronal CD200 with CD200R leads to inactivation of microglia and maintains them in a "resting" phenotype (Fig. 2). In fact, some works on MS reported that in CD200-deficient mice microglia has a less ramified morphology and up-regulate CD45 (leukocyte common antigen) and CD11b, which are also markers of activation [46].

Another important regulator of microglial surveillance is the chemokine receptor CX_3CR1. Its ligand, CX_3CL1, is expressed on different neuronal subsets in the adult CNS [47] and can be found in a secreted form as well as in a membrane-bound variant [48]. In the healthy and diseased brain, the binding of neuronal CX_3CL1 to the microglial CX_3CR1 seems to play a fundamental role in the interaction of neurons and microglia [49] and seems to be a fundamental signalling pair in neurophysiology (Fig. 2). In particular, in the post natal brain CX_3CR1 allows microglial cells to remove redundant synapses by phagocytosis, promoting the formation of mature neural circuits [50].

There are many other inhibitory surface receptors or molecules on the surface of microglial cells, which are mostly interacting with ligands secreted or expressed on the surface of neurons, such as CD47. CD47 is expressed ubiquitously, including on neurons, and transmits a "do not eat me" signal to the microglia via CD172α (Sirpα) [30], [51] (Fig. 2).

Another glycoprotein identified on microglia is the triggering receptor expressed on myeloid cells 2 (TREM2), which is related to an anti-inflammatory phenotype [52]. TREM2 is associated with DAP12 (the adaptor protein DNAX-activating protein of 12-kDa), which activates the intracellular signalling cascade (Fig. 2). TREM2 is crucial for phagocytosis of apoptotic cell membranes by microglia [53] and has beneficial effects in autoimmune CNS demyelination [54]. Moreover, mutations in TREM2 or DAP12 are associated to polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL), a severe neurodegenerative disease [55], [56].

Microglia also express receptors that activate developmental signals and essential cellular survival. One of these receptors is the receptor for colony stimulating factor 1 (CSF-1R), which is also known to regulate the differentiation and survival of peripheral macrophages [57]. CSF-1R binds CSF-1, its specific ligand, which is essential for the maintenance of many macrophage subsets but can also bind IL-34 that compensates and has similar signalling functions to CSF-1. [58]–[61] (Fig. 2). Recent studies show that the signalling of both IL-34 and CSF-1 is important for microglial proliferation upon neurodegeneration during prion infection and AD. Indeed, their

administration could induce microglial proliferation with a stronger proliferative capacity for IL-34 than CSF-1 [62].

Lastly, there are proteins that inhibit signal transduction pathways (e.g., SOCS proteins), induction of transcriptional repressors and transrepressors (e.g., ATF3 and Nurr1), as well as the production of soluble or cell-surface mediators with anti-inflammatory activities (e.g., IL-10, transforming growth factor (TGF)-β, resolvins, and ligands for TAM receptors) [31].

Regulating these inhibitions to keep microglia far from a proinflammatory phenotype has been shown to prevent uncontrolled damage in models of AD, MS, and neurodegeneration [13], [63], [64]. Imminent research will display whether microglial suppression by this mechanism plays a significant role in the steady-state functions and whether loosening of "resting" signals occurs with ageing [6]. Similarly, regulation of these inhibitory proteins may also have beneficial effects on ischemic stroke [13].

TAB. 2: Transcription factors regulating microglia development and homeostasis [28].

Transcription factor	Microglia development	Microglia homeostasis
c-myb	→ Independent of c-myb	→ After induced ablation of c-myb, no change in microglia cell number
Runx-1	→ Expressed on microglia progenitors in the yolk sac	→ Regulating proliferation and tissue homeostasis of postnatal microglia → Transition of amoeboid to ramified morphology
Pu.1	→ Absence of microglia progenitors in the yolk sac; no development of microglia	→ No direct evidence yet for a functional role during microglia cell homeostasis
Irf8	→ Reduced number of microglia and microglial progenitors in the yolk sac	→ Dysregulation of microglial cell morphology and function → Defects in microglial activation
Hoxb8	→ No direct evidence yet for a functional role during microglia development	→ Mutant Hoxb8 microglia induce pathological grooming in mice

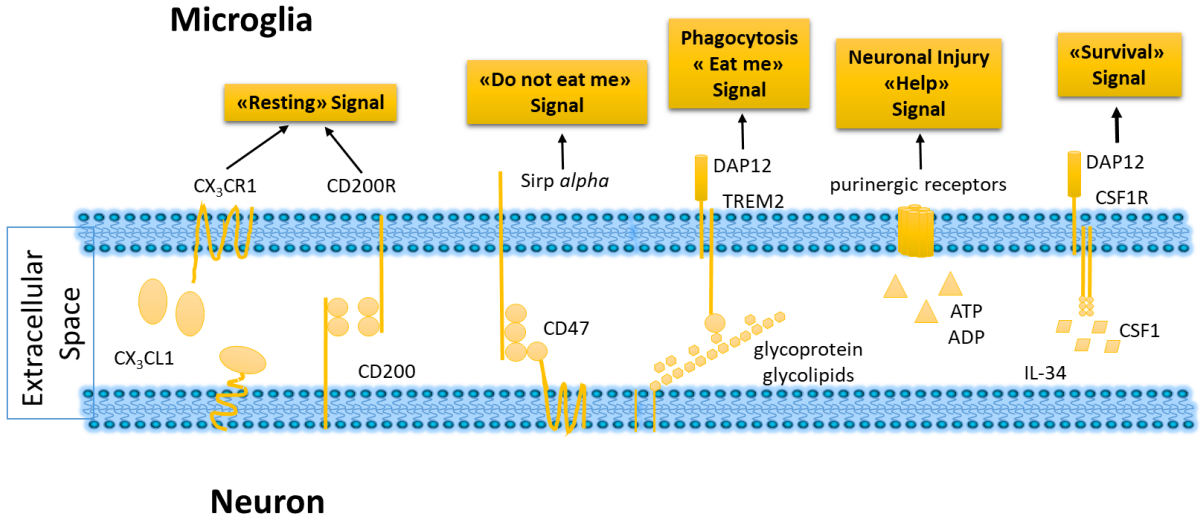


FIG. 2: Exogenous signals and their receptors on microglia. Image reproduced from [28].

Microglia and inflammatory responses

In the brain, stressed or damaged neurons produce specific factors, such as matrix metalloproteinase-3 (MMP-3), neuromelanin, Aβ peptide, ATP and α-synuclein, that stimulate the up-regulation of a variety of surface receptors and the production of multiple secreted factors including cytokines, NO, ROS, glutamate, and growth factors by microglia [31]. Microglia functions related to an innate immune response are principally associated with TNF-α signalling and its regulation of both inflammation and apoptosis. In parallel with the production of pro-inflammatory cytokines, microglial inflammation is usually associated with the production of ROS signalling, that is considered as a unifying mechanism underlying multiple forms of neuroinflammation and microglia responses [31].

Inflammatory responses can be initiated by pattern recognition receptors (PRRs) that include the TLRs, the scavenger receptors and the RAGE (receptor for advanced glycation end-products). Moreover, microglia can identify ligands for CD40, CD91, and the intracellular NOD-like receptors (NLRs). These receptors initiate the signaling process by binding to pathogen associated molecular patterns (PAMPs) [65]. Binding to PRRs activates the intracellular signal transduction pathways and the regulation of different transcriptional and post-transcriptional factors including NF-kB, MAPKs, activator protein 1 (AP-1), caspases, and interferon regulator factor families, which modulate pro-inflammatory target genes encoding cytokines, chemokines, enzymes, and other molecules essential for pathogen elimination [66] (Fig. 3).

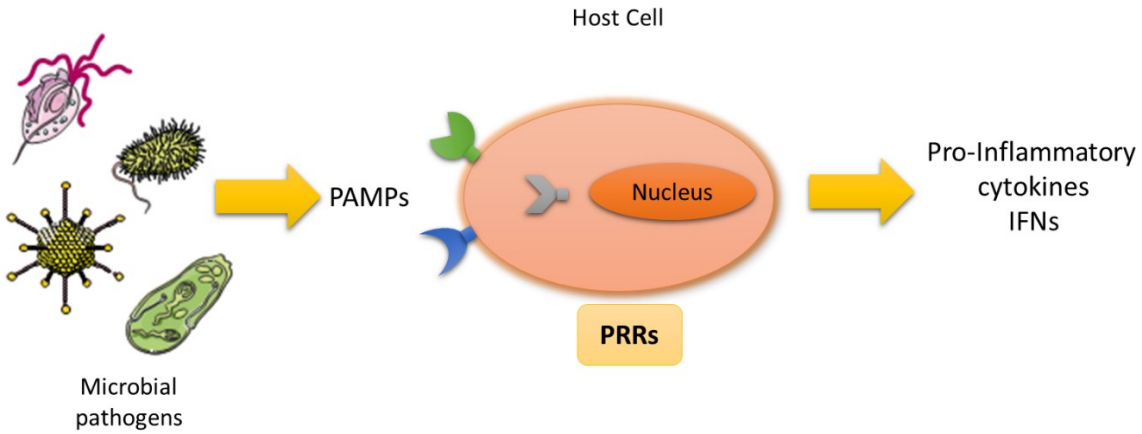


FIG. 3: Pattern Recognition Receptors (PRRs). Image reproduced from [67].

The main family of PRRs is represented by the TLRs. TLRs and their correlated signalling proteins are expressed in the CNS [68], [69], with astrocytes expressing TLR3 and microglia expressing TLR1-9 [70]. In particular, microglia derived from human white matter and primary rat cell cultures express the TLR4, which recognizes LPS and initiates innate immune responses to infection in mammals [71], [72]. TLR signalling can have both detrimental and beneficial effects. TLRs are necessary for pathogen elimination but, at the same time, TLR-induced activation of microglia and the release of pro-inflammatory molecules can contribute to neurotoxicity. For example, in ischemic injury, there is evidence that TLR2 and TLR4 are capable of sensing injury induced by ischemia and, augment the pro-inflammatory response with consequent infarct size increased [71], [73], [74]. On the contrary, the release of inflammatory mediators such as TNF-α and IL-1β by TLR4-induced activation of microglia can operate on astrocytes to induce a secondary inflammatory or growth factor repair response [75]. With an acute damage, the release of TNF-α from microglial cells may provide to combat any secondary injury [76], while the activation of microglia to eliminate cellular debris may prevent consequent tissue inflammation [77], [78].

Scavenger receptors expressed in microglia and astrocytes regulate the uptake of several substrates as well as lipids, oxidized proteins and apoptotic cells, and may contribute to downstream cell signalling [79]. Expression of cytokine and chemokine receptors, potassium channels, various glutamate and gamma-aminobutyric acid (GABA) receptors, adrenaline and dopamine receptors, and purinergic receptors allows microglia to coordinate tissue defense responses and to “sense” astrocyte and neuron activity [4], [80]. Purinergic receptors, for example, allow for a microglial response to ATP release upon cell death, traumatic injury, or ischemia [81] and their activation helps to regulate microglial release of pro-inflammatory cytokines like IL-6 and TNF-α [82], [83], and may operate as a sensor for the phagocytosis process [84].

PRRs also include the cell surface receptor RAGE that belongs to the immunoglobulin superfamily [85], [86]. This receptor is expressed in microglia, astrocytes, vascular endothelial cells, and neurons and its activation is characterized by the production of advanced glycation end-products (AGEs) in pro-oxidant and inflammatory environments. This receptor contributes to the clearance of Aβ and is implicated in apolipoprotein E (apoE)-mediated cellular processing and signalling [87].

Soluble and cytoplasmic PRRs are represented by NLRs that are important sensors of cellular damage. These receptos activate a process that is the basis for the concept of the inflammasome, which is involved in regulating microglial neuroinflammatory responses to select pathogens. In

particular, the NACHT, LRR and PYD Domains-Containing Proteins (NALPs) stimulate downstream signalling proteins, such as apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), inducing apoptosis but also contributing to the maturation of pro-inflammatory cytokines such as IL-1β and IL-18 [88].

Finally, there are additional receptors that recognize apoptotic cellular material (e.g. phosphatidylserine) and that are essential for phagocyte clearance and anti-inflammatory processes [89]. These receptors include T-cell immunoglobulin-and mucin-domain-containing molecule-1 (Tim4) [90], the metabotropic $P2Y_6$ receptor that recognizes the nucleotide UDP released from injured neurons [91], and the TREM2 [54].

Interleukin-6

IL-6 has been defined as a major multifunctional cytokine in the CNS exerting both pro- and anti-inflammatory functions by regulating differentiation, proliferation, migration and apoptosis of target cells [92].

This cytokine was recognized almost 30 years ago as a B-cell differentiation factor, able of inducing the maturation of B cells into antibody-producing cells. As with many other cytokines, it was soon understood that IL-6 was not a factor only involved in the immune response, but with many critical roles in major physiological systems including the nervous system [97]. Neurons, astrocytes, microglia and endothelial cells are important fonts of IL-6 in the CNS. Several studies performed on different animal models have allowed to prove that CNS IL-6 is up-regulated whenever neuroinflammation is expected, such as following CNS infection or injury or in a number of CNS diseases like AD, PD, MS and ischemic stroke, suggesting that IL-6 could have a role in the observed neuropathology and that therefore it is a clear target of strategic therapies [93], [94] (Fig. 4).

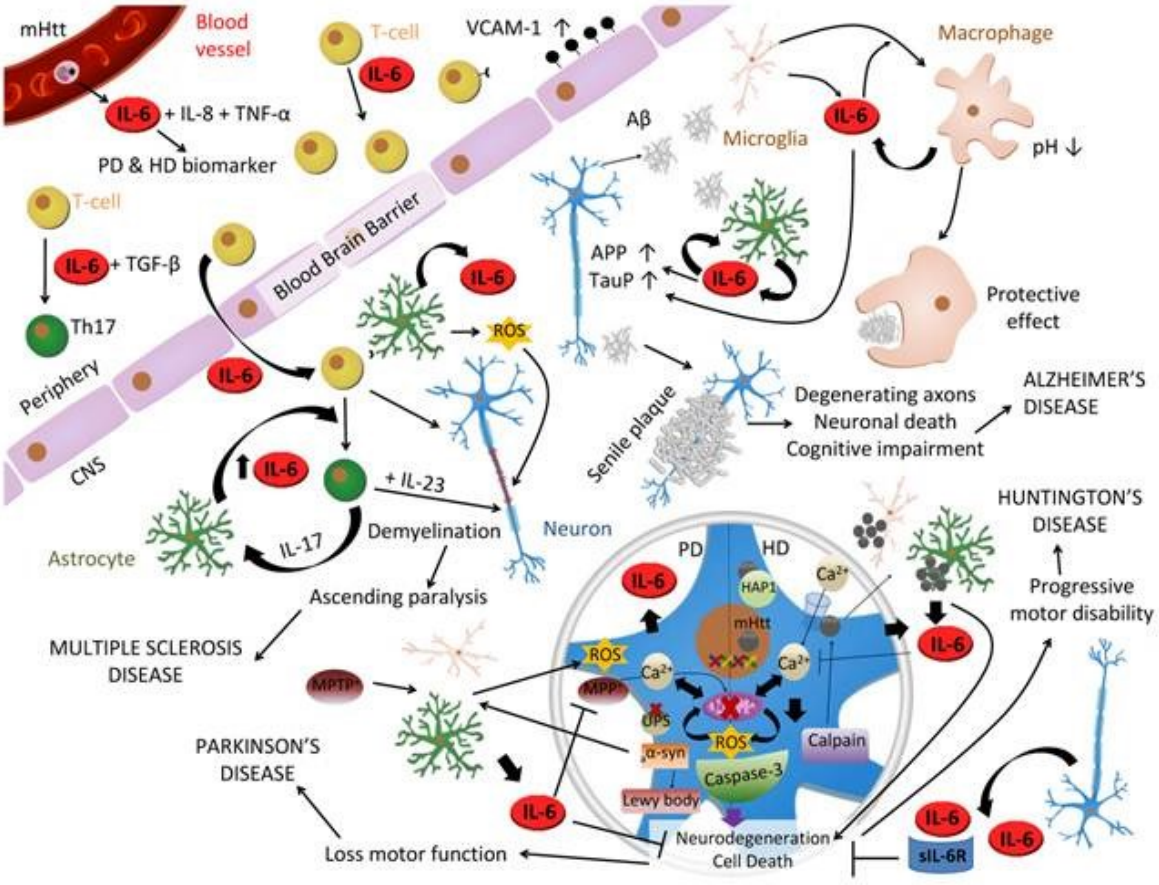


FIG. 4: Role of IL-6 in some CNS diseases [92].

IL-6 is a four-helix bundle cytokine belonging to the family of "neuropoietins" that bind to class I cytokine receptors, membrane proteins with a typical modular architecture that do not have intrinsic enzymatic activity, and that for signalling often need to recruit extra receptor proteins shared by different cytokines: gp130, βc or γc. The IL-6 family of cytokines recruits gp130 for signalling [95], [96]. Specifically for IL-6, a hexamer forms (two IL-6, two IL-6R and two gp130) can activate intracellular tyrosin-kinases such as Janus kinase (JAK) which, in turn, activate a number of proteins including the signal transducer and activator of transcription (STAT), or the RAS-RAF-MAPK pathway, PI3 kinase, or insulin receptor substrate (IRS) [97] (Fig. 5).

The expression of IL-6R is limited to some tissues, while gp130 is ubiquitous, and IL-6 may up-regulate gp130 expression [98]. It is now widely accepted that soluble IL-6R (sIL-6R) is formed physiologically, either by proteolysis of the extracellular domain of membrane IL-6R (mIL-6R) by metalloproteases such as ADAM10 and ADAM17, or by alternative splicing of IL-6R mRNA, and that sIL-6R can bind both IL-6 and gp130 and signal in cells with or without endogenous IL-6R expression, a mechanism known as trans-signalling [94]. It is also known that a soluble form of gp130 (sgp130) is also formed, in this case only by alternative splicing of gp130 mRNA; sgp130 can inhibit trans-signalling but does not affect normal signalling by mIL-6R [99].

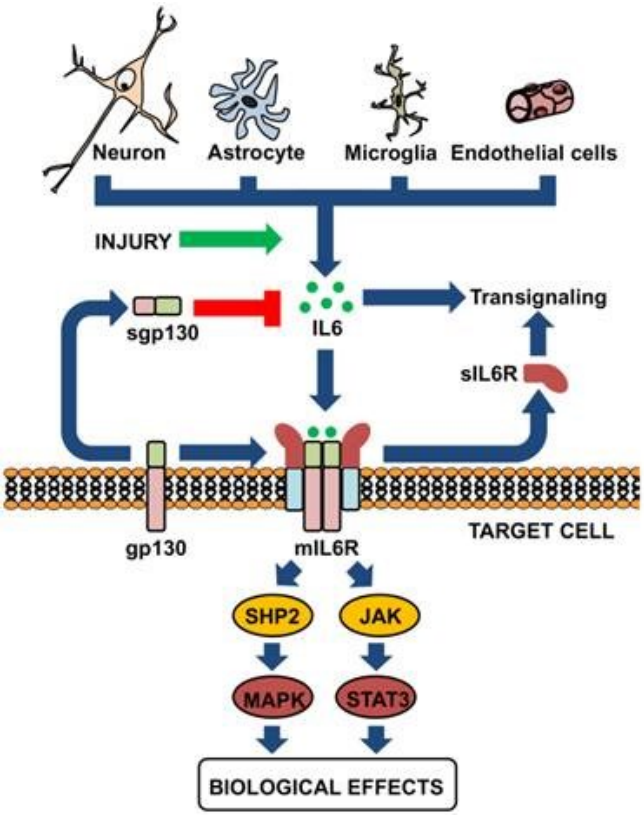


FIG. 5: IL-6 is produced by different brain cells and may signal in a complex manner [92].

Contribution of microglia to neuroinflammation and neurodegeneration

The CNS can be considered as an immune-privileged structure with an innate and acquired immune response that is rigorously controlled in relation with the periphery. Infiltration of leukocytes from the periphery to the CNS with subsequent neuroinflammation and neurodegeneration, is usually generated by a strong inflammatory response in the periphery from infectious pathogens or molecules delivered following tissue injury [12], [100]. A brain damage is associated with the early activation of microglia, which stimulate the release of pro-inflammatory factors that promote the permeabilisation of the BBB, with a possible relevant role of peripheral macrophages in the outcome of neuroinflammation. The presence of activated microglia is often considered as a marker for future neurological diseases. Nevertheless, changes in microglia morphology or functional activation is not always related to neuron loss or damage [31].

Localized activated microglia and neuroinflammation have central parts in the pathophysiology of ischemic stroke, neurodegenerative disease such as PD, AD [101], MS, and psychiatric diseases such as schizophrenia [102]. In general, an acute inflammation has beneficial effect on the CNS, reducing the damage by activating the innate immune system [82], [83]. On the contrary, chronic inflammation is characterized by the long-term activation of microglia that continued release of inflammatory mediators, which maintain and protract the inflammatory cycle, and by the absence of sufficient anti-inflammatory factors to down-regulate the response [84].

For example, the persistent activation can generate a chronic inflammatory through a fibrillar Aβ-induced microglial response. The roles of Aβ and other potential originators of inflammation are uncertain. However, one such role is the activation of caspases and transcription factors such as NF-kB and AP-1, producing the release of inflammatory factors like IL-1β, TNF-α, IL-6, that can act on astrocytes and start the production of factors to modify the neighboring environment or activate microglial cells [103], [104], or might act on neurons to induce apoptosis [20]. A benefical role for neuroinflammation and microglial activation has been proved in several transgenic mouse models and chimeras showing that both complement activation and microglial phagocytosis are crucial for amyloid clearance [105], [106].

Furthermore, some research reported the direct effects of ischemic conditions (low levels of oxygen and glucose) on the microglial phenotype and genomic shape. In particular, there are several data focusing on the role of specific molecular signalling systems in modulating the

microglial response *in vitro* and *in vivo* ischemia. These studies have revealed that treatment of peripheral myeloid cells with LPS, leads to induction of HIF-1 activity that is dependent on the activation of TLR4 [107]–[109].

Hypoxia-inducible factor

Multiple signalling molecules are activated in response to LPS in microglia. Some of them include HIF-1α, a master regulator of the transcriptional response of mammalian cells to hypoxia [110], [111] and important also for TLR4-dependent expression of proinflammatory cytokines [112].

Initially identified as a regulator of erythropoietin (EPO) production, HIF is recognized as a key modulator of the transcriptional response to hypoxic stress. Besides its adaptive function in cellular stress responses, recent work has also revealed important roles for HIF in both physiological and pathological processes [113].

HIFs are obligate heterodimers consisting of an O_2-labile α subunit and a stable β subunit. Mammals possess three isoforms of HIFα, of which HIF-1α and HIF-2α (also known as EPAS1) are the most structurally similar and best characterized. HIF-3α (or IPAS) exists as multiple splice variants, some of which inhibit HIF-1α and HIF-2α activity in a dominant negative fashion [114]. HIF-1α is expressed ubiquitously in all cells, whereas HIF-2α and HIF-3α are selectively expressed in certain tissues, including vascular endothelial cells, type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage [115].

HIF-1 is a heterodimeric transcription factor, composed of a HIF-1 subunit (HIF-1α or HIF-2α) and a HIF-1β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) [116].

Unlike HIF-1β, which is constitutively expressed, the HIF1-α subunit is rapidly degraded under normoxic conditions by a mechanism involving hydroxylation of two conserved proline residues by prolyl-hydroxylase domain enzymes (PHDs) and proteasomal degradation by the direct interaction of von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex and the oxygen-dependent degradation domain (ODD) of HIF-1α [117], [118].

Another key enzyme controlling the oxygen-dependent modification of HIF-1α is asparagine hydroxylase [119]. Under normoxia, the conserved asparagines in the carboxyterminal transactivation domain (CAD) are hydroxylated and interfere with the interaction of CAD with the p300 transcription coactivator. There are several theories how molecular oxygen regulates the hydroxylases. One of them implicates ROS such as superoxide and peroxide in the signal transduction process [120].

Under hypoxic conditions, proteasomal degradation is inhibited, after entering the nucleus, HIF-1α heterodimerizes with HIF-1β and the resulting HIF-1 complex binds to a specific sequence called the HIF-1 binding site (HBS) present in the hypoxia-response elements (HRE) of all HIF-dependent targets [121]. An increase in nuclear HIF-1 concentration leads to elevated

transcriptional activation of the HIF-1 target genes, whose products are involved in vascular tone maintenance, angiogenesis, glucose metabolism, cell survival, invasion and metastasis [122] (Fig. 6).

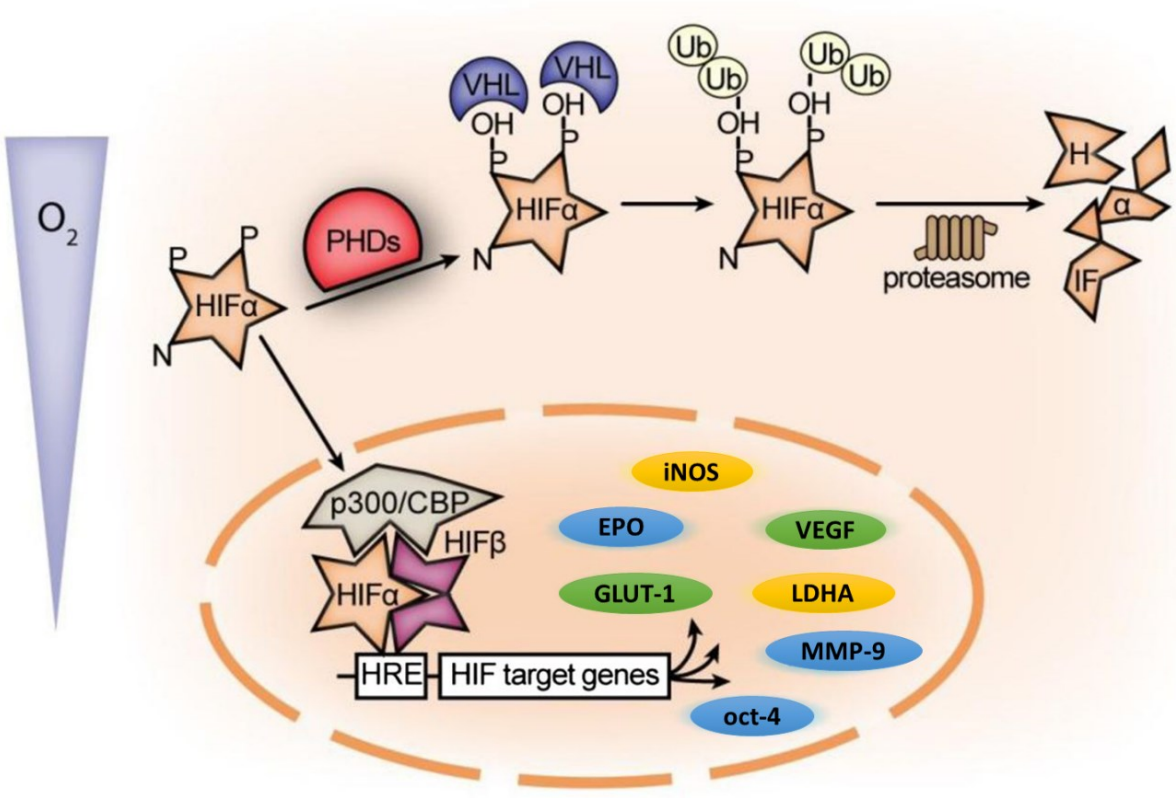


FIG. 6: Oxygen-dependent regulation of HIFα and its target genes. Image reproduced from [123].

There is growing evidence which shows that activation of HIF-1α offer protection in case of cerebral ischemia [112], [124]. For example, during ischemia, the disturbance in vascular tone is one of the major consequences, which results in neuronal cell death. The "inducible" iNOS is one such gene which is regulated by HIF-1 and helps the cells to cope up with these adverse conditions [125]. iNOS is expressed by glia cells, and acts in a Ca^{2+}-independent manner unlike the others NOS isoforms (neuronal and endothelial) that act in a Ca^{2+}-dependent manner [126]. Several studies over the years have pointed to a weak constitutive expression of iNOS in various brain regions, and raised the possibility of glial-derived NO participating in physiological brain processes [127].

Furthermore, the human and rodent VEGF genes [128] and their activation are also triggered by the HIF-1α binding [129]. During cerebral ischemia, the damaged tissue tries to increase oxygen delivery by the induction of angiogenesis *via* VEGF. The VEGF and its receptors are up-regulated in the brain by HIF-1α and 2α during 6 to 24 h post-ischemia [130].

Finally, GLUTs and glycolytic flow as a result of HIF-1α activation by hypoxia has been linked to cell survival [131]. It has been shown that there is an increased expression of HIF-1α dependent glycolytic enzymes in cingulate/retrosplenial cortex following permanent middle cerebral artery occlusion (MCAO) [132].

It's clear that the phosphorylation of HIF-1α is a mechanism that is essential for a normal HIF-1 function resulting in increased transcription rate of the HIF-1 target genes. Additionally, phosphorylation inhibitors can prevent the up-regulation of HIF-1 dependent target genes. Although O_2 induces mainly the stabilisation of HIF-1α, there is also an O_2-independent HIF-1α protein stabilisation. It is known that many factors such as IL-1, angiotensin II, insulin and insulin-like growth factors induce the accumulation of HIF-1α under normoxic conditions [121]. Indeed, recent studies have shown that insulin-like growth factor-1 stimulates increased HIF-1α expression as well as VEGF secretion in human retinal pigment epithelial cell line [133].

Adenosine receptors as target for modulating activated microglia

It is well known that ARs are expressed in microglia affecting several cell functions, including proliferation, process extension, retraction, migration and cytokines production [134]–[136].

ARs are represented by classical seven-transmembrane (TM7) spanning G protein-coupled receptors (GPCRs) and are subclassified into A_1, A_{2A}, A_{2B}, and A_3 ARs with distinct pharmacological and functional properties. [137]. A_1 and A_3 subtypes, coupled to $G_{i/o}$ proteins, inhibit AC activity, while A_{2A} and A_{2B} increase it, through coupling to G_s proteins. In addition A_{2A} ARs are coupled to G_{olf} proteins which have similar activity to G_s proteins, while A_{2B} and A_3 ARs are associated to stimulation of PLC activity, through G_q proteins [138], [139] (Fig. 7).

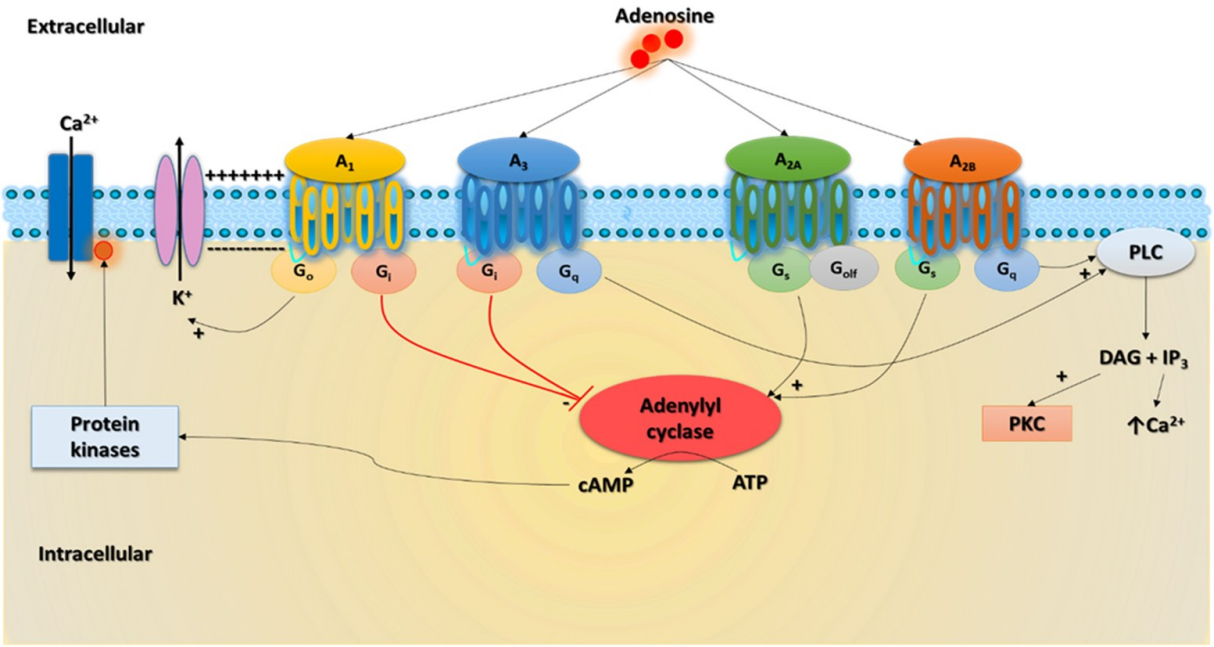


FIG. 7: ARs and and their signal transduction pathways.

Structurally, ARs have a central core domain consisting of seven transmembrane helices (TM1-7), with each TM composed of 20–27 amino acids, connected by three intracellular (IL1, IL2, and IL3) and three extracellular (EL1, EL2, and EL3) loops. Two cysteine residues (one in TM3 and one in EL2) form a disulfide link which is crucial for the packing and for the stabilization of a restricted number of conformations of these seven TMs. GPCRs also differ in the length and function of their C-terminal intracellular domain, their N-terminal extracellular domain and their intracellular loops, which provides specific properties to these receptor proteins. Particularly, consensus sites for N-linked glycosylation exist on the extracellular regions of ARs, although the precise location of the sites for this post-translational modification varies amongst the AR

subtypes. The carboxyl-terminal tails of the A_1 AR, A_{2B} AR, and A_3 AR, but not A_{2A} AR, has a conserved cysteine residue that may serve as a site for receptor palmitoylation and permit the formation of a fourth intracellular loop [140].

Furthermore, G coupled ARs are capable of communicating with diverse MAPK and affect MAPK cascades, which could give them a role in cell growth, survival, death and differentiation. Though each of the ARs can trigger one or more of the MAPKs, the mechanisms appear to differ significantly, both between receptor subtypes in the same cell type and between the same receptor in different cell types [135]. Chinese hamster ovary (CHO) cells transfected with the human ARs, in particular, were studied intensively [135]. The MAPK protein family consists of three main groups, ERK, the stress-activated protein kinases (SAPK) p38 and (SAPK) JNK [141]. MAPKs are proline-directed serine/threonine kinases, which can be doubly phosphorylated at tyrosine and serine/threonine residues and, thus, activated by upstream dualspecificity kinases. The common phosphoacceptor motif (Thr–Xaa–Tyr or TXY) of the MAPKK phosphorylation site is located in the activation loop of the MAPK. One criterion used to classify MAPKs into three main groups is based on their phosphoacceptor motif: ERK, p38 and JNK carrying T/SEY, TGY and TPY motifs, respectively. The group of mammalians ERK comprises ERK1 (p44 MAPK), ERK2 (p42 MAPK), ERK3a (p63 or rat ERK3), ERK3h (human ERK3), ERK5 (BMK1) and ERK7. There are at least four different subforms of p38: p38a, p38h, p38g and p38y. The group of JNK consists of at least three isoforms, the JNK1-3 [142]. Concerning the network of signalling pathways that may be triggered by the four ARs, it is likely that they follow the pattern of other GPCRs and are able of communicating with different MAPK cascades [143], [144].

Ado is the endogenous ligand of ARs released from almost all cell types [145], and is an ubiquitous nucleoside composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) via a β-N9-glycosidic bond (Fig. 8) with important immunomodulatory functions [146].

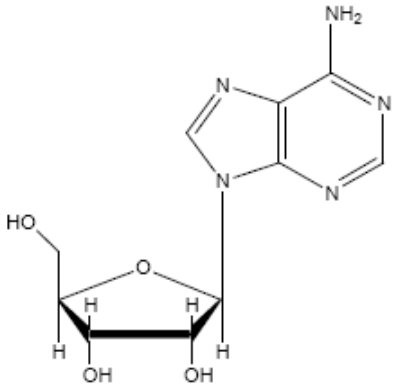


FIG. 8: Chemical structure of Ado.

Ado increases in conditions of ischemia, hypoxia, trauma, asthma, neurodegenerative disorders, chronic inflammatory diseases and cancer [139], [147]–[149]. This nucleoside is continuously formed intracellularly as well as extracellularly. Generally, the concentrations of extracellular Ado are in the nanomolar range unstressed tissues, while Ado levels in inflamed or ischemic tissues can be as high as 100 µM [150]–[152]. The fast release of Ado in response to tissue-disturbing stimuli has a dual role in modulating homeostasis. First, extracellular Ado represents a pre-eminent alarm molecule that reports tissue damage in a paracrine and an autocrine manner to neighboring tissue. Second, extracellular Ado creates several tissue responses that can be normally considered as organ protective thereby mediating homeostasis. [153].

Ado derives from the breakdown of its precursor ATP in both the intracellular and extracellular spaces. Under metabolic stress e.g. hypoxia or ischemia, the increased dephosphorylation of ATP to Ado by the metabolic enzyme 5'-Nucleotidase is paralleled by a suppression of the activity of the salvage enzyme Ado kinase, which avoids the rephosphorylation of Ado [139]. Once Ado reaches high concentrations inside the cell, it is then shunted into the extracellular space through the operation of specialized nucleoside transporters [154]. The other major pathway that contributes to high extracellular Ado levels during metabolic stress is the release of ATP, ADP and AMP from the cell followed by extracellular catabolism to Ado by a cascade of ectonucleotidases, which include CD39 (nucleoside triposphate dephosphorylase) and CD73 (5'-ectonucleotidase). Ado bioavailabilty is limited by its catabolism to inosine by Ado deaminase (ADA), which is then further degraded to the stable end product uric acid [155] (Fig. 9).

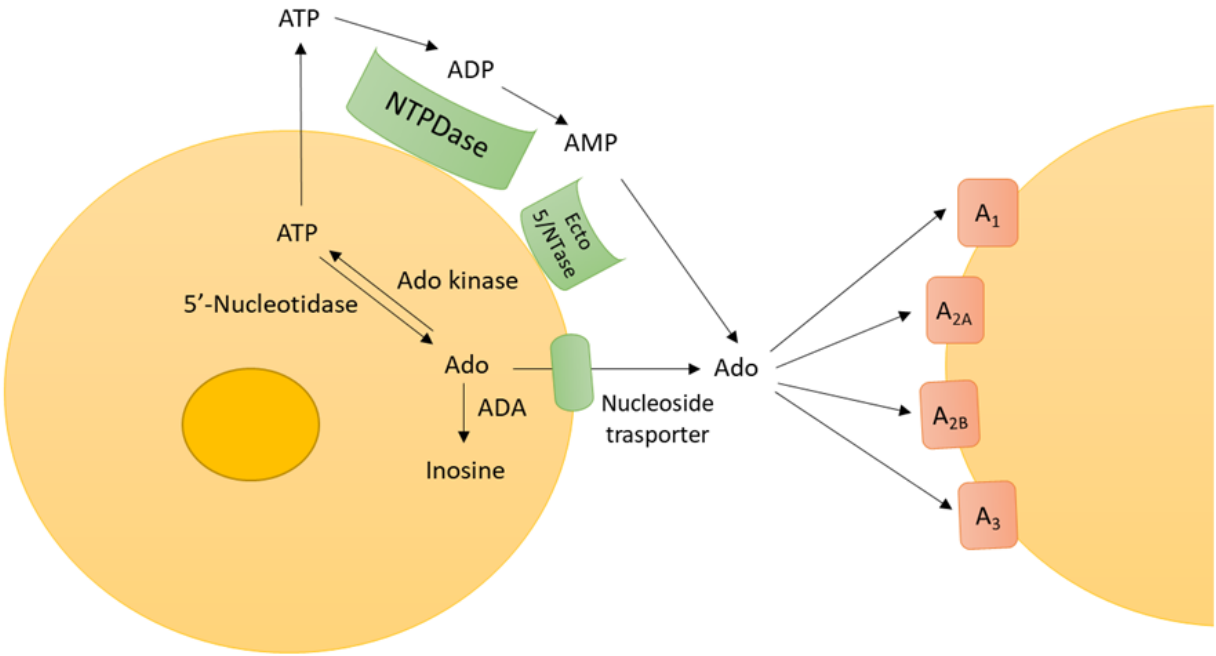


FIG. 9: Major pathways involved in Ado metabolism. Image reproduced from [155].

Physiological actions of Ado almost exclusively result from its occupancy of cell surface ARs and the activation of downstream intracellular pathways. Each AR subtype has a different pattern of tissue properties and expression. In cell-based systems, A_1 ARs have the highest affinity for Ado (Ki=10 nmol/L). The Ki values of Ado for A_{2A} AR, A_{2B} AR, and A_3 AR are 200, 2000, and 10000 nmol/L, respectively, for the human receptors. A_3 ARs are also activated by the Ado metabolite inosine (Ki=2300 nmol/L) [156].

All four types of ARs were identified at the mRNA level in cultured rat microglia [157]. Recent works suggest that A_1ARs inhibit cell proliferation after traumatic brain injury, protect hippocampal neurons against cell death [158] and reduce morphological cell activation [159]. A_{2A}ARs subtypes seem efficacious in the release of NGF and reduction of TNF-α secretion, but they increase BDNF and proliferation [160]–[162]. Furthermore, A_{2A} ARs stimulation potentiates cyclooxygenase-2 and NO release by activated microglia [163], [164], is associated with microglia process retraction [18], [165] and is involved in the LPS-induced neuroinflammation [166]. Interestingly, the switch of A_{2A} ARs activation from anti- to proinflammatory effects has been attributed to glutamate concentration [167]. As for A_{2B} ARs they have been shown to increase the neuroprotective IL-10 and to reduce pro-inflammatory TNF-α secretion [168]. Furthermore, Ado has been found to modulate IL-6 through activation of A_{2B} ARs in different cellular types e.g. astrocytes, pituitary folliculostellate cells, bronchial smooth muscle cells, lung fibroblasts, astrocytoma cells, mouse striatum, peritoneal macrophages and cardiac fibroblasts through various signal transduction pathways, including PKC and MAPKs [169]–[177]. Pharmacological assays have also identified functional A_3 ARs in primary mouse microglial cultures. Specifically, A_3 Ars, are involved in ERK1/2 phosphorylation [178], inhibit LPS-induced TNF-α production [179], and modulate the ADP-induced microglia process extension and migration [180].

It is clear that Ado exerts important neuroimmunomodulatory effects by regulating different cellular types, including neurons and glial cells, and increases in pathophysiological conditions characterized by hypoxia, ischemia and inflammation [146]. Therefore, modulating the mediators of inflammation produced by activated microglia through the activation/block of ARs may help to attenuate the progression of neuroinflammatory and neurodegenerative diseases.

References

[1] F. Ginhoux and M. Prinz, “Origin of microglia: current concepts and past controversies,” *Cold Spring Harb. Perspect. Biol.*, vol. 7, no. 8, p. a020537, Aug. 2015.

[2] S. E. Taylor, C. Morganti-Kossmann, J. Lifshitz, and J. M. Ziebell, “Rod microglia: a morphological definition.,” *PLoS One*, vol. 9, no. 5, p. e97096, 2014.

[3] P. Del Rìo-Hortega, “Microglia. in: Cytology and cellular pathology of the nervous system,” *Penfield W. New York: Hoeber.* vol. 2, pp. 482–534. 1932.

[4] A. Wolf, H. W. G. M. Boddeke, and H. Kettenmann, “Microglia in physiology and disease,” *Annu. Rev. Physiol.*, vol. 79, no. 1, pp. 619–643, Feb. 2017.

[5] G. W. Kreutzberg, “Microglia: a sensor for pathological events in the CNS.,” *Trends Neurosci.*, vol. 19, no. 8, pp. 312–318, Aug. 1996.

[6] H. Kettenmann, U. K. Hanisch, M. Noda, and A. Verkhratsky, “Physiology of microglia.,” *Physiol. Rev.*, vol. 91, no. 2, pp. 461–553, Apr. 2011.

[7] Z. Chen *et al.*, “Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4,” *J. Neurosci.*, vol. 32, no. 34, pp. 11706–11715, Aug. 2012.

[8] M. Bsibsi, R. Ravid, D. Gveric, and J. M. van Noort, “Broad expression of Toll-like receptors in the human central nervous system.,” *J. Neuropathol. Exp. Neurol.*, vol. 61, no. 11, pp. 1013–1021, Nov. 2002.

[9] J. K. Olson and S. D. Miller, “Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs.,” *J. Immunol.*, vol. 173, no. 6, pp. 3916–3924, Sep. 2004.

[10] H. Akiyama and P. L. McGeer, “Brain microglia constitutively express beta-2 integrins.,” *J. Neuroimmunol.*, vol. 30, no. 1, pp. 81–93, Nov. 1990.

[11] B. Almolda, B. González, and B. Castellano, “Are microglial cells the regulators of lymphocyte responses in the CNS?,” *Front. Cell. Neurosci.*, vol. 9, p. 440, 2015.

[12] H. González and R. Pacheco, “T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases.,” *J. Neuroinflammation*, vol. 11, p. 201, Dec. 2014.

[13] A. R. Patel, R. Ritzel, L. D. McCullough, and F. Liu, “Microglia and ischemic stroke: a double-edged sword.,” *Int. J. Physiol. Pathophysiol. Pharmacol.*, vol. 5, no. 2, pp. 73–90,

2013.

[14] A. D. Dick, A. L. Ford, J. V. Forrester, and J. D. Sedgwick, “Flow cytometric identification of a minority population of MHC class II positive cells in the normal rat retina distinct from CD45lowCD11b/c+CD4low parenchymal microglia.,” *Br. J. Ophthalmol.*, vol. 79, no. 9, pp. 834–840, Sep. 1995.

[15] B. Becher and J. P. Antel, “Comparison of phenotypic and functional properties of immediately ex vivo and cultured human adult microglia.,” *Glia*, vol. 18, no. 1, pp. 1–10, Sep. 1996.

[16] M. A. Cuadros and J. Navascués, “The origin and differentiation of microglial cells during development.,” *Prog. Neurobiol.*, vol. 56, no. 2, pp. 173–189, Oct. 1998.

[17] A. Nimmerjahn, F. Kirchhoff, and F. Helmchen, “Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo,” *Science,* vol. 308, no. 5726, pp. 1314–1318, May 2005.

[18] S. Gyoneva *et al.*, “Systemic inflammation regulates microglial responses to tissue damage in vivo.,” *Glia*, vol. 62, no. 8, pp. 1345–1360, Aug. 2014.

[19] Z. Chen and B. D. Trapp, “Microglia and neuroprotection,” *J. Neurochem.*, vol. 136, pp. 10–17, Jan. 2016.

[20] K. Saijo and C. K. Glass, “Microglial cell origin and phenotypes in health and disease,” *Nat. Rev. Immunol.*, vol. 11, no. 11, pp. 775–787, Oct. 2011.

[21] A. Mantovani, S. K. Biswas, M. R. Galdiero, A. Sica, and M. Locati, “Macrophage plasticity and polarization in tissue repair and remodelling,” *J. Pathol.*, vol. 229, no. 2, pp. 176–185, Jan. 2013.

[22] H. Qin *et al.*, “Signal transducer and activator of transcription-3/suppressor of cytokine signaling-3 (STAT3/SOCS3) axis in myeloid cells regulates neuroinflammation.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 13, pp. 5004–5009, Mar. 2012.

[23] M.-È. Tremblay, “The role of microglia at synapses in the healthy CNS: novel insights from recent imaging studies,” *Neuron Glia Biol.*, vol. 7, no. 1, pp. 67–76, Feb. 2011.

[24] G. A. Garden and A. R. La Spada, “Intercellular (mis)communication in neurodegenerative disease.,” *Neuron*, vol. 73, no. 5, pp. 886–901, Mar. 2012.

[25] C. Arcuri, C. Mecca, R. Bianchi, I. Giambanco, and R. Donato, “The pathophysiological role of microglia in dynamic surveillance, phagocytosis and structural remodeling of the

developing CNS.," *Front. Mol. Neurosci.*, vol. 10, p. 191, 2017.

[26] C. Kaur, A. J. Hao, C. H. Wu, and E. A. Ling, "Origin of microglia," *Microsc. Res. Tech.*, vol. 54, no. 1, pp. 2–9, Jul. 2001.

[27] F. Alliot, I. Godin, and B. Pessac, "Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain.," *Brain Res. Dev. Brain Res.*, vol. 117, no. 2, pp. 145–152, Nov. 1999.

[28] K. Kierdorf and M. Prinz, "Factors regulating microglia activation.," *Front. Cell. Neurosci.*, vol. 7, p. 44, 2013.

[29] X. Hu *et al.*, "Microglial and macrophage polarization—new prospects for brain repair.," *Nat. Rev. Neurol.*, vol. 11, no. 1, pp. 56–64, Nov. 2015.

[30] K. Biber, H. Neumann, K. Inoue, and H. W. G. M. Boddeke, "Neuronal 'On' and 'Off' signals control microglia.," *Trends Neurosci.*, vol. 30, no. 11, pp. 596–602, Nov. 2007.

[31] A. D. Kraft and G. J. Harry, "Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity," *Int. J. Environ. Res. Public Health*, vol. 8, no. 12, pp. 2980–3018, Jul. 2011.

[32] T. H. Mogensen, "Pathogen recognition and inflammatory signaling in innate immune defenses.," *Clin. Microbiol. Rev.*, vol. 22, no. 2, p. 240–273, Apr. 2009.

[33] D. Tang, R. Kang, C. B. Coyne, H. J. Zeh, and M. T. Lotze, "PAMPs and DAMPs: signal 0s that spur autophagy and immunity.," *Immunol. Rev.*, vol. 249, no. 1, pp. 158–175, Sep. 2012.

[34] K. Inoue, "Microglial activation by purines and pyrimidines," *Glia*, vol. 40, no. 2, pp. 156–163, Nov. 2002.

[35] D. Davalos *et al.*, "ATP mediates rapid microglial response to local brain injury in vivo," *Nat. Neurosci.*, vol. 8, no. 6, pp. 752–758, Jun. 2005.

[36] S. E. Haynes *et al.*, "The $P2Y_{12}$ receptor regulates microglial activation by extracellular nucleotides," *Nat. Neurosci.*, vol. 9, no. 12, pp. 1512–1519, Dec. 2006.

[37] K. Ohsawa, Y. Irino, Y. Nakamura, C. Akazawa, K. Inoue, and S. Kohsaka, "Involvement of $P2X_4$ and $P2Y_{12}$ receptors in ATP-induced microglial chemotaxis," *Glia*, vol. 55, no. 6, pp. 604–616, Apr. 2007.

[38] L. Ulmann *et al.*, "Up-regulation of $P2X_4$ receptors in spinal microglia after peripheral

nerve injury mediates BDNF release and neuropathic pain," *J. Neurosci.*, vol. 28, no. 44, pp. 11263–11268, Oct. 2008.

[39] F. Ginhoux *et al.*, "Fate mapping analysis reveals that adult microglia derive from primitive macrophages.," *Science*, vol. 330, no. 6005, pp. 841–845, Nov. 2010.

[40] G. Raivich, "Like cops on the beat: the active role of resting microglia.," *Trends Neurosci.*, vol. 28, no. 11, pp. 571–573, Nov. 2005.

[41] I. Napoli and H. Neumann, "Microglial clearance function in health and disease.," *Neuroscience*, vol. 158, no. 3, pp. 1030–1038, Feb. 2009.

[42] H. Kimoto, R. Eto, M. Abe, H. Kato, and T. Araki, "Alterations of glial cells in the mouse hippocampus during postnatal development," *Cell. Mol. Neurobiol.*, vol. 29, no. 8, pp. 1181–1189, Dec. 2009.

[43] H. Neumann, "Control of glial immune function by neurons.," *Glia*, vol. 36, no. 2, pp. 191–199, Nov. 2001.

[44] J. M. Pocock and H. Kettenmann, "Neurotransmitter receptors on microglia," *Trends Neurosci.*, vol. 30, no. 10, pp. 527–535, Oct. 2007.

[45] A. N. Barclay, G. J. Wright, G. Brooke, and M. H. Brown, "CD200 and membrane protein interactions in the control of myeloid cells.," *Trends Immunol.*, vol. 23, no. 6, pp. 285–290, Jun. 2002.

[46] R. M. Hoek *et al.*, "Down-regulation of the macrophage lineage through interaction with OX2 (CD200).," *Science*, vol. 290, no. 5497, pp. 1768–1771, Dec. 2000.

[47] Y. Liu *et al.*, "CX_3CL1/CX_3CR1-mediated microglia activation plays a detrimental role in ischemic mice brain via p38MAPK/PKC pathway," *J. Cereb. Blood Flow Metab.*, vol. 35, no. 10, pp. 1623–1631, Oct. 2015.

[48] P. M. Hughes, M. S. Botham, S. Frentzel, A. Mir, and V. H. Perry, "Expression of fractalkine (CX_3CL1) and its receptor, CX_3CR1, during acute and chronic inflammation in the rodent CNS.," *Glia*, vol. 37, no. 4, pp. 314–327, Mar. 2002.

[49] J. K. Harrison *et al.*, "Role for neuronally derived fractalkine in mediating interactions between neurons and CX_3CR1-expressing microglia.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 95, no. 18, pp. 10896–10901, Sep. 1998.

[50] R. C. Paolicelli, K. Bisht, and M.-È. Tremblay, "Fractalkine regulation of microglial physiology and consequences on the brain and behavior.," *Front. Cell. Neurosci.*, vol. 8,

p. 129, 2014.

[51] E. M. van Beek, F. Cochrane, A. N. Barclay, and T. K. van den Berg, “Signal regulatory proteins in the immune system.,” *J. Immunol.*, vol. 175, no. 12, pp. 7781–7787, Dec. 2005.

[52] M. Colonna, “TREMs in the immune system and beyond,” *Nat. Rev. Immunol.*, vol. 3, no. 6, pp. 445–453, Jun. 2003.

[53] H. Neumann and K. Takahashi, “Essential role of the microglial triggering receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue immune homeostasis,” *J. Neuroimmunol.*, vol. 184, no. 1–2, pp. 92–99, Mar. 2007.

[54] K. Takahashi, M. Prinz, M. Stagi, O. Chechneva, and H. Neumann, “TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis,” *PLoS Med.*, vol. 4, no. 4, p. e124, Apr. 2007.

[55] J. Paloneva *et al.*, “CNS manifestations of Nasu-Hakola disease: a frontal dementia with bone cysts.,” *Neurology*, vol. 56, no. 11, pp. 1552–1558, Jun. 2001.

[56] H. H. Klunemann *et al.*, “The genetic causes of basal ganglia calcification, dementia, and bone cysts: DAP12 and TREM2,” *Neurology*, vol. 64, no. 9, pp. 1502–1507, May 2005.

[57] V. Chitu and E. R. Stanley, “Colony-stimulating factor-1 in immunity and inflammation,” *Curr. Opin. Immunol.*, vol. 18, no. 1, pp. 39–48, Feb. 2006.

[58] M. G. Cecchini *et al.*, “Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse.,” *Development*, vol. 120, no. 6, pp. 1357–1372, Jun. 1994.

[59] J. H. Lin, “CSF as a surrogate for assessing CNS exposure: an industrial perspective.,” *Curr. Drug Metab.*, vol. 9, no. 1, pp. 46–59, Jan. 2008.

[60] S. Wei *et al.*, “Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells,” *J. Leukoc. Biol.*, vol. 88, no. 3, pp. 495–505, Sep. 2010.

[61] H. Wang *et al.*, “Cerebrospinal fluid BAFF and APRIL levels in neuromyelitis optica and multiple sclerosis patients during relapse,” *J. Clin. Immunol.*, vol. 32, no. 5, pp. 1007–1011, Oct. 2012.

[62] D. Gómez-Nicola, N. L. Fransen, S. Suzzi, and V. H. Perry, “Regulation of microglial proliferation during chronic neurodegeneration,” *J. Neurosci.*, vol. 33, no. 6, pp. 2481-

2493, Feb. 2013.

[63] M. E. Lull and M. L. Block, “Microglial activation and chronic neurodegeneration.,” *Neurotherapeutics*, vol. 7, no. 4, pp. 354–365, Oct. 2010.

[64] D. L. Krause and N. Müller, “Neuroinflammation, microglia and implications for anti-inflammatory treatment in Alzheimer’s disease.,” *Int. J. Alzheimers. Dis.*, vol. 2010, Jun. 2010.

[65] S. Akira, S. Uematsu, and O. Takeuchi, “Pathogen recognition and innate immunity.,” *Cell*, vol. 124, no. 4, pp. 783–801, Feb. 2006.

[66] A. ElAli and S. Rivest, “Microglia ontology and signaling.,” *Front. cell Dev. Biol.*, vol. 4, p. 72, 2016.

[67] O. Takeuchi and S. Akira, “Pattern recognition receptors and inflammation.,” *Cell*, vol. 140, no. 6, pp. 805–820, Mar. 2010.

[68] C. C. Bowman, A. Rasley, S. L. Tranguch, and I. Marriott, “Cultured astrocytes express toll-like receptors for bacterial products,” *Glia*, vol. 43, no. 3, pp. 281–291, Sep. 2003.

[69] T. Kielian, P. Mayes, and M. Kielian, “Characterization of microglial responses to Staphylococcus aureus: effects on cytokine, costimulatory molecule, and Toll-like receptor expression.,” *J. Neuroimmunol.*, vol. 130, no. 1–2, pp. 86–99, Sep. 2002.

[70] C. Farina, M. Krumbholz, T. Giese, G. Hartmann, F. Aloisi, and E. Meinl, “Preferential expression and function of Toll-like receptor 3 in human astrocytes,” *J. Neuroimmunol.*, vol. 159, no. 1–2, pp. 12–19, Feb. 2005.

[71] S. Lehnardt *et al.*, “Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway,” *Proc. Natl. Acad. Sci.*, vol. 100, no. 14, pp. 8514–8519, Jul. 2003.

[72] A. Visintin, A. Mazzoni, J. H. Spitzer, D. H. Wyllie, S. K. Dower, and D. M. Segal, “Regulation of Toll-like receptors in human monocytes and dendritic cells.,” *J. Immunol.*, vol. 166, no. 1, pp. 249–255, Jan. 2001.

[73] J. R. Caso, J. M. Pradillo, O. Hurtado, J. C. Leza, M. A. Moro, and I. Lizasoain, “Toll-like receptor 4 is involved in subacute stress-induced neuroinflammation and in the worsening of experimental stroke,” *Stroke*, vol. 39, no. 4, pp. 1314–1320, Apr. 2008.

[74] U. Kilic, E. Kilic, C. M. Matter, C. L. Bassetti, and D. M. Hermann, “TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration,”

Neurobiol. Dis., vol. 31, no. 1, pp. 33–40, Jul. 2008.

[75] K. Saijo *et al.*, "A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death," *Cell*, vol. 137, no. 1, pp. 47–59, Apr. 2009.

[76] H. Nawashiro, K. Tasaki, C. A. Ruetzler, and J. M. Hallenbeck, "TNF-α pretreatment induces protective effects against focal cerebral ischemia in mice," *J. Cereb. Blood Flow Metab.*, vol. 17, no. 5, pp. 483–490, May 1997.

[77] I. Glezer, A. R. Simard, and S. Rivest, "Neuroprotective role of the innate immune system by microglia," *Neuroscience*, vol. 147, no. 4, pp. 867–883, Jul. 2007.

[78] C. A. McPherson, A. D. Kraft, and G. J. Harry, "Injury-induced neurogenesis: Consideration of resident microglia as supportive of neural progenitor cells," *Neurotox. Res.*, vol. 19, no. 2, pp. 341–352, Feb. 2011.

[79] J. Husemann, J. D. Loike, R. Anankov, M. Febbraio, and S. C. Silverstein, "Scavenger receptors in neurobiology and neuropathology: Their role on microglia and other cells of the nervous system," *Glia*, vol. 40, no. 2, pp. 195–205, Nov. 2002.

[80] K. Färber, U. Pannasch, and H. Kettenmann, "Dopamine and noradrenaline control distinct functions in rodent microglial cells," *Mol. Cell. Neurosci.*, vol. 29, no. 1, pp. 128–138, May 2005.

[81] F. Di Virgilio, S. Ceruti, P. Bramanti, and M. P. Abbracchio, "Purinergic signalling in inflammation of the central nervous system," *Trends Neurosci.*, vol. 32, no. 2, pp. 79–87, Feb. 2009.

[82] K. Färber and H. Kettenmann, "Purinergic signaling and microglia," *Pflügers Arch. - Eur. J. Physiol.*, vol. 452, no. 5, pp. 615–621, Aug. 2006.

[83] H. Kettenmann, "Neuroscience: The brain's garbage men," *Nature*, vol. 446, no. 7139, pp. 987–989, Apr. 2007.

[84] S. Koizumi, K. Ohsawa, K. Inoue, and S. Kohsaka, "Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors.," *Glia*, vol. 61, no. 1, pp. 47–54, Jan. 2013.

[85] M. Neeper *et al.*, "Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins.," *J. Biol. Chem.*, vol. 267, no. 21, pp. 14998–15004, Jul. 1992.

[86] A. M. Schmidt *et al.*, “Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface.,” *J. Biol. Chem.*, vol. 267, no. 21, pp. 14987–14997, Jul. 1992.

[87] G. Bu, “Apolipoprotein E and its receptors in Alzheimer’s disease: pathways, pathogenesis and therapy,” *Nat. Rev. Neurosci.*, vol. 10, no. 5, pp. 333–344, May 2009.

[88] V. Compan *et al.*, “Apoptosis-associated speck-like protein containing a CARD Forms specks but does not activate caspase-1 in the absence of NLRP3 during macrophage swelling,” *J. Immunol.*, vol. 194, no. 3, pp. 1261–1273, Feb. 2015.

[89] K. S. Ravichandran, “Recruitment signals; from apoptotic cells: invitation to a quiet meal.,” *Cell*, vol. 113, no. 7, pp. 817–820, Jun. 2003.

[90] M. Miyanishi, K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, and S. Nagata, “Identification of Tim4 as a phosphatidylserine receptor,” *Nature*, vol. 450, no. 7168, pp. 435–439, Nov. 2007.

[91] S. Koizumi *et al.*, “UDP acting at $P2Y_6$ receptors is a mediator of microglial phagocytosis,” *Nature*, vol. 446, no. 7139, pp. 1091–1095, Apr. 2007.

[92] M. Erta, A. Quintana, and J. Hidalgo, “Interleukin-6, a major cytokine in the central nervous system.,” *Int. J. Biol. Sci.*, vol. 8, no. 9, pp. 1254–1266, 2012.

[93] W. Y. Wang, M. S. Tan, J. T. Yu, and L. Tan, “Role of pro-inflammatory cytokines released from microglia in Alzheimer’s disease.,” *Ann. Transl. Med.*, vol. 3, no. 10, p. 136, Jun. 2015.

[94] S. Rose-John, G. H. Waetzig, J. Scheller, J. Grötzinger, and D. Seegert, “The IL-6/sIL-6R complex as a novel target for therapeutic approaches.,” *Expert Opin. Ther. Targets*, vol. 11, no. 5, pp. 613–624, May 2007.

[95] P. C. Heinrich, I. Behrmann, S. Haan, H. M. Hermanns, G. Müller-Newen, and F. Schaper, “Principles of interleukin IL-6-type cytokine signalling and its regulation.,” *Biochem. J.*, vol. 374, no. Pt 1, pp. 1–20, Aug. 2003.

[96] S. Bauer, B. J. Kerr, and P. H. Patterson, “The neuropoietic cytokine family in development, plasticity, disease and injury.,” *Nat. Rev. Neurosci.*, vol. 8, no. 3, pp. 221–232, Mar. 2007.

[97] M. J. Boulanger, D. Chow, E. E. Brevnova, and K. C. Garcia, “Hexameric structure and assembly of the interleukin-6/IL-6-receptor/gp130 complex,” *Science*, vol. 300, no. 5628,

pp. 2101–2104, Jun. 2003.

[98] M. Saito, K. Yoshida, M. Hibi, T. Taga, and T. Kishimoto, “Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo.,” *J. Immunol.*, vol. 148, no. 12, pp. 4066–4071, Jun. 1992.

[99] M. Narazaki *et al.*, “Soluble forms of the interleukin-6 signal-transducing receptor component gp130 in human serum possessing a potential to inhibit signals through membrane-anchored gp130.,” *Blood*, vol. 82, no. 4, pp. 1120–1126, Aug. 1993.

[100] T. Kielian, “Toll-like receptors in central nervous system glial inflammation and homeostasis.,” *J. Neurosci. Res.*, vol. 83, no. 5, pp. 711–730, Apr. 2006.

[101] C. K. Glass, K. Saijo, B. Winner, M. C. Marchetto, and F. H. Gage, “Mechanisms underlying inflammation in neurodegeneration,” *Cell*, vol. 140, no. 6, pp. 918–934, Mar. 2010.

[102] J. S. Rao, G. J. Harry, S. I. Rapoport, and H. W. Kim, “Increased excitotoxicity and neuroinflammatory markers in postmortem frontal cortex from bipolar disorder patients,” *Mol. Psychiatry*, vol. 15, no. 4, pp. 384–392, Apr. 2010.

[103] M. K. McCoy and M. G. Tansey, “TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease,” *J. Neuroinflammation*, vol. 5, no. 1, p. 45, Oct. 2008.

[104] A. Simi, N. Tsakiri, P. Wang, and N. J. Rothwell, “Interleukin-1 and inflammatory neurodegeneration,” *Biochem. Soc. Trans.*, vol. 35, no. 5, pp. 1122–1126, Nov. 2007.

[105] C. Y. D. Lee and G. E. Landreth, “The role of microglia in amyloid clearance from the AD brain,” *J. Neural Transm.*, vol. 117, no. 8, pp. 949–960, Aug. 2010.

[106] G. Naert and S. Rivest, “The role of microglial cell subsets in Alzheimer’s disease.,” *Curr. Alzheimer Res.*, vol. 8, no. 2, pp. 151–155, Mar. 2011.

[107] C. Peyssonnaux, P. Cejudo-Martin, A. Doedens, A. S. Zinkernagel, R. S. Johnson, and V. Nizet, “Cutting edge: Essential role of hypoxia inducible factor-1alpha in development of lipopolysaccharide-induced sepsis.,” *J. Immunol.*, vol. 178, no. 12, pp. 7516–7519, Jun. 2007.

[108] J. Jantsch *et al.*, “Hypoxia and hypoxia-inducible factor-1 alpha modulate lipopolysaccharide-induced dendritic cell activation and function.,” *J. Immunol.*, vol. 180, no. 7, pp. 4697–4705, Apr. 2008.

[109] P. Y. Leung, A. E. Packard, and M. P. Stenzel-Poore, “It’s all in the family: multiple Toll-like receptors offer promise as novel therapeutic targets for stroke neuroprotection.,” *Future Neurol.*, vol. 4, no. 2, pp. 201–208, 2009.

[110] M. L. de Lemos *et al.*, “Evaluation of hypoxia inducible factor expression in inflammatory and neurodegenerative brain models.,” *Int. J. Biochem. Cell Biol.*, vol. 45, no. 7, pp. 1377–1388, Jul. 2013.

[111] Y. T. Oh *et al.*, “Lipopolysaccharide induces hypoxia-inducible factor-1 alpha mRNA expression and activation via NADPH oxidase and Sp1-dependent pathway in BV2 murine microglial cells.,” *Neurosci. Lett.*, vol. 431, no. 2, pp. 155–160, Jan. 2008.

[112] H. Z. Imtiyaz and M. C. Simon, “Hypoxia-inducible factors as essential regulators of inflammation.,” *Curr. Top. Microbiol. Immunol.*, vol. 345, pp. 105–120, 2010.

[113] A. J. Majmundar, W. J. Wong, and M. C. Simon, “Hypoxia-inducible factors and the response to hypoxic stress,” *Mol. Cell*, vol. 40, no. 2, pp. 294–309, Oct. 2010.

[114] W. G. Kaelin and P. J. Ratcliffe, “Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway.,” *Mol. Cell*, vol. 30, no. 4, pp. 393–402, May 2008.

[115] J. A. Bertout, S. A. Patel, and M. C. Simon, “The impact of O_2 availability on human cancer.,” *Nat. Rev. Cancer*, vol. 8, no. 12, pp. 967–975, Dec. 2008.

[116] G. L. Wang, B. H. Jiang, E. A. Rue, and G. L. Semenza, “Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 92, no. 12, pp. 5510–5514, Jun. 1995.

[117] P. H. Maxwell *et al.*, “The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis,” *Nature*, vol. 399, no. 6733, pp. 271–275, May 1999.

[118] M. E. Cockman *et al.*, “Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein,” *J. Biol. Chem.*, vol. 275, no. 33, pp. 25733–25741, Aug. 2000.

[119] D. Lando, D. J. Peet, D. A. Whelan, J. J. Gorman, and M. L. Whitelaw, “Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch.,” *Science*, vol. 295, no. 5556, pp. 858–861, Feb. 2002.

[120] J. Duranteau, N. S. Chandel, A. Kulisz, Z. Shao, and P. T. Schumacker, “Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes.,” *J. Biol. Chem.*,

vol. 273, no. 19, pp. 11619–11624, May 1998.

[121] G. Höpfl, O. Ogunshola, and M. Gassmann, “Hypoxia and high altitude. The molecular response.,” *Adv. Exp. Med. Biol.*, vol. 543, pp. 89–115, 2003.

[122] G. L. Semenza, “Expression of hypoxia-inducible factor 1: mechanisms and consequences.,” *Biochem. Pharmacol.*, vol. 59, no. 1, pp. 47–53, Jan. 2000.

[123] K. Franke, M. Gassmann, and B. Wielockx, “Erythrocytosis: the HIF pathway in control.,” *Blood*, vol. 122, no. 7, pp. 1122–1128, Aug. 2013.

[124] G. S. Sugga, M. U. Khan, and R. Khanam, “Protective role of fibrates in cardiac ischemia/reperfusion.,” *J. Adv. Pharm. Technol. Res.*, vol. 3, no. 3, pp. 188–192, Jul. 2012.

[125] R. Schulz, M. Kelm, and G. Heusch, “Nitric oxide in myocardial ischemia/reperfusion injury,” *Cardiovasc. Res.*, vol. 61, no. 3, pp. 402–413, Feb. 2004.

[126] F. Aktan, “iNOS-mediated nitric oxide production and its regulation,” *Life Sci.*, vol. 75, no. 6, pp. 639–653, Jun. 2004.

[127] C. S. Park *et al.*, “Differential and constitutive expression of neuronal, inducible, and endothelial nitric oxide synthase mRNAs and proteins in pathologically normal human tissues,” *Nitric Oxide*, vol. 4, no. 5, pp. 459–471, Oct. 2000.

[128] Y. Liu, S. R. Cox, T. Morita, and S. Kourembanas, “Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5’ enhancer.,” *Circ. Res.*, vol. 77, no. 3, pp. 638–643, Sep. 1995.

[129] J. A. Forsythe *et al.*, “Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1.,” *Mol. Cell. Biol.*, vol. 16, no. 9, pp. 4604–4613, Sep. 1996.

[130] S. D. Croll, J. H. Goodman, and H. E. Scharfman, “Vascular endothelial growth factor (VEGF) in seizures: a double-edged sword.,” *Adv. Exp. Med. Biol.*, vol. 548, pp. 57–68, 2004.

[131] M. Kilic, H. Kasperczyk, S. Fulda, and K.-M. Debatin, “Role of hypoxia inducible factor-1 alpha in modulation of apoptosis resistance,” *Oncogene*, vol. 26, no. 14, pp. 2027–2038, Mar. 2007.

[132] Z. Zhang, J. Yan, S. Taheri, K. J. Liu, and H. Shi, “Hypoxia-inducible factor 1 contributes to N-acetylcysteine’s protection in stroke,” *Free Radic. Biol. Med.*, vol. 68, pp. 8–21, Mar. 2014.

[133] M. G. Slomiany and S. A. Rosenzweig, “IGF-1–Induced VEGF and IGFBP-3 secretion correlates with increased HIF-1α expression and activity in retinal pigment epithelial cell line D407,” *Investig. Opthalmology Vis. Sci.*, vol. 45, no. 8, p. 2838, Aug. 2004.

[134] M. Koistinaho and J. Koistinaho, “Role of p38 and p44/42 mitogen-activated protein kinases in microglia,” *Glia*, vol. 40, no. 2, pp. 175–183, Nov. 2002.

[135] G. Schulte and B. B. Fredholm, “Signalling from adenosine receptors to mitogen-activated protein kinases.,” *Cell. Signal.*, vol. 15, no. 9, pp. 813–827, Sep. 2003.

[136] C. Hammarberg, B. B. Fredholm, and G. Schulte, “Adenosine A_3 receptor-mediated regulation of p38 and extracellular-regulated kinase ERK1/2 via phosphatidylinositol-3’-kinase.,” *Biochem. Pharmacol.*, vol. 67, no. 1, pp. 129–134, Jan. 2004.

[137] P. A. Borea, S. Gessi, S. Merighi, and K. Varani, “Adenosine as a multi-signalling guardian angel in human diseases: When, where and how does it exert its protective effects?,” *Trends Pharmacol. Sci.*, vol. 37, no. 6, pp. 419–434, Jun. 2016.

[138] P. A. Borea *et al.*, “The A_3 adenosine receptor: history and perspectives.,” *Pharmacol. Rev.*, vol. 67, no. 1, pp. 74–102, 2015.

[139] B. B. Fredholm, A. P. IJzerman, K. A. Jacobson, J. Linden, and C. E. Müller, “International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors--an update.,” *Pharmacol. Rev.*, vol. 63, no. 1, pp. 1–34, Mar. 2011.

[140] D. Dal Ben, C. Lambertucci, S. Vittori, R. Volpini, and G. Cristalli, “GPCRs as therapeutic targets: a view on adenosine receptors structure and functions, and molecular modeling support,” *J. Iran. Chem. Soc.*, vol. 2, no. 3, pp. 176–188, Sep. 2005.

[141] Z. Chen *et al.*, “MAP kinases.,” *Chem. Rev.*, vol. 101, no. 8, pp. 2449–2476, Aug. 2001.

[142] C. Widmann, S. Gibson, M. B. Jarpe, and G. L. Johnson, “Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human.,” *Physiol. Rev.*, vol. 79, no. 1, pp. 143–180, Jan. 1999.

[143] J. S. Gutkind, “The pathways connecting G protein-coupled receptors to the nucleus through divergent mitogen-activated protein kinase cascades.,” *J. Biol. Chem.*, vol. 273, no. 4, pp. 1839–1842, Jan. 1998.

[144] M. J. Marinissen and J. S. Gutkind, “G-protein-coupled receptors and signaling networks: emerging paradigms.,” *Trends Pharmacol. Sci.*, vol. 22, no. 7, pp. 368–376, Jul. 2001.

[145] S. Gessi, S. Merighi, D. Fazzi, A. Stefanelli, K. Varani, and P. A. Borea, "Adenosine receptor targeting in health and disease," *Expert Opin. Investig. Drugs*, vol. 20, no. 12, pp. 1591–1609, Dec. 2011.

[146] B. B. Fredholm, "Rethinking the purinergic neuron-glia connection.," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 16, pp. 5913–5914, Apr. 2012.

[147] J. Linden, "Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection," *Annu. Rev. Pharmacol. Toxicol.*, vol. 41, no. 1, pp. 775–787, Apr. 2001.

[148] S. Gessi, S. Merighi, V. Sacchetto, C. Simioni, and P. A. Borea, "Adenosine receptors and cancer," *Biochim. Biophys. Acta - Biomembr.*, vol. 1808, no. 5, pp. 1400–1412, May 2011.

[149] S. Gessi, S. Merighi, A. Stefanelli, D. Fazzi, K. Varani, and P. A. Borea, "A_1 and A_3 adenosine receptors inhibit LPS-induced hypoxia-inducible factor-1 accumulation in murine astrocytes," *Pharmacol. Res.*, vol. 76, pp. 157–170, 2013.

[150] G. Haskó, P. Pacher, E. S. Vizi, and P. Illes, "Adenosine receptor signaling in the brain immune system.," *Trends Pharmacol. Sci.*, vol. 26, no. 10, pp. 511–516, Oct. 2005.

[151] B. B. Fredholm, "Adenosine receptors as drug targets," *Exp. Cell Res.*, vol. 316, no. 8, pp. 1284–1288, May 2010.

[152] G. Burnstock, "Purinergic Signalling: Therapeutic Developments.," *Front. Pharmacol.*, vol. 8, p. 661, 2017.

[153] G. Haskó, J. Linden, B. Cronstein, and P. Pacher, "Adenosine receptors: therapeutic aspects for inflammatory and immune diseases.," *Nat. Rev. Drug Discov.*, vol. 7, no. 9, pp. 759–770, Sep. 2008.

[154] M. Pastor-Anglada, F. J. Casado, R. Valdés, J. Mata, J. García-Manteiga, and M. Molina, "Complex regulation of nucleoside transporter expression in epithelial and immune system cells.," *Mol. Membr. Biol.*, vol. 18, no. 1, pp. 81–85, Jan. 2001.

[155] G. Haskó and B. N. Cronstein, "Adenosine: an endogenous regulator of innate immunity.," *Trends Immunol.*, vol. 25, no. 1, pp. 33–39, Jan. 2004.

[156] S. A. Rivkees and C. C. Wendler, "Regulation of cardiovascular development by adenosine and adenosine-mediated embryo protection," *Arterioscler. Thromb. Vasc. Biol.*, vol. 32, no. 4, pp. 851–855, Apr. 2012.

[157] P. Fishman *et al.*, "Adenosine receptors and cancer.," *Handb. Exp. Pharmacol.*, no. 193, pp. 399–441, 2009.

[158] C. Lauro *et al.*, "Adenosine A_1 receptors and microglial cells mediate CX_3CL1-induced protection of hippocampal neurons against Glu-induced death.," *Neuropsychopharmacology*, vol. 35, no. 7, pp. 1550–1559, Jun. 2010.

[159] L. Luongo *et al.*, "The A_1 adenosine receptor as a new player in microglia physiology.," *Glia*, vol. 62, no. 1, pp. 122–132, Jan. 2014.

[160] C. Gomes *et al.*, "Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia," *J. Neuroinflammation*, vol. 10, no. 1, p. 780, Dec. 2013.

[161] C. van der Putten *et al.*, "Differential expression of adenosine A_3 receptors controls adenosine A_{2A} receptor-mediated inhibition of TLR responses in microglia.," *J. Immunol.*, vol. 182, no. 12, pp. 7603–7612, Jun. 2009.

[162] K. Heese, B. L. Fiebich, J. Bauer, and U. Otten, "Nerve growth factor (NGF) expression in rat microglia is induced by adenosine A_{2A}-receptors.," *Neurosci. Lett.*, vol. 231, no. 2, pp. 83–86, Aug. 1997.

[163] B. L. Fiebich *et al.*, "Cyclooxygenase-2 expression in rat microglia is induced by adenosine A_{2A}-receptors.," *Glia*, vol. 18, no. 2, pp. 152–160, Oct. 1996.

[164] J. Saura, J. M. Tusell, and J. Serratosa, "High-yield isolation of murine microglia by mild trypsinization.," *Glia*, vol. 44, no. 3, pp. 183–189, Dec. 2003.

[165] A. G. Orr, A. L. Orr, X.-J. Li, R. E. Gross, and S. F. Traynelis, "Adenosine A_{2A} receptor mediates microglial process retraction.," *Nat. Neurosci.*, vol. 12, no. 7, pp. 872–878, Jul. 2009.

[166] N. Rebola *et al.*, "Adenosine A_{2A} receptors control neuroinflammation and consequent hippocampal neuronal dysfunction.," *J. Neurochem.*, vol. 117, no. 1, pp. 100–111, Apr. 2011.

[167] S. S. Dai *et al.*, "Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury.," *J. Neurosci.*, vol. 30, no. 16, pp. 5802–5810, Apr. 2010.

[168] B. Koscsó *et al.*, "Adenosine augments IL-10 production by microglial cells through an

A_{2B} adenosine receptor-mediated process.," *J. Immunol.*, vol. 188, no. 1, pp. 445–453, Jan. 2012.

[169] M. Schwaninger, M. Neher, E. Viegas, A. Schneider, and M. Spranger, "Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine.," *J. Neurochem.*, vol. 69, no. 3, pp. 1145–1150, Sep. 1997.

[170] M. Schwaninger, N. Petersen, S. Prinz, S. Sallmann, M. Neher, and M. Spranger, "Adenosine-induced expression of interleukin-6 in astrocytes through protein kinase A and NF-IL-6.," *Glia*, vol. 31, no. 1, pp. 51–58, Jul. 2000.

[171] D. A. Rees, B. M. Lewis, M. D. Lewis, K. Francis, M. F. Scanlon, and J. Ham, "Adenosine-induced IL-6 expression in pituitary folliculostellate cells is mediated via A_{2B} adenosine receptors coupled to PKC and p38 MAPK.," *Br. J. Pharmacol.*, vol. 140, no. 4, pp. 764–772, Oct. 2003.

[172] H. Zhong, L. Belardinelli, T. Maa, I. Feoktistov, I. Biaggioni, and D. Zeng, "A_{2B} adenosine receptors increase cytokine release by bronchial smooth muscle cells.," *Am. J. Respir. Cell Mol. Biol.*, vol. 30, no. 1, pp. 118–125, Jan. 2004.

[173] H. Zhong, L. Belardinelli, T. Maa, and D. Zeng, "Synergy between A_{2B} adenosine receptors and hypoxia in activating human lung fibroblasts.," *Am. J. Respir. Cell Mol. Biol.*, vol. 32, no. 1, pp. 2–8, Jan. 2005.

[174] B. L. Fiebich *et al.*, "IL-6 expression induced by adenosine A_{2B} receptor stimulation in U373 MG cells depends on p38 mitogen activated kinase and protein kinase C.," *Neurochem. Int.*, vol. 46, no. 6, pp. 501–512, May 2005.

[175] S. Ryzhov *et al.*, "Effect of A_{2B} adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines.," *J. Pharmacol. Exp. Ther.*, vol. 324, no. 2, pp. 694–700, Feb. 2008.

[176] J. F. Vazquez, H.-W. Clement, O. Sommer, E. Schulz, and D. van Calker, "Local stimulation of the adenosine A_{2B} receptors induces an increased release of IL-6 in mouse striatum: an in vivo microdialysis study.," *J. Neurochem.*, vol. 105, no. 3, pp. 904–909, May 2008.

[177] W. Feng, Y. Song, C. Chen, Z. Z. Lu, and Y. Zhang, "Stimulation of adenosine A_{2B} receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-delta-P38 signalling pathway.," *Br. J. Pharmacol.*, vol. 159, no. 8, pp. 1598–1607, Apr. 2010.

[178] C. Hammarberg, G. Schulte, and B. B. Fredholm, “Evidence for functional adenosine A_3 receptors in microglia cells.,” *J. Neurochem.*, vol. 86, no. 4, pp. 1051–1054, Aug. 2003.

[179] J. Y. Lee *et al.*, “Activation of adenosine A_3 receptor suppresses lipopolysaccharide-induced TNF-α production through inhibition of PI3-kinase/Akt and NF-kB activation in murine BV2 microglial cells,” *Neurosci. Lett.*, vol. 396, no. 1, pp. 1–6, Mar. 2006.

[180] K. Ohsawa, T. Sanagi, Y. Nakamura, E. Suzuki, K. Inoue, and S. Kohsaka, “Adenosine A_3 receptor is involved in ADP-induced microglial process extension and migration.,” *J. Neurochem.*, vol. 121, no. 2, pp. 217–227, Apr. 2012.

CHAPTER 1: A_{2A} AND A_{2B} ADENOSINE RECEPTORS AFFECT HIF-1α SIGNALING IN ACTIVATED PRIMARY MICROGLIAL CELLS

Aim of the study

Multiple signalling molecules are activated in response to LPS in microglia. Some of them include HIF-1α. It is well recognized that Ado affects HIF-1α induction in peripheral inflammatory cells like tumor cells [1]–[3], macrophages [4], [5] and foam cells [6] recruiting different receptor subtypes. As the effects of ARs and HIF-1α modulation in glial cells resident in the CNS have not been addressed, we recently investigated Ado modulation of HIF-1α accumulation following hypoxic/inflammatory insult in murine astrocytes. In these cells we found that A_1 and A_3 ARs reduce the LPS-mediated HIF-1α accumulation, resulting in a downregulation of genes involved in inflammation and hypoxic injury [7].

Taking this background into account, the aim of this study was to evaluate the effect induced by ARs modulation on HIF-1α accumulation in activated microglial cells, that are considered in the CNS the functional equivalent of macrophages [8].

Materials and Methods

Materials

HIF-1α siRNA and anti-A_3 ARs rabbit polyclonal antibody were from Santa Cruz DBA (Milan, Italy). RNAiFect Transfection Kit was purchased from Qiagen (Milan, Italy). Rabbit polyclonal antibody against A_1 and A_{2A} were obtained from Alpha Diagnostic, Vinci Biochem (Florence, Italy). Rabbit polyclonal anti-HIF-1α was from Cayman, Vinci Biochem (Florence, Italy). SH5 was from Enzo-Life, Vinci Biochem (Florence, Italy). SB202190 was from Adipogen, Vinci Biochem (Florence, Italy). U0126 was from Promega (Milan, Italy). Anti-beta Actin (cod. 04-1116) and chetomin were from Millipore (Milan, Italy). GFAP and anti-CD11b antibodies were from Becton Dickinson (Milan, Italy). Rabbit monoclonal anti-phospho-pERK1/2 MAPK (Thr202/Tyr204), anti-phospho-p38 MAPK (T180/Y182), rabbit polyclonal phospho-Akt (Ser473), Akt, ERK1/2 MAPK, p38 MAPK and iNOS were from Cell Signalling, Euroclone (Milan, Italy). The Assays-on- demand™ Gene expression Products for HIF-1 (Mm00468869-m1), GLUT-1 (Mm00441480_m1), VEGF (Mm00437304_m1), iNOS (Mm00440485_m1), TNF-α (Mm00443260_m1), A_1 (Mm01308023_m1), A_{2A} (Mm00802075_m1), A_{2B} (Mm00839292_m1) and A_3 (Mm00802076_m1) were purchased from Life Technologies Italia (Monza, Italy). PSB 36, Cl-IB-MECA, BAY 60-6583 and PSB 603 were purchased from Tocris, Space Import-Export (Milan, Italy). SCH 442416, MRS 1523, CGS 21680, CHA were purchased from Sigma. Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

Animals

One-day-old Balb/c mice were obtained from Charles River (Calco, Italy). Animal care procedures were in accordance with the guidelines of the European Council directives (86/609/EEC) and were approved by the local Animal Care and Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals [9].

Primary microglia cultures

Briefly, after anaesthesia (Zoletil 100, 30 mg/kg, Virbac Laboratories, France) and decapitation, forebrains from newborn Balb/c mice were excised, meninges were removed and tissue was dissociated mechanically in 0.25% trypsin solution (Gibco, Life Technologies, Italy). Confluent mixed glial cultures were subjected to mild trypsinization (0.06%) as previously described [10]. This results in the detachment of an intact layer of cells containing virtually all the astrocytes and

leaves a population of firmly attached cells identified as >98% microglia. Cultures were characterized by immunostaining with Mac-1 anti-CD11b antibody and the absence of astrocytes was confirmed using GFAP antibody.

Cloned A_1, A_{2A}, A_{2B}, and A_3 ARs

CHO cells transfected with human A_1, A_{2A}, and A_3 ARs and HEK293 cells transfected with the human A_{2B} have been previously described [11]. The cells were grown adherently and maintained in DMEM medium with nutrient mixture F12 (DMEM:F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), L-glutamine (2 mM), and Geneticin (G418, 0.2 mg/ml) at 37°C in 5% CO_2/95% air.

ARs ligands and LPS treatment

LPS, a cell wall component of Gram-negative bacteria, is a potent activator of glia. Hence, microglial cells were treated with 1 μg/ml LPS (from *Escherichia coli*, serotype 055:B5) (soluble in cell culture medium) following incubation with Ado ligands. In more detail, all treatments to the cells with Ado were carried out in the presence of the ADA inhibitor, EHNA 5 μM. Under the normoxic conditions in monolayer culture phosphorylation of intracellular Ado through Ado kinase, which has a lower Km (40 nM), would take preference. Under hypoxic conditions, the generation rate of Ado through AMP nucleotidase would be much higher, shifting the major role in intracellular breakdown over to ADA, which has a much higher Km (70 μM) but far greater capacity for Ado metabolism than Ado kinase [12]. In our experimental condition, EHNA was added to avoid metabolism of micromolar concentrations of Ado similar to those obtained in hypoxia, that is due preferentially by ADA. Experiments with Ado agonists were performed in the presence of ADA. Microglial cells were treated with Ado (100 μM) or ARs agonists for 30 min prior to LPS treatment. Antagonists, EHNA and ADA were preincubated 30 min prior to the treatment with Ado or ARs agonists. Microglial cells were then maintained in DMEM:F12 containing Ado ligands or their vehicle, and harvested after treatment at the indicated times. LPS and Ado solutions were performed in cell culture medium. Ado receptor agonists and antagonists, inhibitors of kinases, chetomin and CLI-095 were made up in DMSO and then diluted in cell culture medium (0.1 max 0.2% of DMSO). An equal amount of DMSO was used in control.

Hypoxic treatment

Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2 (MiniGalaxy, RSBiotech, Irvine, Scotland).

Immunofluorescence analysis

For HIF-1α detection, cells were treated with Ado and LPS for 4 h. Microglial cells were washed two times with PBS, fixed in 10% paraformaldehyde for 10 min, permeabilized in a PBS solution containing 0.1% of Triton X-100 and incubated for 30 min with PBS plus 5% goat serum. The cells were then incubated O.N. at 4°C in a humidified chamber with anti-HIF-1α Ab solutions (1:50) containing 0.5% of goat serum. Excessive antibody was washed away with PBS and rabbit antibodies were detected with fluorescein isothiocyanate-labeled goat anti-rabbit IgG. Coverslips were stained with DAPI, mounted in DABCO glycerol-PBS and observed on Nikon fluorescent microscope (Eclipse 50i) as previously described [6]. Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Milan, Italy). Levels of HIF-1 in microglial cells were the basis for calculation of the additional LPS-mediated increase and for the stimulation played by Ado agonists. The mean intensity of each cell was obtained from the cells pixels that had a higher intensity than that of the mean background intensity. A mean of 150 cells was analyzed for each condition at 40X magnification, at fixed time exposure.

Real-time RT-PCR

Total cytoplasmic RNA was extracted by the acid guanidinium thiocyanate phenol method. Quantitative real-time RT-PCR assays of A_1, A_{2A}, A_{2B}, A_3, HIF-1, VEGF, GLUT-1, iNOS, TNF-α, mRNA were carried out using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) in a MX3000P Stratagene Real-Time PCR System (M-Medical, Milan, Italy) [6]. For the real-time RT-PCR of the reference gene the endogenous control mouse β-actin kit was used, and the probe was fluorescent-labeled with VIC™ (Life Technologies, Italy). Reactions were normalized to β-actin mRNA within the same sample using the ΔΔCT method.

Western blotting

Whole cell lysates from murine microglial cells were prepared as described previously [6], whilst nuclear extracts were obtained by using the Nuclear Extract Kit (Active Motif, Vinci Biochem, Florence, Italy) according to the manufacturer's recommendations. The protein concentration was determined using a protein assay kit (Bio-Rad, Milan, Italy). Equivalent amounts of protein (40 μg) were subjected to electrophoresis on sodium dodecyl sulfate-acrylamide gel. The gel was then electroblotted onto a nitrocellulose membrane. The membranes were probed with anti-A_1 antibody (1:1000 dilution), anti-A_{2A} antibody (1:1000 dilution), anti-A_{2B} (1:1000 dilution), anti-A_3 antibody (1:1000 dilution), anti-HIF-1α (1:1000 dilution), anti-iNOS (1:1000 dilution) and anti-GLUT1 (1:500 dilution). Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against rabbit IgG

(1:2000 dilution). For detection of phosphorylated proteins antibodies specific for phosphorylated or total ERK1/2 MAPK (1:1000 dilution), phosphorylated or total Akt (1:1000 dilution) and phosphorylated or total p38 (1:1000 dilution) were used. Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Perkin Elmer, Milan, Italy). Actin (1:1000) and anti-HIF-1β (1:1000 dilution) were used to ensure equal protein loading.

Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Mean densitometry data from independent experiments were normalized to the results in control cells.

HIF-1 DNA binding activity

Nuclear extracts from microglial cells were prepared by using the Nuclear Extract Kit (Cayman, Cabru, Italy) and HIF-1 binding activities in the nuclear extracts were detected by using an ELISA-based HIF binding kit (HIF-1 transcription factor assay kit, Cayman, Cabru, Italy) according to the manufacturer's recommendations.

siRNA treatment

Microglial cells were plated in six-well plates and grown to 50-70% confluence before transfection. Transfection of (siRNA)-HIF-1α, was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit for 72 h. Cells were cultured in complete media, and RNA and total proteins were isolated at 24, 48 and 72 h for mRNA and Western blot analysis of HIF-1α proteins. A randomly chosen non-specific siRNA was used under identical conditions as control [7].

Nitrite assay

NOS activity was assessed indirectly by measuring nitrite (NO_2^-) accumulation in the cell culture media using a colorimetric kit (Cayman, Cabru, Italy). At the end of the treatment period, the nitrite concentration in the conditioned media was determined according to a modified Griess method [7]. Briefly, the NADH-dependent enzyme nitrate reductase was used to convert the nitrate to nitrite prior to quantification of the absorbance, measured at 540 nm by a spectrophotometric microplate reader (Multiskan Ascent, Labsystems, Sweden). Sodium nitrite was used as the standard compound.

ELISA

The levels of VEGF and TNF-α protein secreted by the cells in the medium were determined by ELISA kits (R&D Systems). In brief, subconfluent cells were changed into fresh medium in the presence of solvent or various concentrations of drugs. The medium was collected, and VEGF and TNF-α protein concentrations were measured by ELISA according to the manufacturer's instructions. The results were normalized to the number of cells per plate. The data are presented as mean ± SE from four independent experiments performed in triplicate.

Statistical analysis

All values in the figures and text are expressed as mean ± standard error of mean (SEM) of independent experiments. Each experiment was performed by using the microglial cells derived from one single mouse, and was performed in triplicate. The experiments were repeated at least four times as indicated from n values that represent the number of mice used [13]. Data sets were examined by one-way ANOVA and *post hoc* Tukey or Dunnett's test (when required). Two-way ANOVA and Bonferroni's *post hoc* test were used when LPS was tested with Ado ligands. In each figure legends are reported the F values for the main effects of each factor and of the interaction. Since, in general, interaction is statistically highly significant, we can't say anything about the main effect of each factor, just focus on the multiple comparison *post hoc* tests. A P value less than 0.05 was considered statistically significant. In all the figures only the most important significant results were indicated.

Results

Microglia cells morphology

Nonstimulated microglia cells showed a morphology characterized by bipolar or unipolar processes and elongated cell bodies, while in the presence of LPS presented amoeboid cell bodies with retraction of extensions (Fig. 1). Furthermore, LPS did not significantly affect cell number during the duration of the protocols.

Expression of ARs in nonstimulated and LPS-activated primary microglial cells

The expression of ARs in nonstimulated and LPS-activated primary murine microglial cells is illustrated in Fig. 2. By means of real-time RT-PCR experiments we observed that the most expressed Ado subtype in microglial cells was the A_{2B} followed by A_1, A_{2A} and A_3. To evaluate in primary microglia the previously reported effect of LPS-induced modulation of ARs we investigated their level over 8 h of LPS treatment. We observed a strong reduction of A_1 and A_3 mRNAs at 8 h, while a marked up-regulation of A_{2A} and A_{2B} mRNA starting at 2 h of LPS incubation (Fig. 2A-D). Accordingly, A_1 and A_3 significantly decreased following 24 h, while A_{2A} and A_{2B} protein levels strongly increased after 8 h of LPS treatment (Fig. 3A-D). The additional bands found in A_{2B} and A_3 Western blots may account for the presence of homo- or hetero-oligomers.

LPS induces HIF-1α accumulation in primary microglial cells

The effect of LPS on HIF-1α accumulation in BV2 murine and primary rat microglial cells has been previously reported [14]. Accordingly, we found that LPS significantly increased HIF-1α mRNA and protein accumulation also in primary murine microglial cells (Fig. 4A-B). A kinetic study revealed that a significant HIF-1α mRNA induction could be observed at 2 h stimulation with LPS and maximal effect was attained at 4 h. Incubation with LPS maximally increased HIF-1α protein accumulation at 4 h and declined at 8 h. The effect of LPS on HIF-1α protein accumulation was observed at concentrations from 0.01 to 1 μg/ml (Fig. 4C). The addition of the pharmacologic agent CLI-095, which blocks TLR4-mediated signalling, abrogated the LPS-induced HIF-1α accumulation, suggesting the involvement of TLR4 receptors in this effect (Fig. 4D). HIF-1α-DNA-binding activity in the nuclear extracts was evaluated by transcription factor assay. As shown in Fig. 4E, LPS induced stimulation of DNA binding activity, but at a lesser extent than hypoxia, used as positive control.

Ado increases LPS-induced HIF-1α accumulation in primary microglial cells

We evaluated the effect of Ado on LPS-induced HIF-1α protein accumulation by means of Western blotting assay in nuclear extracts obtained from microglial cells. Ado did not modulate HIF-1α protein level, when given alone (data not shown), while it increased the LPS-induced HIF-1α protein accumulation in a concentration-dependent way, with maximum effect reached at 100 μM (Fig. 5A). The effect of a supramaximal concentration of Ado (300 μM) was attenuated by either SCH 442416 200 nM or PSB 603 100 nM (A_{2A} and A_{2B} AR antagonists, respectively) and blocked in the simultaneous presence of both type of antagonists (Fig. 5B). A_1 and A_3 AR blockers did not affect this response (PSB 36 10 nM and MRS 1523 1 μM, respectively, data not shown). To confirm the role of ARs in this effect, we evaluated the response induced by Ado agonists. Accordingly, CGS 21680 100 nM (A_{2A} AR agonist) and BAY 60-6583 100 nM (A_{2B} AR agonist) lead to an increase in LPS induced HIF-1α protein accumulation in nuclear extracts from microglial cells; their effect was antagonized by A_{2A} and A_{2B} selective AR antagonists, respectively (Fig. 5C-D, respectively). Antagonists alone do not change HIF-1α LPS-stimulated levels (Fig. 5C-D). To evaluate a possible cross-talk between A_{2A} and A_{2B} ARs we treated the cells with both CGS 21680 and BAY 60-6583 and as shown in Fig. 5E we found an additive effect on HIF-1α levels. Furthermore, we did not observe a cross-inhibition of the A_{2B} AR antagonist on the A_{2A} AR agonists and *vice versa* (data not shown).

We analyzed the nuclear level of HIF-1α protein after treatment with LPS without and with A_{2A} and A_{2B} selective receptor agonists also by immunofluorescence. As demonstrated by Fig. 6, LPS increased nuclear HIF-1α immunofluorescence intensity (Panel B) and the effect was further raised by CGS 21680 and BAY 60-6583 (Panels C and D, respectively).

Finally, to evaluate whether stimulation of HIF-1α protein accumulation was reflected in a transcriptional effect, DNA binding activity of HIF-1α was investigated at 4 h of incubation. LPS alone caused only a slight induction (125% of control), whereas Ado in the presence of LPS caused an increase of 180% of HIF-1α-DNA binding activity (Fig. 6F). The same effect was obtained with A_{2A} and A_{2B} AR agonists, CGS 21680 and BAY60-6583, respectively and was blocked by preincubation with selective receptor antagonists (Fig. 6G). This suggests that Ado in the presence of LPS increased an activated form of HIF-1α that was able of transcriptional activity.

Ado mediates induction of HIF-1α mRNA expression in primary microglia activated with LPS

To elucidate the mechanism involved in the enhanced LPS-induced HIF-1α accumulation by Ado, we evaluated the effect of the nucleoside on HIF-1α gene expression in microglial cells by TaqMan real-time PCR. Ado did not modulate, when given alone, whilst it increased the LPS-stimulated HIF-1α mRNA level (Fig. 7A). This effect was strongly reduced following incubation with the A_{2B} antagonist PSB 603 (100 nM) and partly with the A_{2A} antagonist, SCH 442416 (200 nM), respectively. Antagonists alone do not change HIF-1α basal or LPS-stimulated levels. To confirm the role of ARs in this effect we evaluated the response induced by Ado agonists. Our data reveal an increase in HIF-1α mRNA following addition of BAY 60-6583 (100 nM) and CGS 21680 (100 nM), but not CHA (30 nM), Cl-IBMECA (100 nM) suggesting a role for A_{2A} and A_{2B} subtypes in this effect (Fig. 7B).

In order to see whether the increase of HIF-1α mRNA level was essential for the increase in protein accumulation, cells were incubated with the transcriptional inhibitor ActD. As shown in Fig. 7C the effect of LPS on HIF-1α protein increase was completely abrogated after incubation with the inhibitor of transcription.

Ado affects LPS-modulated downstream HIF-1α target genes

Given the increase in the proportion of nuclear localized HIF-1α and DNA binding activity after treatment with LPS, we looked for a series of genes involved in angiogenesis, cell metabolism and inflammation that are known to be regulated by HIF-1 like VEGF, GLUT-1, iNOS, and TNF-α. LPS increased the expression of these genes in a time-dependent way, with a peak at 16 h for VEGF, at 8 h for GLUT-1 and iNOS, while at 4 h for TNF-α (Fig. 8A-G). All these genes appeared to be regulated by LPS, at least in part, through HIF-1α activation as demonstrated by the inhibitory effect induced by both HIF-1 inhibitor chetomin 10 nM, that inhibits HIF-1 binding to the transcriptional coactivator p300, and HIF-1 silencing (Fig. 8B-H). The efficacy of the HIF-targeting siRNA is shown in Fig. 9.

Then we went on to evaluate the effect of Ado on LPS-induced VEGF, GLUT-1, iNOS, and TNF-α gene expression. Treatment with Ado before LPS activation increased the level of VEGF mRNA. Accordingly, LPS induced VEGF secretion was increased by Ado and the effect was reduced following incubation with the A_{2B} antagonist. To further confirm the involvement of ARs on VEGF levels the effect of selective agonists was evaluated. We found that the A_{2B} AR agonist BAY 60-6583 induced an increase in VEGF secretion and no stimulation was observed with CHA, CGS 21680, and Cl-IB-MECA (Fig. 10). However, treatment of the cells with siRNA

of HIF-1α, before stimulation with Ado failed to block the effect of BAY 60-6583 on VEGF mRNA levels, suggesting that HIF-1α was not triggered by A_{2B} AR to rise VEGF levels (Fig. 10D).

A role for A_{2A} and A_{2B} ARs was observed in the increase in LPS-induced GLUT-1 expression. No modulation was found following CHA or Cl-IB-MECA treatment (Fig. 11A). CGS 21680 and BAY 60-6583 were able to increase GLUT-1 mRNA expression and the effect was also observed at protein level in a concentration-dependent way (Fig. 11A-C). Incubation of microglia with siRNA of HIF-1α, before stimulation with CGS 21680 and BAY 60-6583 reduced their effect on GLUT-1 mRNA, suggesting that HIF-1α was involved in the capability of A_{2A} and A_{2B} ARs to rise GLUT-1 levels (Fig. 11D).

Ado increased LPS-induced iNOS mRNA level (Fig. 12A). A similar effect was obtained by incubating the cells with A_{2A} and A_{2B} AR agonists (Fig. 12B). Accordingly, CGS 21680 and BAY 60-6583, but not CHA and Cl-IB-MECA raised the level of iNOS protein stimulated by LPS (Fig. 12C). This effect was blocked by incubation with selective A_{2A} and A_{2B} antagonists (Fig. 12D-E, respectively). Finally, the level of extracellular nitrite in LPS-induced microglial cells was evaluated by Griess reaction. Treatment with LPS raised nitrite level and the effect was further increased by Ado. Nitrite production was strongly reduced by A_{2A} and A_{2B} antagonists PSB 603 and SCH 442416, respectively, suggesting the involvement of A_{2A} and A_{2B} AR in this effect (Fig. 12F). iNOS stimulation induced by ARs activation was HIF-1α dependent, as addition of HIF-1α siRNA to the cells inhibited stimulation of iNOS mRNA levels by Ado (Fig. 12G). In contrast we found that Ado strongly reduced TNF-α gene expression. The receptor involved in this effect was the A_{2B} as demonstrated by the inhibitory effect induced by the A_{2B} selective receptor antagonist (Fig. 13A). Furthermore, evaluation of the agonists effect revealed that BAY 60-6583 was the most potent compound able to inhibit TNF-α mRNA and protein levels, followed by high doses of CHA, CGS 21680 and Cl-IB-MECA, strongly suggesting the involvement of A_{2B} ARs in this response (Fig. 13B-C, respectively).

Signalling pathways involved in LPS-Induced HIF-1α and downstream genes increase and effect of A_{2A} and A_{2B} Ado agonists

To examine whether MAPK and PI3K/Akt pathways were involved in the stimulatory effect mediated by LPS on HIF-1α accumulation, primary microglial cells were pretreated with U0126, SB202190, and SH5, inhibitors of ERK1/2 (or p44/42), p38, and Akt, respectively, before exposure to LPS. U0126, SB202190, and SH5 strongly reduced the LPS stimulation of HIF-1 α level (Fig. 14A). The involvement of these kinases was also observed in the downstream HIF-1α dependent genes like iNOS and GLUT-1 protein levels, while VEGF was mainly p38-dependent

(Fig. 14A-B). Phosphorylation of ERK, p38, and Akt was assessed at 30 min following LPS incubation in the presence of A_{2A} and A_{2B} AR agonists. Interestingly, we found that CGS 21680 was able to increase ERK1/2 (Fig. 14C), while BAY 60-6583 induced a stimulation of p38 and Akt phosphorylation (Fig. 14D-E). Finally, both Akt and p38 kinases were involved in A_{2B}-induced HIF-1α modulation (Fig. 14F), while ERK1/2 in the effect induced by A_{2A} AR activation (Fig. 14G).

Discussion

Endogenous microglia in the CNS safeguard organ integrity by constantly scanning the tissue and rapidly responding to danger signals [15]. All CNS diseases involve microglia, which typically convert from the resting/surveillant cell type to an activated form producing proinflammatory cytokines as well as potentially neurotoxic substances, commonly associated with neuroinflammation. Therefore, pharmacological modulation of microglial function may be of therapeutic relevance in the clinical management of diverse neurological diseases associated with inflammatory processes such as MS, stroke, and AD [16].

In the present study we add a novel regulatory pathway mediated by Ado in microglial cells, showing for the first time to our knowledge, that it enhances LPS-induced HIF-1α accumulation with consequent increase in some HIF-1α target genes. The main receptor subtypes involved in this response appear to be A_{2A} and A_{2B} as demonstrated by the inhibitory effect exerted by selective receptor antagonists SCH 442416 and PSB 603, respectively, on the stimulation evoked by Ado on LPS-induced HIF-1α accumulation. Antagonists did not affect LPS-induced responses suggesting that microglia cells per se are unable to generate enough extracellular Ado to trigger a modulation of LPS responses, excluding the possibility that this purinergic system is acting as an autocrine modulation system. The alternative scenario is that Ado is derived from other cells, namely from astrocytes and/or neurons, implying that this modulation system fulfills a paracrine role. However, there are works in literature reporting effect of antagonists alone on LPS in N9 microglial cells and also in primary microglia [17]. So, we do not exclude the possibility that in *in vivo* situation during inflammatory insult there may be a more consistent damage responsible for a generation of Ado also in microglial cells, supporting the possibility that the purinergic system may act as an autocrine modulation system. In addition, the stimulatory effect of selective A_{2A} and A_{2B} ARs agonists CGS 21680 and BAY 60-6583 on LPS-induced HIF-1α accumulation, confirm that these receptors are the most important Ado subtypes involved in this effect. Interestingly, a similar behavior of Ado involving A_{2A} or both A_{2A} and A_{2B} subtypes has been reported in macrophages [18], [19] CHA and Cl-IB-MECA were unable to produce any stimulatory effect on LPS-induced HIF-1α accumulation and antagonists of A_1 and A_3 ARs did not reverse the result of Ado suggesting that in microglial cells they were not involved in LPS-induced HIF-1α accumulation. This was in contrast to data found in murine astrocytes prompting us to hypothesize that Ado exhibits different effects depending on the cerebral cell type involved [7]. Indeed, according to a primary role for A_{2A} and A_{2B} subtypes, mRNA and protein levels of these receptors were increased after LPS treatment of microglial cells, while those of A_1 and A_3 were reduced. These findings agree with other studies of ARs in

murine microglia stimulated with LPS [17], [20]–[23]. A similar trend of modulation has been also observed in macrophages, where it has been suggested that LPS induces a receptor expression pattern switch, that can selectively modify the macrophage phenotype [18], [19]. Importantly, we found that also in unstimulated microglia the A_{2B} AR was the most expressed Ado subtype according to data found in BV2 cells [24].

We confirmed the previously described effect of LPS as non-hypoxic stimulator of the HIF-1α at the transcriptional level in microglia, suggesting that it may play a critical role in brain damages during infection, trauma and ischemia [14]. Indeed, we observed that LPS increases some HIF-1α target genes like VEGF, GLUT-1, iNOS, and TNF-α. A similar modulation has been also observed in macrophages and, as HIF-1-dependent response is critical for providing cells and tissues undergoing ischemia with sufficient energy supplies and allows them to resist cell death, it has been suggested that this effect induced by LPS possibly prepares the cells for a better survival and activity in the inflamed environment [25].

However, it is also important to add that ARs, HIF-1α activation and its downstream genes regulation play an important role not only in microglia cells, but in the stem cell niche too. Both cell specimen, together with neurons, contribute to neuroinflammatory phenomena. In particular, recent evidence has identified a broader spectrum of stem cells influenced by hypoxia that includes neuronal and cancer stem cells as well as induced pluripotent stem cells [26]. Furthermore, it was demonstrated that HIF-1α levels controlled the switch of neuronal stem cells expansion to differentiation. Indeed, hypoxia and HIF-1α produce a regulatory loop that guarantees initial radial glia expansion during early cortical development and subsequent induction of angiogenesis promoting a shift to radial glia asymmetrical neurogenic divisions allowing neurogenesis [27]. A recent study on cancer stem cells isolated from human glioblastoma multiforme shows that the differential effects of the A_1 and A_{2B} ARs agonists on the survival and/or differentiation of these cells is ascribed to their distinct regulation of the kinetics of ERK/Akt phosphorylation and HIF expression [28].

Ado has long been known to stimulate VEGF production and angiogenesis [29], [30]. This effect has been previously attributed to both A_{2A} and A_3-A_{2B} ARs activation through HIF-1α stimulation in macrophages and foam cells, respectively [5], [6], [18]. However, in the present study we have shown that the A_{2B} subtype is involved in the VEGF secretion not through HIF-1α stimulation. Several works in literature reported a role for A_{2B} ARs in VEGF secretion in various cell types in a HIF-1α-independent way and recently a role of Jun B in A_{2B}-mediated VEGF secretion has been demonstrated [31], [32]. Therefore, it is likely that different pathways may be activated by the same receptor depending on the cellular system investigated or the stimulus

applied. In addition to VEGF, we observed a stimulatory effect of A_{2A} and A_{2B} subtypes on GLUT-1 and iNOS mRNA and protein expression. Interestingly, it has been reported that A_{2A} and A_{2B} ARs cooperates to increase glycolytic flux in macrophages by enhancing pfkfb3 expression through a pathway involving Sp1 and HIF-1 activity [19]. As microglia/macrophages derive most of their ATP from glycolysis, particularly when activated, this effect induced by ARs may be important for energy supply and for microglial activation. As for the modulation of Ado on iNOS contrasting results have been reported in literature in microglial cells. Some works reported an inhibitory effect mediated through A_1 and A_{2A} AR activation [33], others demonstrated a stimulatory effect induced via both receptor binding as well as downstream metabolites of Ado [34], others showed an increase due to A_{2A} AR activation [35], other explain these contradictory findings on the basis of glutamate concentrations in cell culture [36]. We found that iNOS was stimulated by Ado through A_{2B} and A_{2A} AR activation and this effect was induced through HIF-1α modulation. Our results are consistent with that found in macrophages, where an anti-inflammatory role for A_{2A} has been suggested [19]. However, iNOS increase may be considered a double-edged sword able to kill pathogens in the acute phase of inflammation, but also responsible of neuron damage or even cell death in a chronic activation. So further studies in *in vivo* animal models are necessary to elucidate whether the increase in NO production induced by Ado may be considered beneficial or dangerous.

Downstream signalling of the TLR4 complex triggers activation of a wide variety of phosphorylation cascades including MAPK and PI3K/Akt pathways [37]. In our cells we found that LPS induced a significant increase in pERK1/2, p38 and Akt phosphorylation, in order to stimulate HIF-1α accumulation. Interestingly, we observed that A_{2A} and A_{2B} ARs triggered different pathway in order to increase the LPS effect, being A_{2A} subtypes involved in pERK1/2 phosphorylation, while A_{2B} in p38 and Akt activation. This pattern of kinases was also used by A_{2B} subtype in microglia in order to increase IL-10 with anti-inflammatory activity [20].

So far conflicting conclusions have been reported on the role of A_{2B} ARs in inflammation with some publications indicating proinflammatory effects and others suggesting the opposite [38]. It has been previously shown that A_{2B} ARs knock-out (KO) mice are anti-inflammatory [39] and several protective aspects of the A_{2B} ARs against metabolic and cardiovascular diseases have been reported, suggesting that manipulation of macrophage A_{2B} ARs, may be a promising therapeutic approach [40]. In the present study we observed that the A_{2B} was the main subtype involved in the inhibition of TNF-α production induced by LPS in microglia. Accordingly, it has been found an augmentation of TNF-α in A_{2B} null mice and inhibition of TNF-α release was demonstrated in mouse macrophages by A_{2B} AR activation [39], [41], [42]. However, other

studies reported conflicting results concerning TNF-α modulation in microglia, showing an inhibition mediated by A_3 or A_{2A} subtypes in BV2 and rhesus monkey microglia, respectively [23], [43]. Our data revealed that only high doses of A_1, A_{2A}, and A_3 agonists were able to significantly reduce TNF-α secretion, thus supporting a primary role for A_{2B} ARs in this effect.

In conclusion, as illustrated in Fig. 15 we found that Ado through A_{2A} and A_{2B} ARs increases the effect of LPS on GLUT-1 and iNOS gene expression in a HIF-1α-dependent way while through the A_{2B} subtypes rises VEGF in a HIF-1α-independent way. As HIF-1α is involved in inflammatory gene activation our results suggest a role triggered by A_{2A} and A_{2B} ARs in the modulation of microglial cells function following injury. Furthermore, inhibition of TNF-α adds an important anti-inflammatory effect only for the A_{2B} subtype.

Figures and figure legends

FIG. 1

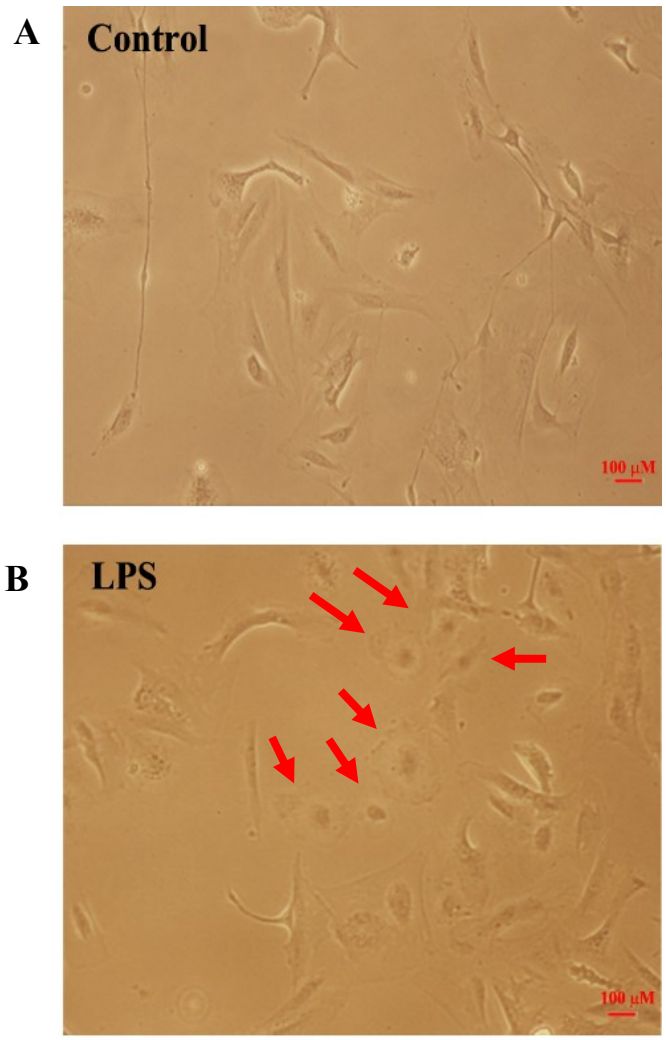


FIG. 1: Effect of LPS on microglia. Microglia in the absence (A) and in the presence (B) of LPS (1µg/ml, 4 h). Representative images of the microglial morphology are shown, arrows indicate changed morphology. Scale bar, 100 µm.

FIG. 2

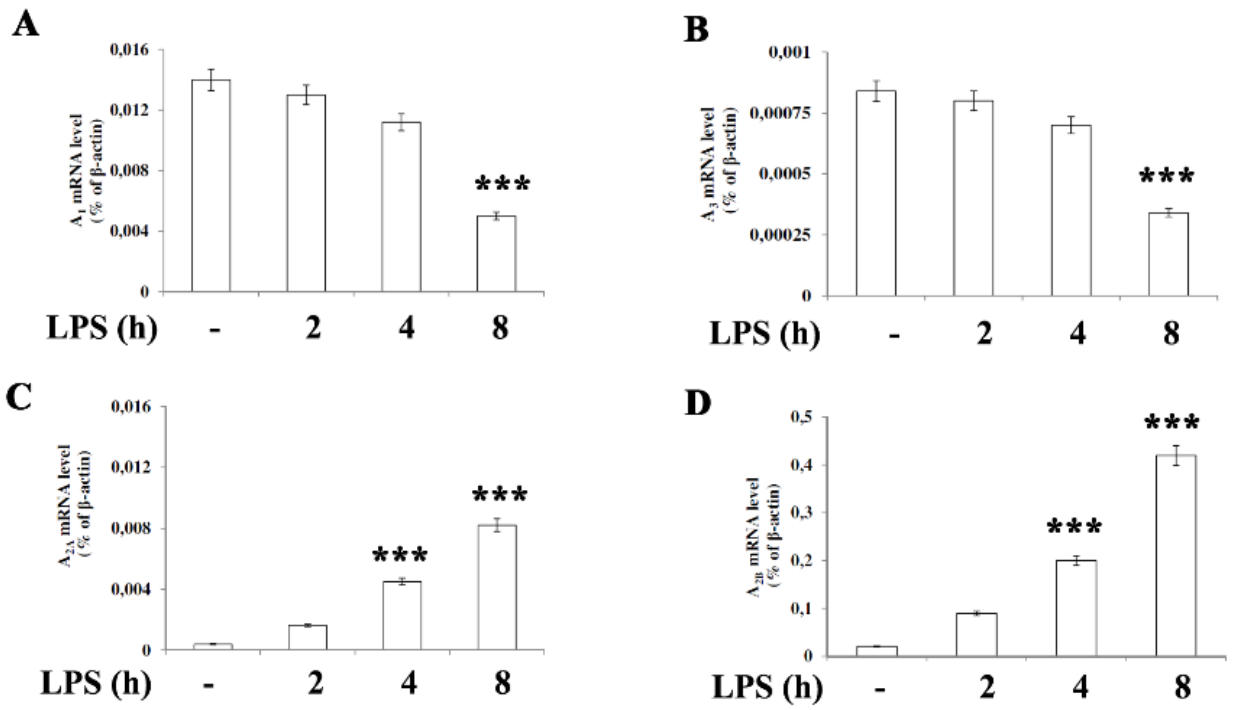


FIG. 2: Expression of ARs in resting and LPS-activated primary microglial cells. Cells were incubated for up to 8 h in the absence or presence of LPS (1 μg/ml) and mRNA levels of A_1, A_{2A}, A_{2B}, and A_3 ARs were evaluated by real-time RT-PCR. Data were expressed as % of β-actin. Each point is the mean ± SEM of four independent experiments (N = 4; A–D).

FIG. 3

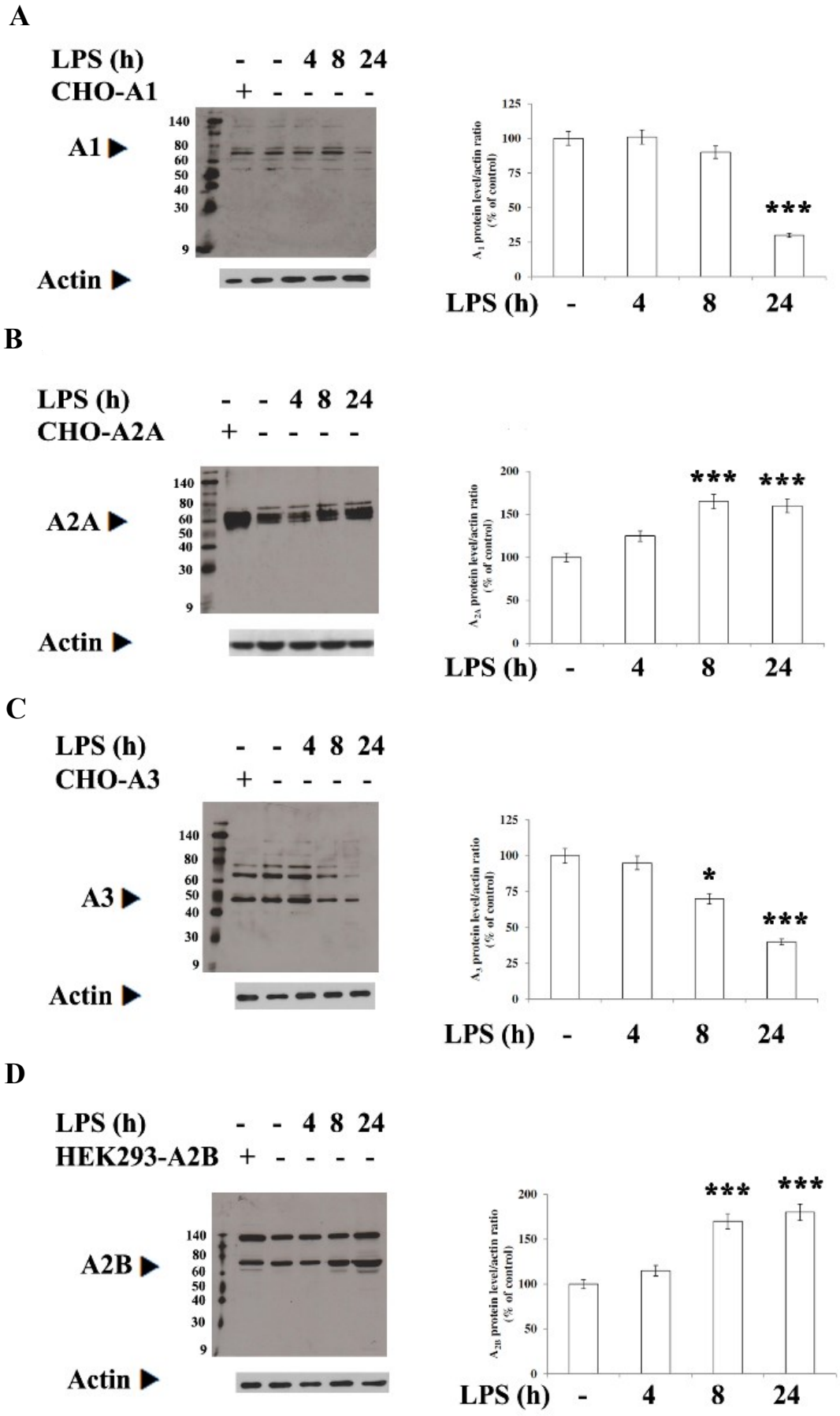

A
LPS (h) - - 4 8 24
CHO-A1 + - - - -
A1
Actin
140
80
60
50
40
30
9
A1 protein level/actin ratio (% of control)
125
100
75
50
25
0
***
LPS (h) - 4 8 24
B
LPS (h) - - 4 8 24
CHO-A2A + - - - -
A2A
Actin
A2A protein level/actin ratio (% of control)
200
150
100
50
0
***
***
LPS (h) - 4 8 24
C
LPS (h) - - 4 8 24
CHO-A3 + - - - -
A3
Actin
A3 protein level/actin ratio (% of control)
*
***
LPS (h) - 4 8 24
D
LPS (h) - - 4 8 24
HEK293-A2B + - - - -
A2B
Actin
A2B protein level/actin ratio (% of control)
***
***
LPS (h) - 4 8 24

FIG. 3: Expression of ARs in resting and LPS-activated primary microglial cells. Cells were incubated in the presence of LPS for 4, 8, and 24 h and protein levels of A_1, A_{2A}, A_{2B}, and A_3 ARs were detected by immunoblotting in whole cell lysates (A–D). Actin shows equal loading protein. Densitometric quantification is the mean ± SEM of four independent experiments (N = 4); $^{*}P < 0.05$, $^{***}P < 0.001$ compared with cells in the absence of LPS (Control); analysis was by one-way ANOVA followed by Dunnett's test.

FIG. 4

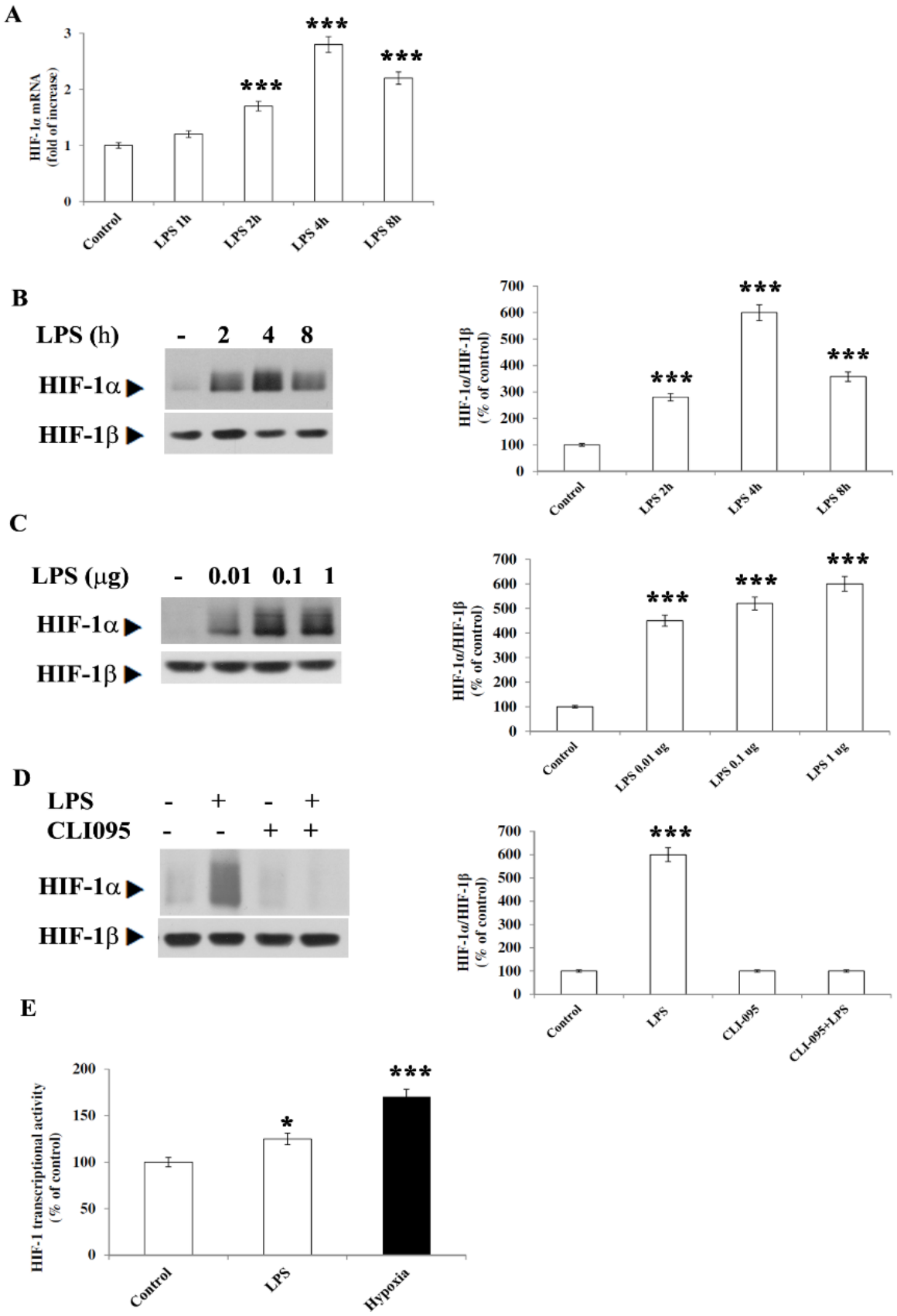

A
HIF-1α mRNA (fold of increase)
Control
LPS 1h
LPS 2h
LPS 4h
LPS 8h
B
LPS (h) - 2 4 8
HIF-1α
HIF-1β
HIF-1α/HIF-1β (% of control)
C
LPS (μg) - 0.01 0.1 1
D
LPS - + - +
CLI095 - - + +
E
HIF-1 transcriptional activity (% of control)
LPS
Hypoxia
LPS 0.01 ug
LPS 0.1 ug
LPS 1 ug
CLI-095
CLI-095+LPS

FIG. 4: LPS induces HIF-1α accumulation in primary microglial cells. Cells were incubated for up to 8 h in the absence or presence of LPS (1 μg/ml) and mRNA levels of HIF-1α were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs cells in the absence of LPS (Control), arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4; A). Microglial cells were incubated in the absence and in the presence of LPS for 2, 4, and 8 h (B) and for 4 h in the presence of increasing concentrations of LPS (0.01, 0.1, and 1 μg/ml) or CLI-095 (1 μM; C and D, respectively). The protein levels of HIF-1α were determined by Western blotting in whole cell lysates and HIF-1β served as control for equal protein loading. Densitometric quantification is the mean ± SEM of four independent experiments (N = 4); control was set to 100%. HIF-1α-DNA-binding activity was evaluated in the absence or in the presence of LPS or under hypoxia for 4 h (E). Values are the mean ± SEM of four independent experiments (N = 4); control was set to 100%. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with control; analysis was by one-way ANOVA followed by Dunnett's test.

FIG. 5

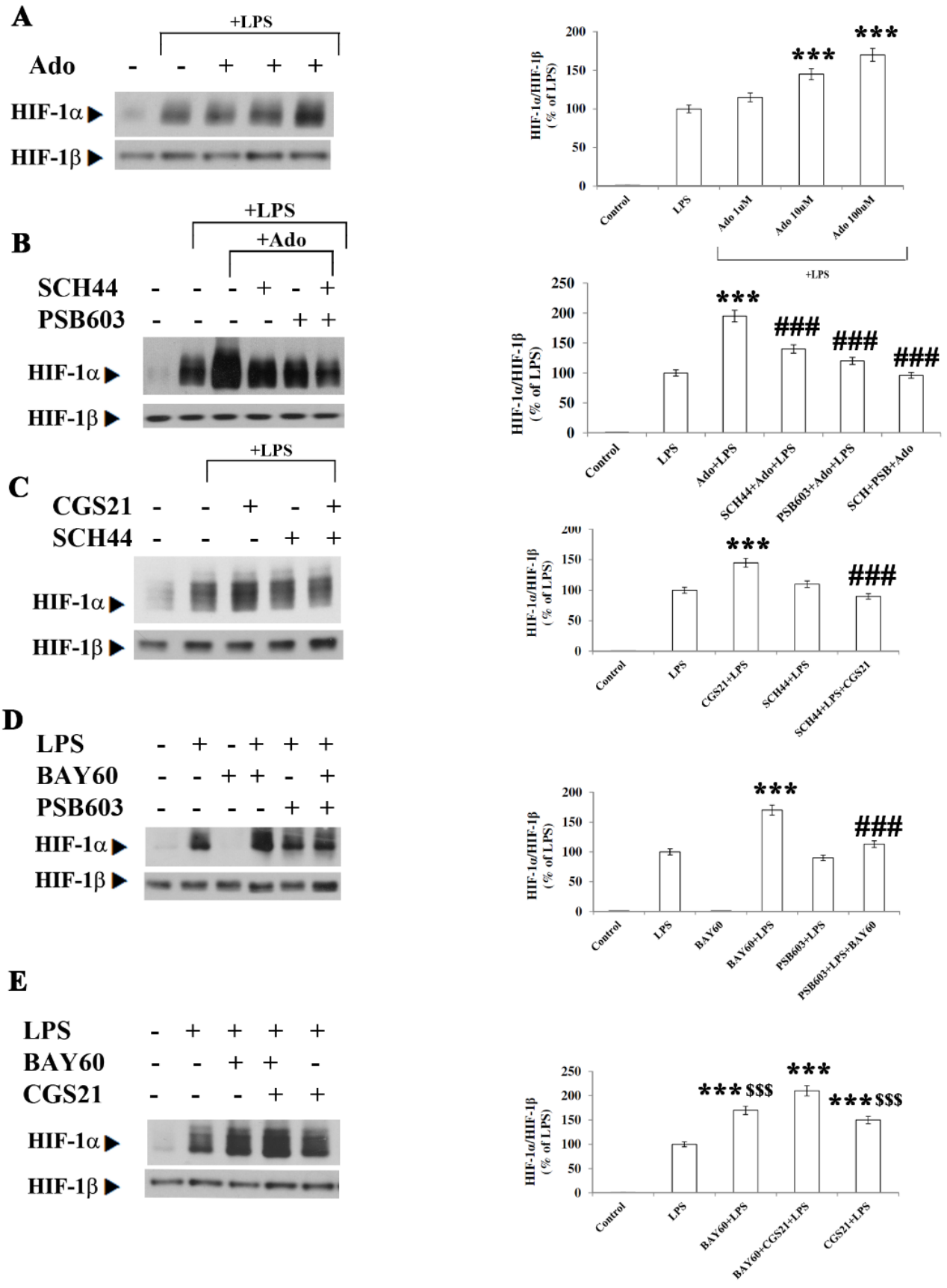

A
+LPS
Ado - - + + +
HIF-1α
HIF-1β
B
+LPS
+Ado
SCH44 - - - + - +
PSB603 - - - - + +
HIF-1α
HIF-1β
C
+LPS
CGS21 - - + - +
SCH44 - - - + +
HIF-1α
HIF-1β
D
LPS - + - + + +
BAY60 - - + + - +
PSB603 - - - - + +
HIF-1α
HIF-1β
E
LPS - + + + +
BAY60 - - + + -
CGS21 - - - + +
HIF-1α
HIF-1β
HIF-1α/HIF-1β (% of LPS)
Control
LPS
Ado 1uM
Ado 10uM
Ado 100uM
***
+LPS
Ado+LPS
SCH44+Ado+LPS
PSB603+Ado+LPS
SCH+PSB+Ado
###
CGS21+LPS
SCH44+LPS
SCH44+LPS+CGS21
BAY60
BAY60+LPS
PSB603+LPS
PSB603+LPS+BAY60
$$$
BAY60+CGS21+LPS
CGS21+LPS

FIG. 5: Ado increases LPS-induced HIF-1α accumulation in primary microglial cells. Cells were pretreated for 30 min with increasing concentrations (1, 10, and 100 μM) of Ado (Ado), before stimulation with LPS (1μg/ml) for 4 h (A). Microglial cells were incubated with SCH 442416 (200 nM), PSB 603 (100 nM), and SCH442416 + PSB603 before treatment with 300 μM Ado (B). Cells were treated with CGS 21680 (100 nM) with or without SCH 442416 (C) or BAY 60-6583 (100 nM) with or without PSB 603 (D) or CGS 21680 + BAY 60-6583 (E) in the presence of LPS for 4 h. The protein levels of HIF-1α were determined by Western blotting in nuclear extracts and HIF-1β served as control for equal protein loading. Densitometric quantification is the mean ± SEM of four independent experiments (N = 4); cells in the presence of LPS were set to 100%; $^{***}P < 0.001$ compared with cells treated with LPS; $^{###}P < 0.001$ compared with cells treated with LPS + Ado (B), CGS 21680 (C), and BAY 60-6583 (D), respectively; $^{\$\$\$}P < 0.001$ compared with cells treated with LPS + CGS 21680 + BAY 60-6583 (E); analysis was by two-way ANOVA followed by Bonferroni's test. (A) Interaction $F_{(3,24)} = 20.42$, $P < 0.0001$; Ado $F_{(3,24)} = 20.42$, $P < 0.0001$; LPS $F_{(1,24)} = 1448.56$, $P < 0.0001$. (B) Interaction $F_{(4,30)} = 36.73$, $P < 0.0001$; Ado ligands $F_{(4,30)} = 36.73$, $P < 0.0001$; LPS $F_{(1,30)} = 1944$, $P < 0.0001$. (C) Interaction $F_{(3,24)} = 16.97$, $P < 0.0001$; Ado ligands $F_{(3,24)} = 16.97$, $P < 0.0001$; LPS $F_{(1,24)} = 1440.49$, $P < 0.0001$. (D) Interaction $F_{(3,24)} = 34.10$, $P < 0.0001$; Ado ligands $F_{(3,24)} = 34.10$, $P < 0.0001$; LPS $F_{(1,24)} = 1466.31$, $P < 0.0001$. (E) Interaction $F_{(3,24)} = 24.34$, $P < 0.0001$; Ado ligands $F_{(3,24)} = 24.31$, $P < 0.0001$; LPS $F_{(1,24)} = 1139.13$, $P < 0.0001$.

FIG. 6

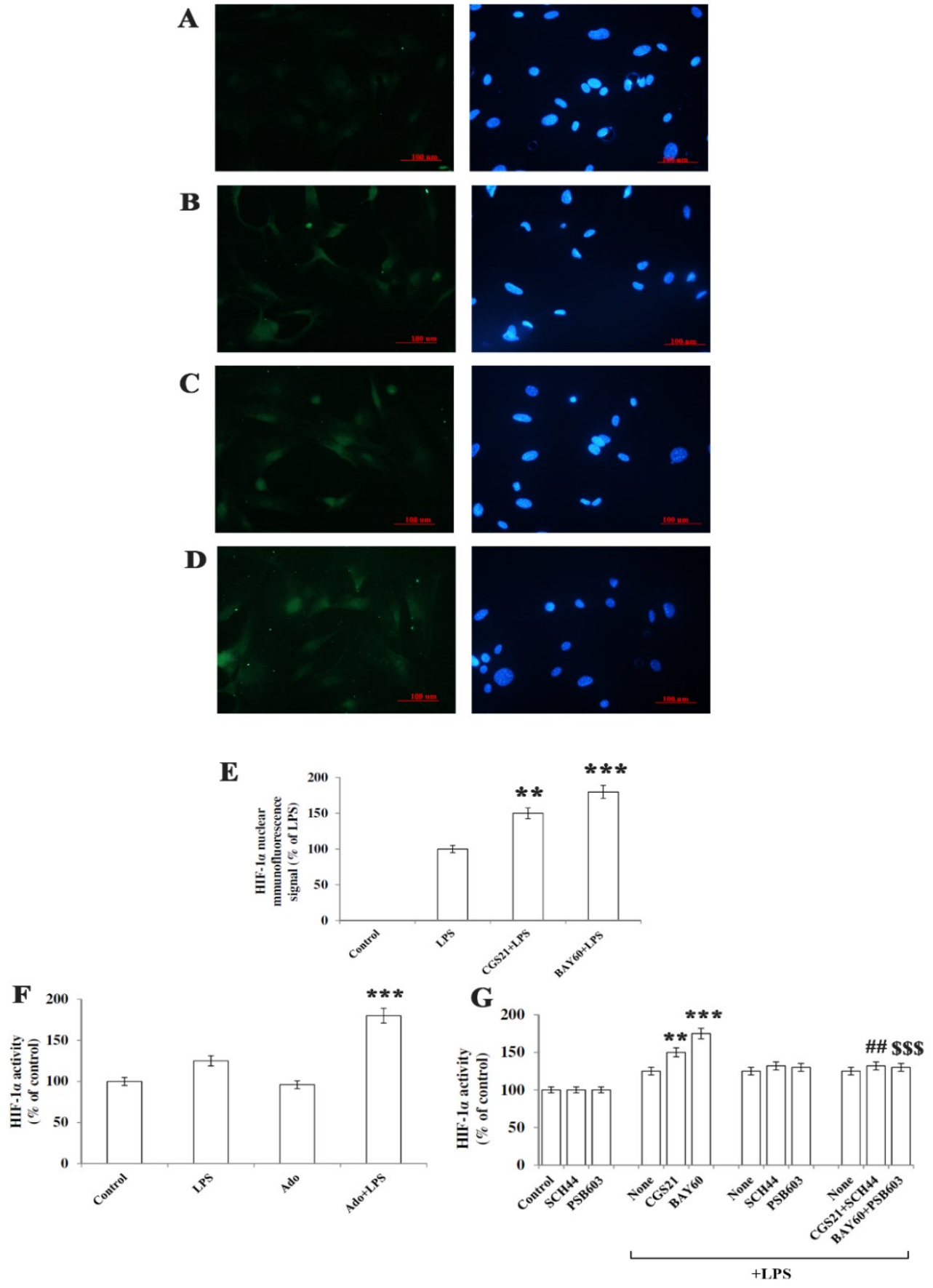

A
B
C
D
E
HIF-1α nuclear immunofluoresence signal (% of LPS)
200
150
100
50
0
**
***
Control
LPS
CGS21+LPS
BAY60+LPS
F
HIF-1α activity (% of control)
200
150
100
50
0
***
Control
LPS
Ado
Ado+LPS
G
HIF-1α activity (% of control)
200
150
100
50
0
**
***
$$$
Control
SCH44
PSB603
None
CGS21
BAY60
None
SCH44
PSB603
None
CGS21+SCH44
BAY60+PSB603
+LPS

FIG. 6: A_{2A} and A_{2B} ARs increase HIF-1α nuclear localization and transcriptional activity induced by LPS stimulation in primary microglial cells. Cells were incubated in the presence of LPS (4 h; B–D), CGS 21680 100 nM (C), and BAY 60-6583 100 nM (D). HIF-1α nuclear accumulation was evaluated by immunofluorescence analysis; (E) bargraph data are expressed as mean ± SEM percentage of HIF-1α staining from four independent experiments (N = 4); cells in the presence of LPS were set to 100%. The fluorescent dye DAPI was used to stain the nuclei of all microglial cells. Fig. shows one representative experiment. Cells were pretreated for 30 min with Ado (Ado; 100 μM; F), or with SCH 442416 (200 nM) and PSB 603 (100 nM) before treatment with CGS 21680 (100 nM) and BAY 60-6583 (100 nM; G) in the presence of LPS for 4 h. HIF-1α-DNA-binding activity is the mean ± SEM of four independent experiments (N = 4); cells in the absence of LPS were set to 100%. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with cells in the presence of LPS, respectively. $^{\#\#}P < 0.01$, $^{\$\$\$}P < 0.001$ compared with cells in the presence of LPS + CGS 21680 and LPS + BAY 60-6583, respectively; analysis was by two-way ANOVA followed by Bonferroni's test. (E) Interaction $F_{(2,18)} = 9.80$, $P < 0.0013$; Ado ligands $F_{(2,18)} = 9.80$, $P < 0.0013$; LPS $F_{(1,18)} = 364.66$, $P < 0.0001$. (F) Interaction $F_{(1,12)} = 29.75$, $P < 0.0001$; Ado $F_{(1,12)} = 22.23$, $P < 0.0005$; LPS $F_{(1,18)} = 101.55$, $P < 0.0001$. (G) Interaction $F_{(6,42)} = 7.79$, $P < 0.0001$; Ado ligands $F_{(6,42)} = 7.79$, $P < 0.0001$; LPS $F_{(1,42)} = 138.66$, $P < 0.0001$.

FIG. 7

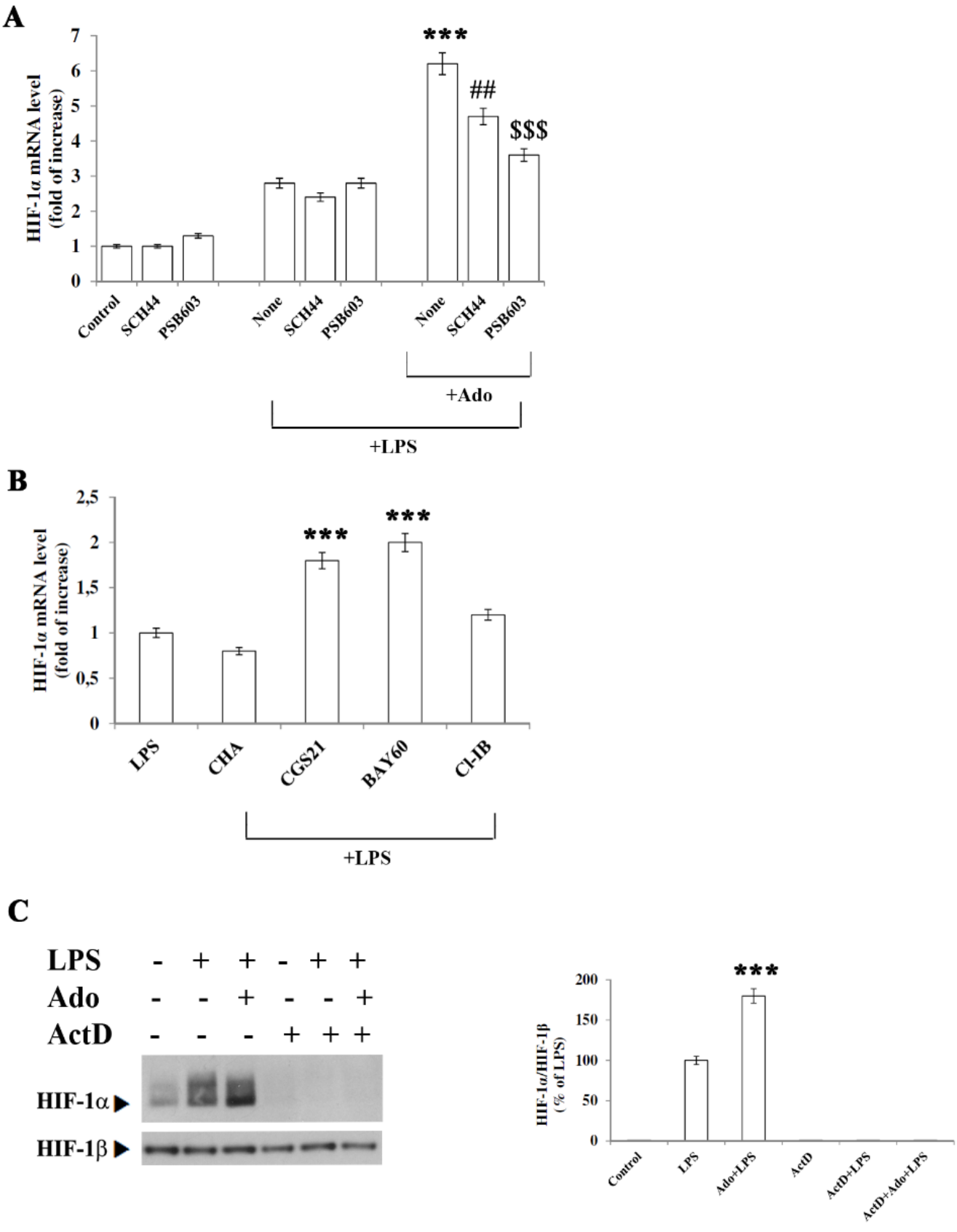

A
HIF-1α mRNA level (fold of increase)
***
##
$$$
Control
SCH44
PSB603
None
SCH44
PSB603
None
SCH44
PSB603
+Ado
+LPS
B
HIF-1α mRNA level (fold of increase)
***
***
LPS
CHA
CGS21
BAY60
Cl-IB
+LPS
C
LPS - + + - + +
Ado - - + - - +
ActD - - - + + +
HIF-1α
HIF-1β
***
HIF-1α/HIF-1β (% of LPS)
Control
LPS
Ado+LPS
ActD
ActD+LPS
ActD+Ado+LPS

FIG. 7: Ado mediates induction of HIF-1α mRNA expression in primary microglia activated with LPS. Cells were pretreated for 30 min with SCH 442416 (200 nM) and PSB 603 (100 nM) before stimulation with Ado (Ado) and LPS (1 μg/ml) for 4 h (A). Data were expressed as fold of increase compared with cells in the absence of LPS (Control), arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). Cells were incubated with CHA (30 nM), CGS 21680 (100 nM), BAY 60-6583 (100 nM), and Cl-IB-MECA (100 nM) in the presence of LPS stimulation (B). mRNA levels of HIF-1α were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs LPS, arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). Microglial cells were incubated with ActD (10 μM) before treatment with Ado in the presence of LPS for 4 h (C). The protein levels of HIF-1α were determined by Western blotting and HIF-1β served as control for equal protein loading. Densitometric quantification of HIF-1α Western blot is the mean ± SEM of four independent experiments (N = 4); LPS was set to 100% in (C). $^{***}P < 0.001$ compared with cells in the presence of LPS, $^{\#\#}P < 0.01$, $^{\$\$\$}P < 0.001$ compared with cells in the presence of LPS + Ado; analysis was by two-way ANOVA followed by Bonferroni's test. (A) Interaction $F_{(5,36)} = 5.69$, $P < 0.0006$; Ado ligands $F_{(5,36)} = 4.70$, $P < 0.0021$; LPS $F_{(1,36)} = 112.19$, $P < 0.0001$. (B) Interaction $F_{(4,30)} = 21.60$, $P < 0.0001$; Ado ligands $F_{(4,30)} = 35.83$, $P < 0.0001$; LPS $F_{(1,30)} = 66.24$, $P < 0.0001$. (C) Interaction $F_{(3,24)} = 282.51$, $P < 0.0001$; Ado $F_{(3,24)} = 283.96$, $P < 0.0001$; LPS $F_{(1,24)} = 728.44$, $P< 0.0001$.

FIG. 8

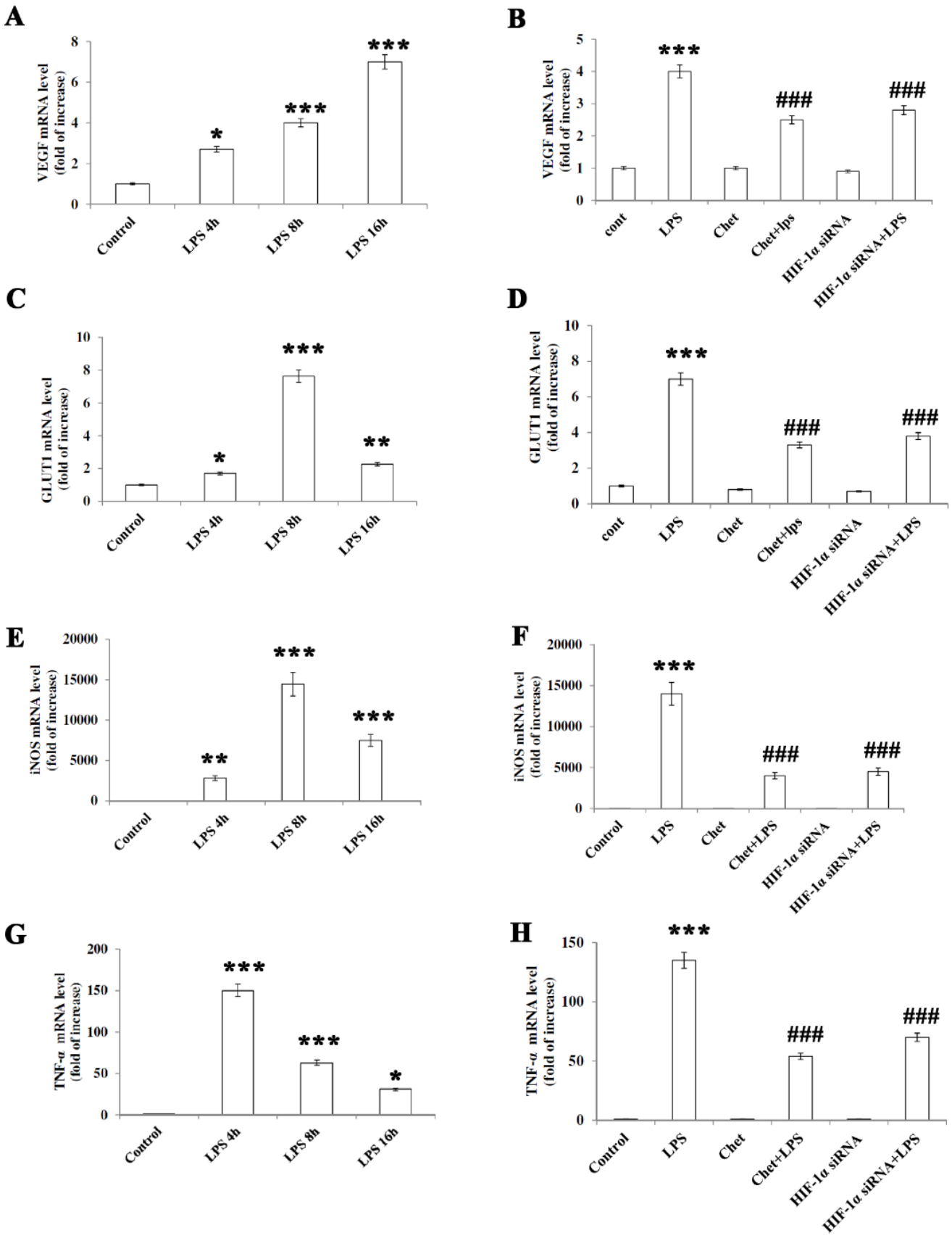

A
VEGF mRNA level (fold of increase)
Control
LPS 4h
LPS 8h
LPS 16h
B
VEGF mRNA level (fold of increase)
cont
LPS
Chet
Chet+lps
HIF-1α siRNA
HIF-1α siRNA+LPS
C
GLUT1 mRNA level (fold of increase)
D
GLUT1 mRNA level (fold of increase)
E
iNOS mRNA level (fold of increase)
F
iNOS mRNA level (fold of increase)
G
TNF-α mRNA level (fold of increase)
H
TNF-α mRNA level (fold of increase)

FIG. 8: Time course of LPS on HIF-1α target genes mRNA levels and effect of HIF-1α inhibition by chetomin or siRNA on HIF-1α target genes mRNA levels. Microglial cells were incubated in the presence of LPS (1μg/ml) stimulation for 0-16 h and mRNA levels of VEGF, GLUT-1, iNOS, and TNF-α were evaluated by real-time RT-PCR (A, C, E, and G). Data were expressed as fold of increase vs cells in the absence of LPS (control), arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). Microglial cells were incubated in the presence of LPS stimulation for 8 h, in the presence of chetomin (10 nM) or HIF-1α siRNA and mRNA levels of VEGF, GLUT-1, iNOS, and TNF-α (4 h) were evaluated by real-time RT-PCR (B, D, F, and H). Data were expressed as fold of increase vs cells in the absence of LPS (control), arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control; $^{\#\#\#}P < 0.001$versus cells in the presence of LPS; analysis was by one-way ANOVA followed by Dunnett's test (A, C, E, and G) and Tukey's test (B, D, F, and H).

FIG. 9

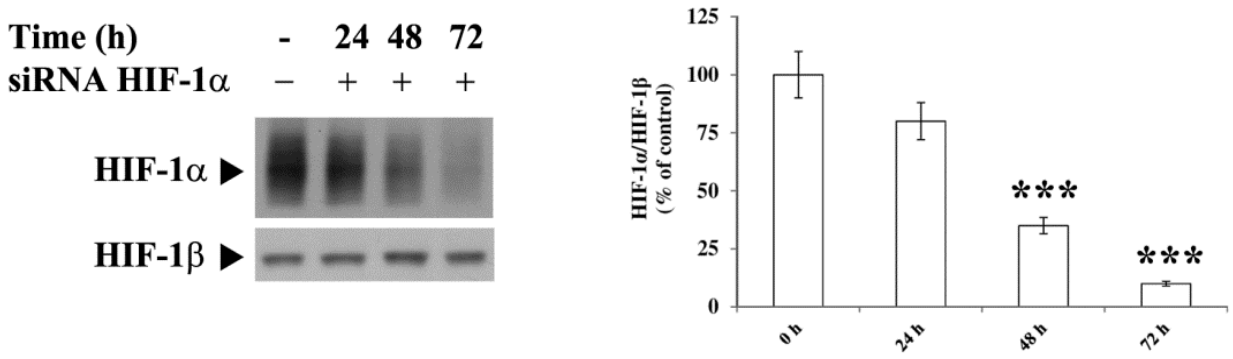


FIG. 9: HIF-1α silencing by siRNA transfection in microglial cells. Western blot analysis using anti HIF-1 polyclonal antibodies of protein extracts from microglia treated with siRNA and cultured for 24, 48 and 72 h. HIF-1β shows equal loading protein. Densitometric quantification is the mean ± SEM of four independent experiments (N = 4); control was set to 100% $^{***}P<0.001$ compared with Control; analysis was by one-way ANOVA followed by Dunnett's test.

FIG. 10

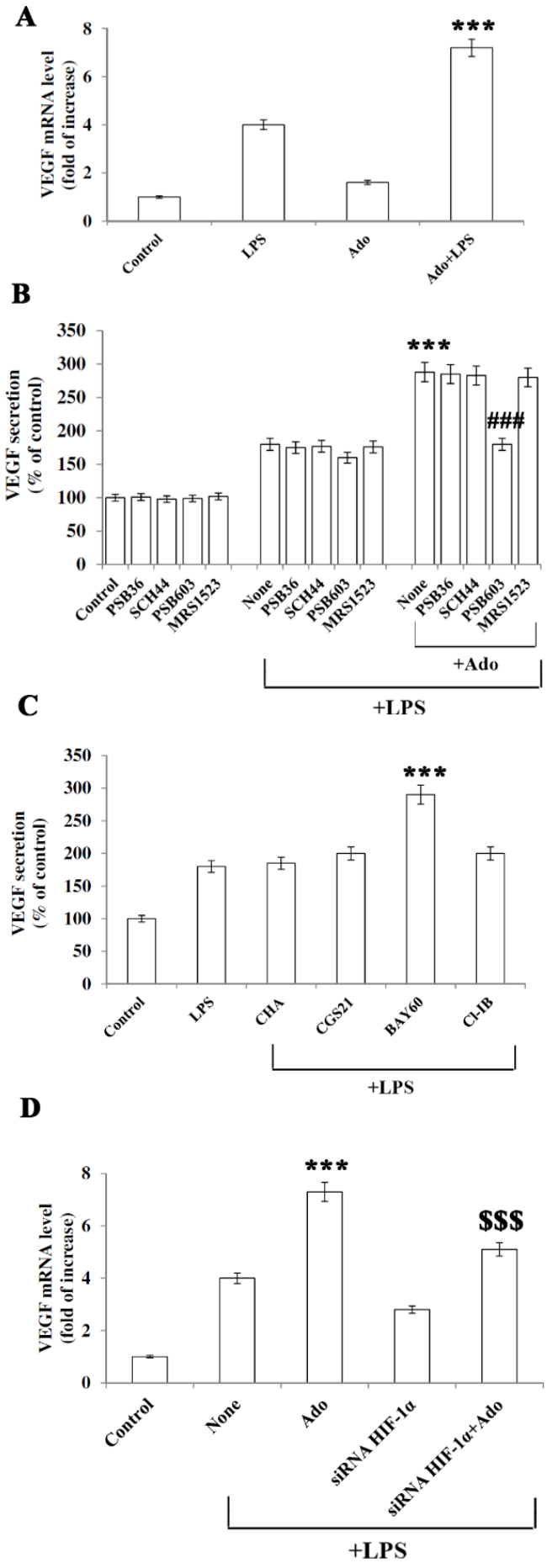

A
VEGF mRNA level (fold of increase)
***
Control
LPS
Ado
Ado+LPS
B
VEGF secretion (% of control)
***
###
Control
PSB36
SCH44
PSB603
MRS1523
None
PSB36
SCH44
PSB603
MRS1523
None
PSB36
SCH44
PSB603
MRS1523
+Ado
+LPS
C
VEGF secretion (% of control)
***
Control
LPS
CHA
CGS21
BAY60
Cl-IB
+LPS
D
VEGF mRNA level (fold of increase)
***
$$$
Control
None
Ado
siRNA HIF-1α
siRNA HIF-1α+Ado
+LPS

FIG. 10: Downstream HIF-1α target genes: VEGF. Microglial cells were incubated with Ado (Ado; 100 μM) before stimulation with LPS (1 μg/ml) for 16 h and mRNA levels of VEGF were evaluated by real-time RT-PCR (A). Data were expressed as fold of increase vs control, arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments. Cells were pretreated for 30 min with PSB36 (10 nM), SCH 442416 (200 nM), PSB603 (100 nM), and MRS 1523 (1 μM) before treatment with Ado in the presence of LPS (1 μg/ml) for 24 h (B). Microglial cells were incubated with CHA (30 nM), CGS 21680 (100 nM), BAY 60-6583 (100 nM), and Cl-IB-MECA (100 nM) in the presence of LPS stimulation for 24 h (C) and levels of secreted VEGF were evaluated by ELISA kit. Bar graph is the mean ± SEM values (N = 4). Cells in the absence of LPS (Control) were set to 100%. Microglial cells were incubated with Ado (Ado) (100 μM) in the presence of HIF-1a siRNA before stimulation with LPS (1 μg/ml) and mRNA levels of VEGF were evaluated by real-time RT-PCR (D). Data were expressed as fold of increase vs control, arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments. $^{***}P < 0.001$ compared with cells in the presence of LPS; $^{###}P < 0.001$ compared with cells in the presence of LPS + Ado; $^{\$\$\$}P < 0.01$ compared with cells in the presence of LPS + HIF-1α siRNA; analysis was by two-way ANOVA followed by Bonferroni's test. (A) Interaction $F_{(1,12)} = 37.87$, $P < 0.0001$; Ado $F_{(1,12)} = 80.90$, $P < 0.0001$; LPS $F_{(1,12)} = 414.3$, $P < 0.0001$. (B) Interaction $F_{(9,60)} = 21.53$, $P < 0.0001$; Ado ligands $F_{(9,60)} = 21.88$, $P < 0.0001$; LPS $F_{(1,60)} = 1045.01$, $P < 0.0001$. (C) Interaction $F_{(4,30)} = 13.01$, $P < 0.0001$; Ado ligands $F_{(4,30)} = 13.80$, $P < 0.0001$; LPS $F_{(1,30)} = 406.4$, $P < 0.0001$. (D) Interaction $F_{(3,24)} = 32.93$, $P < 0.0001$; Ado $F_{(3,24)} = 46.25$, $P < 0.0001$; LPS $F_{(1,24)} = 569.53$, $P < 0.0001$.

FIG. 11

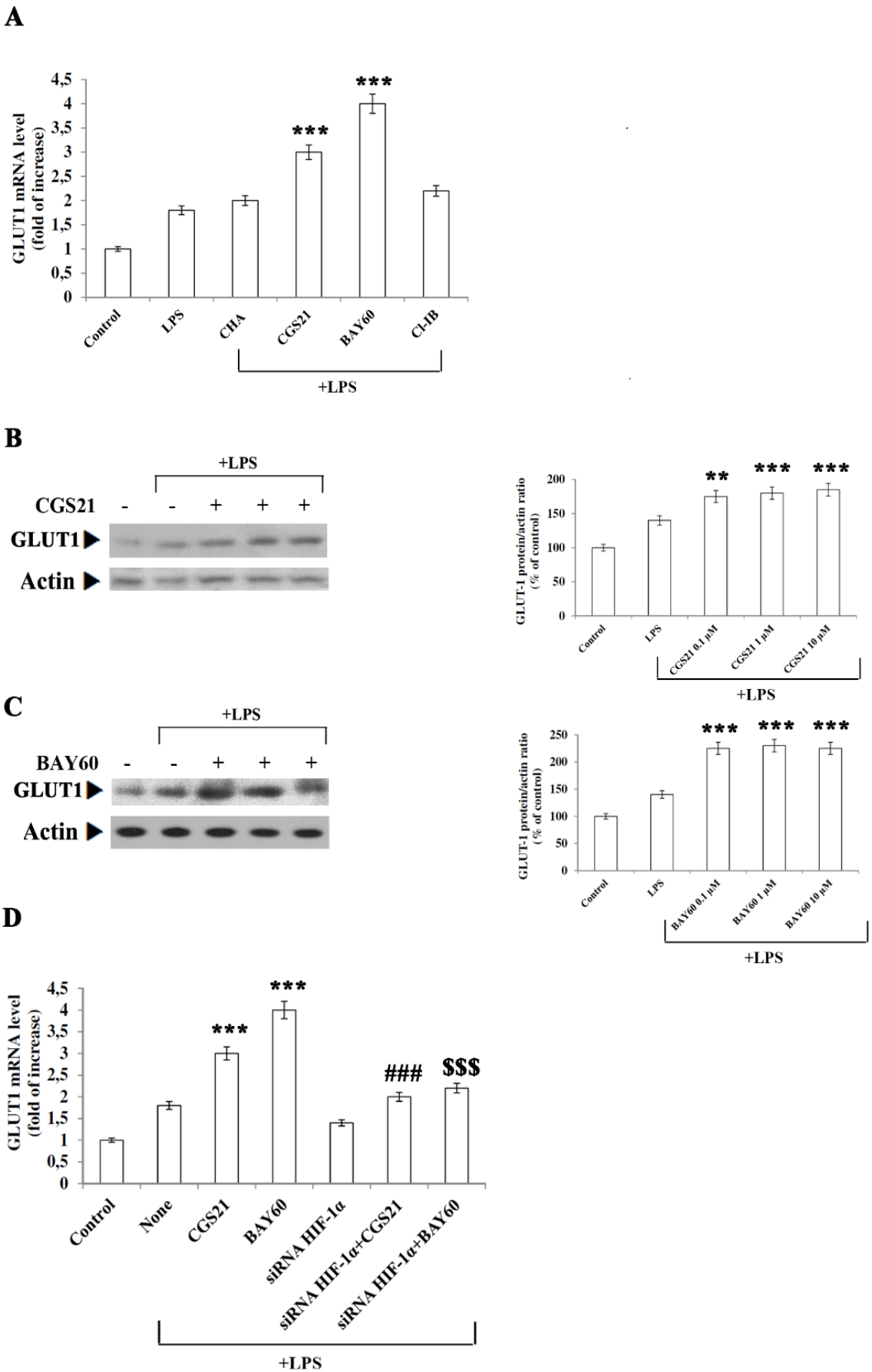
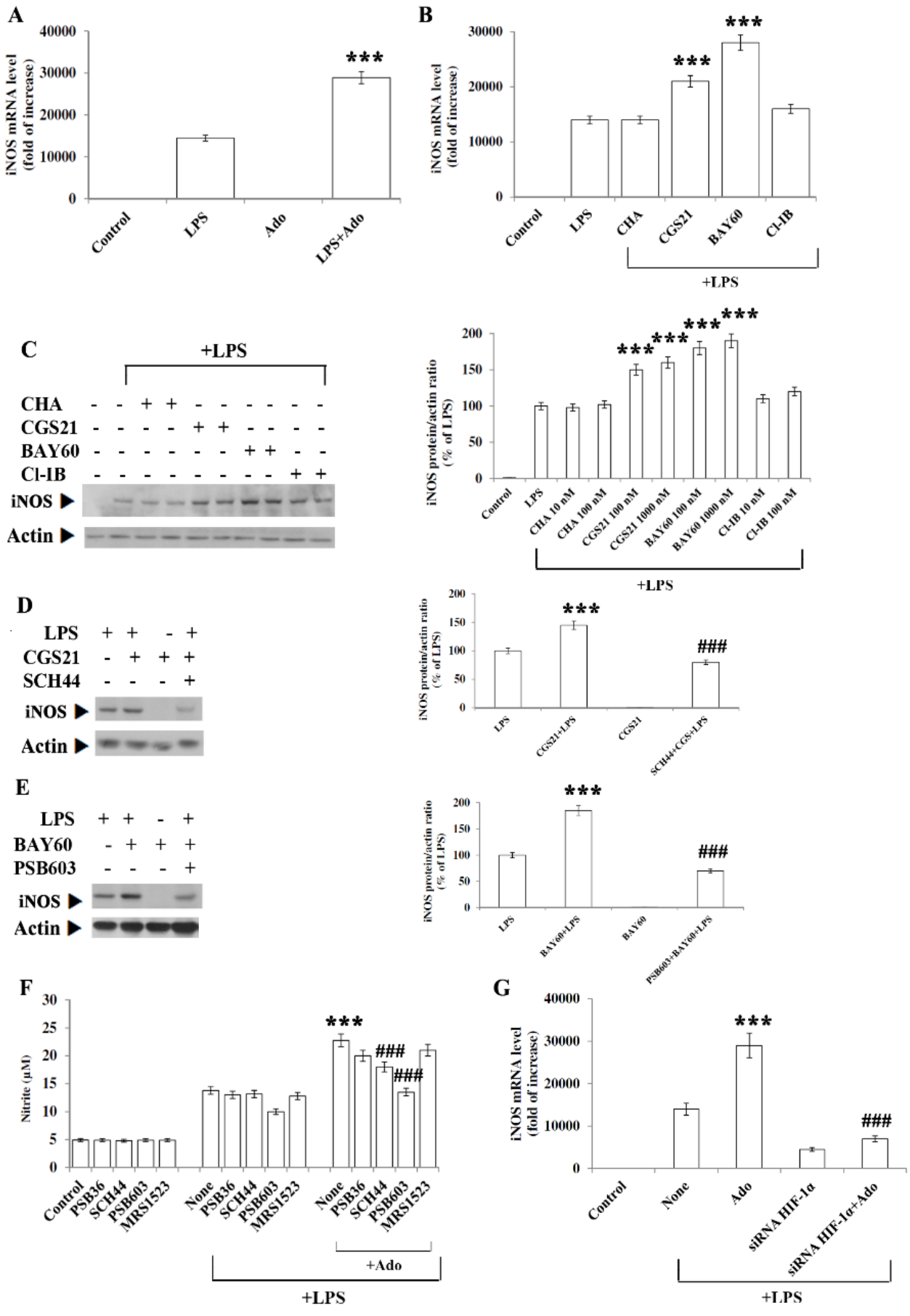

A
GLUT1 mRNA level (fold of increase)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
***
***
Control
LPS
CHA
CGS21
BAY60
Cl-IB
+LPS
B
+LPS
CGS21 - - + + +
GLUT1
Actin
GLUT-1 protein/actin ratio (% of control)
200
150
100
50
0
**
***
***
Control
LPS
CGS21 0.1 µM
CGS21 1 µM
CGS21 10 µM
+LPS
C
+LPS
BAY60 - - + + +
GLUT1
Actin
GLUT-1 protein/actin ratio (% of control)
250
200
150
100
50
0
***
***
***
Control
LPS
BAY60 0.1 µM
BAY60 1 µM
BAY60 10 µM
+LPS
D
GLUT1 mRNA level (fold of increase)
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
***
***
###
$$$
Control
None
CGS21
BAY60
siRNA HIF-1α
siRNA HIF-1α+CGS21
siRNA HIF-1α+BAY60
+LPS

FIG. 11: Downstream HIF-1α target genes: GLUT-1. Microglial cells were incubated with CHA (30 nM), CGS 21680 (100 nM), BAY 60-6583 (100 nM), and Cl-IB-MECA (100 nM) in the presence of LPS stimulation for 8 h and mRNA levels of GLUT-1 were evaluated by real time RT-PCR (A). Data were expressed as fold of increase vs control, arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). Microglial cells were pretreated for 30 min with increasing concentrations (0.1, 1, and 10 μM) of CGS 21680 (B) or BAY 60-6583 (C) in presence of LPS for 24 h. The protein levels of GLUT-1 were determined by Western blotting in whole cell lysates and actin served as control for equal protein loading. Densitometric quantification is the mean ± SEM of four independent experiments (N = 4); cells in the absence of LPS (control) were set to 100%. Microglial cells were incubated with CGS 21680 (100 nM) and BAY 60-6583 (100 nM) in the presence of HIF-1α siRNA before stimulation with LPS for 8 h and mRNA levels of GLUT-1 were evaluated by real-time RT-PCR (D). Data were expressed as fold of increase vs control, arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with cells in the presence of LPS; $^{###,\$\$\$}P < 0.001$ compared with cells in the presence of LPS + CGS 21680 and LPS + BAY 60-6583, respectively; analysis was by two-way ANOVA followed by Bonferroni's test. (A) Interaction $F_{(4,30)} = 39.08$, $P < 0.0001$; Ado $F_{(4,30)} = 41.55$, $P < 0.0001$; LPS $F_{(1,30)}$ 5577.78, $P < 0.0001$. (B) Interaction $F_{(3,24)} = 4.67$, $P < 0.0104$; Ado ligands $F_{(3,24)} = 4.90$, $P < 0.0085$; LPS $F_{(1,24)} = 222.09$, $P < 0.0001$. (C) Interaction $F_{(3,24)} = 12.93$, $P < 0.0001$; Ado ligands $F_{(3,24)} = 12.79$, $P < 0.0001$; LPS $F_{(1,24)} = 348.3$, $P < 0.0001$. (D) Interaction $F_{(5,36)} = 25.36$, $P < 0.0001$; Ado ligands $F_{(5,36)} = 24.05$, $P < 0.0001$; LPS $F_{(1,36)} = 329.91$, $P < 0.0001$.

FIG. 12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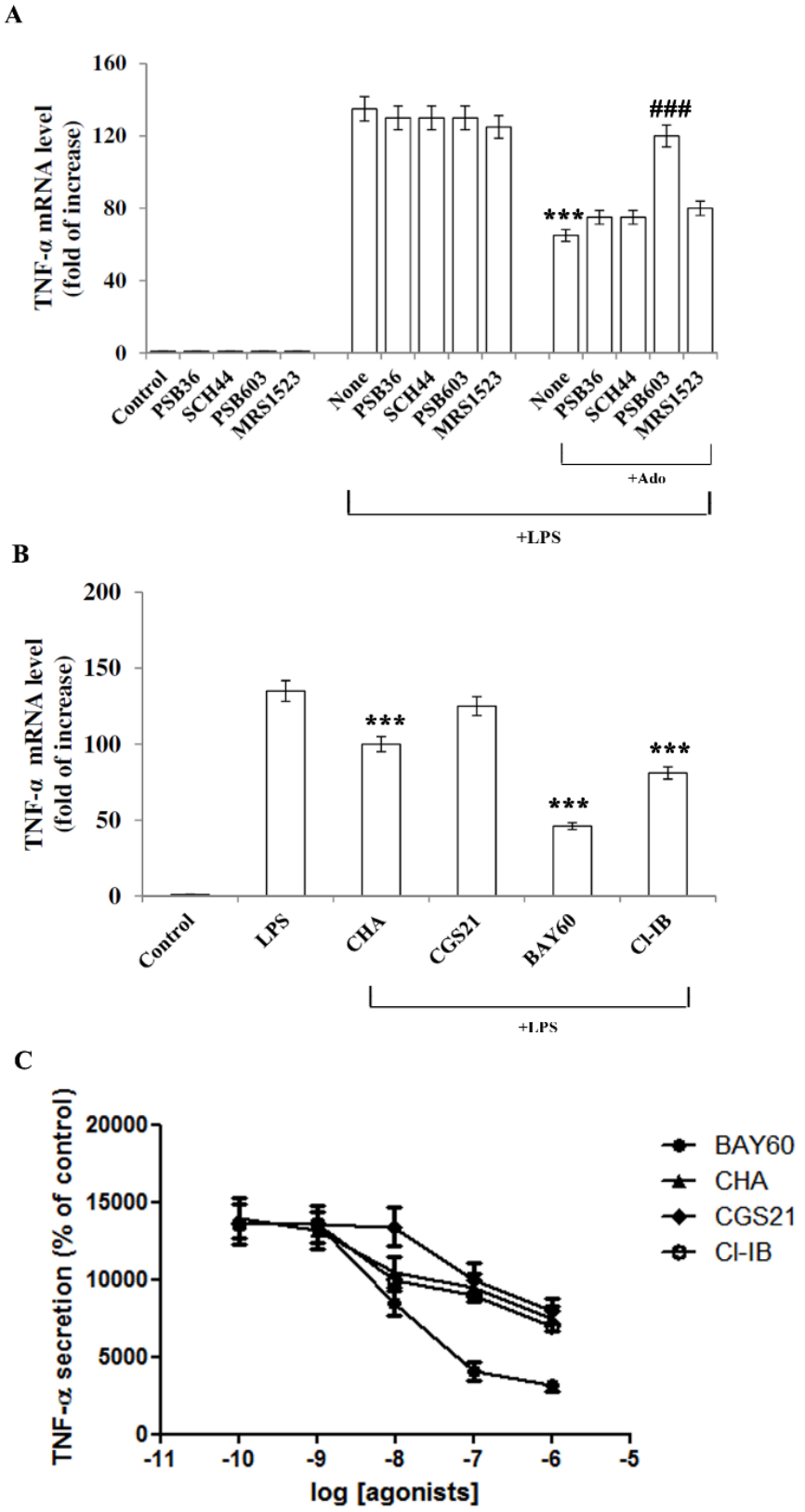
40000
30000
20000
10000
0
***
Control
LPS
Ado
LPS+Ado
B
iNOS mRNA level (fold of increase)
30000
20000
10000
0
***
***
Control
LPS
CHA
CGS21
BAY60
Cl-IB
+LPS
C
+LPS
CHA - - + + - - - - - -
CGS21 - - - - + + - - - -
BAY60 - - - - - - + + - -
Cl-IB - - - - - - - - + +
iNOS
Actin
iNOS protein/actin ratio (% of LPS)
200
150
100
50
0
***
***
***
***
***
Control
LPS
CHA 10 nM
CHA 100 nM
CGS21 100 nM
CGS21 1000 nM
BAY60 100 nM
BAY60 1000 nM
Cl-IB 10 nM
Cl-IB 100 nM
+LPS
D
LPS + + - +
CGS21 - + + +
SCH44 - - - +
iNOS
Actin
iNOS protein/actin ratio (% of LPS)
200
150
100
50
0
***
###
LPS
CGS21+LPS
CGS21
SCH44+CGS+LPS
E
LPS + + - +
BAY60 - + + +
PSB603 - - - +
iNOS
Actin
iNOS protein/actin ratio (% of LPS)
200
150
100
50
0
***
###
LPS
BAY60+LPS
BAY60
PSB603+BAY60+LPS
F
Nitrite (μM)
30
25
20
15
10
5
0
***
###
###
Control
PSB36
SCH44
PSB603
MRS1523
None
PSB36
SCH44
PSB603
MRS1523
None
PSB36
SCH44
PSB603
MRS1523
+Ado
+LPS
G
iNOS mRNA level (fold of increase)
40000
30000
20000
10000
0
***
###
Control
None
Ado
siRNA HIF-1α
siRNA HIF-1α+Ado
+LPS

FIG. 12: Downstream HIF-1α target genes: iNOS. Microglial cells were incubated with Ado (Ado; (100 μM; A) or with CHA (30 nM), CGS 21680 (100 nM), BAY 60-6583 (100 nM), and Cl-IB-MECA (100 nM; B) before stimulation with LPS (1 μg/ml) for 8 h and mRNA levels of iNOS were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs control (cells in the absence of LPS), arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). Microglial cells were incubated with CHA (10-100 nM), CGS 21680 (100- 1000 nM), BAY 60-6583 (100-1000 nM), and Cl-IB-MECA (10-100 nM) in the presence of LPS stimulation for 24 h (C) and the protein levels of iNOS were evaluated by immunoblotting in whole cell lysates and actin served as control for equal protein loading. Cells were pretreated for 30 min with SCH 442416 (200 nM) or PSB 603 (100 nM) before CGS 21680 (D) or BAY 60-6583 (E) in the presence of LPS for 24 h. The protein levels of iNOS were determined by Western blotting in whole cell lysates and actin served as control for equal protein loading. Densitometric quantification of iNOS Western blots is the mean ± SEM of four independent experiments (N = 4); cells in the presence of LPS were set to 100%. Cells were pretreated for 30 min with PSB 36 (10 nM), SCH 442416 (200 nM), PSB 603 (100 nM), and MRS 1523 (1 μM) before treatment with Ado in the presence of LPS (1 μg/ml) for 24 h (F) and levels of secreted nitrite were evaluated by Greiss reaction. Bargraph of colorimetric nitrite assay is the mean ± SEM of four independent experiments (N = 4). Microglial cells were incubated with Ado (Ado; 100 μM) in the presence of HIF-1α siRNA before stimulation with LPS (1 μg/ml) and mRNA levels of iNOS were evaluated by real-time RT-PCR (G). Data were expressed as fold of increase vs control, arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments. $^{***}P< 0.001$ compared with cells in the presence of LPS; $^{###}P< 0.001$ compared with cells in the presence of LPS + CGS 21680 (D), LPS + BAY 60-6583 (E), and LPS + Ado (F and G); analysis was by two-way ANOVA followed by Bonferroni's test. (A) Interaction $F_{(1,12)} = 80.02$, $P< 0.0001$; Ado $F_{(1,12)} = 80.02$, $P< 0.0001$; LPS $F_{(1,12)} = 720.13$, $P< 0.0001$. (B) Interaction $F_{(4,30)} = 39.49$, $P< 0.0001$; Ado ligands $F_{(4,30)} = 39.49$, $P< 0.0001$; LPS $F_{(1,30)} = 1908.01$, $P< 0.0001$. (C) Interaction $F_{(8,54)} = 28.69$, $P< 0.0001$; Ado ligands $F_{(8,54)} = 28.69$, $P< 0.0001$; LPS $F_{(1,54)} = 3040.74$, $P< 0.0001$. (D) Interaction $F_{(3,24)} = 17.16$, $P< 0.0001$; Ado ligands $F_{(3,24)} = 16.99$, $P< 0.0001$; LPS $F_{(1,24)} = 1125.25$, $P< 0.0001$. (E) Interaction $F_{(3,24)} = 64.73$, $P< 0.0001$; Ado ligands $F_{(3,24)} = 64.40$, $P< 0.0001$; LPS $F_{(1,24)} = 1383.58$, $P< 0.0001$. (F) Interaction $F_{(9,60)} = 18.62$, $P< 0.0001$; Ado ligands $F_{(9,60)} = 18.72$, $P< 0.0001$; LPS $F_{(1,60)} = 1194.01$, $P< 0.0001$. (G) Interaction $F_{(3,24)} = 51.13$, $P< 0.0001$; Ado $F_{(3,24)} = 51.13$, $P< 0.0001$; LPS $F_{(1,24)} = 313.15$, $P< 0.0001$.

FIG. 13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160
120
80
40
0
###
***
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SCH44
PSB603
MRS1523
None
PSB36
SCH44
PSB603
MRS1523
None
PSB36
SCH44
PSB603
MRS1523
+Ado
+LPS
B
TNF-α mRNA level (fold of increase)
200
150
100
50
0
***
***
***
Control
LPS
CHA
CGS21
BAY60
Cl-IB
+LPS
C
TNF-α secretion (% of control)
20000
15000
10000
5000
0
-11
-10
-9
-8
-7
-6
-5
log [agonists]
BAY60
CHA
CGS21
Cl-IB

FIG. 13: Downstream HIF-1α target genes: TNF-α. Cells were pretreated for 30 min with PSB 36 (10 nM), SCH 442416 (200 nM), PSB 603 (100 nM), and MRS 1523 (1 μM) before treatment with Ado (Ado) in the presence of LPS (1 μg/ml) for 4 h (A). Microglial cells were incubated with CHA (30 nM), CGS 21680 (100 nM), BAY 60-6583 (100 nM), and Cl-IB-MECA (100 nM; B) before stimulation with LPS (1μg/ml) for 4 h and mRNA levels of TNF-α were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs control (cells in the absence of LPS), arbitrarily fixed as 1. Each point is the mean ± SEM of four independent experiments (N = 4). Microglial cells were pretreated for 30 min with increasing concentrations (0.1-1000 nM) of CHA, CGS 21680, BAY 60-6583, and Cl- IB-MECA (C) in presence of LPS for 24 h and levels of secreted TNF-α were evaluated by ELISA kit. Bargraph is the mean ± SEM of four independent experiments (N = 4). $^{***}P< 0.001$ compared with cells in the presence of LPS; $^{###}P< 0.001$ compared with cells in the presence of LPS + Ado; analysis was by two-way ANOVA followed by Bonferroni's test. (A) Interaction $F_{(9,60)} = 37.25$, $P< 0.0001$; Ado ligands $F_{(9,60)} = 37.25$, $P< 0.0001$; LPS $F_{(1,12)} = 720.13$, $P< 0.0001$. (B) Interaction $F_{(4,30)} = 54.55$, $P< 0.0001$; Ado ligands $F_{(4,30)} = 54.27$, $P< 0.0001$; LPS $F_{(1,30)} = 1984.34$, $P< 0.0001$.

FIG. 14

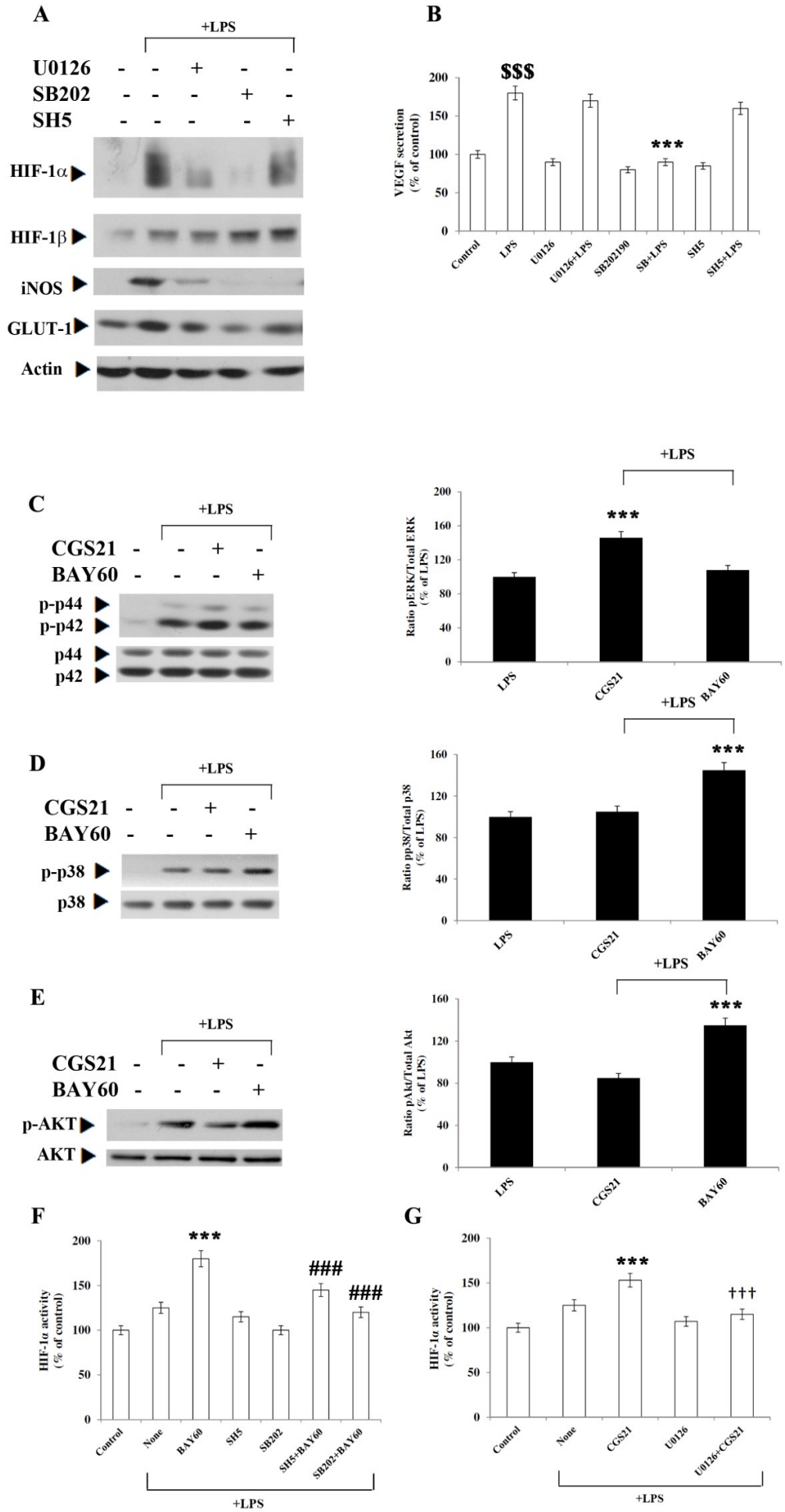

A
+LPS
U0126 - - + - -
SB202 - - - + -
SH5 - - - - +
HIF-1α
HIF-1β
iNOS
GLUT-1
Actin
B
$$$
***
VEGF secretion (% of control)
Control
LPS
U0126
U0126+LPS
SB202190
SB+LPS
SH5
SH5+LPS
C
+LPS
CGS21 - - + -
BAY60 - - - +
p-p44
p-p42
p44
p42
Ratio pERK/Total ERK (% of LPS)
***
LPS
CGS21
BAY60
D
+LPS
CGS21 - - + -
BAY60 - - - +
p-p38
p38
Ratio pp38/Total p38 (% of LPS)
***
LPS
CGS21
BAY60
E
+LPS
CGS21 - - + -
BAY60 - - - +
p-AKT
AKT
Ratio pAkt/Total Akt (% of LPS)
***
LPS
CGS21
BAY60
F
HIF-1α activity (% of control)
***
###
###
Control
None
BAY60
SH5
SB202
SH5+BAY60
SB202+BAY60
+LPS
G
HIF-1α activity (% of control)
***
†††
Control
None
CGS21
U0126
U0126+CGS21
+LPS

FIG. 14: Signalling pathways involved in LPS-induced HIF-1α and downstream genes increase and effect of A_{2A} and A_{2B} Ado agonists. Cells were preincubated with the ERK1/2 (or p44/42) inhibitor 10 μM U0126, p38 inhibitor 10 μM SB202190, and the Akt blocker 10 μM SH5, before addition of LPS for 4 h for HIF-1α and 24 h for iNOS, GLUT-1 (A) and VEGF (B). HIF-1α, HIF-1β, iNOS, GLUT-1, and actin were detected by immunoblotting in whole cell lysates, VEGF levels were evaluated by ELISA kit. Microglial cells were preincubated with CGS 21680 (100 nM) and BAY 60-6583 (100 nM) before stimulation with LPS (1 μg/ml) for 30 min. Phosphorylated as well as total pERK1/2 MAPK (C), p38 (D), and pAkt (E) were detected by immunoblot. Cells were preincubated with 10 μM SH5 and SB202190, before treatment with BAY 60-6583 (100 nM; F) and with 10 μM U0126 before treatment with CGS 21680 (100 nM; G) in the presence of LPS for 4 h. HIF-1α-DNA binding activity is the mean ± SEM of four independent experiments (N = 4); cells in the absence of LPS were set to 100%. Densitometric quantification of Western blots is the mean ± SEM of four independent experiments (N = 4); cells in the presence of LPS were set to 100% in C-E. $^{\$\$\$}P< 0.001 compared with cells in the absence of LPS, $^{***}P< 0.001$, compared with cells in the presence of LPS, $^{\#\#\#,\dagger\dagger\dagger}P< 0.001$ compared with cells in the presence of LPS + BAY 60-6583 (F), LPS + CGS 21680 (G), respectively; analysis was by two-way ANOVA followed by Bonferroni's test. (C) Interaction $F_{(2,18)} = 16.58$, P< 0.0001; Ado ligands $F_{(2,18)} = 16.37$, P< 0.0001; LPS $F_{(1,18)} = 1119.92$, P< 0.0001. (D) Interaction $F_{(2,18)} = 16.73$, P< 0.0001; Ado ligands $F_{(2,18)} = 16.45$, P< 0.0001; LPS $F_{(1,18)} = 1093.86$, P< 0.0001. (E) Interaction $F_{(2,18)} = 21.99$, P< 0.0001; Ado ligands $F_{(2,18)} = 21.89$, P< 0.0001; LPS $F_{(1,18)} = 1114.25$, P< 0.0001. (F) Interaction $F_{(3,24)} = 64.73$, P< 0.0001; Ado ligands $F_{(3,24)} = 64.40$, P< 0.0001; LPS $F_{(1,24)} = 1383.58$, P< 0.0001. (G) Interaction $F_{(9,60)} = 18.62$, P< 0.0001; Ado ligands $F_{(9,60)} = 18.72$, P< 0.0001; LPS $F_{(1,60)} = 1194.01$, P< 0.0001. (H) Interaction $F_{(5,36)} = 12.76$, P< 0.0001; Ado ligands $F_{(5,36)} = 8.30$, P< 0.0001; LPS $F_{(1,30)} = 66.26$, P< 0.0001. (I) Interaction $F_{(3,24)} = 7.54$, P< 0.0001; Ado ligands $F_{(3,24)} = 4.24$, P< 0.0001; LPS $F_{(1,24)} = 38.49$, P< 0.0001.

FIG. 15

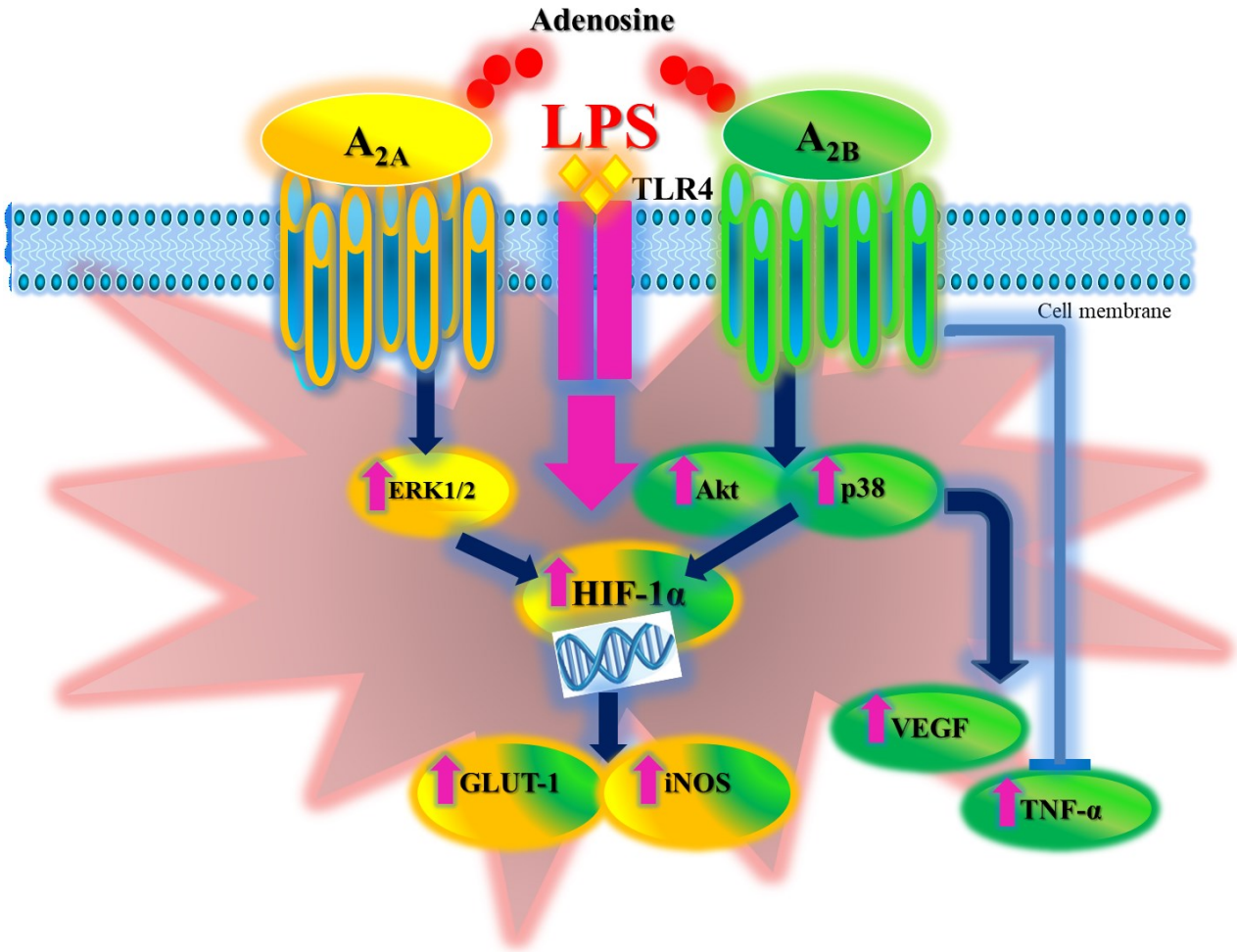


FIG. 15: Schematic summary of Ado-triggered signal transduction cascade in murine microglia. Ado through A_{2A}-ERK1/2 and A_{2B}-Akt-p38 receptor pathways increases the effect of LPS on GLUT-1 and iNOS gene expression in a HIF-1α-dependent way, while through A_{2B} receptor subtypes rises VEGF and decreases TNF-α in a HIF-1α-independent way.

References

[1] S. Merighi *et al.*, "A_3 adenosine receptors modulate hypoxia-inducible factor-1alpha expression in human A375 melanoma cells.," *Neoplasia*, vol. 7, no. 10, pp. 894–903, Oct. 2005.

[2] S. Merighi *et al.*, "Adenosine modulates vascular endothelial growth factor expression via hypoxia-inducible factor-1 in human glioblastoma cells.," *Biochem. Pharmacol.*, vol. 72, no. 1, pp. 19–31, Jun. 2006.

[3] S. Merighi *et al.*, "Caffeine inhibits adenosine-induced accumulation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and interleukin-8 expression in hypoxic human colon cancer cells.," *Mol. Pharmacol.*, vol. 72, no. 2, pp. 395–406, Aug. 2007.

[4] C. De Ponti *et al.*, "Adenosine A_{2A} receptor-mediated, normoxic induction of HIF-1 through PKC and PI-3K-dependent pathways in macrophages.," *J. Leukoc. Biol.*, vol. 82, no. 2, pp. 392–402, Aug. 2007.

[5] M. Ramanathan, G. Pinhal-Enfield, I. Hao, and S. J. Leibovich, "Synergistic up-regulation of vascular endothelial growth factor (VEGF) expression in macrophages by adenosine A_{2A} receptor agonists and endotoxin involves transcriptional regulation via the hypoxia response element in the VEGF promoter.," *Mol. Biol. Cell*, vol. 18, no. 1, pp. 14–23, Jan. 2007.

[6] S. Gessi *et al.*, "Adenosine modulates HIF-1{alpha}, VEGF, IL-8, and foam cell formation in a human model of hypoxic foam cells.," *Arterioscler. Thromb. Vasc. Biol.*, vol. 30, no. 1, pp. 90–97, Jan. 2010.

[7] S. Gessi, S. Merighi, A. Stefanelli, D. Fazzi, K. Varani, and P. A. Borea, "A_1 and A_3 adenosine receptors inhibit LPS-induced hypoxia-inducible factor-1 accumulation in murine astrocytes," *Pharmacol. Res.*, vol. 76, pp. 157–170, 2013.

[8] J. Ock *et al.*, "Regulation of Toll-like receptor 4 expression and its signaling by hypoxia in cultured microglia.," *J. Neurosci. Res.*, vol. 85, no. 9, pp. 1989–1995, Jul. 2007.

[9] J. C. McGrath, G. B. Drummond, E. M. McLachlan, C. Kilkenny, and C. L. Wainwright, "Guidelines for reporting experiments involving animals: the ARRIVE guidelines.," *Br. J. Pharmacol.*, vol. 160, no. 7, pp. 1573–1576, Aug. 2010.

[10] J. Saura, J. M. Tusell, and J. Serratosa, "High-yield isolation of murine microglia by mild

trypsinization.," *Glia*, vol. 44, no. 3, pp. 183–189, Dec. 2003.

[11] S. Gessi *et al.*, "Adenosine receptors in colon carcinoma tissues and colon tumoral cell lines: focus on the A_3 adenosine subtype.," *J. Cell. Physiol.*, vol. 211, no. 3, pp. 826–836, Jun. 2007.

[12] M. Mujoomdar, D. Hoskin, and J. Blay, "Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism.," *Biochem. Pharmacol.*, vol. 66, no. 9, pp. 1737–1747, Nov. 2003.

[13] S. Merighi, S. Gessi, K. Varani, D. Fazzi, P. Mirandola, and P. A. Borea, "Cannabinoid CB_2 receptor attenuates morphine-induced inflammatory responses in activated microglial cells," *Br. J. Pharmacol.*, vol. 166, no. 8, pp. 2371–2385, Aug. 2012.

[14] Y. T. Oh *et al.*, "Lipopolysaccharide induces hypoxia-inducible factor-1 alpha mRNA expression and activation via NADPH oxidase and Sp1-dependent pathway in BV2 murine microglial cells.," *Neurosci. Lett.*, vol. 431, no. 2, pp. 155–160, Jan. 2008.

[15] H. Kettenmann, F. Kirchhoff, and A. Verkhratsky, "Microglia: new roles for the synaptic stripper.," *Neuron*, vol. 77, no. 1, pp. 10–18, Jan. 2013.

[16] H. Kettenmann, U.-K. Hanisch, M. Noda, and A. Verkhratsky, "Physiology of Microglia," *Physiol. Rev.*, vol. 91, no. 2, pp. 461–553, Apr. 2011.

[17] C. Gomes *et al.*, "Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia," *J. Neuroinflammation*, vol. 10, no. 1, p. 780, Dec. 2013.

[18] I. Ernens, F. Léonard, M. Vausort, M. Rolland-Turner, Y. Devaux, and D. R. Wagner, "Adenosine up-regulates vascular endothelial growth factor in human macrophages," *Biochem. Biophys. Res. Commun.*, vol. 392, no. 3, pp. 351–356, Feb. 2010.

[19] A. Ruiz-García *et al.*, "Cooperation of adenosine with macrophage Toll-4 receptor agonists leads to increased glycolytic flux through the enhanced expression of PFKFB3 gene.," *J. Biol. Chem.*, vol. 286, no. 22, pp. 19247–19258, Jun. 2011.

[20] B. Koscsó *et al.*, "Adenosine augments IL-10 production by microglial cells through an A_{2B} adenosine receptor-mediated process.," *J. Immunol.*, vol. 188, no. 1, pp. 445–453, Jan. 2012.

[21] L. Luongo *et al.*, "The A_1 adenosine receptor as a new player in microglia physiology.," *Glia*, vol. 62, no. 1, pp. 122–132, Jan. 2014.

[22] A. G. Orr, A. L. Orr, X.-J. Li, R. E. Gross, and S. F. Traynelis, "Adenosine A_{2A} receptor mediates microglial process retraction.," *Nat. Neurosci.*, vol. 12, no. 7, pp. 872–878, Jul. 2009.

[23] C. van der Putten *et al.*, "Differential expression of adenosine A_3 receptors controls adenosine A_{2A} receptor-mediated inhibition of TLR responses in microglia.," *J. Immunol.*, vol. 182, no. 12, pp. 7603–7612, Jun. 2009.

[24] M. L. Haselkorn *et al.*, "Adenosine A_1 receptor activation as a brake on the microglial response after experimental traumatic brain injury in mice.," *J. Neurotrauma*, vol. 27, no. 5, pp. 901–910, May 2010.

[25] S. Frede, C. Stockmann, P. Freitag, and J. Fandrey, "Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB.," *Biochem. J.*, vol. 396, no. 3, pp. 517–527, Jun. 2006.

[26] A. Mohyeldin, T. Garzón-Muvdi, and A. Quiñones-Hinojosa, "Oxygen in Stem Cell Biology: A Critical Component of the Stem Cell Niche," *Cell Stem Cell*, vol. 7, no. 2, pp. 150–161, Aug. 2010.

[27] C. Lange *et al.*, "Relief of hypoxia by angiogenesis promotes neural stem cell differentiation by targeting glycolysis," *EMBO J.*, vol. 35, no. 9, pp. 924–941, May 2016.

[28] S. Daniele, E. Zappelli, L. Natali, C. Martini, and M. L. Trincavelli, "Modulation of A_1 and A_{2B} adenosine receptor activity: a new strategy to sensitise glioblastoma stem cells to chemotherapy.," *Cell Death Dis.*, vol. 5, no. 11, p. e1539, Nov. 2014.

[29] T. H. Adair, "Growth regulation of the vascular system: an emerging role for adenosine.," *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, vol. 289, no. 2, pp. R283–R296, Aug. 2005.

[30] I. Feoktistov, I. Biaggioni, and B. N. Cronstein, "Adenosine receptors in wound healing, fibrosis and angiogenesis.," *Handb. Exp. Pharmacol.*, no. 193, pp. 383–397, 2009.

[31] I. Feoktistov *et al.*, "Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A_{2B} angiogenic phenotype.," *Hypertens. (Dallas, Tex. 1979)*, vol. 44, no. 5, pp. 649–654, Nov. 2004.

[32] S. Ryzhov *et al.*, "Role of JunB in adenosine A_{2B} receptor-mediated vascular endothelial

growth factor production.," *Mol. Pharmacol.*, vol. 85, no. 1, pp. 62–73, Jan. 2014.

[33] G. Haskó, C. Szabó, Z. H. Németh, V. Kvetan, S. M. Pastores, and E. S. Vizi, "Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice.," *J. Immunol.*, vol. 157, no. 10, pp. 4634–4640, Nov. 1996.

[34] H. W. Min, S. Moochhala, and K. H. Eng, "Adenosine and its receptor agonists regulate nitric oxide production and RAW 264.7 macrophages via both receptor binding and its downstream metabolites-inosine.," *Life Sci.*, vol. 66, no. 19, pp. 1781–1793, Mar. 2000.

[35] J. Saura *et al.*, "Adenosine A_{2A} receptor stimulation potentiates nitric oxide release by activated microglia.," *J. Neurochem.*, vol. 95, no. 4, pp. 919–929, Nov. 2005.

[36] S.-S. Dai *et al.*, "Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury.," *J. Neurosci.*, vol. 30, no. 16, pp. 5802–5810, Apr. 2010.

[37] A. Kuschel, P. Simon, and S. Tug, "Functional regulation of HIF-1α under normoxia--is there more than post-translational regulation?," *J. Cell. Physiol.*, vol. 227, no. 2, pp. 514–524, Feb. 2012.

[38] I. Feoktistov and I. Biaggioni, "Role of adenosine A_{2B} receptors in inflammation.," *Adv. Pharmacol.*, vol. 61, pp. 115–144, 2011.

[39] D. Yang *et al.*, "The A_{2B} adenosine receptor protects against inflammation and excessive vascular adhesion.," *J. Clin. Invest.*, vol. 116, no. 7, pp. 1913–1923, Jul. 2006.

[40] H. Johnston-Cox, A. S. Eisenstein, M. Koupenova, S. Carroll, and K. Ravid, "The macrophage A_{2B} adenosine receptor regulates tissue insulin sensitivity.," *PLoS One*, vol. 9, no. 6, p. e98775, Jun. 2014.

[41] H. Chen, D. Yang, S. H. Carroll, H. K. Eltzschig, and K. Ravid, "Activation of the macrophage A_{2B} adenosine receptor regulates tumor necrosis factor-alpha levels following vascular injury.," *Exp. Hematol.*, vol. 37, no. 5, pp. 533–538, May 2009.

[42] L. M. Kreckler, T. C. Wan, Z.-D. Ge, and J. A. Auchampach, "Adenosine inhibits tumor necrosis factor-alpha release from mouse peritoneal macrophages via A_{2A} and A_{2B} but not the A_3 adenosine receptor.," *J. Pharmacol. Exp. Ther.*, vol. 317, no. 1, pp. 172–180, Apr. 2006.

[43] J. Y. Lee *et al.*, "Activation of adenosine A_3 receptor suppresses lipopolysaccharide-induced TNF-α production through inhibition of PI 3-kinase/Akt and NF-κB activation in murine BV2 microglial cells," *Neurosci. Lett.*, vol. 396, no. 1, pp. 1–6, Mar. 2006.

CHAPTER 2: A_{2B} ADENOSINE RECEPTORS STIMULATE IL-6 PRODUCTION IN PRIMARY MURINE MICROGLIA THROUGH p38 MAPK KINASE PATHWAY

Aim of the study

IL-6 has been defined as a major multifunctional cytokine in the CNS exerting both pro- and anti-inflammatory functions by regulating differentiation, proliferation, migration and apoptosis of target cells [1]. Ado has been found to modulate IL-6 through activation of A_{2B} ARs in different cellular types e.g. astrocytes, pituitary folliculostellate cells, bronchial smooth muscle cells, lung fibroblasts, astrocytoma cells, mouse striatum, peritoneal macrophages and cardiac fibroblasts through various signal transduction pathways, including PKC and MAPKs [2]–[10]. However, no evidences have been reported about the potential regulation of IL-6 in microglial cells by ARs stimulation and the consequent mechanism involved.

Therefore, in this study we examined whether ARs activation could modulate IL-6 secretion and cell proliferation in primary microglial cells. In addition, as inflammation and hypoxic damage may coexist in stroke, ischemia and other CNS disorders the same effects have been investigated also in the presence of 1% oxygen.

Materials and Methods

Materials

A_{2B} ARs siRNAs (sc-29643), and anti-A_{2B} ARs rabbit polyclonal antibodies were from Santa Cruz DBA (Milan, Italy). Rabbit polyclonal anti-HIF-1α was from Cayman, Vinci Biochem (Florence, Italy). Anti-beta Actin was from Millipore (Milan, Italy). RNAiFect Transfection Kit was purchased from Qiagen (Milan, Italy). U0126 was from Promega (Milan, Italy). Anti-beta Actin was from Millipore (Milan, Italy). GFAP and anti-CD11b antibodies were from Becton Dickinson (Milan, Italy). The Assays-on-demand™ Gene expression Products for IL-6 (Mm00446190_m1), A_{2B} ARs (Mm00839292_m1) and β-actin (VIC-MGB 452341E-1301018) (were purchased from Life Technologies Italia (Monza, Italy). PSB 603, Cl-IB-MECA and BAY 60-6583 were purchased from Tocris, Space Import-Export (Milan, Italy). SB202190 was purchased by Adipogen (Florence, Italy). SH5 and SP600125 were from Enzo Life (Florence, Italy). U73122 was from Cayman (Florence, Italy). AlphaScreen SureFire phospho(p)ERK1/2(Thr202/Tyr204), pJNK1/3(pThr183/Tyr185), and p-p38αMAPK (pThr180/Tyr182) assay kits were from PerkinElmer (Milan, Italy). PKC-ε translocation inhibitor peptide was purchased by Calbiochem (Milan, Italy). IL-6 ELISA kit was obtained from R&D, Space Import-Export (Milan, Italy). Unless otherwise noted, all other reagents were purchased from Sigma (Milan, Italy).

Animals

One-day-old Balb/c mice were obtained from Charles River (Calco, Italy). Animal care procedures were in accordance with the guidelines of the European Council directives (86/609/EEC) and were approved by the local Animal Care and Ethics Committee [11].

Primary microglia cultures

Briefly, after anaesthesia (Zoletil 100, 30 mg/kg, Virbac Laboratories, France) and decapitation, forebrains from newborn Balb/c mice were excised, meninges were removed and tissue was dissociated mechanically in 0.25% Trypsin–ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Life Technologies, Milan, Italy) for 5 min at 37 °C. An equal volume of culture medium with 10% of fetal bovine serum (FBS) was added to stop trypsinization. Cells were centrifuged, supernatant was discarded and pellet resuspended in 75 cm^2 flasks with DMEM with nutrient mixture F12 (DMEM:F12), supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), at 37 °C in 5% CO_2/95% air. After 21 days in vitro (DIV) confluent mixed glial cultures were subjected to mild trypsinization (0.06%) as previously

described [12]. In particular mixed glial cells were washed for 1 min in DMEM:F12 to eliminate serum, then treated for 30 min with 0.06% of trypsin in the presence of 0.25 mM of EDTA and 0.5 mM of Ca^{2+}. This results in the detachment of an intact layer of cells containing virtually all the astrocytes and leaves a population of firmly attached cells identified as >98% microglia. Cultures were characterized by immunostaining with Mac-1 anti-CD11b antibody and the absence of astrocytes was confirmed using GFAP antibody.

Microglial cell cultures

Microglial cells were grown adherently in either 75 cm^2 flasks, 6-well, 24-well or 96-well dishes in the presence of DMEM:F12, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), at 37 °C in 5% CO_2/95% air. The medium was changed every two days and the cells were observed under light microscope to ensure their were free from any contamination. The cells were sub-cultured and maintained appropriately at 60–70% confluency.

Cloned A_{2B} AR

HEK293 cells transfected with the human A_{2B} have been previously described [15]. The cells were grown adherently and maintained in DMEM:F12 without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and Geneticin (G418, 0.2 mg/ml) at 37 °C in 5% CO_2/95% air.

Hypoxic treatment

Hypoxic exposures were done in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2 (MiniGalaxy, RSBiotech, Irvine, Scotland).

Cell viability exclusion assay

Microglia (20,000 cells in 24-well dishes) were collected after trypsinization into Eppendorf tubes and centrifuged at 2000 rpm for 15 min at 4 °C. Supernatants were discarded and the pellets were re-suspended in PBS solution mixed with 0.4% trypan blue for 5 min at room temperature, before being examined under the microscope using a Burker chamber. The number of viable cells was evaluated by trypan blue exclusion. In particular, the dead cells that stained blue were scored positive and counted against the total number of cells to determine the percentage of cell death. The results are expressed as the percentage of cells, which excludes the vital trypan blue/total cells.

Immunofluorescence analysis

Expression of CD11b on primary microglial cells was checked by immunofluorescence analysis as previously described [15]. Briefly, 100-200 cells/mm^2 were seeded on cover slips, and after overnight incubation, fixed with 4% paraformaldehyde for 20 min, permeabilized with cold methanol for 5 min and washed with PBS. Cells were blocked with 3% bovine serum albumin (BSA) in PBS added with Tween-20 0.2% (PBST) for 30 min and incubated for 1 h in PBST with 1% BSA and anti-CD11b and GFAP (1:50). For negative controls, a series of cover slips was also incubated under similar conditions without the primary antibodies. Coverslips were stained with DAPI, mounted in DABCO glycerol-PBS and observed on Nikon fluorescent microscope (Eclipse 50i) as previously described [13]. Images were analyzed using NIS Elements BR 3.0 software (Nikon Instruments Inc., Milan, Italy).

Real-time RT-PCR

Microglial cells were plated in 60 mm diameter petri dishes at a density of 700,000 cells/petri and allowed to attach overnight before pharmacological treatments. Total cytoplasmic RNA was extracted from microglial cells by the acid guanidinium thiocyanate phenol method, using the Trizol reagent (Fisher Molecular Biology) according to the manufacturer's instructions. RNA concentration was determined using a spectrophotometer (Beckman DU520) at 260 nm. Residual DNA was removed by treatment with 1 U DNase/1 μg RNA (RQ1 RNase-free DNase, Promega) at 37 °C for 10 min. Identical amounts of RNA (1 μg) were reversely transcribed into cDNA using a commercial RT-PCR kit Improm II Reverse transcription system (Promega) and random primers in 20 μl at the following conditions 25 °C 5 min, 42 °C 50 min, 70 °C 15 min. Quantitative real-time RT-PCR assays of IL-6 and A_{2B} ARs were carried out in triplicate using gene-specific fluorescently labelled TaqMan MGB probe (minor groove binder) purchased from Applied Biosystems (IL-6 Mm00446190_m1, A_{2B} ARs Mm00839292_m1 and β-actin VIC-MGB 4352341E-1301018) on a MX3000P Stratagene Real-Time PCR System (M-Medical, Milan, Italy) [14]. For the real-time RT-PCR of the reference gene the endogenous control human β-actin kit was used, and the probe was fluorescent-labelled with VIC™ (Life Technologies Italia, Monza, Italy). Reactions were normalized to β-actin mRNA within the same sample using the ΔΔCT method. A negative control reaction without template DNA yielded no PCR product. The thermal cycling conditions were 30 s at 95 °C, followed by 40 cycles that consisted of a denaturation step at 95 °C for 5 s, annealing and extension step at 60 °C for 34 s.

ELISA

The levels of IL-6 protein secreted by the cells in the medium were determined by mouse Quantikine ELISA kits (R&D Systems, Milan, Italy). In brief, subconfluent microglia, 20,000 cells in 24-well dishes, were plated and allowed to attach overnight, then changed into fresh medium in the presence of solvent or various concentrations of drugs. The medium was collected, and IL-6 protein concentrations were measured by ELISA according to the manufacturer's instructions. The results were normalized to the number of cells per well. The data are presented as mean ± SE from four independent experiments performed in triplicate.

Western blotting

Microglia were plated in six-well plates at a density of 250,000 cells/well and allowed to attach overnight before pharmacological treatments. Whole cell lysates from murine primary microglia were prepared as described previously [28]. In brief, cells were then lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM $Na3VO_4$, 1 mM NaF, 1 mM EDTA) containing protease inhibitors (aprotinin, leupeptin, pepstatin: 1 μg/ml each) and 10 μM PMSF. The protein concentration was determined using a BCA protein assay kit (Pierce, Euroclone, Milan, Italy). Proteins were denaturated at 100 °C for 10 min then equivalent amounts of protein (40 μg) were subjected to electrophoresis on sodium dodecyl sulfate-acrylamide gel (7.5% for HIF-1α and 10% for A_{2B} AR and β-actin). The gel was then electroblotted onto a nitrocellulose membrane and blocked with 5% defatted milk powder in TBST (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature to reduce aspecific binding. Subsequently the membranes were probed with anti-HIF-1α or anti-A_{2B} AR antibody (1:1000 dilution) overnight at 4 °C. Filters were washed and incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies against rabbit IgG (1:2000 dilution). Specific reactions were revealed with the Enhanced Chemiluminescence Western blotting detection reagent (Perkin Elmer, Milan, Italy). Actin (1:1000) was used to ensure equal protein loading.

Densitometry analysis

The intensity of each band in immunoblot assay was quantified using a VersaDoc Imaging System (Bio-Rad, Milan, Italy). Mean densitometry data from independent experiments were normalized to the results in control cells.

siRNA treatment

Microglia were plated in 60 mm diameter petri dishes at a density of 500,000 cells/petri or in six-well plates containing 200,000 cells/well, for RNA and Western blotting analysis, respectively,

before transfection. Transfection of siRNA-A_{2B} was performed at a concentration of 100 nM using RNAiFectTM Transfection Kit. Cells were cultured in complete media, and RNA and total proteins were isolated at 24, 48 and 72 h for mRNA and Western blot analysis of A_{2B} AR proteins. A randomly chosen non-specific siRNA was used under identical conditions as control [14]. A time of 72 h of transfection was chosen for the experiments.

AlphaScreen SureFire pMAPK assays

AlphaScreen SureFire pERK1/2 (Thr202/Tyr204), pJNK1/3 (pThr183/Tyr185), and p-p38 MAPK (pThr180/Tyr182) assay kits (Perkin Elmer, Milan, Italy) were utilized. Upon MAPK phosphorylation and excitation at 680 nm, fluorescent signals at 615 nm are emitted. Microglial cells were seeded in 100 µl culture medium into 96-well plates (4000/well), and incubated at 37°C, in both normoxic and hypoxic conditions, for 24 h. Cells were pretreated with various inhibitors for 30 min. Then, receptors were maximally stimulated using 100 nM BAY 60-6583 and incubated for 5–15 min (ERK1/2, p38MAPK) or 5–30 min (JNK) at 37 °C. After agonist removal, lysis buffer was added, then donor and acceptor beads linked to specific anti-p-MAPK- and anti-MAPK-antibodies were dispensed, according to manufacturer instructions. Finally, fluorescent signals were detected through an Ensight Perkin Elmer multimode plate reader (Perkin Elmer, Milan, Italy). Data were normalized to fold activation above basal pMAPK levels (=100). For pp38 inhibitor graphs, raw data were transformed into percentages relative to controls (basal level = 100%) in order to merge data from several experiments.

MTS assay

The MTS assay was performed to determine microglial cell proliferation according to the manufacturer's protocol from the CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Milan, Italy). Cells were plated in 96-multiwell plates (4000 cells/well), allowed to attach overnight, then 100 µl of complete medium was added to each well in the absence and in the presence of the A_{2B} AR agonist/antagonist and inhibitors of cell signalling. The cells were then incubated for 0, 12, 24, 48 and 72 h in normoxia and hypoxia. At the end of the incubation period, MTS solution was added to each well. The optical density of each well was read on a spectrophotometer at 570 nm.

Statistical analysis

All values in the figures and text are expressed as mean ± standard error (SEM) of independent experiments. Each experiment was performed by using the microglial cells derived from one single mouse, and was performed in triplicate. The experiments were repeated at least four times as indicated from n values that represent the number of mice used [15]. Data sets were examined

by one-way ANOVA and Dunnett's test (when required). Two-way ANOVA and Bonferroni's *post hoc* test were used when time and treatment were compared (Fig. 6B). A P-value less than 0.05 was considered statistically significant.

Results

Microglia cells morphology

Primary microglial cells showed a morphology characterized by bipolar or unipolar processes and elongated cell bodies, as shown by the myeloid cell surface antigen CD11b staining. The purity of the cells was verified through the lack of astrocyte-specific protein GFAP staining (Fig.1).

BAY 60-6583 stimulates IL-6 secretion in primary murine microglial cells

We evaluated the effect of treatment with 100 nM ARs agonists for 24 h on IL-6 secretion in normoxia and hypoxia in murine primary microglial cells. As shown in Fig 2A the A_{2B} agonist BAY 60-6583 but not the A_1 agonist CHA, the A_{2A} CGS 21680, and the A_3 agonist Cl-IB-MECA produced an increase of IL-6 secretion in both normoxic and hypoxic conditions. The effect of BAY 60-6583 was time and dose-dependent, with maximal stimulation obtained after 24 h of treatment with BAY 60-6583 1 μM (Fig. 2B-C, respectively). To confirm the involvement of A_{2B} ARs in this effect, microglia were treated with 300 nM PSB 603 for 30 min, before addition of 100 nM BAY 60-6583 for 24 h in normoxia and hypoxia. As shown in Fig 2D, the stimulatory effect on IL-6 production induced by BAY 60-6583 was blocked by PSB 603 in both conditions. Antagonist alone did not modify IL-6 basal levels in microglial cells. No significant difference between normoxia and hypoxia was observed (Fig. 2).

BAY 60-6583 increases IL-6 transcription in primary murine microglial cells

Activation of microglial cells with 100 nM BAY 60-6583 produced a stimulation of IL-6 mRNA transcription after 4 and 8 h of treatment. The effect was investigated in both normoxic and hypoxic conditions with a fold increase of 1.7 ± 0.1 and 2.3 ± 0.2 in normoxic and 1.8 ± 0.1 and 2.6 ± 0.2 in hypoxic conditions, respectively (Fig. 3).

Hypoxia up-regulates hypoxia-inducible factor HIF-1α and A_{2B} ARs in a time-dependent way

Microglial cells were exposed to 1% O_2 for 4, 8 and 24 h and HIF-1α and A_{2B} AR modulation were assessed through Western blot analysis. As shown in Fig. 4 both proteins HIF-1α (Fig. 4A) and A_{2B} AR (Fig. 4B) were up-regulated in a time-dependent way by hypoxia thus confirming the effect of a hypoxic environment in our experimental conditions. The additional band in A_{2B} Western blot may be due to homo- or hetero-oligomers as previously observed [15].

Signalling pathways involved in the IL-6 production induced by BAY 60-6583 in primary murine microglial cells

The involvement of AC, PLC, PKC-ε and PKC-δ in the IL-6 increase due to A_{2B} activation was investigated. Microglial cells were incubated with 50 μM SQ22,536 as inhibitor of AC, 10 μM U73122 as inhibitor of PLC, 10 μM translocation inhibitor peptide of PKC-ε as blocker of PKC-ε and 10 μM rottlerin as blocker of PKC-δ. As shown in Fig. 5A, all inhibitors alone did not significantly affect IL-6 levels, whilst inhibitors of PLC, PKC-ε and PKC-δ were able to antagonize the stimulatory effect of BAY 60-6583 on IL-6 secretion, suggesting the involvement of these molecules in the A_{2B} agonist effect. Furthermore, to examine whether MAPK and Akt pathways were involved in the stimulatory effect mediated by BAY 60-6583 on IL-6 secretion, primary microglia were pretreated with 1 μM SB202190, U0126, SP600125 and SH5, inhibitors of p38, ERK1/2, JNK1/2 MAPK kinases and Akt respectively, before exposure to 100 nM BAY 60-6583 in normoxia and hypoxia. As shown in Fig. 5B, only SB202190 strongly reduced the effect of BAY 60-6583 on IL-6 secretion, whilst U0126, SP600125 and SH5 did not. All inhibitors alone did not significantly regulate IL-6 levels in microglia.

Effect of BAY 60-6583 on p38 MAPK kinase

To confirm our data, phosphorylation of p38 was assessed at 0-15 min in the absence and in the presence of 100 nM BAY 60-6583 as stimulator of A_{2B} ARs following normoxia and hypoxia (Fig. 6A-B, respectively). The extent of p38 phosphorylation was strictly similar in normoxic and hypoxic conditions with a maximal effect after 5 min of incubation that decreased at 15 min in both conditions. Phosphorylation of p38 was maximal after 100 nM BAY 60-6583 for 5 min both in normoxia and hypoxia (Fig. 6C-D respectively). The effect of 100 nM BAY 60-6583 was potently antagonized by pretreatment of cells with 300 nM PSB603 for 30 min, in both normoxic and hypoxic conditions (Fig. 6E-F, respectively). BAY 60-6583 did not alter significantly ERK1/2, JNK1/2 and Akt phosphorylation (data not shown). p38 phosphorylation induced by 100 nM BAY 60-6583 for 5 min in microglia was reduced in the presence of PLC, PKC-ε and PKC-δ inhibitors in normoxia and hypoxia, but not after incubation with AC inhibitor (Fig. 6G-H, respectively).

Effect of BAY 60-6583 on microglial cell proliferation

The effect of 100 nM BAY 60-6583 on microglial cells proliferation was assessed after 12, 24 and 48 and 72 h of treatment. Hypoxia did not significantly affect cell health, viability and number of these cells. Cell proliferation started to increase after 12 h and reached a maximum effect after 48 h of incubation (Fig. 7A). This effect was antagonized by addition of 300 nM PSB

603, 1μM SB202190, U73122, inhibitor of PKC-ε and rottlerin, thus suggesting the involvement of A_{2B} ARs, PLC, PKC-ε, PKC-δ and p38 signalling in cell proliferation (Fig. 7B).

Effect of A_{2B} AR silencing on p38 MAPK kinase, IL-6 and cell proliferation

In order to further verify the role of A_{2B} ARs in the p38 phosphorylation, IL-6 protein secretion and cell proliferation we knocked-down it. Microglial cells were transfected with siRNA targeting A_{2B} ARs. After 48 and 72 h posttransfection, A_{2B} ARs mRNA and protein levels were significantly reduced in comparison to control cells transfected with non-specific random control ribonucleotides (siRNA scrambled, siRNActr) (Fig. 8A-B, respectively). Then the stimulatory effect of BAY 60-6583 on p38 phosphorylation, IL-6 secretion and cell proliferation was evaluated in primary microglia exposed to A_{2B} ARs siRNA. In this condition A_{2B} ARs siRNA treatment for 72 h inhibited the increase of p38 phosphorylation induced by 100 nM BAY 60-6583 applied for 5 min (Fig. 9A). Furthermore, A_{2B} ARs stimulation for 24 h did not increase IL-6 secretion in microglial cells preincubated with A_{2B} ARs siRNA for 48 h, suggesting the involvement of A_{2B} subtypes in this effect (Fig. 9B). Similarly, cell proliferation was not more affected by 100 nM BAY 60-6583 added for 48 h to the cells transfected with A_{2B} ARs siRNA (Fig. 9C). No significant difference between normoxia and hypoxia was observed.

Viability of microglial cells following pharmacological treatments

To evaluate the status of the cells following the different pharmacological treatments used during the study we have checked their viability through the trypan blue exclusion assay. In particular, microglia were treated with 100 nM CHA, CGS 21680, Cl-IB-MECA, 50 μM SQ22,536, 1 μM SB202190, U0126, SP600125 and SH5 for 24 h or with 100 nM BAY 60-6583, 300 nM PSB 603, 10 μM U73122, 10 μM PKC-ε translocation inhibitor peptide, 10 μM rottlerin for 48 h or with siRNA-ctr and A_{2B} ARs siRNA for 72 h, in both normoxia and hypoxia. As shown in Fig. 10 hypoxia by itself and the various pharmacological treatments in normoxia and hypoxia did not significantly affect cell viability of these cells.

Discussion

Microglia represent a significant fraction of the CNS cells, including 5% to 20% of the total glial population, and are as numerous as neurons [15]. Microglia are crucial in maintaining tissue homeostasis, very sensitive to environmental changes in the brain and in case of tissue damage or invading microorganisms e.g in infection, traumatic injury, ischemia, neurodegenerative disease become activated [16]. This implies a change of morphology and functions, cell proliferation, increase in the production of a variety of cytotoxic mediators contributing to the damage of surrounding healthy neurons [17], [18]. For these reasons microglial responses must be tightly regulated since their sustained activation results in chronic neuroinflammation. Therefore, the reduction of inflammation induced by activated microglia may slow the progression of several CNS pathologies [19].

In addition to pro-inflammatory functions through M1 polarization, myeloid cells exert protective effects by M2 phenotype resulting in the neuroprotection after brain injury [20]–[22]. Extracellular nucleotides/nucleosides and the related purinergic receptors are important actors into the transformation process of microglia in pathological conditions, where the autacoid ado increases its levels in a significant way [23]. Ado affects almost all functions of microglia from process extension and retraction, migration, proliferation and cytokine production, recruiting different ARs subtypes, that may represent important targets for drug development [24].

This work aimed to evaluate whether ARs contribute to neuroinflammation induced following microglia activation, in both normoxic and hypoxic conditions. We have demonstrated that A_{2B} ARs activation increased both mRNA and protein levels of IL-6, as well as cell proliferation through PLC, PKC-ε, PKC-δ and p38 signalling. Inhibition of A_{2B} ARs either pharmacologically by the A_{2B} ARs antagonist PSB 603 or by siRNA-induced A_{2B} ARs knockdown was associated with a reduction in p38 phosphorylation, IL-6 secretion and microglial cells proliferation. Microglial cells express all ARs but only the A_{2B} AR subtype was involved in this effect [13], [23], [25], [26]. Surprisingly, IL-6 was not differentially modulated in normoxia and hypoxia even though both HIF-1α and the A_{2B} AR were increased by 1% O_2. Therefore, as microglia are less sensitive to hypoxia than any other brain parenchymal cell population it might be speculated that i) more severe hypoxic stimuli should be applied to reveal possible differences between normoxia and hypoxia; ii) the IL-6 increase observed following A_{2B} AR stimulation has already reached its plateau. As IL-6 is pivotal in inflammation by regulating a number of cell function, including cell proliferation, it is possible to hypothesize that the increased microglial cell proliferation observed in our study, dependent on A_{2B} ARs, PLC, PKC-ε, PKC-δ, p38, involves also IL-6 signalling. Our results are in agreement with literature

data reporting the modulation of IL-6 through A_{2B} ARs activation, in a variety of cells such as astrocytes, pituitary folliculostellate cells, bronchial smooth muscle cells, lung fibroblasts, astrocytoma cells, mouse striatum, peritoneal macrophages and cardiac fibroblasts, indicating that A_{2B} ARs represent an important target in neuroinflammation [2]–[10]. Accordingly, the signal transduction pathway linked to A_{2B} AR modulation of IL-6 was attributed to PLC, PKC and p38 in murine pituitary folliculostellate cells, U373 human astroglioma cells and murine cardiac fibroblasts but not in rat astrocytes where it was associated to PKA and cAMP signalling [3], [4], [7], [10]. Indeed the involvement of PKC in the regulation of IL-6 formation has been widely reported and in particular the IL-6 secretion induced by A_{2B} ARs activation has been earlier attributed to the enrollment of PKC-ε and PKC-δ isoforms [7], [10]. Therefore, in our study we took in consideration these particular isoforms by using specific inhibitors, thus confirming their role in the pathway triggered by A_{2B} ARs in increase IL-6 production and microglial cell proliferation. Anyway, we cannot exclude the involvement of other PKC isoforms in this effect modulated by A_{2B} ARs in microglia.

It is well known that three independent subgroups of MAPK and Akt are expressed in microglia affecting several cell functions and that ARs are all coupled to them [27]–[29]. Therefore, by using specific and selective inhibitors we investigated the roles of MAPK and Akt in the pathway triggered by A_{2B} AR activation. We found that only p38 was responsible for the stimulatory effect of A_{2B} AR on IL-6 secretion and cell proliferation. Accordingly, an association between p38 and A_{2B} AR activation has been already found in the increase of IL-10, HIF-1α, iNOS, VEGF and GLUT-1 in activated microglial cells [13], [25].

Interestingly, this is the first time that an increase in cell proliferation was observed in microglial cells related to A_{2B} ARs stimulation, suggesting a role of this ado subtype in one of the early step in microglial activation with possible implication in microglia protective functions.

Previous data confirmed a stimulatory effect of AR agonists on microglial cell proliferation, attributing this role to A_1 and A_2 ARs [30]. Then a contribution of A_{2A} AR was observed in cell proliferation induced by LPS stimulation in both N9 and primary microglial cells, through a pathway dependent on brain derived neurotrophic factor [31]. In contrast it has been reported a role of A_1 and possibly A_{2B} ARs in the reduction of proliferation in microglial cells [32]. It would be possible that the high and not more selective doses of AR agonists used to investigate thymidine incorporation in the BV2 microglia cell line may explain the different effect observed in that study.

However, it has to be reported that the in vitro approach in the study of microglial functions retains some limitations linked to the microglia limited motility, difficulty to acquire a ramified

morphology and the lack of exposure to signals from other cell types e.g. neurons, suggesting that *in vivo* studies are necessary to corroborate our findings. In summary, our results show for the first time that ado by activating A_{2B} ARs increases IL-6 protein levels and cell proliferation through a pathway dependent on PLC, PKC-ε, PKC-δ, and p38 signalling (Fig. 11). These findings add a new important piece of knowledge on the role of ARs in microglia activation and neuroinflammation.

Figures and figure legends

FIG. 1

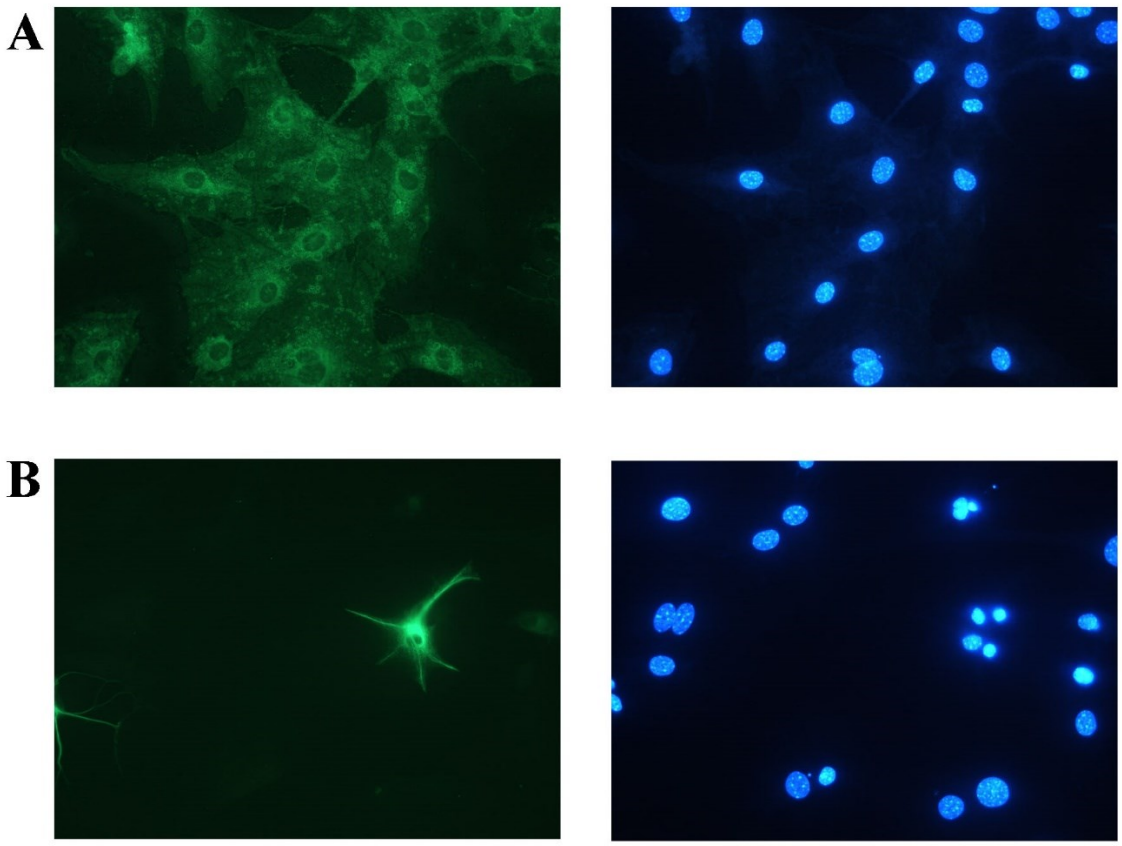


FIG. 1: Microglial cells morphology by immunofluorescence analysis. Staining of microglia with the myeloid cell surface antigen CD11b (A) and astrocyte-specific protein GFAP (B). The blue-fluorescent DAPI nucleic acid stain is shown.

FIG. 2

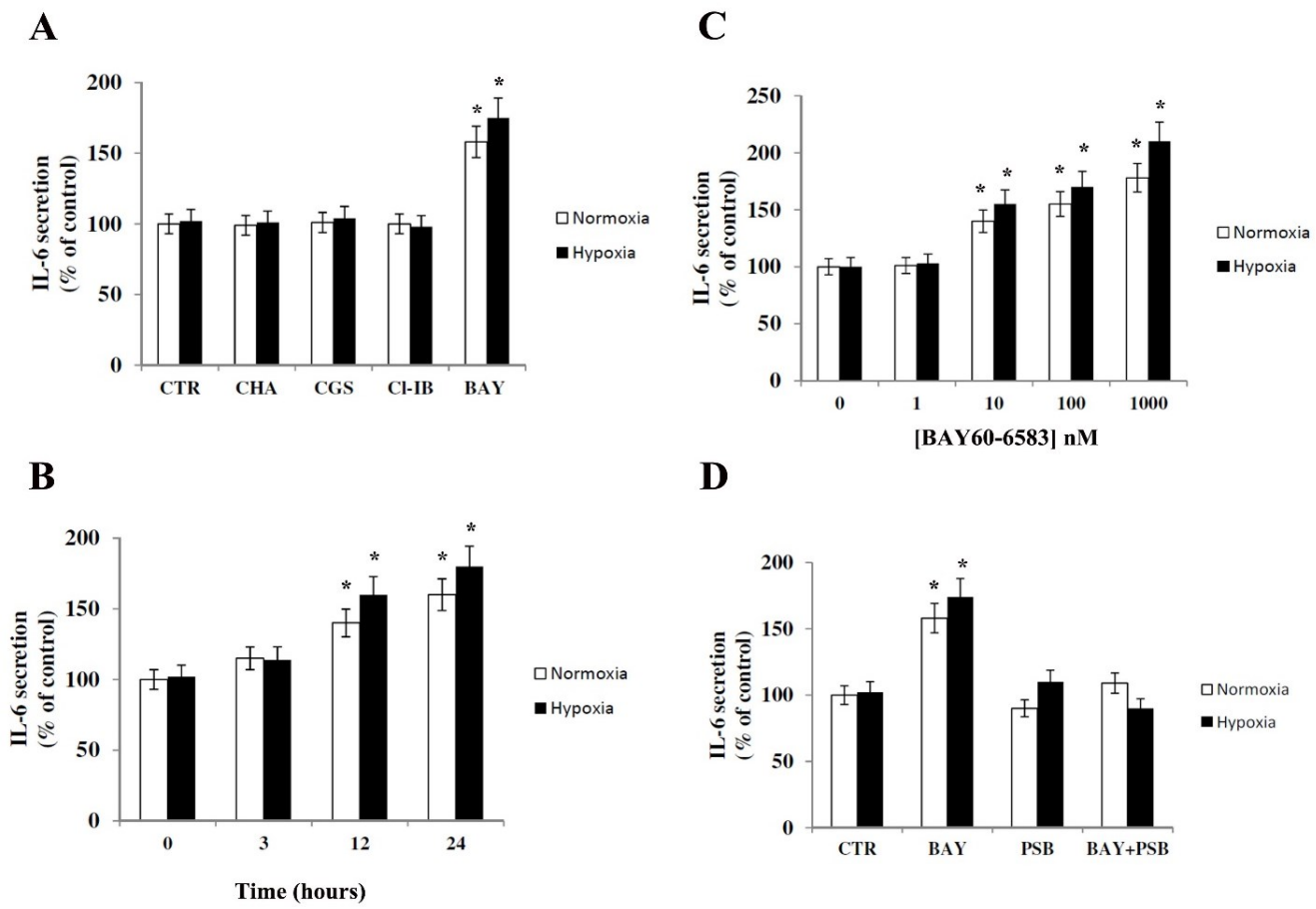


FIG. 2: BAY 60-6583 stimulates IL-6 secretion in primary murine microglial cells. (A) Cells were incubated in normoxia and hypoxia for 24 h in the absence (CTR, 100%) and in the presence of 100 nM CHA, CGS 21680 (CGS), Cl-IB-MECA (Cl-IB) and BAY 60-6583 (BAY) and IL-6 protein secretion was evaluated by ELISA. (B) Cells were incubated in the presence of 100 nM BAY 60-6583 under normoxia and hypoxia for 0, 3, 12 and 24 h and IL-6 protein secretion was evaluated by ELISA. (C) Cells were incubated with increasing concentrations of BAY 60-6583 (0-1000 nM) for 24 h under normoxia and hypoxia and IL-6 protein secretion was evaluated by ELISA. (D) Cells in the absence or in the presence of 300 nM PSB 603 (PSB) were exposed to 100 nM BAY 60-6583 for 24 h in normoxia and hypoxia and IL-6 protein secretion was evaluated by ELISA. *P<0.01 compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one-way ANOVA, followed by Dunnett's test.

FIG. 3

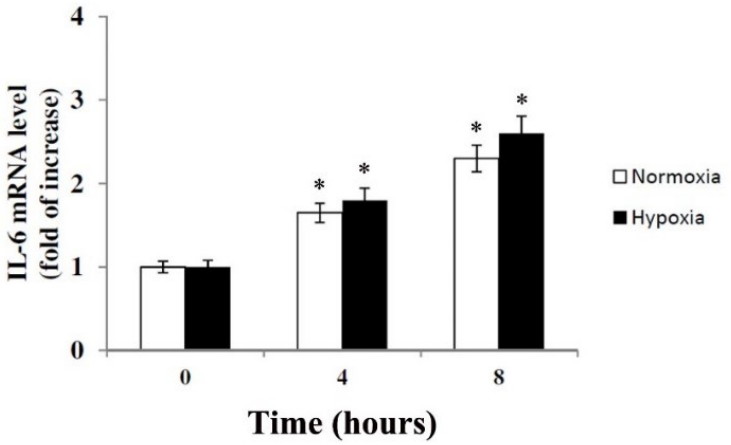


FIG. 3: BAY 60-6583 increases IL-6 transcription in primary murine microglial cells. Cells were incubated in normoxia and hypoxia for 0, 4, 8 h with 100 nM BAY 60-6583 and IL-6 mRNA levels were evaluated by real-time RT-PCR. Data were expressed as fold of increase vs cells in the absence of BAY 60-6583, arbitrarily fixed as 1. *P<0.01 compared with 0. Each point is the mean ± SEM of four independent experiments (N = 4). Analysis was by one-way ANOVA, followed by Dunnett's test.

FIG. 4

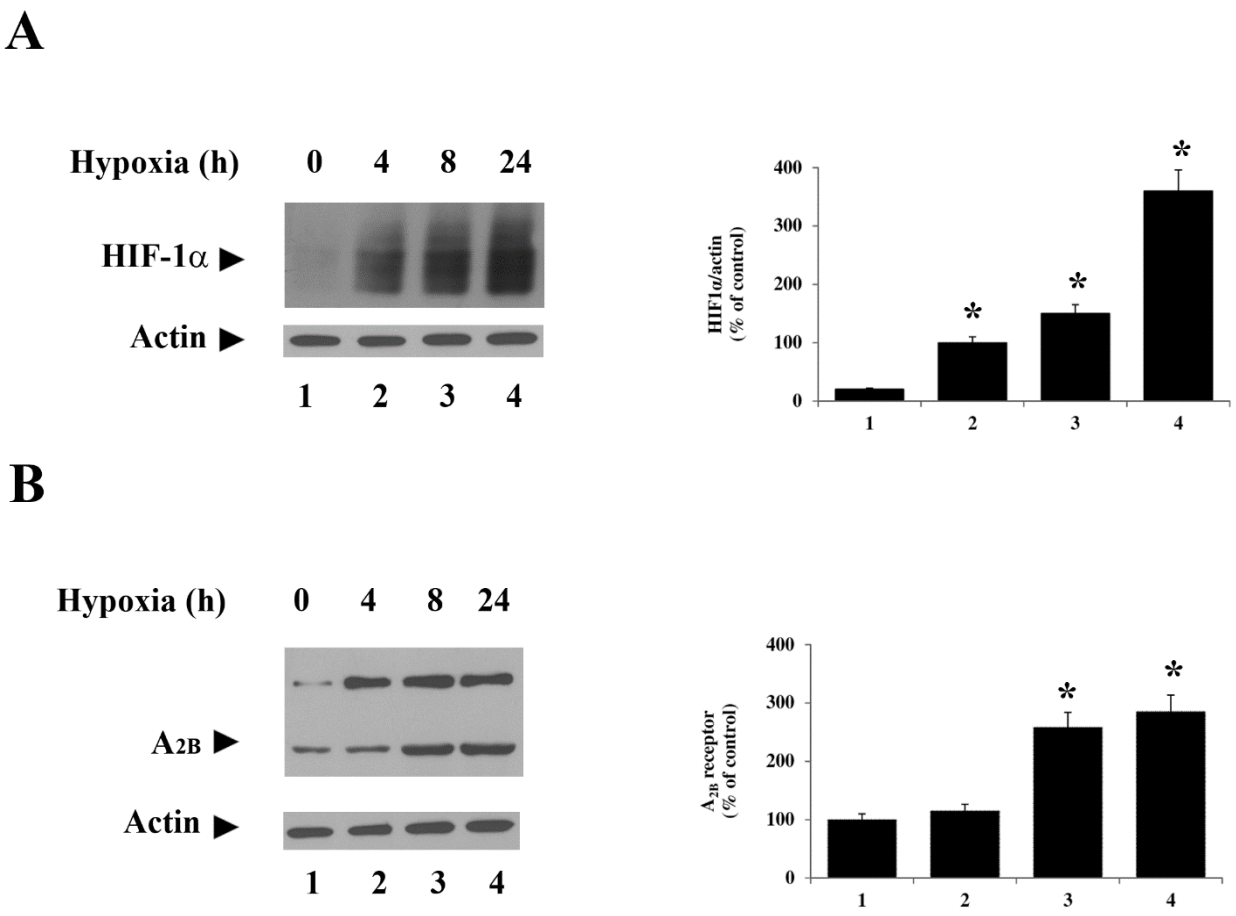


FIG. 4: Hypoxia induces HIF-1α accumulation and A_{2B} AR up-regulation in primary microglial cells. Cells were incubated for up to 24 h in the absence or presence of hypoxia (1%) and the protein levels of HIF-1α (A) and A_{2B} AR (B) were determined by Western blotting in whole cell lysates (Lane 1-4) and actin served as control for equal protein loading. Densitometric quantification is the mean ± SEM of four independent experiments (N=4); control [cells in the presence of 4 h hypoxia (A), cells in normoxia (B)] was set to 100%. Values are the mean ± S.E. of four independent experiments (N = 4); $^{*}P<0.05$ compared with control; analysis was by one-way ANOVA followed by Dunnett's test.

FIG. 5

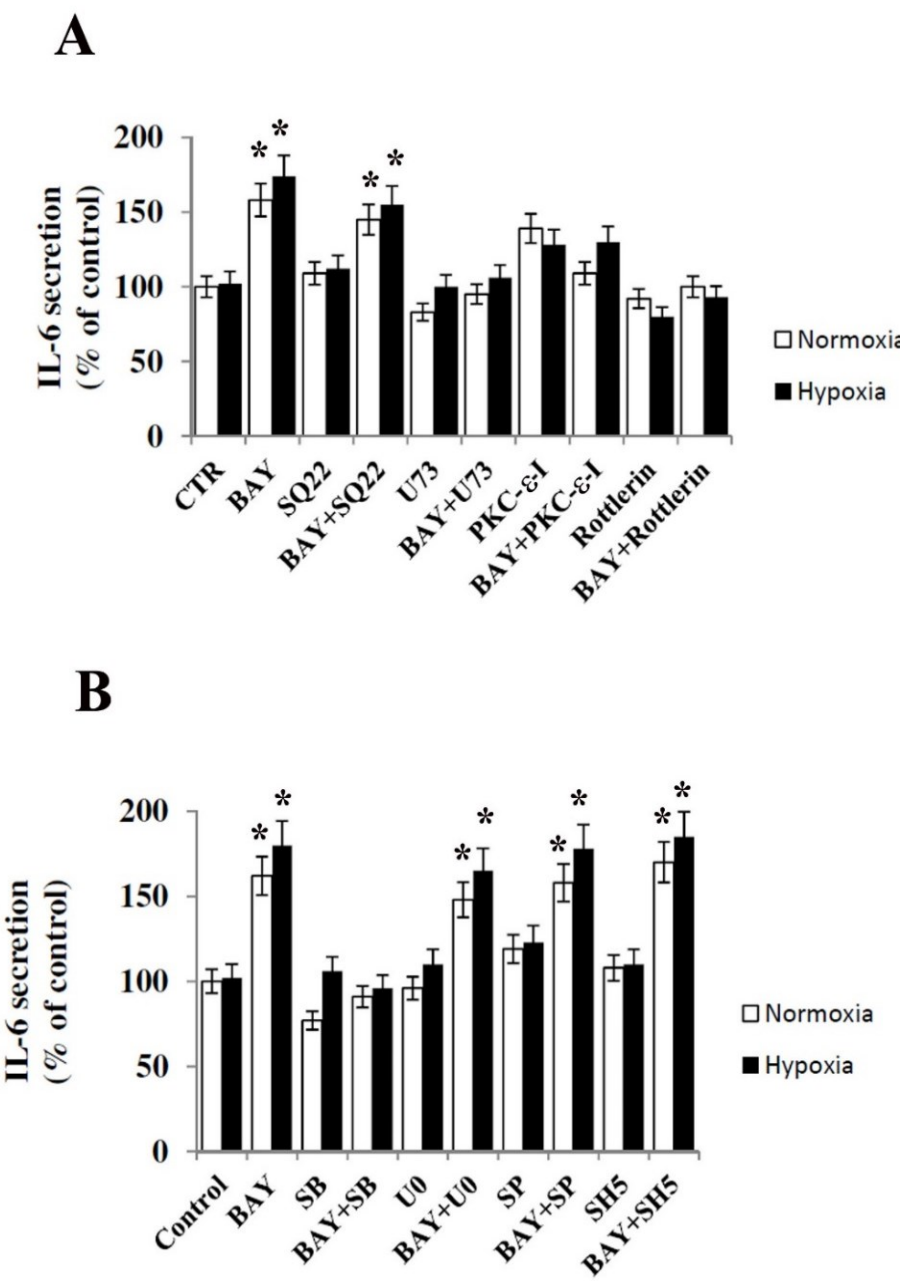


FIG. 5: Signalling pathways involved in the IL-6 production induced by BAY 60-6583 in primary murine microglial cells. Effect of 100 nM BAY 60-6583 on IL-6 protein secretion under normoxia and hypoxia (24 h) and antagonism by 50 μM SQ22,536, 10 μM U73122, PKC-ε blocker and rottlerin (A), 1 μM SB202190, U0126, SP600125 and SH5 (B) evaluated by ELISA. $^{*}P<0.01$ compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one-way ANOVA, followed by Dunnett's test.

FIG. 6

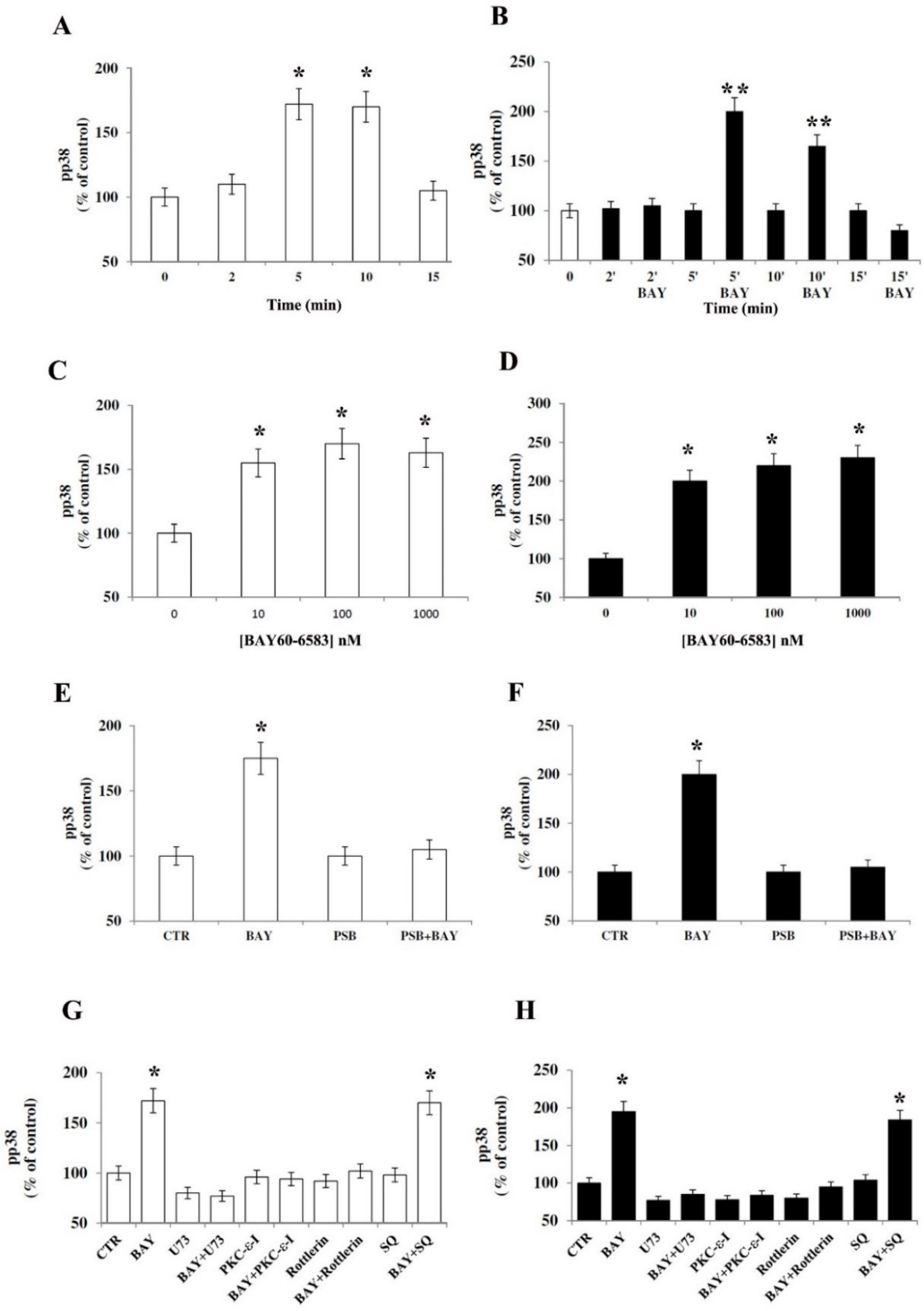

A
B
C
D
E
F
G
H
pp38
(% of control)
Time (min)
[BAY60-6583] nM
CTR
BAY
PSB
PSB+BAY
U73
BAY+U73
PKC-ε-I
BAY+PKC-ε-I
Rottlerin
BAY+Rottlerin
SQ
BAY+SQ

FIG. 6: Effect of BAY 60-6583 on p38 MAPK activation. Cells were incubated in the presence of 100 nM BAY 60-6583 for 0, 2, 5, 10 and 15 min and pp38 protein levels were evaluated by AlphaScreen SureFire pMAPK assays under normoxia (A) and hypoxia (B). (C) Cells were incubated with increasing concentrations of BAY 60-6583 (0-1000 nM) for 5 min and pp38 protein levels were evaluated by AlphaScreen SureFire pMAPK assays under normoxia (C) and hypoxia (D). Cells in the absence or in the presence of 300 nM PSB 603 (PSB) were exposed to 100 nM BAY 60-6583 for 5 min and pp38 protein levels were evaluated by AlphaScreen SureFire pMAPK assays in normoxia (E) and hypoxia (F). Effect of 100 nM BAY 60-6583 on pp38 protein levels under normoxia (G) and hypoxia (H) (5 min) and antagonism by 10 μM U73122, PKC-ε blocker, rottlerin and 50 μM SQ22,536 evaluated by AlphaScreen SureFire pMAPK assays. $^{*}P<0.01$ compared with CTR. Analysis was by one-way ANOVA followed by Dunnett's test; $^{**}P<0.001$ compared with cells without BAY 60-6583 in normoxia. Analysis was by two-way ANOVA followed by Bonferroni's test. Interaction $F_{(4,20)} = 40.82$, $P<0.0001$; Time $F_{(4,20)} = 40.02$, $P<0.0001$; BAY 60-6583 $F_{(1,20)} = 69.76$, $P<0.0001$. Means ± S.E. values from four experiments are shown.

FIG. 7

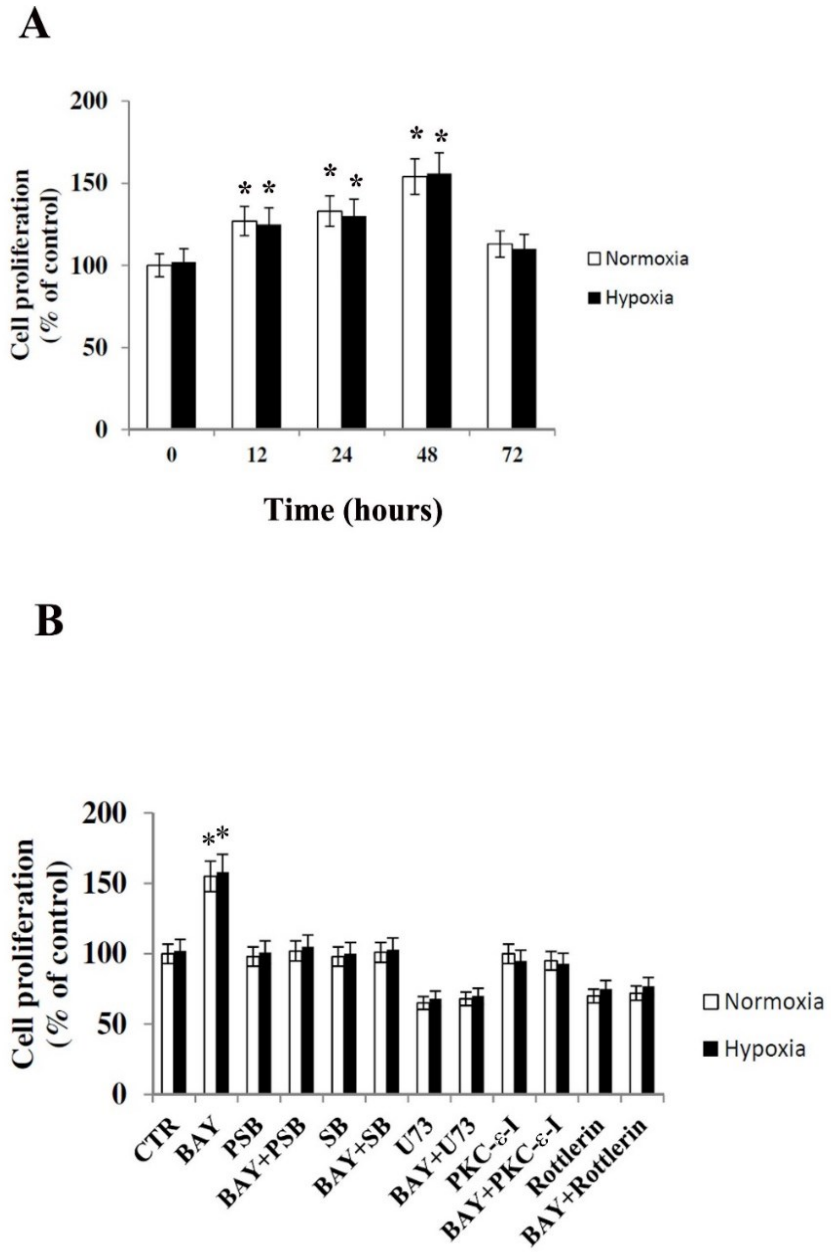


FIG. 7: Effect of BAY 60-6583 on microglial cells proliferation. Cells were incubated in the presence of 100 nM BAY 60-6583 for 12, 24, 48 and 72 h and cell proliferation was evaluated by MTS assay under normoxia and hypoxia (A). Cells in the absence or in the presence of 300 nM PSB 603, 10 μM U73122, PKC-ε blocker and rottlerin were exposed to 100 nM BAY 60-6583 for 48 h and cell proliferation was evaluated by MTS assay under normoxia and hypoxia (B). $^{*}P<0.01$ compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one-way ANOVA, followed by Dunnett's test.

FIG. 8

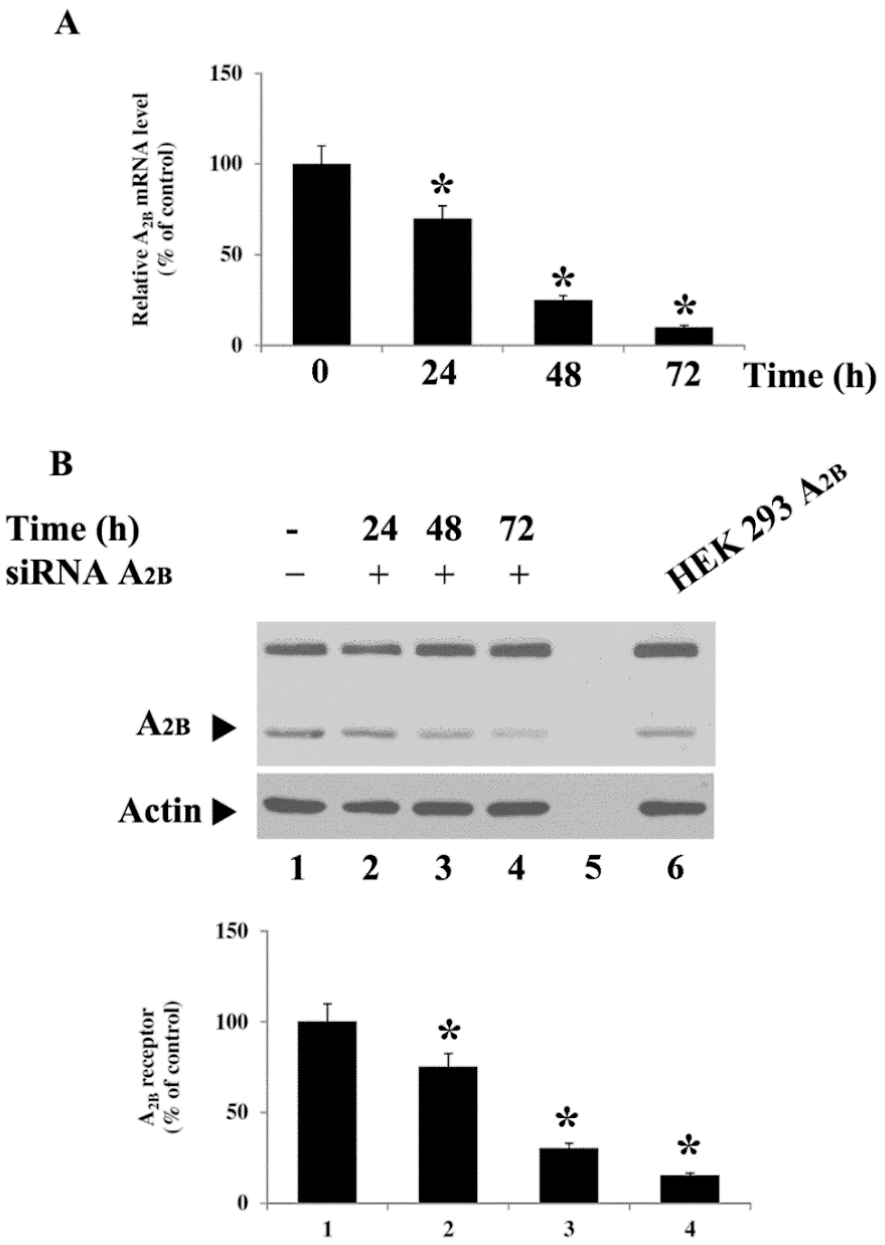
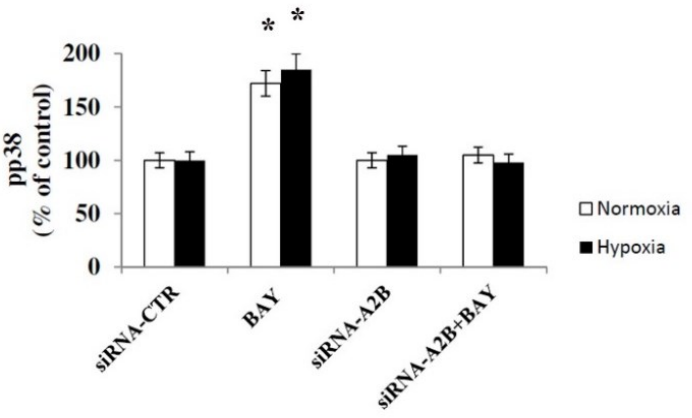


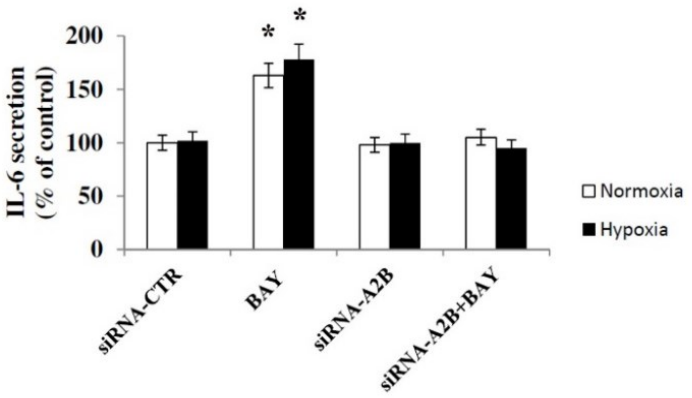
FIG. 8: A_{2B} AR silencing by siRNA transfection. (A) A_{2B} AR mRNA quantification, related to β-actin mRNA, by real-time RT-PCR. Microglial cells were transfected with siRNA-A_{2B} by RNAiFectTM Transfection reagent and cultured for 24, 48 and 72 h. Plots are mean ± S.E. (N = 4); $^{*}P< 0.01$ compared with the control (time = 0). (B) Western blot analysis using anti A_{2B} AR polyclonal antibodies of protein extracts from microglial cells treated with siRNA-A_{2B} and cultured for 24, 48 and 72 h; cells transfected with siRNA-ctr for 72 h and HEK293 cells transfected with A_{2B} AR as positive control (line 1 and 6, respectively). Actin shows equal loading protein. $^{*}P<0.05$ vs cells transfected with siRNA-ctr for 72 h. Analysis was by one-way ANOVA, followed by Dunnett's test.

FIG. 9

A


pp38
(% of control)
200
150
100
50
0
* *
siRNA-CTR
BAY
siRNA-A2B
siRNA-A2B+BAY
Normoxia
Hypoxia

B


IL-6 secretion
(% of control)
200
150
100
50
0
* *
siRNA-CTR
BAY
siRNA-A2B
siRNA-A2B+BAY
Normoxia
Hypoxia

C

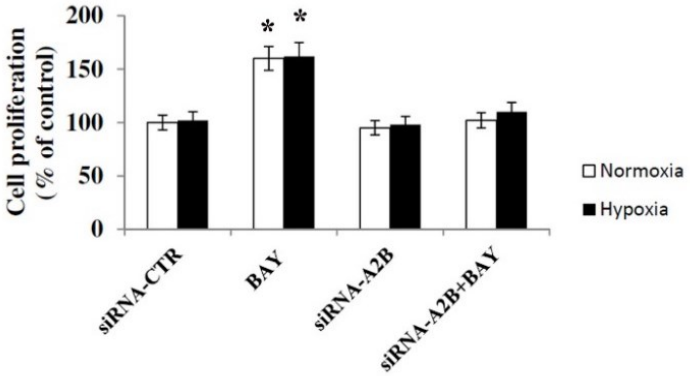

Cell proliferation
(% of control)
200
150
100
50
0
* *
siRNA-CTR
BAY
siRNA-A2B
siRNA-A2B+BAY
Normoxia
Hypoxia

FIG. 9: pp38, IL-6 protein levels and proliferation in microglia, A_{2B} ARs silenced, stimulated by BAY 60-6583. (A) Microglial cells were incubated with both siRNA scrambled (siRNA-ctr) and siRNA of A_{2B} ARs (siRNA-A_{2B}) and cultured for 72 h before addition of 100 nM BAY 60-6583 for 5 min and pp38 levels were evaluated by AlphaScreen SureFire pMAPK assays under normoxia and hypoxia. (B) Microglial cells were incubated with both siRNA-ctr and siRNA-A_{2B} and cultured for 48 h before addition of 100 nM BAY 60-6583 for 24 h and IL-6 levels were evaluated by ELISA under normoxia and hypoxia. (C) Microglial cells were incubated with both siRNA-ctr and siRNA-A_{2B} and cultured for 24 h before addition of 100 nM BAY 60-6583 for 48 h and cell proliferation was evaluated by MTS under normoxia and hypoxia. $^{*}P<0.01$ compared with CTR. Means ± S.E. values from four experiments are shown. Analysis was by one-way ANOVA, followed by Dunnett's test.

FIG. 10

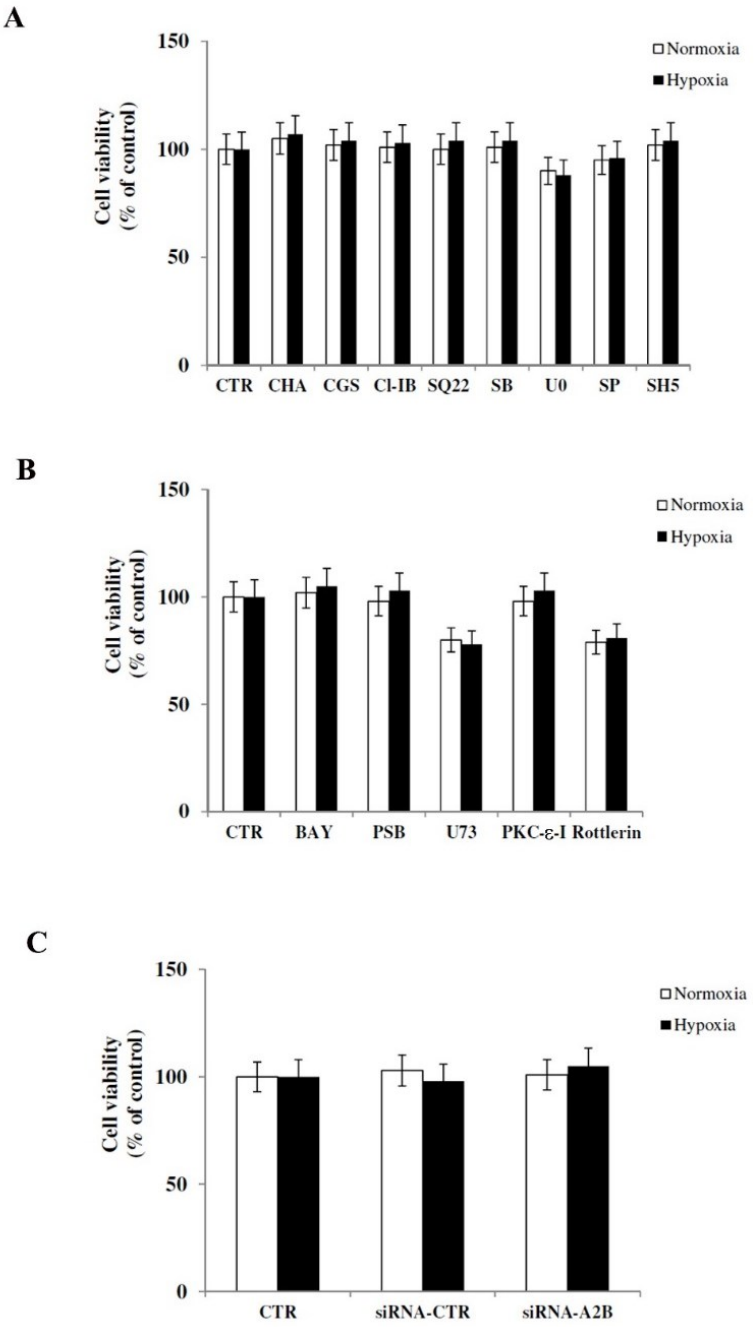


FIG. 10: Microglia viability assessed by Trypan Blue Exclusion assay. Cells were treated with 100 nM CHA, CGS 21680 (CGS), Cl-IB-MECA (Cl-IB), 50 μM SQ22,536 (SQ22), 1 μM SB202190 (SB), U0126 (U0), SP600125 (SP) and SH5 for 24 h (A) or with 100 nM BAY 60-6583 (BAY), 300 nM PSB 603 (PSB), 10 μM U73122 (U73), 10 μM PKC-ε translocation inhibitor peptide (PKC-ε-I), 10 μM rottlerin for 48 h (B) or with siRNA-ctr and or A_{2B} ARs siRNA for 72 h (C) in both normoxia and hypoxia (A-C). The results are expressed as the percentage of cell viability observed in different treatment conditions compared with untreated cells (considered as 100%). Means ± S.E. values from four experiments are shown. Analysis was by one-way ANOVA.

FIG. 11

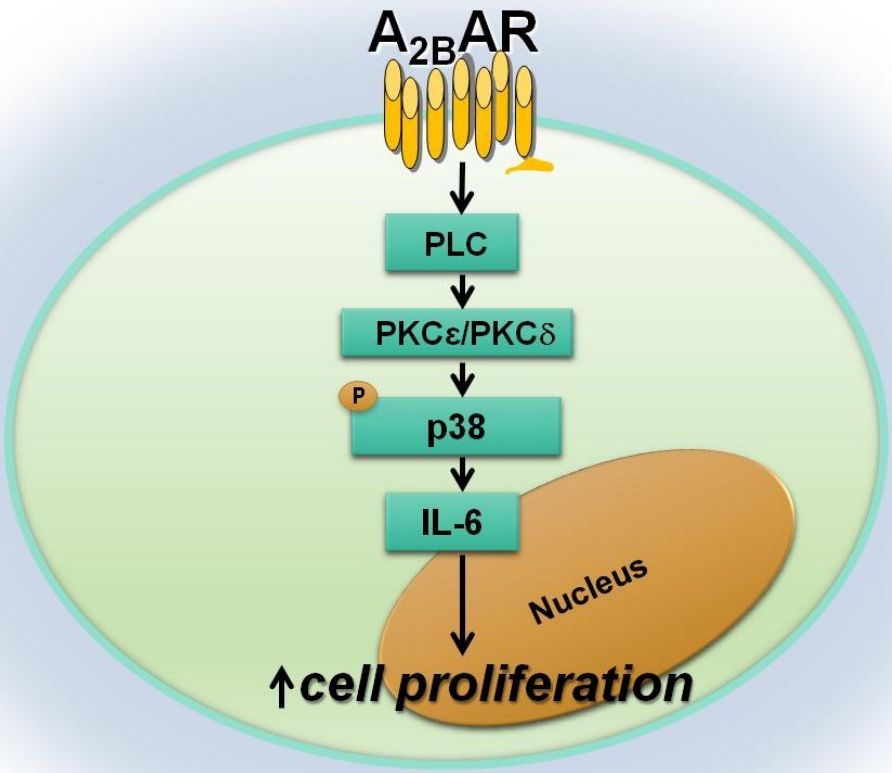


FIG. 11: Schematic summary of Ado-triggered signal transduction cascade in murine microglia. Ado by activating A_{2B} ARs increases IL-6 protein levels and cell proliferation through a pathway dependent on PLC, PKC-ε, PKC-δ, and p38 signalling

References

[1] M. Erta, A. Quintana, and J. Hidalgo, “Interleukin-6, a major cytokine in the central nervous system.,” *Int. J. Biol. Sci.*, vol. 8, no. 9, pp. 1254–1266, 2012.

[2] M. Schwaninger, M. Neher, E. Viegas, A. Schneider, and M. Spranger, “Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine.,” *J. Neurochem.*, vol. 69, no. 3, pp. 1145–1150, Sep. 1997.

[3] M. Schwaninger, N. Petersen, S. Prinz, S. Sallmann, M. Neher, and M. Spranger, “Adenosine-induced expression of interleukin-6 in astrocytes through protein kinase A and NF-IL-6.,” *Glia*, vol. 31, no. 1, pp. 51–58, Jul. 2000.

[4] D. A. Rees, B. M. Lewis, M. D. Lewis, K. Francis, M. F. Scanlon, and J. Ham, “Adenosine-induced IL-6 expression in pituitary folliculostellate cells is mediated via A_{2B} adenosine receptors coupled to PKC and p38 MAPK.,” *Br. J. Pharmacol.*, vol. 140, no. 4, pp. 764–772, Oct. 2003.

[5] H. Zhong, L. Belardinelli, T. Maa, I. Feoktistov, I. Biaggioni, and D. Zeng, “A_{2B} adenosine receptors increase cytokine release by bronchial smooth muscle cells.,” *Am. J. Respir. Cell Mol. Biol.*, vol. 30, no. 1, pp. 118–125, Jan. 2004.

[6] H. Zhong, L. Belardinelli, T. Maa, and D. Zeng, “Synergy between A_{2B} adenosine receptors and hypoxia in activating human lung fibroblasts.,” *Am. J. Respir. Cell Mol. Biol.*, vol. 32, no. 1, pp. 2–8, Jan. 2005.

[7] B. L. Fiebich *et al.*, “IL-6 expression induced by adenosine A_{2B} receptor stimulation in U373 MG cells depends on p38 mitogen activated kinase and protein kinase C.,” *Neurochem. Int.*, vol. 46, no. 6, pp. 501–512, May 2005.

[8] S. Ryzhov *et al.*, “Effect of A_{2B} adenosine receptor gene ablation on adenosine-dependent regulation of proinflammatory cytokines.,” *J. Pharmacol. Exp. Ther.*, vol. 324, no. 2, pp. 694–700, Feb. 2008.

[9] J. F. Vazquez, H.-W. Clement, O. Sommer, E. Schulz, and D. van Calker, “Local stimulation of the adenosine A_{2B} receptors induces an increased release of IL-6 in mouse striatum: an in vivo microdialysis study.,” *J. Neurochem.*, vol. 105, no. 3, pp. 904–909, May 2008.

[10] W. Feng, Y. Song, C. Chen, Z. Z. Lu, and Y. Zhang, "Stimulation of adenosine A_{2B} receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-delta-p38 signalling pathway.," *Br. J. Pharmacol.*, vol. 159, no. 8, pp. 1598–1607, Apr. 2010.

[11] J. C. McGrath, G. B. Drummond, E. M. McLachlan, C. Kilkenny, and C. L. Wainwright, "Guidelines for reporting experiments involving animals: the ARRIVE guidelines.," *Br. J. Pharmacol.*, vol. 160, no. 7, pp. 1573–1576, Aug. 2010.

[12] J. Saura, J. M. Tusell, and J. Serratosa, "High-yield isolation of murine microglia by mild trypsinization.," *Glia*, vol. 44, no. 3, pp. 183–189, Dec. 2003.

[13] S. Merighi *et al.*, "A_{2A} and A_{2B} adenosine receptors affect HIF-1α signaling in activated primary microglial cells," *Glia*, vol. 63, no. 11, pp. 1933–1952, Nov. 2015.

[14] S. Gessi, S. Merighi, A. Stefanelli, D. Fazzi, K. Varani, and P. A. Borea, "A_1 and A_3 adenosine receptors inhibit LPS-induced hypoxia-inducible factor-1 accumulation in murine astrocytes," *Pharmacol. Res.*, vol. 76, pp. 157–170, Oct. 2013.

[15] I. Yang, S. J. Han, G. Kaur, C. Crane, and A. T. Parsa, "The role of microglia in central nervous system immunity and glioma immunology," *J. Clin. Neurosci.*, vol. 17, no. 1, pp. 6–10, Jan. 2010.

[16] J. D. Cherry, J. A. Olschowka, and M. K. O'Banion, "Neuroinflammation and M2 microglia: the good, the bad, and the inflamed.," *J. Neuroinflammation*, vol. 11, no. 1, p. 98, Jun. 2014.

[17] M. T. Heneka, M. P. Kummer, and E. Latz, "Innate immune activation in neurodegenerative disease.," *Nat. Rev. Immunol.*, vol. 14, no. 7, pp. 463–477, Jul. 2014.

[18] D. J. Loane and A. Kumar, "Microglia in the TBI brain: The good, the bad, and the dysregulated.," *Exp. Neurol.*, vol. 275, pp. 316–327, Jan. 2016.

[19] S. Gessi, P. A. Borea, S. Bencivenni, D. Fazzi, K. Varani, and S. Merighi, "The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia.," *FEBS Lett.*, vol. 590, no. 17, pp. 2813–2826, Sep. 2016.

[20] S. Girard, D. Brough, G. Lopez-Castejon, J. Giles, N. J. Rothwell, and S. M. Allan, "Microglia and macrophages differentially modulate cell death after brain injury caused by oxygen-glucose deprivation in organotypic brain slices.," *Glia*, vol. 61, no. 5, pp. 813–

824, May 2013.

[21] C. Benakis, L. Garcia-Bonilla, C. Iadecola, and J. Anrather, “The role of microglia and myeloid immune cells in acute cerebral ischemia,” *Front. Cell. Neurosci.*, vol. 8, p. 461, Jan. 2015.

[22] S. Fumagalli, C. Perego, F. Pischiutta, E. R. Zanier, and M.-G. De Simoni, “The ischemic environment drives microglia and macrophage function.,” *Front. Neurol.*, vol. 6, p. 81, Apr. 2015.

[23] S. Koizumi, K. Ohsawa, K. Inoue, and S. Kohsaka, “Purinergic receptors in microglia: functional modal shifts of microglia mediated by P2 and P1 receptors.,” *Glia*, vol. 61, no. 1, pp. 47–54, Jan. 2013.

[24] E. Daré, G. Schulte, O. Karovic, C. Hammarberg, and B. B. Fredholm, “Modulation of glial cell functions by adenosine receptors,” *Physiol. Behav.*, vol. 92, no. 1–2, pp. 15–20, Sep. 2007.

[25] B. Koscsó *et al.*, “Adenosine augments IL-10 production by microglial cells through an A_{2B} adenosine receptor-mediated process.,” *J. Immunol.*, vol. 188, no. 1, pp. 445–453, Jan. 2012.

[26] C. Hammarberg, G. Schulte, and B. B. Fredholm, “Evidence for functional adenosine A_3 receptors in microglia cells.,” *J. Neurochem.*, vol. 86, no. 4, pp. 1051–1054, Aug. 2003.

[27] M. Koistinaho and J. Koistinaho, “Role of p38 and p44/42 mitogen-activated protein kinases in microglia,” *Glia*, vol. 40, no. 2, pp. 175–183, Nov. 2002.

[28] G. Schulte and B. B. Fredholm, “Signalling from adenosine receptors to mitogen-activated protein kinases.,” *Cell. Signal.*, vol. 15, no. 9, pp. 813–827, Sep. 2003.

[29] C. Hammarberg, B. B. Fredholm, and G. Schulte, “Adenosine A_3 receptor-mediated regulation of p38 and extracellular-regulated kinase ERK1/2 via phosphatidylinositol-3’-kinase.,” *Biochem. Pharmacol.*, vol. 67, no. 1, pp. 129–134, Jan. 2004.

[30] P. J. Gebicke-Haerter, F. Christoffel, J. Timmer, H. Northoff, M. Berger, and D. Van Calker, “Both adenosine A1- and A2-receptors are required to stimulate microglial proliferation.,” *Neurochem. Int.*, vol. 29, no. 1, pp. 37–42, Jul. 1996.

[31] C. Gomes *et al.*, “Activation of microglial cells triggers a release of brain-derived

neurotrophic factor (BDNF) inducing their proliferation in an adenosine A_{2A} receptor-dependent manner: A_{2A} receptor blockade prevents BDNF release and proliferation of microglia," *J. Neuroinflammation*, vol. 10, no. 1, p. 780, Dec. 2013.

[32] M. L. Haselkorn *et al.*, "Adenosine A_1 receptor activation as a brake on the microglial response after experimental traumatic brain injury in mice.," *J. Neurotrauma*, vol. 27, no. 5, pp. 901–910, May 2010.

CURRICULUM VITAE

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- Internship in Prof. Stefania Gessi's laboratory from December 2013 to October 2014 at University of Ferrara Dep. of Medical Science, Section of Pharmacology;
- PhD student from November 2014 to November 2017 in Molecular Medicine and Pharmacology at the University of Ferrara, tutor Prof. Stefania Gessi.

Skills acquired:

Principal molecular and cellular biology techniques, primary and secondary cell cultures, isolation of figurate elements from the peripheral blood, electrophoresis and Western blot, enzyme-linked immunosorbent assay (ELISA), cell viability and proliferation assays, nucleic acids extraction and quantification, real time RT-PCR, immunofluorescence and microscopy, dosage of cyclic AMP, Alpha technology for the quantitative detection of phospho-kinase and cytokines in cellular lysates and supernatant, techniques for the study of the receptor functionality, in particular the measurement of intracellular calcium (Calcium Imaging, HTS) and the receptor binding assay. Ability to work in sterility by the use of biological laminar flow hoods and instruments for sterilization. Planning of experimental activities, statistical data

analysis and problem solving; technical reports writing and presentation of results. Knowledge of techniques for the study of pain and epilepsy *in vivo* models. Supervisor for undergraduate students and correlator of First and Master's degree thesis.

LIST OF PUBLICATIONS AND PATENT

PUBLICATIONS

1. S. Merighi P. A. Borea, A. Stefanelli, **S. Bencivenni**, C.A. Castillo, K. Varani, and S. Gessi, "A_{2A} and A_{2B} adenosine receptors affect HIF-1α signaling in activated primary microglial cells," *Glia*, vol. 63, no. 11, pp. 1933–1952, Nov. 2015.
2. S. Gessi, P. A. Borea, **S. Bencivenni**, D. Fazzi, K. Varani, and S. Merighi, "The activation of μ-opioid receptor potentiates LPS-induced NF-kB promoting an inflammatory phenotype in microglia.," *FEBS Lett.*, vol. 590, no. 17, pp. 2813–2826, Sep. 2016.
3. A. Dore, B. Asproni, A. Scampuddu, S. Gessi, G. Murineddu, E. Cichero, P. Fossa, S. Merighi, **S. Bencivenni**, and G. A. Pinna, "Synthesis, molecular modeling and SAR study of novel pyrazolo[5,1-f][1,6]naphthyridines as CB_2 receptor antagonists/inverse agonists," *Bioorg. Med. Chem.*, vol. 24, no. 21, pp. 5291–5301, Nov. 2016.
4. S. Merighi, **S. Bencivenni**, F. Vincenzi, K. Varani, P. A. Borea, and S. Gessi, "A_{2B} adenosine receptors stimulate IL-6 production in primary murine microglia through p38 MAPK kinase pathway," *Pharmacol. Res.*, vol. 117, pp. 9–19, Mar. 2017.
5. S. Gessi, **S. Bencivenni**, E. Battistello, F. Vincenzi, V. Colotta, D. Catarzi, F. Varano, S. Merighi, P. A. Borea, and K. Varani. " Inhibition of A_{2A} adenosine receptor signalling in cancer cells proliferation by the novel antagonist TP455," *Front Pharmacol.,* vol. 8, pp. 1-13. Dec. 2017.
6. G. Ragusa, **S. Bencivenni**, P. Morales, T. Callaway, D. P. Hurst, B. Asproni, S. Merighi, G. Loriga, G. A. Pinna, P. H. Reggio, S. Gessi, and G. Murineddu. "Synthesis, pharmacological evaluation and docking studies of novel pyridazinone-based cannabinoid receptor type-2 ligands," *Eur. J. Med. Chem*. Submitted. 2017.

PATENT

1. F. Fruttarolo, M. G. Pavani, **S. Bencivenni**, R. Gatti and M. Napoletano, 2014. "Novel sulfonamide TRPA1 receptor anatagonists," (EP 13158046).

POSTERS AND MEETINGS

1. SYNTHESIS AND BIOLOGICAL EVALUATION OF DIAZABICYCLO[3.3.1]NONANES WITH A THIOETHER BRIDGE. Autors: G. Pinna, B. Asproni, G. Murineddu, S. Gessi, S. Merighi, **S. Bencivenni**, M.M. Curzu, G.A. Pinna. "Bioheterocycles 2015 – XVI International Conference on Heterocycles in Bioorganic Chemistry", Metz (France), 8-11 June 2015.
2. PYRAZOLO-NAPHTHYRIDINES: DESIGN AND SYNTHESIS OF POTENTIAL CB-LIGAND. Autors: A. Dore, B. Asproni, G. Murineddu, S. Gessi, S. Merighi, **S. Bencivenni**, M.M. Curzu, G.A. Pinna. "Bioheterocycles 2015 – XVI International Conference on Heterocycles in Bioorganic Chemistry", Metz (France), 8-11 June 2015.
3. THE ACTIVATION OF μ-OPIOID RECEPTOR POTENTIATES THE LPS-INDUCED ACTIVATION OF NF-kB PROMOTING AN INFLAMMATORY PHENOTYPE IN CULTURED MICROGLIA. Autors: S. Gessi, P.A. Borea, **S. Bencivenni**, K. Varani K, S. Merighi. "Nuovi orizzonti della ricerca farmacologica: tra etica e scienza" 37th SIF National Congress, Naples, 27-30 October 2015.
4. A_{2A} AND A_{2B} ADENOSINE RECEPTORS AFFECT HIF-1α SIGNALLING IN ACTIVATED PRIMARY MICROGLIAL CELLS. Autors: S. Merighi, P.A. Borea, A. Stefanelli, **S. Bencivenni**, C.A. Castillo, K. Varani, S. Gessi. "Nuovi orizzonti della ricerca farmacologica: tra etica e scienza" 37th SIF National Congress, Naples, 27-30 October 2015.
5. GLIAL CELLS AND NEUROINFLAMMATION: THE ADENOSINERGIC PERSPECTIVE. Autors: S. Gessi, S. Merighi, **S. Bencivenni**, E. Battistello, F. Vincenzi, S. Pasquini, A. Ravani, P. A. Borea. "Advances in basic and translational purinergic research" 7th Purine Club Meeting, Rome, 20-22 July 2017.
6. A_{2B} ADENOSINE RECEPTORS STIMULATE IL-6 PRODUCTION IN PRIMARY MURINE MICROGLIA THROUGH p38 MAPK KINASE PATHWAY. Autors: S. Merighi, **S. Bencivenni**, E. Battistello, F. Vincenzi, S. Pasquini, A. Ravani, K. Varani, P. A. Borea, S. Gessi. *"FARMACI, SALUTE E QUALITÀ DELLA VITA"* 38th SIF National Congress, Rimini, 25-28 October 2017.

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