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Microglia control adenosine A_{2A}-receptor mediated astrogliosis

Diploma thesis

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DECLARATION

I declare that this thesis is my original author work. All literature and other sources of information, that I used while processing, are listed in the bibliography section and they are properly cited.

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ABSTRACT

In the central nervous system, astrocytes and microglia are the main cells coordinating the inflammatory response. During inflammation, dying or temporarily damaged cells release ATP, as a danger-associated signal molecule, that contributes to the induction of astrogliosis and promotes clearance of the debris by immune cells such as microglia. Adenosine that results from ATP metabolism also stimulates astrogliosis. However, the effects of adenosine on astrogliosis may be more complex, since it also modulates microglia phenotype and microglia have been shown to prevent excessive astroglial proliferation mediated by nucleotides. In this context, ATP and adenosine are assumed as relevant signalling molecules in the control of astrogliosis and its modulation by microglia. However, it is still unknown whether and how microglia modulate adenosine-mediated astrogliosis. The present study aims to clarify the impact of microglia in the control of adenosine-induced astrogliosis.

Two types of primary glial cultures were prepared from cortical hemispheres of newborn rats (age: 0-2 days): co-cultures of astrocytes containing approximately 15% of microglia and "pure" cultures of astrocytes, where microglia were almost absent (< 1%). These cultures were used to evaluate the effect of P1 agonists on methyl-[^{3}H]-thymidine incorporation and to evaluate A_{2A} receptors expression by Western blot.

In "pure" cultures of astrocytes adenosine (0.001-0.3 mM) increased astroglial proliferation up to 172 ± 5% (n=7; P<0.05), but the effect was attenuated to 131 ± 5% (n=5; P<0.05) by 30 nM of the selective A_{2A} antagonist SCH 58261 or to 125 ± 6% (n=5; P<0.05) by 10 nM of the selective A_{2B} antagonist MRS 1706. The selective agonists of A_{2A} receptor CGS 21680 (1-100 nM) induced astroglial proliferation up to 155 ± 3% (n=4; P<0.05), while the A_1 agonist CPA (1-100 nM) and the A_3 agonist 2-Cl-IB-MECA (1-100 nM) had no effect. Furthermore, the proliferative effect of adenosine (100 microM; 179 ± 4%; n=5, P<0.05) was attenuated to 107 ± 7% (n=3; P<0.05) by inhibition of protein kinase A (PKA) with 1 μM of H-89 and to 120 ± 6% (n=4, P<0.05) by inhibition of mitogen-activated protein kinase kinase 1/2 (MEK1/2) with 10 μM of U0126.

In co-cultures, the proliferative effects induced by adenosine and CGS 21680 (concentrations as above) were lower than those obtained in “pure” cultures. Adenosine increased the proliferation to 142 ± 8% (n=4; $P<0.05$) and CGS 21680 to 126 ± 5% (n=4; $P<0.05$).

Western blot indicated that A_{2A} receptors are expressed either in pure cultures of astrocytes and in co-cultures being present in both types of cells.

The results show that astroglial proliferation induced by adenosine is mediated by A_{2A} and A_{2B} receptors coupled to the intracellular PKA-ERK pathway and this effect can be attenuated by microglia.

ABSTRAKT

V centrální nervové soustavě mají astrocyty a mikroglie stěžejní funkci při řízení zánětlivé odpovědi. Během zánětu uvolňují umírající a poškozené buňky do okolí molekuly ATP, které slouží jako „signál nebezpečí“, podílející se na rozvoji astrogliosy a podporující odstraňování poškozené tkáně mikrogliemi a dalšími buňkami imunitního systému. Produkt metabolizace ATP, adenosin, také zvyšuje proliferaci astrocytů. Jeho působení na astrogliosu je však pravděpodobně mnohem komplexnější, protože zároveň ovlivňuje fenotyp mikroglií, které, jak se zdá, mohou zamezovat nadměrné proliferaci astrocytů zprostředkované nukleotidy. V těchto souvislostech jsou ATP a adenosin pokládány za důležité signální molekuly, které řídí astrogliosu a její modulaci mikrogliemi, nicméně se stále neví, zda a jak mikroglie ovlivňují astrogliosu zprostředkovanou adenosinem. Tato studie si klade za cíl objasnit roli mikroglií při kontrole adenosinem navozené astrogliosy.

Z mozkové kůry novorozených potkanů (stáří: 0-2 dny) byly připraveny dva druhy primárních buněčných kultur: kultury astrocytů obsahující asi 15% mikroglií a „čisté“ kultury astrocytů, které byly téměř bez mikroglií (<1%). Tyto kultury byly použity ke zkoumání účinku P1 agonistů na inkorporaci methyl-[^{3}H]-thymidinu a ke zjištění přítomnosti A_{2A} receptorů pomocí Western blot analýzy.

V „čistých“ astrocytárních kulturách adenosin (0.001-0.3 mM) zvýšil proliferaci astrocytů až na 172 ± 5% (n=7; P<0.05), ale účinek byl zeslaben na 131 ± 5% (n=5; P<0.05) po přidání 30 nM selektivního antagonisty A_{2A} receptoru SCH 58261 a na 125 ± 6% (n=5; P<0.05) po přidání 10 nM selektivního antagonisty A_{2B} receptoru MRS 1706. Selektivní agonista A_{2A} receptoru CGS 21680 (1-100 nM) vyvolal nárůst proliferace astrocytů na 155 ± 3% (n=4; P<0.05), zatímco agonista A_1 receptoru CPA (1-100 nM) a agonista A_3 receptoru 2-Cl-IB-MECA (1-100 nM) neměli žádný účinek. Kromě toho se proliferační účinek adenosinu (100 μM; 179 ± 4%; n=5, P<0.05) snížil na 107 ± 7% (n=3; P<0.05) inhibicí proteinkinázy A (PKA) pomocí 1 μM H-89 a na 120 ± 6% (n=4, P<0.05) inhibicí mitogenem aktivované proteinkinázy kinázy 1/2 (MEK1/2) pomocí 10 μM U0126.

V kulturách obsahujících 15% mikroglií byl proliferační efekt vyvolaný adenosinem a CGS 21680 (v koncentracích uvedených výše) nižší než ten který byl naměřen v "čistých" kulturách astrocytů. Adenosin zvýšil proliferaci jen na 142 ± 8% ($n=4$; $P<0.05$) a CGS 21680 na 126 ± 5% ($n=4$; $P<0.05$).

Výsledky Western blot analýzy svědčí o tom, že A_{2A} receptory se vyskytují jak v čistých astrocytárních kulturách, tak i v kulturách astrocytů s mikrogliemi a jsou přítomny v obou typech těchto buněk.

Výsledky ukázaly, že proliferace astrocytů vyvolaná adenosinem je zprostředkovaná A_{2A} a A_{2B} receptory spojenými s vnitrobuněčnou dráhou PKA-ERK a že tento efekt může být oslabený působením mikroglií.

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LIST OF ABREVIATION

ADA - Adenosine deaminase

ADP - Adenosine diphosphate

AMP - Adenosine monophosphate

ATP - Adenosine triphosphate

cAMP - Cyclic adenosine monophosphate

BBB - Blood brain barrier

CNS - Central nervous system

COX-2 - Cyclooxygenase-2

DPBS - Dulbecco's phosphate-buffered saline

ERK - Extracellular signal-regulated kinase

FBS - Fetal bovine serum

GABA - γ-aminobutyric acid

GFAP - Glial fibrillary acidic protein

IL - Interleukin

MAPK - Mitogen-activated protein kinase

NO - Nitric oxide

PBS - Phosphate buffered saline

PKA - Protein kinase A

PKC - Protein kinase C

ROS - Reactive oxygen species

SDS - Sodium dodecyl sulfate

SEM - Standard errors of the mean

TCA - trichloroacetic acid

TRITC - Tetramethylrodamine isothiocyanate

1 INTRODUCTION

1.1 Glial cells

The central nervous system (CNS) is formed by two categories of cells: glial cells and neurons. Glial cells outnumber the neurons and they occupy about half of the brain's tissue volume (Jessen, 2004). The term neuroglia was originally introduced by Rudolf Ludwig Karl Virchow to describe glial cells and it was derived from the Greek word "glue" which means sticky, because Virchow considered glial cells to be the glue that keeps neurons together (Verkhratsky and Butt, 2007). Nowadays, many functions of glial cells have been discovered and their roles are recognized to be very important in the maintenance of the CNS homeostasis.

In terms of morphology and function, glial cells are a very heterogeneous cell population (Jessen, 2004). According to their origin they can be divided into two major groups: macroglial and microglial cells. Macroglial cells have neuronal origin and include astrocytes, oligodendrocytes and ependymal cells. Microglia cells have mesodermal origin and are derived from macrophages that migrate into the brain during the early development (Verkhratsky and Butt, 2007).

1.1.1 Astrocytes

Astrocytes are the most numerous cells in the CNS. The term "astrocyte" comes from the star-shaped appearance even though this shape is not typical for all the subtypes (Verkhratsky and Butt, 2007). Most of the astrocytes in normal adult brain can be classified as fibrous or protoplasmic astrocytes. Protoplasmic astrocytes are found throughout all the grey matter, are more branched and their end-feet surround blood vessels. Fibrous astrocytes are found in the white matter and have fewer branches with long processes that contact nodes of Ranvier and blood vessels (Choudhury and Ding, 2016). Astrocytes usually do not work as individual units since they are connected via gap junctions, which allow them to form a functional net (Pekny and Pekna, 2016).

A common feature of astrocytes is the expression of intermediate filament proteins, such as glial fibrillary acidic protein (GFAP) and vimentin that form their cytoskeleton. The level of GFAP expression differs according to the subtype of astrocyte and brain area (Verkhratsky and Butt, 2007). The process of reactive astrogliosis and glial scar formation is associated with increased expression of this protein. Therefore, GFAP is frequently used as a marker of astrocytes and of reactive astrogliosis. Its detection is based on immunological methods (Eng et al., 2000). Moreover, there are others immunological markers, including S100β (Gonçalves et al., 2008) and glutamine synthetase (Norenberg, 1979) that can be used for the identification of astrocytes.

Apart from providing structural support, astrocytes have many other functions that contribute to the regulation of normal brain function. Astrocytes are essentially important in maintaining homeostasis in the CNS. They are involved in the regulation of fluids and ions concentration, including those of K^+, Ca^{2+} and H^+, which can contribute to changes in neuronal excitability (Rose, 2010). By reuptake and recycling of neurotransmitters, such as glutamate and γ-aminobutyric acid (GABA), astrocytes can control their concentration in the synaptic cleft (Sibson et al., 1998).

There is a growing body of evidence suggesting that astrocytes are directly involved in the regulation of synaptic transmission and neuronal activity in so-called tripartite synapses. They are able to release neuronal active substances, including glutamate, GABA and purines, which can directly influence synaptic transmission (Sofroniew and Vinters, 2010). As a response to the neuronal activity intracellular Ca^{2+} concentration can increase and induce release of these compounds from astrocytes (Araque et al., 1999).

Astrocytes are involved in brain development and synaptic plasticity. By creating molecular boundaries they can control growth of axons growth and synaptic formation (Powell and Geller, 1999). It was demonstrated that astrocytes have essential functions in synapse formation, maturation (Ullian et al., 2001) and also in their pruning. Synapses, which should be eliminated, are tagged by the protein of complement cascade C1q. Expression of this protein is influenced by astrocytic signalling (Stevens et al., 2007).

They are also very important in the brain nutrition and have the largest glycogen reserve in the CNS. During hypoglycaemia, glycogen can be metabolised alternatively to produce lactate that is transported to axons and aerobically turned into energy (Brown and Ransom, 2007). Another way how astrocytes affect neuronal nutrition is through regulation of local blood flow by releasing vasoactive molecules from their end-feet that contact with blood vessels (MacVicar and Newman, 2015). Astrocytes participate in the blood brain barrier (BBB) formation. Their end-feet surround endothelial cells with tight junction and influence the development and function of BBB. (Pit'ha, 2014).

Apart from these physiological functions in healthy brain, in case of injury astrocytes are also involved in the brain inflammatory response by a process called astrogliosis.

1.1.2 Microglia

Microglial cells form a population of resident macrophages in the brain and the spinal cord. They are fulfilling essential role in both innate and adaptive immunity in the CNS (Glass and Saijo, 2011). They form about 10% of total glial cells population (Verkhratsky and Butt, 2007). Like gatekeepers microglia constantly scan the brain tissue and in the case of detection of any pathological marker, such as foreign antibodies, cytokines, surface structures of microorganisms, complement factors and others potentially toxic signals, they become activated and trigger and command the defensive immune response of the brain (Glass and Saijo, 2011). They also act as antigen-presenting cells for T-cells activation (Nakajima and Kohsaka, 2001). The relation of these cells with macrophages and monocytes is demonstrated by the presence of similar markers, such as the proteins CD11b or CD14. Antibodies directed to these proteins can be used for microglia identification in the brain tissue (Glass and Saijo, 2011).

Microglia are essential for the CNS development and functionality in physiological conditions. They participate in maturation, pruning and elimination of synapses in a complement-dependent manner. Trophic factors released by microglia are also involved in hippocampal neurogenesis. Besides that microglia can produce growth factors participating in the brain development (Crotti and Ransohoff, 2016). There are also some evidences that microglia can contribute to the regulation of neuronal activity.

In vivo experiments in zebra fish showed that the projections of resting microglia are attracted by highly active neurons and their contact can reduce spontaneous neural activity (Li et al., 2012).

Most of microglial cells found in the healthy mature brain are in a resting state. This state is characterized by a ramified morphology with a small cell body and many long branched projections (Nakajima and Kohsaka, 2001). Despite of this, resting microglia are not inactive, their projections randomly move and each individual microglial cell is responsible for monitoring a defined territory (Verkhratsky and Butt, 2007). If they detect any change in the environment that can constitute a signal of danger, they undergo a transformation into a more active phenotype. This activated phenotype is characterized by several cellular changes that allow them to protect the neural tissue (Nakajima and Kohsaka, 2001).

1.2 Role of glial cells in brain injury

The primary impact of the brain injury is cell death followed by oxidative stress, excitotoxicity, BBB disruption and neuroinflammation. Even though the brain tissue is not able to regenerate itself as other tissues in the body, such as liver or skin, it has a significant ability to limit the spread of the damage and adapt its function. Acute inflammation is a normal response to any noxious agent (Provencio and Badjatia, 2014).

In the condition of a CNS injury glial cells trigger a series of nonspecific reactions, which include their activation and production of inflammation-promoting mediators. The earliest response is generally mediated by microglial cells and infiltrated macrophages from blood that migrate to the lesioned area. After 3-5 days, precursors of oligodendrocytes start the process of demyelination followed by astrogliosis, which, in more severe cases, can result in glial scar formation (Fawcett and Asher, 1999).

1.2.1 Microgliosis

Microglia activation or microgliosis is commonly the first stage of reactive gliosis triggered by the brain damage (Figure 1). This change of the phenotype affects the cell size, number of cells and their molecular expression. Activated microglia cells lose their processes and their cell bodies hypertrophy and acquire an amoeboid morphology. Moreover, they became more mobile and actively migrate towards the area of lesion. The number of microglia in the damaged region is also enhanced by their increased proliferation (Kettenmann et al., 2011). If the damage remains and the brain cells start to die, also their phagocytic activity increases to remove the cell debris. The process of transformation from resting through activated microglia into phagocytes is gradual and there are many intermediate stages between these phenotypes (Verkhratsky and Butt, 2007).

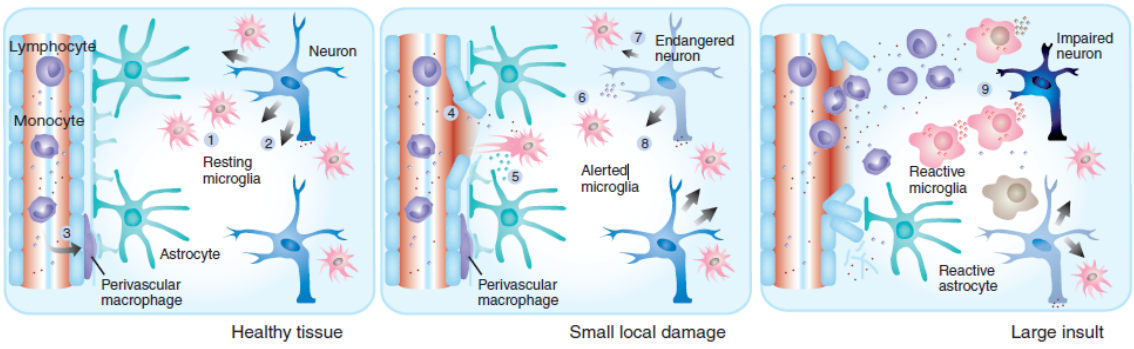

Fig. 1 The activation of microglia after detection of an insult (Hanisch and Kettenmann, 2007)

The individual aspects of microgliosis occur in a time-dependent manner. Cell hypertrophy and cytokine production begin within the first day, but cell proliferation can be observed after 3-4 days. This suggests that different aspects of microglia activation are triggered by different signalling pathways (Lindsay, 1986).

Depending on the type of injury there are two major ways of microglia transformation resulting into different phenotypes of activated microglia. Classical activation is usually evoked by agonists of toll-like receptor, such as lipopolysaccharide or interferon-γ (INF-γ), associated with bacterial or viral infection, resulting in the development of a phenotype, which is characterized by the secretion of cytotoxic compounds, such as oxidative metabolites, pro-inflammatory cytokines and proteases (Ghosh et al., 2016). Parasitic infections or tissue damage (Glass and Saijo, 2011) are

accompanied by production of anti-inflammatory cytokines, such as interleukin-4 (IL-4), IL-10 or IL-13, which trigger another type of microglia activation. The outcome of this activation is the development of a phenotype that helps to repair the tissue by secretion of growth factors and anti-inflammatory mediators (Ghosh et al., 2016).

As mentioned before, microglia communicate with other brain cells, like astrocytes, by releasing soluble messengers. Some of them play an essential role in the activation of astrocytes (Verkhratsky and Butt, 2007).

1.2.2 Astrogliosis

Astrogliosis is a mechanism whereby the nervous tissue protects itself against damage. In response to an insult, such as infection, ischemia, trauma, stroke or inflammation, astrocytes become activated and trigger series of molecular, structural and functional changes (Pekny and Pekna, 2016), whose main aim is the isolation of the damaged area, regeneration of the BBB and facilitation of reconstruction of impaired brain circuits (Verkhratsky and Butt, 2007). Regulation of this defensive brain response is mediated by intercellular and extracellular signalling molecules that have impact on astrocytes. Astrogliosis is characterized by hypertrophy and morphological changes of astrocytes, modification in expression of signalling molecules, cell proliferation and glial scar formation. An increase in GFAP expression is the hallmark for these changes (Sofroniew, 2009).

1.2.2.1 Classification of astrogliosis

Traditional classification of astrogliosis has been based on structural changes of brain tissue and its reversibility. It can be classified into two major types: isomorphic (reversible, retaining original morphology of astrocytes) and anisomorphic astrogliosis (irreversible, changing original morphology of astrocytes) (Fernaud-Espinosa et al., 1993). Recently this point of view has been changed because the number of possible responses is larger and can differ depending on the type and intensity of the brain insult (Sofroniew, 2009).

To simplify, astrogliosis can be classified as mild, moderate and severe, when the formation of glial scar occurs. Mild and moderate astroglioses are temporary reactions,

which contribute to the elimination of the insult, after which the affected brain tissue may be able to fully recover (Sofroniew, 2009). During this phases astrocytes have enhanced GFAP expression, they are hypertrophied but there is just a minimal cell proliferation (Sofroniew and Vinters, 2010). A more serious damage can involve a pronounced glial reaction that leads to irreversible changes in tissue architecture known as glial scar formation. In contrast to milder forms, severe astrogliosis is characterized by excessive proliferation of astrocytes. Even though mature astrocytes in healthy brain do not have the ability to proliferate, activated astrocytes can re-enter the cell cycle. Glial scar is built out of highly branched astrocytes and extracellular matrix molecules and its purpose is to isolate the impaired area from healthy tissue and to prevent the spread of the damage (Figure 2) (Huang et al., 2014).

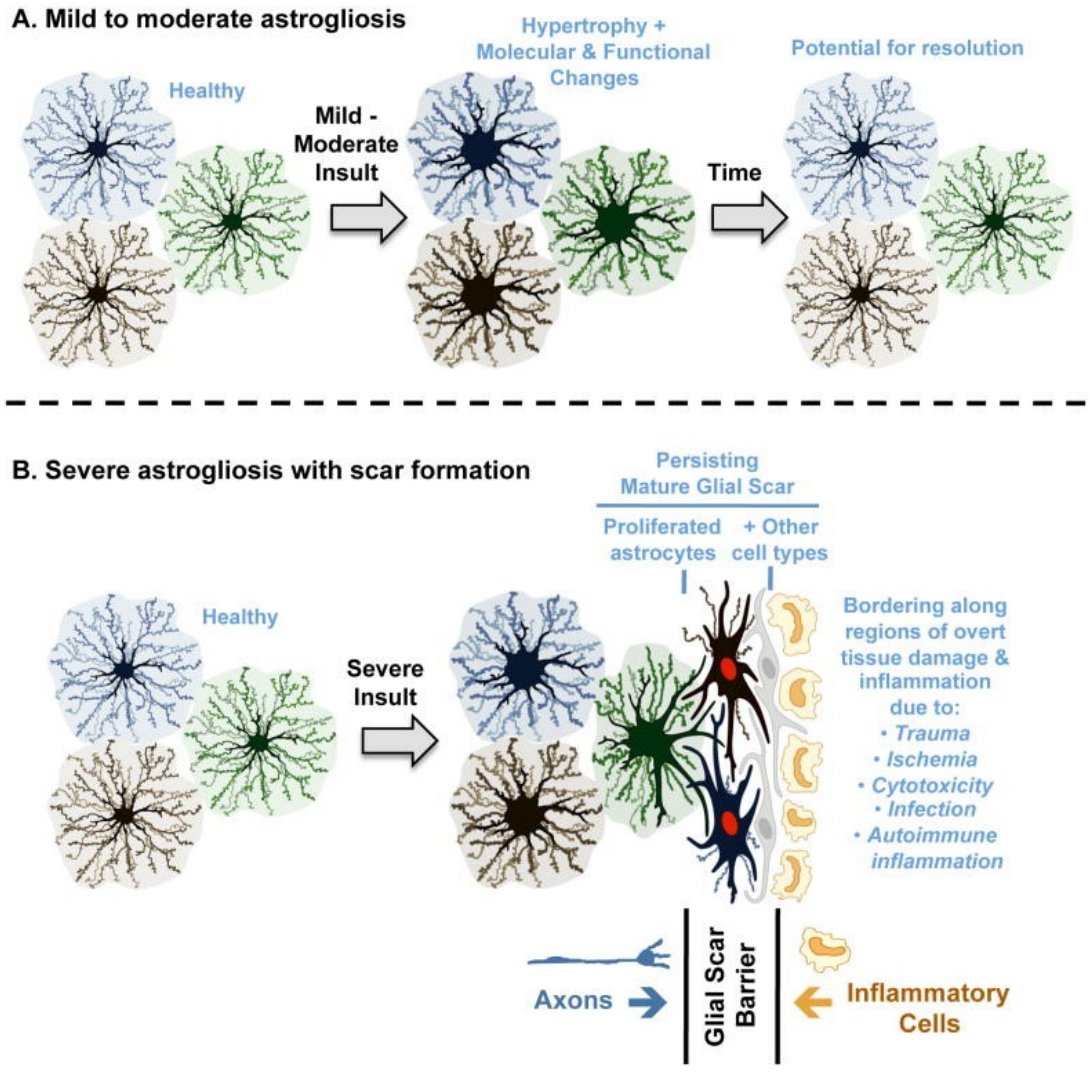

Fig. 2 Stages of astrogliosis and glial scar formation (Sofroniew, 2009)

1.2.2.2 Mediators of astrogliosis

There are plenty of signalling molecules, which can trigger astrogliosis and participate in its regulation such as cytokines and growth factors, mediators of innate immunity, molecules released by disrupted cells, reactive oxygen species (ROS) or messengers that can cause neuronal excitotoxicity (Sofroniew, 2009). These mediators can be released by immune cells, such as leucocytes or microglia, which migrate to the area of lesion, but also by injured neurons and activated astrocytes (Burda and Sofroniew, 2014). Effects of these signalling molecules can be pro-inflammatory or anti-inflammatory. The response to a signal mediated by a specific messenger can vary in a context-dependent manner and in some cases even cause opposite effects by activating different signalling pathways (Sofroniew, 2009).

Astrogliosis depends on amount and type of released signalling molecules. In the beginning there are many cytokines, which activate astrocytes and trigger astrogliosis, including IL-6, ciliary neurotrophic factor or transforming growth factor-α (Pekny and Pekna, 2016). The mediators of the later response that may lead to glial scar formation are not entirely characterized but may include endothelin 1 (Gadea et al., 2008), epidermal growth factor (Levison et al., 2000), fibroblast growth factor and ATP with its metabolites (Neary and Zimmermann, 2009).

1.2.2.3 Pathological aspects of astrogliosis

In spite of the fact that astrogliosis usually has a protective role in the fight against brain injury, under particular circumstances it can become a detrimental response, that leads to neurodegeneration. Astrocytes have many functions, which are essential in physiology and pathophysiology of CNS. During astrogliosis these functions can be disturbed and can contribute to neural dysfunction. An example is the excitotoxicity that leads to neurodegeneration as a consequence of decreased glutamate uptake (Sofroniew, 2009). Under some conditions reactive astrocytes can also cause adverse effects, such as increased expression of pro-inflammatory cytokines, release of neurotoxic concentrations of ROS and glutamate (Sofroniew and Vinters, 2010). A well-known case of detrimental effects of astrogliosis is the inhibition of axon regeneration when a

glial scar is formed. However, more recent studies have shown this is not entirely true and that glial scar can facilitate axonal regeneration (Anderson et al., 2016).

These pathological effects of severe astrogliosis can result in the development of neuropathic pain or chronic inflammation. For this reason astrogliosis may be a potential target for treating neurodegenerative diseases associated with chronic inflammation such as Alzheimer disease, multiple sclerosis or amyotrophic lateral sclerosis (Rossi, 2014).

1.3 Role of purinergic signalling in astrocytes and microglia during brain inflammation

Glial cells communicate with each other using numerous signalling molecules. Damaged cells and other cells, which are involved in the defensive response to a pathological event, release soluble messengers, such as purines and their nucleotides, that serve as mediators of the communication between glial cells during the brain inflammatory process.

Adenosine and ATP are present in the extracellular space of the healthy tissue in low concentrations. Brain injury and other acute pathological episodes, such as ischemia, trauma or infection are followed by rapid increase in the levels of adenosine, ATP and other nucleotides coming from dead or damaged cells. These messenger can modulate astrogliosis, cause phenotypic changes in microglia and their migration to the site of the lesion (Daré et al., 2007). Various neurodegenerative diseases are associated with chronic inflammation and a deeper understanding of purinergic signalling during this processes can help to find new targets for development of new drugs (Boison et al., 2011).

1.3.1 Purinergic receptors in glial cells

The purinergic receptors are a family of the membrane receptors expressed in most cells. Various subtypes of purinergic receptors have been shown to be expressed by glial cells (Chadwick and Goode, 2008). These receptors are involved in control of

their physiological and pathological functions, including cell growth, apoptosis, neurotransmission and inflammatory response (Weisman et al., 2012). Purinergic receptors can be activated by the purine nucleoside adenosine (P1 receptors) or by ATP and other adenine and uridine nucleotides (P2 receptors). P2 receptors are classified as ionotropic (P2X) and metabotropic (P2Y) receptors (Figure 3) (Chadwick and Goode, 2008).

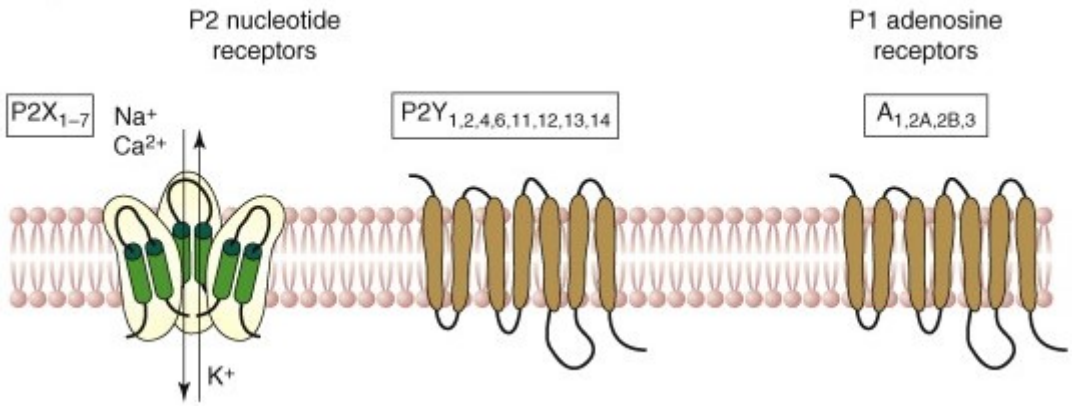

Fig. 3 Basic structure of purinergic receptors (Abbracchio et al., 2009)

1.3.1.1 P1 purinoceptors

There are four known subtypes of adenosine receptors called A_1, A_{2A}, A_{2B} and A_3. Most of them are expressed by astrocytes and microglia (Boison et al., 2011). Common feature of all P1 purinoceptors is that they belong to the family of G protein-coupled receptors and they have seven transmembrane α-helix domains as all members of this family. The N-terminal of the protein is located in extracellular side and C-terminal is on the intracellular side of the membrane (Chadwick and Goode, 2008).

Their endogenous ligand is adenosine but A_1 and A_3 can be also activated by inosine acting as partial agonist (Boison et al., 2011). The affinity of adenosine for A_1 and A_{2A} receptors is much higher compared to that for A_{2B} and A_3 receptors whose effects are manifested only during a significant increase of adenosine concentration (Lusardi, 2009). Both types of A_2 receptors couple to the G_S family and their activation leads to an increase of intercellular levels of cyclic adenosine monophosphate (cAMP) (Chadwick and Goode, 2008). The adenosine A_1 and A_3 subtypes are coupled to G_i proteins and their stimulation inhibits the formation cAMP (Boison et al., 2011) but can also induce the release of Ca^{2+} from intercellular stores. All subtypes of adenosine

receptors are coupled to mitogen-activated protein kinase (MAPK) pathways, including p38 MAPK, extracellular signal-regulated kinase 1 (ERK1) and ERK2 (Antonioli et al., 2013). These receptors can be pharmacologically characterized using selective agonists and antagonists recently developed (see Table 1).

TABLE 1 Examples of specific agonists and antagonists (Chadwick and Goode, 2008)

Receptor	Agonists	Antagonists
$\mathbf{A_1}$	*CCPA, CPA*	*DPCPX, CPX, XAC*
$\mathbf{A_{2A}}$	*CGS 21680*	*KF17837, SCH58261*
$\mathbf{A_{2B}}$	*NECA*	*Enprofylline, MRS1706*
$\mathbf{A_3}$	*DB-MECA, DBX MR, CL-IBMECA*	*MRS1222, L-268,605*

1.3.1.2 P2X purinoceptors

P2X receptors are ligand-gated cationic channel permeable for potassium, sodium and calcium. This family of receptors is responsible for faster receptor-mediated response (Franke et al., 2006). Until now seven P2X subunits ($P2X_{1-7}$) have been identified. They can form several ion channels by combinations of three subunits. After binding of ATP the channel conformation is changed resulting in its opening (North, 2016). All of these seven subunits are expressed in glial cells (Franke et al., 2006).

1.3.1.3 P2Y purinoreceptors

P2Y receptors are metabotropic receptors coupled G-proteins, which can be stimulated by adenine and/or uracil nucleotides or by sugar-nucleotides in the case of $P2Y_{14}$ receptor. Eight subtypes of mammalian P2Y receptors have been identified, $P2Y_{1,2,4,6,11,12,13,14}$, most of them being coupled to $G_{q/11}$ proteins so their activation leads to phospholipase C stimulation followed by diacylglycerol and inositol-(1,4,5)-trisphosphate formation and subsequent increase of intercellular Ca^{2+} concentration. Some of P2Y receptors are also associated with stimulation of the cAMP pathway, such

as the $P2Y_{11}$ subtype, while the $P2Y_{12,13}$ subtypes inhibit the cAMP formation. P2Y receptors can form heterooligomers with adenosine receptors. This process is considered to have an important regulatory function in the CNS (Franke et al., 2006).

1.3.2 Metabolism of adenosine and its nucleotides during the brain injury

In neurons and glial cells ATP is co-stored with neurotransmitters in vesicles and can be physiologically released by exocytosis (Zhang et al., 2007). Brain injury is accompanied by excessive release of ATP from disrupted cells through their damaged plasma membrane resulting in a rapid increase of its extracellular level in the damaged area.

Extracellular ATP undergoes fast degradation followed by the formation of other signalling molecules, including adenosine diphosphate (ADP) and adenosine. These processes are catalysed by enzymes, such as the ectonucleoside triphosphate diphosphohydrolase 1 (NTPDase 1) and ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase 2), which hydrolyse ATP to ADP and then to adenosine monophosphate (AMP), the ectonucleoside triphosphate diphosphohydrolase 3 (NTPDase 3) that metabolizes ATP directly to AMPand the 5'-nucleotidase (CD73), which catalyses the hydrolysis of AMP to adenosine (Lusardi, 2009). These enzymes are highly expressed on the cell surface of microglia whose presence in the area of lesion accelerates the metabolism of ATP causing an increase of extracellular adenosine levels (Boison et al., 2011).

Adenosine can be removed by two different metabolic pathways: deamination into inosine catalysed by enzyme adenosine deaminase (ADA) or conversion into AMP, a reaction catalysed by adenosine kinase that is highly expressed in astrocytes. Both enzymes are considered to be crucial factors in maintaining adenosine homeostasis (Boison, 2013) and their dysregulation is involved in the pathology of several disorders associated with astrogliosis. That makes these enzymes potential therapeutic targets for the development of drugs to control astrogliosis (Figure 4) (Fedele et al., 2005).

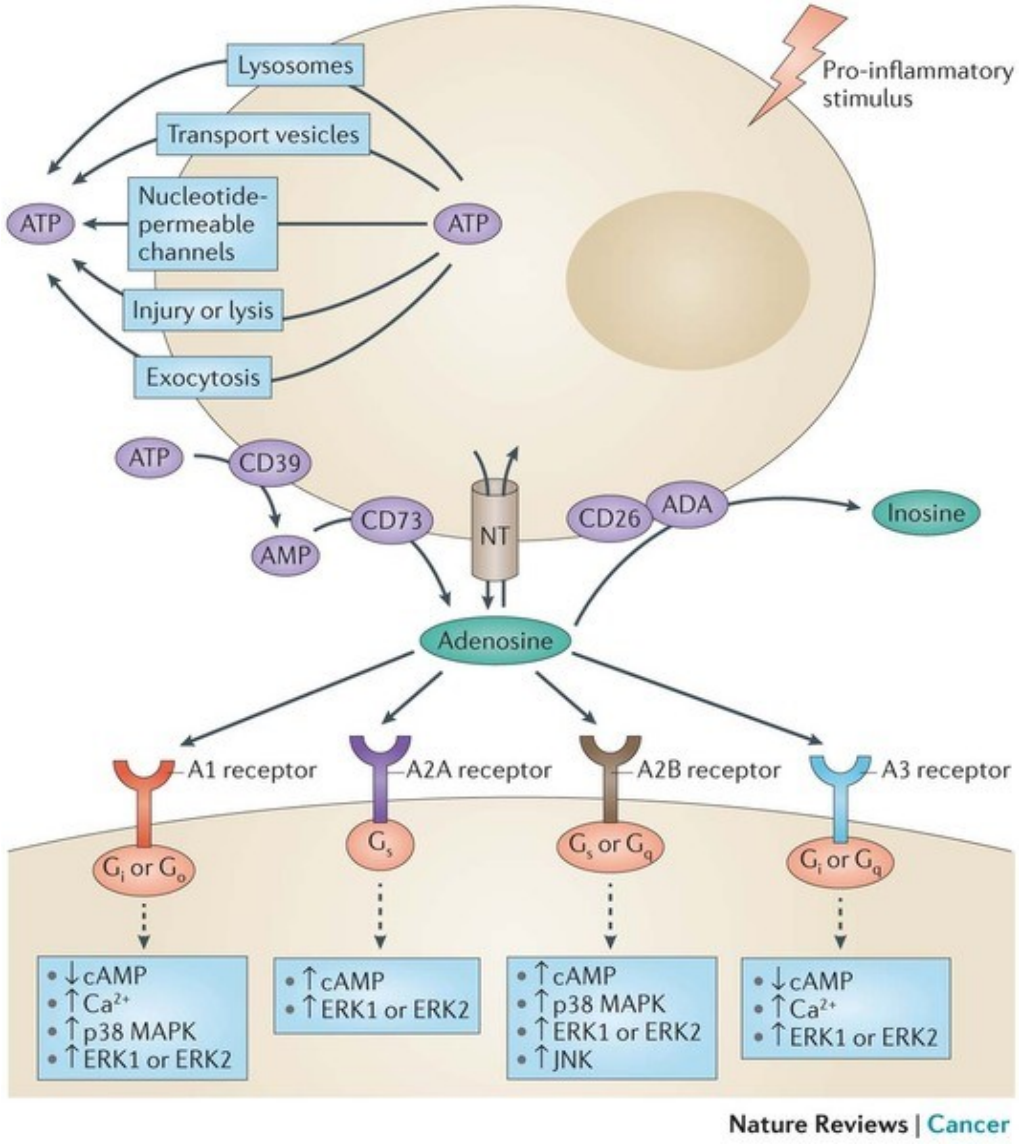

Fig. 4 Adenosine signalling during inflammation (adapted from (Antonioli et al., 2013))

1.3.3 Role of nucleotides in brain inflammation

ATP acts as a signalling molecule in neuron-glia and glia-glia communication in both physiological and pathological context. The effect of ATP is mediated by activating multiple P2 receptors. ATP is involved in response to noxious stimuli during inflammation, mostly through activation of $P2Y_2$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2X_4$ receptor subtypes (Di Virgilio et al., 2009). One of these effects is the contribution of ATP to the reactive gliosis. Overstimulation of immune system by ATP and changes in the expression of its receptors can have cytotoxic consequences and may contribute to pathogenesis of neurodegenerative diseases (Franke et al., 2006).

During the early-phase of the brain injury the extracellular levels of ATP, UTP and their metabolites are elevated for several hours and serve as warning signals. ATP and ADP induce chemotactic migration of microglia to the site of damage by activating $P2Y_{12}$ and $P2X_4$ receptors (Ohsawa et al., 2007). In the meanwhile UDP is a "eat me" signal that facilitates phagocytosis of dead cells through activation of $P2Y_6$ receptors in microglia (Inoue).

Activation of $P2Y_1$ receptors expressed in astrocytes causes an increase in its proliferation, however in presence of microglia this effect is attenuated or abolished. This suggests that there is a regulatory mechanism, which is probably mediated by a soluble messenger, whereby microglia protects the neuronal tissue (Quintas et al., 2011b).

1.3.4 Role of adenosine in brain inflammation

The concentration of extracellular adenosine in the site of damage in the later stage of brain inflammation is elevated as a result of ATP breakdown. By stimulation of A_1, A_{2A}, A_{2B} and A_3 receptors adenosine controls a variety of pathological responses of neurons and glial cells. Adenosine can affect glial proliferation and morphological changes, induce the release of inflammatory mediators and even cell death. The effect of adenosine depends on the subtype of P1 receptor activated (Haskó et al., 2005). In general, A_1 and A_3 subtypes are considered to have neuroprotective effects, whereas the A_{2A} subtype seems to increase the inflammatory response.

The A_1 receptor is highly expressed in physiological conditions. Its activation has been reported to reduce cell proliferation in astrocyte cultures (Ciccarelli et al., 1994). This subtype also facilitates neuronal growth and survival by inducing secretion of nerve growth factor (NGF) by astrocytes (Ciccarelli et al., 1999). In acute brain injury, immediately after harmful stimuli, the A_1 receptor protects against the excessive release of glutamate and other excitatory neurotransmitters by neurons and other cells. At the same time it hyperpolarize the neurons preventing the generation of action potentials (de Mendonça et al., 2000). In microglial cells the A_1 receptor inhibits excessive microglial activation, which can also influence, indirectly, the proliferation of astrocytes (Tsutsui et al., 2004).

In physiological conditions the A_{2A} receptors expressed in astrocytes and microglia are below the detection limit. Trauma, ischemia or inflammation are usually followed by upregulation of A_{2A} receptors (Svenningsson et al., 1999) that stimulate reactive gliosis, promoting astrocytes proliferation (Ciccarelli et al., 1994) and elevating extracellular glutamate concentration stimulating its release from astrocytes (Tsutsui et al., 2004). On the other hand, expression of inducible nitric oxide synthase in astrocytes is reduced by A_{2A} activation in astrocytes. This enzyme catalyses the production of nitric oxide (NO), that contributes to the pathophysiology of some neurodegenerative diseases (Brodie et al., 1998).

The main effect of A_{2A} receptor in microglia is an increase in secretion of some pro-inflammatory substances such as NO (Saura et al., 2005). Also, the expression of cyclooxygenase-2 (COX-2) is up-regulated upon activation of A_{2A} receptors, followed by higher synthesis of prostaglandin E_2 (Fiebich et al., 1996).

The A_{2B} receptor is the only subtype of adenosine receptors, which was not detected in microglial cells. In primary astrocytes it mediates glycogenolysis and enhances glycogen synthesis (Daré et al., 2007). There is also some evidence that adenosine can induce release of IL-6 via activation of A_{2B} receptor in astrocytes (Vazquez et al., 2008).

Stimulation of the A_3 receptor by adenosine during severe metabolic stress induced apoptosis of astrocytes in previous studies (Abbracchio et al., 1997). This process can be helpful when cells are irreversibly injured. The elimination of damaged cells can save energy resources for the less damaged tissue (Haskó et al., 2005). Astrocytic A_3 receptors also stimulate secretion of chemokine ligand 2, which has neuroprotective functions (Wittendorp et al., 2004).

2 AIMS OF STUDY

ATP and other nucleotides modulate the inflammatory response by triggering astrogliosis, a local defence mechanism observed in the brain under pathological states such hypoxia, trauma and neurodegenerative diseases.

The effect of ATP and its analogues on astrogliosis is highly dependent on their metabolism by ectonucleotidases that is increased by the presence of microglia resulting in the formation of high levels of adenosine that also modulates astrogliosis. However, the effects of adenosine receptors on the modulation of astrogliosis and communication between astrocytes and microglia during brain inflammation are poorly understood.

This study aims to investigate the contribution of microglia to the modulation of astroglial proliferation, observed during astrogliosis, mediated by P1 receptors.

3 METHODS AND MATERIALS

3.1 Materials

The following drugs have been used: 2-Chloro-N^6-(3-iodobenzyl)-adenosine-5′-N-methyluronamide (CL-IB-MECA), 2-*p*-(2-Carboxyethyl)phenethylamino-5′-N-ethylcarboxamidoadenosine hydrochloride hydrate (CGS 21680), N^6-cyklopentyladenosine (CPA), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH 58261), N-[2-(p-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H-89), bisindolylmaleimide XI hydrochloride (RO 32-0432), 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene monoethanolate (U0126), 2‘-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5‘-bi-1H-benzimidazole trihydrochloride hydrate (Hoechst 33258), adenosine, penicillin and streptomycin from Sigma-Aldrich (Sintra, Portugal); *N*-(4-Acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS 1706) from Tocris (Bristol, UK); methyl-[^{3}H]-thymidine (specific activity 80–86 Ci/mmol) and enhanced chemiluminescence Western blotting system from Amersham Biosciences (Lisbon, Portugal); Stock solutions were prepared with distilled water and kept at -20°C.

Used antibodies are shown in Table 2 (p.29).

3.2 Cell cultures

Animal handling and experiments were conducted according to the guidelines of the Directive 2010/63/EU of the European Parliament and the Council of the European Union. Two types of primary cell cultures were prepared: co-cultures of astrocytes and microglia and highly enriched cultures of astrocytes (named astrocyte cultures), which were obtained from co-cultures by eliminating most of the microglial cells by the procedure described below. In both types of cultures, astrocytes were the main cell type, but the number of microglia present differed between the two types of cultures.

Primary cortical co-cultures of astrocytes and microglia and cultures of astrocytes were prepared from newborn (age: 0-2 days) Wistar rats (Charles River, Barcelona, Spain) as previously described (Queiroz et al., 1997). The brains were placed in ice-cold Dulbecco's phosphate buffered calcium free saline solution (DPBS) containing 0.2% glucose. The hemispheres were free of meninges and large blood vessels and after washing twice with ice-cold DPBS, they were cut into small pieces in culture medium, that is, Dulbecco's modified Eagle medium containing 3.7 g/L $NaHCO_3$, 1.0 g/L D-glucose and stable glutamine, supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin. Tissue from the two hemispheres was dissociated by triturating in 10 ml culture medium. The cell suspension obtained was passed through a 40 µm pore nylon mesh and then centrifuged at 200g for 5 min and the supernatant was discharged. Centrifugation followed by resuspension was repeated twice and the pellet obtained was suspended in culture medium supplemented with 10% foetal bovine serum (FBS) and seeded at a density of $2x10^5$ cells/ml. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air, 5% CO_2 and the medium was replaced one day after preparation and subsequently twice a week. After 3 weeks of culturing highly enriched astroglial cultures were obtained from confluent co-cultures, which grown attached in monolayer in $75cm^2$ flasks, by removing microglia and cell passaging. After shaking co-cultures overnight at 200 rpm, for removing microglia, culture medium was cleared away and confluent cells were washed twice with DPBS. Then, cultures were incubated with trypsin 0.05% and ethylenediamine tetraacetic acid 0.2% (pH=7.4) for about 5 min at 37°C and cells were released into the medium. Trypsin was then neutralized by adding culture medium containing 10 % FBS, which neutralizes the proteolytic action of trypsin. The cell suspension obtained was transferred to a falcon tube and centrifuged at 800g for 3 min at 4°C. After centrifugation, supernatant was discharged, new medium was added and the cycle of cell suspension followed by centrifugation was repeated twice. The pellet obtained in the final centrifugation was suspended in culture medium containing 10% FBS and cell suspension was seeded at a density of $5x10^5$ cells/ml. The culture medium was changed one day after preparation and then twice a week. Cultures of highly enriched astrocytes were allowed to grow for 3 days to form a monolayer before experimental procedures were undertaken. The cultures obtained were characterized by immunocytochemistry and 99% of the cells identified as astrocytes.

Before experiments both types of culture were synchronized to a quiescent phase of the cell cycle, by shifting serum containing 0.1% FBS and incubating for 48 h.

3.3 Immunocytochemistry

Cell cultures (co-cultures and astrocyte cultures), grown in 4 well glass slides, were fixed with a solution containing 4% formaldehyde and 4% sucrose in phosphate buffered saline (PBS) (100 mM) and then treated with PBS (100 mM) containing 0.3% Triton X-100. For double-labelling astrocytes and microglia, cultures were incubated with the primary antibodies rabbit anti-GFAP (1:600) and mouse anti-CD11b (1:50), overnight at 4°C. Visualization of GFAP and CD11b positive cells was accomplished upon 1 h incubation at room temperature in the absence of light with the secondary antibodies anti-rabbit IgG conjugated to crystalline tetramethylrodamineisothiocyanate (TRITC; 1:100) and anti-mouse IgG conjugated to Alexa Fluor 488 (1:200; Table 2). In negative controls, the primary antibody was omitted. Cell nuclei were labelled with Hoechst 33258 (5 μg/ml) for 30 min at room temperature. To evaluate the percentage of microglia, the two types of cultures were processed in parallel and about 200 cells were counted in each culture. The number of CD11b-positive cells was expressed as percentage of the total number of cells counted. Images were captured with a Digital Sight DS-5Mc camera (Nikon, Japan) coupled to an Eclipse E400 fluorescence microscope (Nikon, Japan).

3.4 Proliferation assay

Influence of adenosine receptors on cell proliferation of co-cultures and astrocyte cultures was evaluated by measuring of the incorporation of radiolabelled DNA precursor methyl-[^{3}H]-thymidine.

Confluent cultures grown in 24-well plates were synchronized and incubated with P1 agonists or solvent for 48 h. In experiments with antagonists or enzyme inhibitors, these were added to the cultures 1 h before the agonists. In the last 24 h methyl-[^{3}H]-thymidine was added at a concentration of 1 μCi/ml. Cells were then rinsed with PBS,

fixed with 10% of trichloroacetic acid (TCA) for 30 min at 4°C, washed with ice-cold 5% TCA and rinsed again with PBS. Protein content and methyl-[^{3}H]-thymidine incorporation were evaluated after cell lysis with 0.2 M NaOH. The amount of tritium incorporated was quantified by liquid scintillation spectrometry (Beckman LS 6500, Beckman Instruments, Fullerton, USA) and normalized by protein content evaluated by the Bradford method (Bradford, 1976).

3.5 Western blot

Cells were rinsed with ice-cold PBS and total cell protein was extracted in lysis buffer with protease inhibitors (1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 2 µg/ml aprotinin and 2 µg/ml leupeptin). After a brief sonication (10 s), the lysate was incubated on ice for 1 h and then centrifuged at 20.000g for 45 min at 4°C. The protein concentration was determined in collected supernatant and proteins were denatured through boiling at 95°C for 5 min in 6X sample buffer (0.35 M Tris–HCl at pH 6.8, 4% sodium dodecyl sulfate (SDS), 30% glycerol, 9.3% dithiothreitol and 0.01% bromophenol blue) with 5% mercaptoethanol.

Samples (80µg of protein) were loaded into 10% precast polyacrylamide gel and electrophoresed at 125 V for 1 h 20 min with running buffer (25 mM Tris base, 250 mM glycine, 0.1% SDS; pH 8.3). Proteins were electro-transferred onto polyvinylidene difluoride membrane for 40 min at 100 V in a transfer buffer (25mM Tris base, 200 mM glycine, 0.037% SDS, 20% methanol). Antibodies in appropriate dilution were used for immunoblotting (Table 2). First, membranes were blocked for 1 h at 4°C with 5% of non-fat dry milk in phosphate buffered saline with tween (0.1% Tween 20 in PBS pH 7.4) and then incubated overnight at room temperature with primary polyclonal antibody: rabbit anti-A_{2A} (1:500) followed by secondary antibody conjugated to horseradish peroxidase (1:5000). Proteins were detected with Novex ECL detection kit (Invitrogen® #WP20005, USA). Subsequently, membranes were reprobed with the primary polyclonal antibody rabbit anti-tubulin (1:1000) for 1 h at room temperature, followed by the secondary antibody. Immunocomplexes were detected by enhanced chemiluminescence. The A_{2A} protein levels were expressed in arbitrary density units,

performed by densitometry analysis using Image Lab software (version 5.2.1; Bio Rad, USA) and the total A_{2A} protein expression was normalized to tubulin.

TABLE 2 Antibodies used in immunocytochemistry and Western blotting

Primary antibodies				
Antigen	**Code**	**Host**	**Dilution**	**Supplier**
A_{2A}	AB3461	Rabbit	1:500	Abcam (Cambridge, USA)
Tubulin	AB4074	Rabbit	1:1000	Abcam (Cambridge, USA)
GFAP	G9269	Rabbit	1:600	Sigma-Aldrich (Sintra, Portugal)
CD11b	sc-53086	Mouse	1:50	Santa Cruz Biotechnology (Santa Cruz, USA)
Secondary antibodies				
Antigen	**Code**	**Host**	**Dilution**	**Supplier**
anti-rabbit conjugated to HRP	sc-2004	Goat	1:5000	Santa Cruz Biotechnology (Santa Cruz, USA)
TRITC anti-rabbit	T6778	Goat	1:100	Sigma-Aldrich (Sintra, Portugal)
Alexa fluor 488 anti-mouse	A-11034	Goat	1:200	Molecular Probes (Eugene, USA)

3.6 Statistical analysis

Data are expressed as means ± standard errors of the mean (SEM) unless otherwise stated. Statistical analysis was carried out using the unpaired Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. The Western blot data analysis was performed by paired t-test. P values lower than 0.05 were considered to indicate significant differences.

4 RESULTS AND DISCUSION

In the previous studies ATP increased astrocytes proliferation by activation of $P2Y_1$ receptors in pure cultures of astrocytes but not in co-cultures. This effect was partially attenuated by ADA an enzyme that metabolises adenosine and by antagonists of A_{2A} and A_{2B} receptors (Figure 5) (Quintas et al., 2011a).

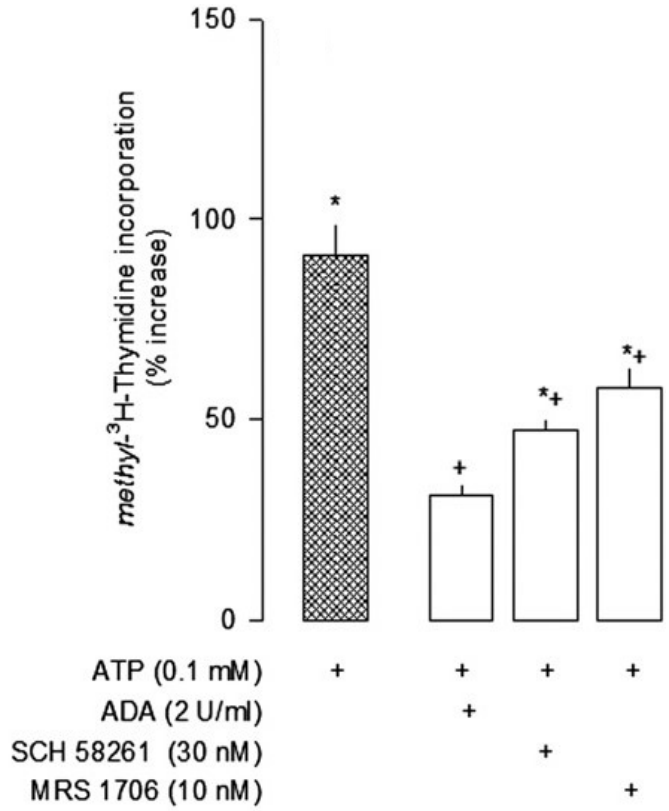

Fig. 5 Pharmacological characterization of adenosine receptors involved in astroglial proliferation induced by ATP in cultures. Cultures of astrocytes were incubated with ATP for 48 h and in the last 24 h methyl-[^{3}H]-thymidine was added to the medium at a concentration of 1 μCi/ml. Adenosine receptor antagonists and ADA were added to the medium 1 h before ATP. Cell proliferation was estimated by methyl-[^{3}H]-thymidine incorporation and expressed as percentage of increase from the respective control. Values are means ± SEM from 7 experiments. *P<0.05, significant differences from the respective control; +P<0.05, significant differences from ATP alone (adapted from Quintas et al. (2011a)).

These results showed that part of the effect of ATP on astroglial proliferation is probably mediated also by its metabolite, adenosine, acting on A_{2A} and A_{2B} receptors. Therefore we decided to explore this mechanism and find out if there is also some regulatory mechanism mediated by microglia (Quintas et al., 2011a).

4.1 Cell cultures characterization

Both types of cell cultures were characterized by immunocytochemical methods and the percentage of microglia was determined. Astrocytes and microglia were visualized by double labelling of astrocytes (GFAP positive cells, shown in red) and microglia (CD11b positive cells, shown in green; Figure 6).

Co-cultures contained about 15% of microglia, a proportion similar to that found in physiological conditions in the brain. Highly enriched astroglial cultures were almost pure with less than 1% of microglia.

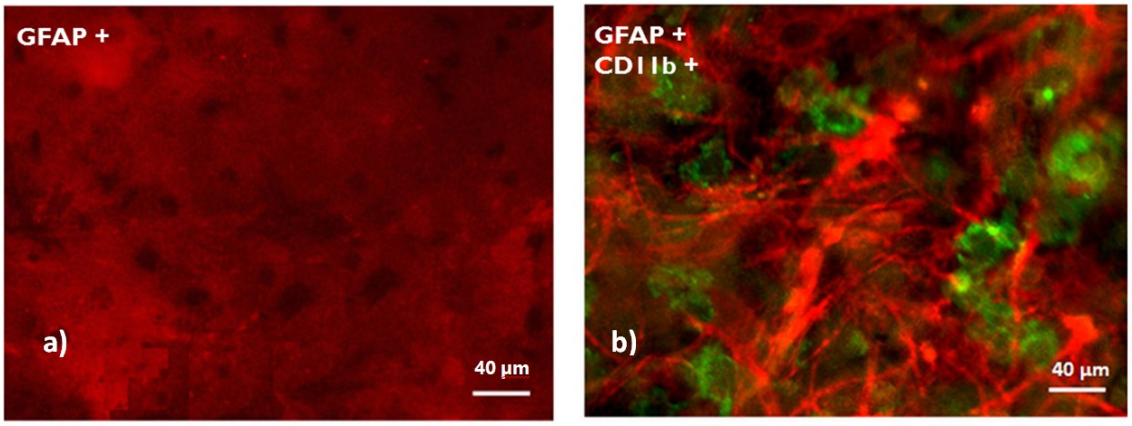

Fig. 6 Characterization of primary astroglial cultures containing different percentage of microglia. Astrocytes were labelled with anti-GFAP (TRITC, red) and microglia with anti-CD11b (Alexa Fluor 488, green). Representative immunofluorescent micrographs of the two types of cultures: astrocyte cultures (a) and co-cultures of astrocytes and microglia (b), double-labelled for GFAP and CD11b.

4.2 Effects of adenosine and A_{2A} receptors activation on cell proliferation

Both types of cell cultures were incubated either with adenosine or selective agonists of A_1, A_{2A} and A_3 adenosine receptors subtypes. In astroglial cultures both adenosine (0.001-0.1 mM) and the selective agonist of A_{2A} receptor CGS 21680 (1-100 nM) caused a concentration dependent increase in cell proliferation (Figure 7), an effect that was significantly attenuated in co-cultures of astrocytes and microglia.

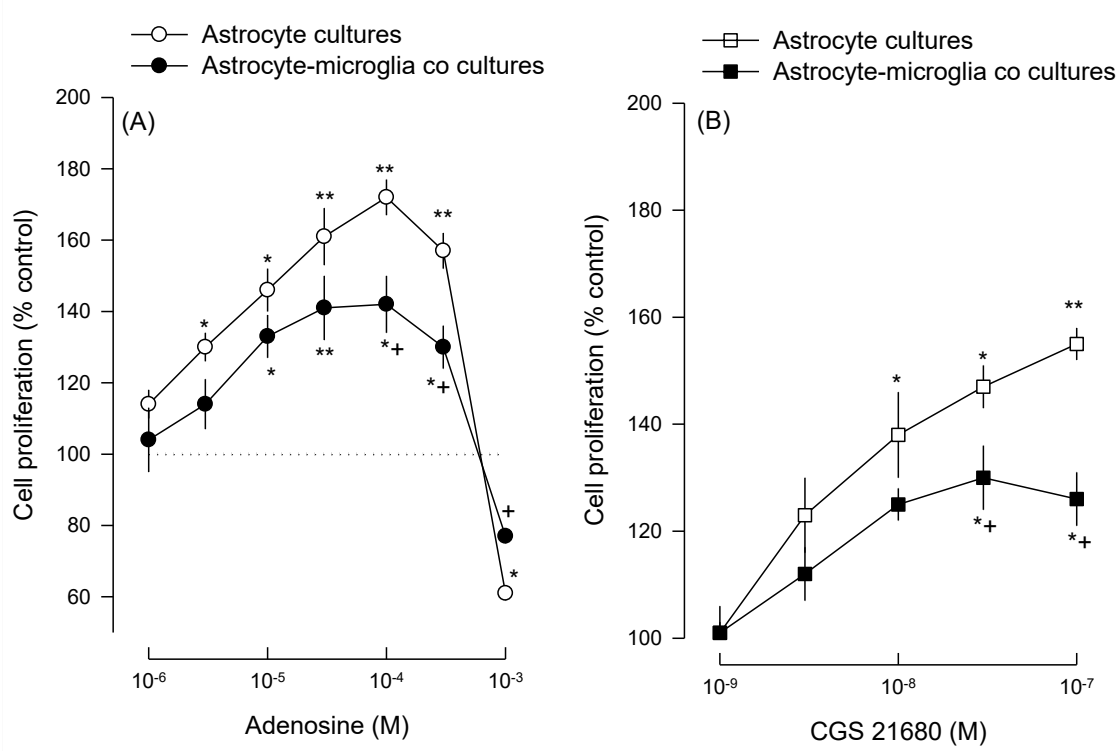

Fig. 7 Modulation of astroglial proliferation in astrocyte cultures and co-cultures of astrocytes and microglia by the P1 receptor agonists (A) adenosine and (B) the selective adenosine A_{2A} receptor agonist CGS 21680. Cultures were incubated with agonists or solvent for 48 h and methyl-[^{3}H]-thymidine (1 μCi/ml) was added in the last 24 h. Cell proliferation was estimated by methyl-[^{3}H]-thymidine incorporation and expressed in percentage of control (100%). Values are means ± SEM from 3-4 different cultures (each tested in triplicate). *P<0.05, significant differences from control (solvent). +P<0.05, significant differences between astrocyte cultures and co-cultures.

These results demonstrate that the proliferative effect of adenosine in astrocyte cultures is partly mediated by activation of A_{2A} receptors and suggest that there is a regulatory mechanism by which microglia control astroglial proliferation mediated by adenosine and A_{2A} receptors. A possible explanation could be faster adenosine degradation in the presence of microglial cells because of enzymes in their cell membrane, but the results do not support this explanation, since a similar result was obtained with the stable agonist CGS 21680, which is resistant to metabolism.

Another possibility could be a lower expression of A_{2A} receptors in co-cultures compared to cultures of astrocytes. The receptor was detected in both types of cultures by Western blot and there was not a significant difference in the expression of these receptors in both types of cultures (Figure 8).

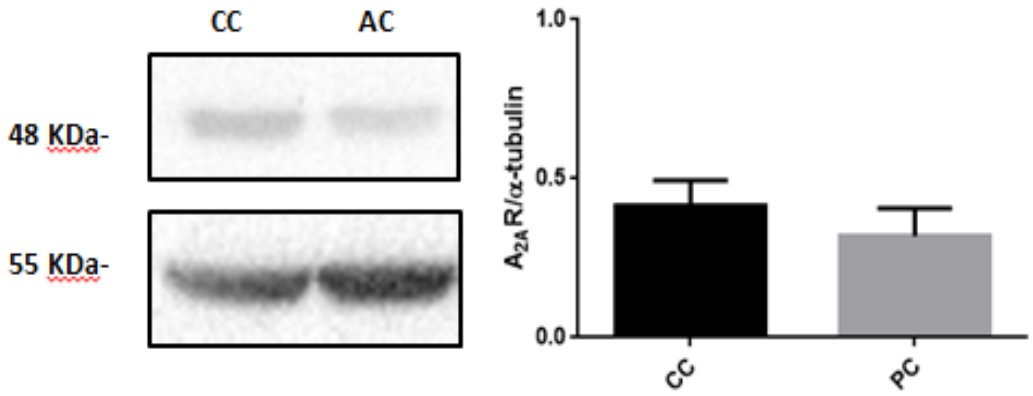

Fig. 8 Representative Western blots and quantification of A_{2A} receptors and a-tubulin expression in astrocytes-microglia co-cultures (CC) and in astrocyte cultures (PC); Total A_{2A} receptors and α-tubulin (loading control) were obtained from the whole cell lysates; One immunoreactive band of ~44 kDa specifically reacted with rabbit anti-A_{2A} antibody; n = 4 independent cell cultures.

Most likely the regulatory mechanism is mediated by soluble messengers, produced by microglia after adenosine receptor stimulation, that decrease astroglial proliferation. One candidate can be NO, which has been reported to be released by activated microglia after A_{2A} receptor activation (Saura et al., 2005).

4.3 Effects of A_1 and A_3 receptors activation on cell proliferation

In both types of cultures the proliferative effect decreased to levels lower than the control at higher concentrations of adenosine (0.3-1 mM; Figure 7). High concentrations of adenosine probably activate other adenosine receptors with opposite effect on the astrocytes proliferation, such as A_1 and A_3 receptors. In our hands neither the A_1 nor the A_3 receptors selective agonists changed cell proliferation either in astrocyte cultures or in co-cultures (Table 3).

TABLE 3 Astroglial proliferation: effect of selective A_1 and A_3 receptor agonists.

P1 receptor agonists	nM	Cell proliferation (% control)	
		Astrocyte Cultures	Co-cultures
Solvent		100 ± 6 (4)	100 ± 6 (3)
CPA	1	100 ± 3 (4)	101 ± 4 (3)
	3	101 ± 2 (4)	104 ± 3 (3)
	10	102 ± 4 (4)	104 ± 5 (3)
	30	101 ± 1 (4)	109 ± 4 (3)
	100	100 ± 3 (4)	106 ± 3 (3)
CL-IBMECA	1	101 ± 1 (4)	104 ± 2 (3)
	3	107 ± 2 (4)	108 ± 3 (3)
	10	108 ± 2 (4)	109 ± 2 (3)
	30	106 ± 3 (4)	110 ± 1 (3)
	100	108 ± 4 (4)	110 ± 7 (3)

Cultures were incubated with agonists or solvent for 48 h and methyl-[^{3}H]-thymidine (1 μCi/ml) was added in the last 24 h. Cell proliferation was estimated by methyl-[^{3}H]-thymidine incorporation and expressed in percentage of control (100%). Values are means ± SEM from 3-4 different cultures (each tested in triplicate). There were no significant differences.

These results are in contrast with our expectations because in previous studies A_1 receptor showed the ability to reduce adenosine mediated astrocyte proliferation and A_3 receptor induced cell death. The antagonist of adenosine A_1 receptor PACPX enhanced astroglial proliferation mediated by adenosine or guanosine (Rathbone et al., 1991). A_3 receptor activated by IB-MECA caused apoptosis of astrocytes (Abbracchio et al., 1997). Therefore more investigation is necessary to understand the role of A_1 and A_3 receptors in astrogliosis.

4.4 Pharmacological characterization of the receptors involved in adenosine mediated cell proliferation

The pharmacological characterization of the adenosine receptor subtypes involved in proliferation of astrocytes have been performed using selective antagonists of A_{2A} and also A_{2B} receptors. Astrocyte cultures were pre-incubated with adenosine and then the selective antagonist of A_{2A} receptor SCH58261 (30 nM) or the selective antagonist of A_{2B} receptor MRS1706 (10 nM) was added.

According to the expectations both antagonists attenuated astroglial proliferation induced by adenosine (Figure 9). These results confirm that the proliferative effect is mediated by A_{2A} receptors and also by A_{2B} receptors.

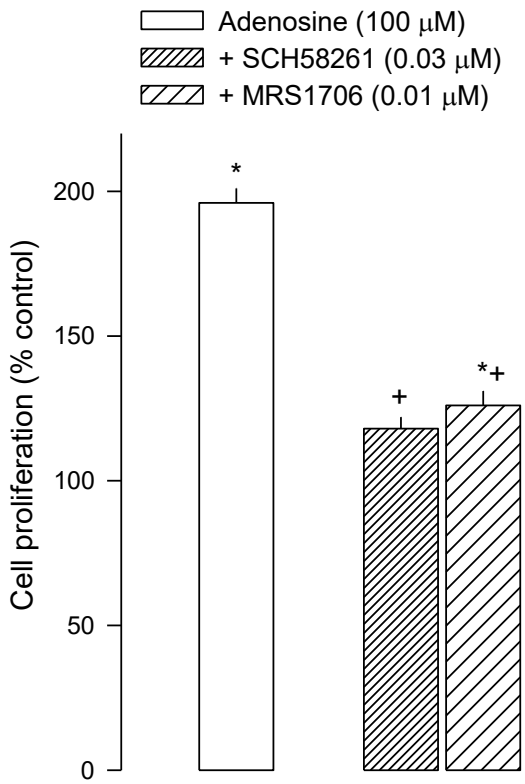

Fig. 9 Pharmacological characterization of P1 receptors involved in astroglial proliferation induced by adenosine. Astrocyte cultures were incubated with adenosine for 48 h and in the last 24 h methyl-[^{3}H]-thymidine was added to the medium at a concentration of 1 μCi/ml. The selective antagonists of A_{2A} receptors SCH58261 and of A_{2B} receptors MRS1706 were added to the medium 1 h before adenosine. Cell proliferation was estimated by methyl-[^{3}H]-thymidine incorporation and expressed as percentage from the respective control (100%). Values are means ± SEM from 3 different cultures (each in tested in triplicate). *P<0.05, significant differences from the respective control (solvent); +P<0.05, significant differences from adenosine alone.

4.5 Identification of signalling pathway involved in astroglial proliferation

For deeper understanding of molecular mechanism of adenosine mediated astrogliosis, the signalling pathways involved were investigated. In this set of experiments, inhibitors of the intracellular signalling pathways, known to be involved in the effect of A_{2A} and A_{2B} receptors, were used (Ciccarelli et al., 1994). The influence of PKA inhibitor H-89 (1 μM), the mitogen-activated protein kinase kinase 1/2 inhibitor U0126 (10 μM) and the protein kinase C (PKC) inhibitor RO 32-0432 (1 μM) has been tested on the adenosine proliferative effect (Figure 10).

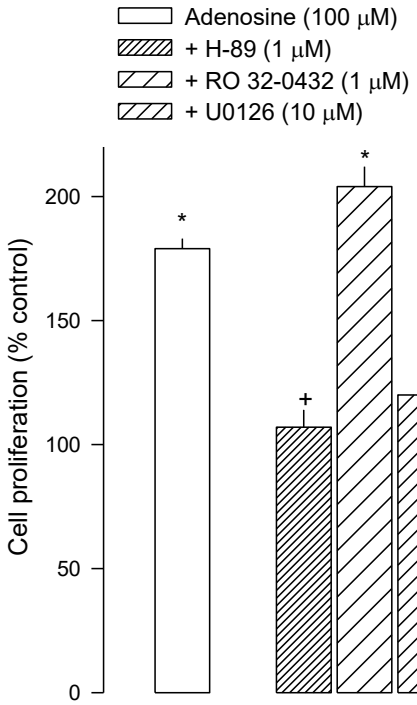

Fig. 10 Signalling pathways of P1 receptors involved in astrocyte proliferation induced by adenosine. Astrocytes cultures were incubated with adenosine for 48 h and in the last 24 h methyl-[^{3}H]-thymidine was added to the medium at a concentration of 1 μCi/ml. The inhibitors H-89, U0126 and RO 32-0432, were added to the medium 1 h before adenosine. Cell proliferation was estimated by methyl-[^{3}H]-thymidine incorporation and expressed as percentage from the respective control (100%). Values are means ± SEM from 3 different cultures (each in tested in triplicate). *P<0.01, significant differences from the respective control (solvent); +P<0.05, significant differences from adenosine alone.

Adenosine induced cell proliferation was attenuated by H-89 and U0126. These results suggest that the proliferative effect is mediated by cAMP-dependent pathway coupled to G_s-protein and ERK pathway. The compound RO 32-0432 did not change cell proliferation induced by A_2 receptors excluding the involvement of PKC in signalling of adenosine-mediated astroglial proliferation.

5 CONCLUSION

The main aim of this work was to investigate the influence of microglia on astroglial proliferation induced by adenosine and specific agonists of P1 receptors. We focused more on the A_{2A} receptor.

The experiments were done in astroglial cultures and co-cultures of astrocytes and microglia to evaluate whether microglia cells could have any influence on adenosine mediated astrogliosis. These cultures were characterized by immunocytochemistry and expression of A_{2A} receptors was evaluated in both types of cultures by Western blot.

The P1 receptors and the signalling pathways involved in the adenosine proliferative effect were also evaluated by using selective antagonists and inhibitors, respectively.

Both, adenosine and the selective agonist of A_{2A} receptor CGS 21680, caused a concentration dependent increase in proliferation of astroglial cultures. The selective agonists of A_1 and A_3 receptors did not affect the cell proliferation. The role of a selective A_{2B} receptor agonist was not investigated directly, but results show that astroglial proliferative effect of adenosine is mediated by both A_{2A} and A_{2B} receptors.

Adenosine induced proliferation is mediated by the cAMP-dependent and ERK signalling pathways, which are involved in transduction mechanism of A_2 receptors and carry the signal leading to astrogliosis.

The decrease in the proliferative effect of adenosine and CGS 21680 observed in the co-cultures suggests, that activation of A_{2A} receptors in microglia might be involved in controlling astroglial proliferation induced by adenosine, possibly by releasing soluble messengers.

Although the model of co-cultures allows the investigation of the communication between astrocytes and microglia, other types of cells and molecules can affect this process. More research in this field is necessary to deeper understanding of the role of adenosine in the modulation of astrogliosis and the contribution of microglia to the process.

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