Purinergic control of neuroinflammation and

neuroprotection by the blockade of P2 receptors

under excitotoxic conditions in the hippocampus

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Universidade de Coimbra

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Purinergic control of neuroinflammation and neuroprotection afforded by the blockade of P2 receptors under excitotoxic conditions in the hippocampus

Controlo da neuroinflamação pelo sistema purinergico e neuroprotecção conferida pelo bloqueio de receptores P2 em condiçoes excitotóxicas no hipocampo

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Abreviations List

$[Ca^{2+}]$ - Free intracellular calcium concentration

2-MeSATP - 2-methylthio-ATP

A_1R - A_1 receptor

$A_{2A}R$ - A_{2A} receptor

$A_{2B}R$ - A_{2B} receptor

A_3R - A_3 receptor

Ach - Acetylcholine

ACSF - Artificial cerebrospinal fluid

ADP - Adenosine-5'-diphosphate

AMP - Adenosine-5'-monophosphate

AMPA - α-amino-3-hidroxi-5-methyl-4-isoxazolopropionic acid

AP - Alkaline phosphatase

ATP - Adenosine-5'-triphosphate

Aβ - β-amyloid

BBG - Brilliant blue G

BSA - Bovine serum albumin

BzATP - 2'-3'-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate

cAMP - Cyclic adenosine-5'-monophosphate

CAPS - 3-(Cyclohexylamino)-1-propanesulfonic acid

CCPA - 2-chloro-N^6-cyclopentyladenosine

CGS21680 - 4-[-2[[6-amino-9-(N-ethyl-b-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzenepropanoic acid

Cl-IB-MECA - 2-chloro-N6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide

CNS - Central nervous system

CPA - N^6-cyclopentyladenosine

DIV - Days *in vitro*

DMSO - Dimethylsulphoxide

DNA - Desoxyribonucleic acid

DPCPX - 1,3-dipropyl-8-cyclopentyladenosine

DTT - Dithiothreitol

EC_{50} - Concentration of a ligand eliciting 50% of the maximal response

Ecto-5'-NT - Ecto-5'-nucleotidase

ABREVIATIONS LIST

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid

E-NPP - Ecto-nucleotide pyrophosphatase

E-NTPDases - Ecto-nucleoside triphosphate diphosphohydrolases

ERK - Extracellular signal-regulated kinases

Extra - Extrasynaptic

GABA - γ-aminobutyric acid

GFAP - Glial fibrillary acidic protein

HEK293 - Human embryonic kidney 293 cells

HEPES - N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

IC$_{50}$ - Concentration of a ligand that causes 50% of the maximal inhibition

ICV - Intracerebroventricular

IL-1β - Interleukin 1-β

IP - Intraperitoneal

IP3 - 1,4,5-inositol triphosphates

JNK - c-Jun amino-terminal kinases

KA - Kainate

Kainate - 2-carboxy-4(1-methylethenyl)-3-pirrolidiacetic acid

K$_D$ - Dissociation constant

KW6002 - (E)-1,3-diethyl-8-(3,4-dimethoxystyryl)-7-methyl-3,7-dihydro-1H-purine-2,6-dione

LDH - Lactate dehydrogenase

LUF5835 - 2-amino-4-(3-hydroxyphenyl)-6-(1H-imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile

LTP - Long-term potentiation

MAP-2 - Microtubules-associated protein 2

MAPK - Mitogen-activated protein kinase

MCAO - Medial cerebral artery occlusion

mEPSP - Miniature excitatory postsynaptic potentials

mIPSC - Miniature inhibitory postsynaptic current

MRE 2029-F20 - N-benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydro-1H-purin-8-yl)-1-methyl-1H-pyrazol-3-yloxy]acetamide

mRNA - Messenger ribonucleic acid

MRS1754 - N-4(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide

MRS2179 - 2'-deoxy-N^6-methyladenosine-3',5'-biphosphate

MRS2500 - (1R*,2S*)-4-[2-Iodo-6-(methylamino)-9H-purin-9-yl]-2(phosphonooxy) bicyclo[3.1.0] hexane-1-methanol dihydrogen phosphate ester tetraammonium salt

MTLE - Mesial temporal lobe epilepsy

MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NECA - adenosine-5'-N-ethylcarboxamide

NeuN - Neuronal nuclei marker

NF023 - 8-(benzamido) naphthalene-1,3,5-trisulfonate

NMDA - N-methyl-D-aspartate

P1R - P1 receptor

P2R - P2 receptor

P2XR - P2X receptor

P2YR - P2Y receptor

PBS - Phosphate buffered saline medium

PI - Propidium iodide

PKC - Protein kinase C

PLC - Phospholipase C

PMSF - Phenylmethanesulfonylfluoride

PNS - Peripheral nervous system

Post - Postsynaptic

PPADS - Pyridoxal phosphate-6-azo(benzene)-2,4-disulfonic acid

Pre - Presynaptic

PSD-95 - Post-synaptic density protein 95

RB2 - Reactive blue 2

RNA - Ribonucleic acid

RT - Room temperature

SAL - Saline

SCH58261 - SCH58261 - 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SDS - Sodium dodecyl sulfate

SNAP-25 - Synaptosomal-associated protein 25

SO - Sham operated

ABREVIATIONS LIST

TBS - Tris buffered saline medium

TBS-T - Tris buffered saline medium with 0.1% Tween-20

TLE - Temporal lobe epilepsy

TNF-α - Tumor necrosis factor-α

TNP-ATP - 2',3'-O-(2,4,6-trinitrophenyl) adenosine-5'-triphosphate

TTC - 2,3,5-Triphenyltetrazolium chloride

UDP - Uridine-5'-phosphate

UTP - Uridine-5'-triphosphate

vGAT - Vesicular GABA transporter

vGLUT1 - Vesicular glutamate transporter 1

VOCCs - Voltage-operated calcium channels

ZM241385 - 4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol

α,β-MeATP - α,β-MethyleneATP

β,γ-ImATP - β,γ-ImidoATP

ABSTRACT

Purine receptors are involved in the pathophysiology of most disorders of the central nervous system (CNS) taking part in the early synaptic dysfunction and being in the genesis and propagation of the inflammatory response in the brain. However, the different ways that ATP and adenosine receptors contribute to the evolution or to the arrest of deleterious conditions are still being unraveled.

The broad spectrum of neuroprotection afforded with the blockade of adenosine A_{2A} receptors under different adverse conditions in the brain can be underlain by a general mechanism through which these receptors may operate. One possibility is the control of neuroinflammation, a common event in most CNS disorders. However, while in the peripheral nervous system A_{2A} receptors have a well established role as a "stop signal" of the inflammatory cascade, it is not clear whether these receptors trigger or arrest the reactivity of microglia, the brain immune-competent cells. To tackle this question, it was tested if the blockade of A_{2A} receptors could prevent biochemical and morphological consequences of neuroinflammation triggered by the systemic administration of lipopolysaccharide (LPS). In this work it is shown that the intracerebroventricular injection of a selective A_{2A} receptor antagonist (SCH58261) was able to prevent the LPS-induced recruitment of activated microglial cells and the release of the pro-inflammatory cytokine interleukin-1β in the hippocampus. Moreover, SCH58261 also prevented the LPS-induced activation of mitogen-activated protein kinases (MAPKs) such as c-Jun N-terminal kinases (JNK) and p38 and the activation of caspase-3, a key mediator of apoptosis. These results indicate a tight control mainly of the genesis of neuroinflammation by the blockade of A_{2A} receptors. Therefore, it was next investigated if A_{2A} receptors were also able to control the direct effects on neurons of the pro-inflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α), known to be important effectors of neuroinflammation-induced deleterious consequences in the hippocampus. To answer this question, cultured hippocampal neurons were exposed for different periods to different concentrations of these cytokines and their activation of MAPKs was evaluated by Western blot and immunocytochemistry. Both TNF-α and IL-1β increased the phosphorylation (i.e. the activation) of p38 in neurons and IL-1β also increased the phosphorylation of JNK. In addition, the exposure of hippocampal neurons to IL-1β just before adding glutamate increased the susceptibility of cells to glutamate-mediated excitotoxicity. The blockade of A_{2A} receptors with SCH58261 abrogated the activation of

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MAPKs induced by IL-1β and prevented the IL-1β-induced exacerbation of excitotoxicity. Taking advantage of single cell calcium imaging it was found that IL-1β increased both the calcium entry and the calcium deregulation caused by glutamate exposure in hippocampal neurons. Pre-incubation of cells with SCH58261 also prevented this effect of IL-1β. Thus, it is concluded that the antagonism of A_{2A} receptors can control not only the genesis of neuroinflammation *in vivo* but also the direct effects of pro-inflammatory cytokines on neurons, which gives a further insight into the mechanisms operated by these receptors under pathological conditions.

In addition to adenosine receptors, the ATP (P2) receptors are involved in the pathophysiology of brain cells in several brain disorders, when the extracellular levels of ATP are significantly raised. In particular, the pharmacological blockade or the genetic deletion of $P2Y_1$ receptors conferred a robust neuroprotection against the toxicity induced by the $A\beta_{1-42}$ peptide (an Alzheimer's disease related peptide) in hippocampal neuronal cultures and against $A\beta_{1-42}$-induced early loss of synaptic markers and mnemonic deficits in rodents. Moreover, the antagonism of $P2Y_1$ receptors ameliorates the consequences of ischaemic/hypoxic insults to hippocampal slices. Given the involvement of neuroinflammation in the deleterious effects of the above conditions, it was hypothesized that the blockade of these receptors could not only control synaptotoxicity but also neuroinflammation. To begin answering this question it was investigated whether blocking the $P2Y_1$ receptors could prevent the direct effects of pro-inflammatory cytokines on hippocampal neuronal cultures. The results showed that the selective antagonism of $P2Y_1$ receptors either prevented or attenuated the IL-1β-mediated effects in hippocampal neurons but, importantly, it prevented the effects of glutamate per se. Thus it was investigated if the same occurred upon different excitotoxic stimuli to hippocampal neurons. The results obtained show that neurons are protected against the toxicity induced by N-methyl-D-aspartic acid (NMDA) or by quinolinic acid when exposed in the presence of a general P2 receptor antagonist or in the presence of a selective $P2Y_1$ receptor antagonist. This suggests a coupling between $P2Y_1$ receptors and the neurodegeneration mediated by glutamate NMDA receptors. An impaired function of glutamate receptors is implicated in the pathophysiology of chronic neurodegenerative diseases such as epilepsy and in the excitotoxic environment of ischaemia. The previous results obtained with the antagonism of $P2Y_1$ receptors prompted the testing of the blockade of these receptors in animal models of temporal lobe epilepsy and stroke.

Epileptic seizures were induced in rats by the intraperitoneal administration of kainate, which results in a clear neurodegeneration of hippocampal neurons. In this work it is shown

that kainate-induced seizures cause a long-term modification of the density of most P2 receptors, in both synaptic and glial membranes, predicting a central role of these receptors in the aberrant neurotransmission observed in the brain of epileptic animals. In this model, the blockade of $P2Y_1$ receptors through the intracerebralventricular administration of a selective antagonist (MRS2500) clearly prevented the early kainate-induced activation of microglia and the loss of synaptic markers in the hippocampus. Moreover, it also attenuated the seizure-induced neurodegeneration in hippocampal circuits. Likewise, in a mice model of focal ischaemia generated by the permanent occlusion of the middle cerebral artery, the general blockade of P2 receptors or the selective antagonism of $P2Y_1$ receptors prevented both the ischaemia-induced lesion in the brain and the resultant mnemonic deficits.

Overall, the work presented in this thesis provides evidence supporting the control of neuroinflammation by the blockade of A_{2A} and of $P2Y_1$ receptors and also the control of excitotoxicity by the antagonism of $P2Y_1$ receptors in the hippocampus, contributing to the unraveling of the mechanisms through which these receptors may operate to aggravate deleterious conditions in the brain.

RESUMO

RESUMO

Os receptores purinérgicos estão envolvidos na patofisiologia da maioria das doenças do sistema nervoso central (SNC), contribuindo para a disfunção sináptica e estando na génese e propagação da resposta inflamatória no cérebro. Contudo, as diferentes formas através das quais estes receptores contribuem para a evolução ou suspensão de condições deletérias estão ainda em estudo.

Tendo em conta o largo espectro de neuroprotecção conferido pelo bloqueamento dos receptors de adenosina do tipo A_{2A} no cérebro, é possível que esteja subjacente um mecanismo geral através do qual estes receptores operam. Esse mecanismo poderá resultar no controlo da neuroinflamação, visto ser um fenómeno comum à maioria das patologias do SNC. Contudo, embora a activação dos receptores A_{2A} seja reconhecidamente um sinal de "stop" da inflamação no sistema periférico, é ainda discutível se o mesmo desencadeia ou diminui a reactividade da microglia, as células imunocompetentes do cérebro. Para responder a esta questão, avaliou-se a influência do bloqueio destes receptores nas alterações bioquímicas e morfológicas resultantes da neuroinflamação induzida pela administração sistémica de lipopolissacarídeo (LPS). Neste trabalho é demonstrado que a injecção intracerebroventricular de um antagonista selectivo dos receptores A_{2A} (SCH58261) é capaz de prevenir o recrutamento de células da microglia induzido por LPS e a libertação da citocina pró-inflamatória interleucina-1β (IL-1β), no hipocampo. Adicionalmente, o SCH58261 preveniu a activação induzida por LPS das proteínas cinases activadas por mitógenos (MAPKs) c-Jun N-terminal (JNK) e p38 e ainda a activação da caspase-3, um mediador-chave da apoptose. Estes resultados estão de acordo com um controlo da génese da neuroinflamação pelo bloqueio dos receptores A_{2A}. Assim, em seguida foi investigado se o mesmo seria capaz de controlar os efeitos directos das citocinas pró-inflamatórias IL-1β e factor de necrose tumoral-α (TNF-α), dois dos principais efectores dos danos causados pela neuroinflamação, nos neurónios. Para responder a esta questão, neurónios de hipocampo em cultura foram expostos por diferentes períodos de tempo a várias concentrações destas citocinas e a consequente activação de MAPKs foi avaliada por Western blot e imunocitoquímica. Tanto o TNF-α como a IL-1β aumentaram a fosforilação (ou seja, a activação) da p38 nos neurónios e a IL-1β ainda aumentou a fosforilação da JNK. Adicionalmente, a exposição dos

neurónios de hipocampo à IL-1β imediatamente antes da exposição a glutamato aumentou a susceptibilidade dos mesmos à excitotoxicidade induzida pelo glutamato. O bloqueio dos receptores A_{2A} com o SCH58261 preveniu a activação das MAPKs despoletada pela IL-1β assim como a exacerbação da excitotoxicidade induzida pela mesma. A análise da variação do cálcio intracelular através da técnica de imagiologia de cálcio em células individuais permitiu demonstrar que a IL-1β aumenta tanto a entrada de cálcio como a sua desregulação causadas pela exposição de culturas neuronais de hipocampo a glutamato. A pré-incubação de células com SCH58261 também preveniu este efeito da IL-1β. Assim, conclui-se que o antagonismo dos receptores A_{2A} pode controlar não só a génese de neuroinflamação mas também os efeitos directos das citocinas pró-inflamatórias em neurónios, permitindo uma maior comprensão dos mecanismos operados por estes receptores em condições patológicas.

Para além dos receptores de adenosina, também os receptores P2 de ATP estão envolvidos na patofisiologia das células cerebrais em condições adversas, precisamente quando os níveis extracelulares de ATP são mais elevados. Em particular, o bloqueio farmacológico ou a delecção genética dos receptores $P2Y_1$ confere uma robusta neuroprotecção contra a toxicidade induzida pelo péptido β-amilóide ($A\beta_{1-42}$), um péptido relacionado com a doença de Alzheimer, em neurónios de hipocampo em cultura e contra a perda inicial de marcadores sinápticos e défices mnemónicos em roedores induzidos pelo mesmo. Adicionalmente, o antagonismo dos receptores $P2Y_1$ é também efectivo na prevenção ou atenuação das alterações morfológicas, bioquímicas e fisiológicas resultantes de um episódio de isquémia/hipóxia em fatias de hipocampo. Dado o envolvimento da neuroinflamação nos efeitos deletérios das condições acima descritas, foi colocada a hipótese do bloqueio destes receptores não só controlarem a sinaptotoxicidade mas também a neuroinflamação. Inicialmente foi investigado se o bloqueio dos receptors $P2Y_1$ era capaz de prevenir os efeitos directos de citocinas pró-inflamatórias em neurónios de hipocampo. Os resultados obtidos demonstram que o antagonismo selectivo destes receptores confere uma prevenção ou atenuação dos efeitos mediados pela IL-1β contudo, previne principalmente a neurotoxicidade induzida por glutamato. Assim, em seguida foi investigado se a mesma protecção ocorria com diferentes estímulos excitotóxicos aos neurónios do hippocampo. Observou-se que os neurónios em cultura estavam protegidos contra a toxicidade induzida por N-metil-D-aspartato (NMDA) ou

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por ácido quinolínico na precença de um antagonista geral de receptores P2 ou de um antagonista selectivo dos receptores $P2Y_1$. Os resultados sugerem uma ligação entre os receptores $P2Y_1$ e a neurodegeneração induzida por glutamato mediada pelos receptores NMDA. A disfunção dos receptores de glutamato está implicada na patofisiologia de doenças neurodegenerativas crónicas como a epilepsia e no ambiente excitotóxico decorrente de episódios isquémicos. Desta forma, o antagonismo dos receptores $P2Y_1$ foi testado num modelo animal de epilepsia do lóbulo temporal e num modelo de isquémia. No primeiro caso, foram induzidas convulsões epilépticas em ratos, através da administração intraperitoneal de cainato, que resultaram numa clara neurodegeneração dos neurónios hipocampais. Neste trabalho foi observado que as convulsões induzidas por cainato alteram a longo termo a densidade da maioria dos receptores P2 em membranas sinápticas e gliais do hipocampo. Estas alterações sugerem um papel central dos receptores P2 na neurotransmissão e inervação aberrantes observadas no hipocampo de animais epilépticos. O bloqueio dos receptores $P2Y_1$, através da administração intracerebroventricular de um antagonista selectivo destes receptores (MRS2500), preveniu a activação inicial da microglia e a perda de proteínas sinápticas no hipocampo induzidas pelas consulsões. Além disso, atenuou também a neurodegeneração nos circuitos hipocampais. Igualmente, num modelo animal de isquémia focal, gerada pela oclusão permanente da artéria cerebral média em ratinhos, o bloqueio geral dos receptores P2 ou o antagonismo selectivo dos receptores $P2Y_1$ preveniram tanto a lesão induzida por isquémia como os défices mnemónicos resultantes da mesma.

No geral, os resultados aqui apresentados suportam o controlo da neuroinflamação pelo bloqueio dos receptores A_{2A} e dos receptores $P2Y_1$ e ainda a prevenção da excitotoxicidade pelo antagonismo dos receptores $P2Y_1$ no hipocampo, contribuindo para a elucidação dos mecanismos através dos quais estes receptores podem operar no agravamento de condições deletérias no cérebro.

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- **Simões AP**, Duarte J, Agasse F, Canas PM, Tomé AR, Cunha RA. Blockade of adenosine A_{2A} receptors prevents the IL-1β-induced exacerbation of neuronal toxicity through a p38 MAPK-dependent pathway. Submitted to Journal of Neuroinflammation.

INTRODUCTION

1.1 ATP AS A NEUROTRANSMITTOR

Adenosine 5'-triphosphate (ATP), first discovered by Karl Lohmann in 1929, is a purine nucleotide composed of an adenine attached to a ribose sugar molecule (adenosine) that links to a chain of three phosphate groups and is the major source of chemical energy used in the metabolism of all living cells (Lipmann, 1941; Yu *et al.*, 2008). Given the importance of ATP within cells, the release and presence of this molecule in the extracellular environment was regarded as a contradiction and an unlikely event. Notwithstanding, the effects of extracellular purines on heart and blood vessels (Drury and Szent-Gyorgyi, 1929), on autonomic ganglia (Feldberg and Hebb, 1948) and their release during antidromic stimulation of sensory nerves (Holton, 1959) were already known when the idea of ATP as a neurotransmitter was first introduced in 1970 by professor Geoffrey Burnstock and collegues, when they found that ATP was the signalling molecule released during non-adrenergic, non-cholinergic neurotransmission in the smooth muscle (Burnstock *et al.*, 1970). The establishment of ATP as a neurotransmitter in the peripheral nervous system (PNS) impelled the search of a similar role in the central nervous system (CNS). Indeed, in the brain, ATP was found to transiently facilitate cortical excitability, an effect not mimicked by other purines (Phillis *et al.*, 1975). However, it was only 10 years later that the depolaring effects of ATP in the CNS were well described in a subpopulation of dorsal horn neurons (Jahr and Jessell, 1983) and in the caudal trigeminal nucleus (Salt and Hill, 1983). The functional data was supported by evidences of a strong expression of ATP receptors in the brain and contributed to the establishment of ATP as a fast neurotransmitter in the CNS (Edwards and Gibb, 1993). Nowadays it is well documented that ATP is released from all cell types in the brain, acting not only as a fast transmitter but also as a modulator of neuronal, glial and neuron-glia communication (Burnstock, 2007a). Moreover, ATP is co-released and sometimes co-stored with classical neurotransmitters both in the PNS and in the CNS (Burnstock, 2004; Burnstock, 2009a) contradicting what became known as the Dale's principle that neurons can only synthesize, store and release a single substance (Strata and Harvey, 1999). Nevertheless, in the brain terminals, ATP is primarily stored and released from a distinct pool of vesicles (Pankratov *et al.*, 2006).

1. INTRODUCTION

1.2 ATP STORAGE, RELEASE AND BREAKDOWN

Under physiological conditions, ATP is found in a concentration range of 2-5 mM in the cytoplasm of cells and at even higher concentrations (up to 100 mM) inside synaptic vesicles (Burnstock, 2007a) whereas it is usually found in the nanomolar range in the extracellular space (Agteresch *et al.*, 1999). Consequently, in normal resting conditions, there is a constant efflux of ATP, which is balanced by its extracellular enzymatic breakdown (Schwiebert, 1999; Lazarowski *et al.*, 2003). Accumulation of ATP into secretory vesicles was found to be mediated by a specific recently identified ADP/ATP translocase (Sawada *et al.*, 2008) or by passive diffusion through non-specific ion channels (Lange and Brandt, 1993). In neurons, the vesicular release of ATP depends on membrane depolarization, sodium channels activation and the presence of calcium (Pankratov *et al.*, 2006). This was first shown in synaptosome preparations from the whole brain (White, 1977; Potter and White, 1982) and later in synaptosomes from specific brain regions like the cortex (White, 1977; Salgado *et al.*, 1997), the mossy fibers (Terrian *et al.*, 1989) and the spinal cord (Sawynok *et al.*, 1993). ATP was also shown to be released in a frequency and Ca^{2+} dependent manner from hippocampal slices (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996c) and in a stimulation-dependent manner from the medial habenula (Sperlagh *et al.*, 1998a) and the hypothalamus (Sperlagh *et al.*, 1998b). In addition to the vesicular release, in non-neuronal cells, the efflux of ATP can be mediated by ATP-binding cassette (ABC) transporters (Schwiebert, 1999; Ballerini *et al.*, 2002), connexin or pannexin hemichannels (Dahl and Locovei, 2006; De Vuyst *et al.*, 2007; Iglesias *et al.*, 2009), plasmalemmal voltage-dependent anion channels (Sabirov and Okada, 2005) and $P2X_7$ channels (Suadicani *et al.*, 2006). Pharmacological relevant amounts of ATP can be released from these cells through mechanical stimulation, membrane swelling and upon activation of membrane receptors by several neurotransmitters, including purines themselves (Lazarowski *et al.*, 2003).

Once in the extracellular space, ATP is rapidly hydrolysed into its metabolites by a group of enzymes denominated ecto-nucleotidases as illustrated in Figure 1, which are ubiquitously expressed in the brain (Zimmermann, 2006). There are four main types of ecto-nucleotidases: the ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) that convert ATP into ADP and ADP into AMP, comprising eight subtypes (E-NTPDases1 to 8) of which the E-NTPDases 1,2,3 and 8 are typically located on the plasmatic membrane (Robson *et al.*, 2006); the ecto-nucleotide pyrophosphatases/phosphodiesterases (E-NPPs), that hydrolyse 5'-phosphodiester bonds in nucleotides

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and their derivatives originating 5´-nucleotide monophosphates, composed of 5 members of which E-NPP 1-3 are the best characterized (Goding *et al.*, 2003); the GPI-anchored alkaline phosphatases (APs) that convert ADP directly into adenosine and finally, the ecto-5'-nucleotidase (Ecto-5´-NT/CD73) which converts AMP into adenosine (Zimmermann *et al.*, 1998; Zimmermann, 2000; Zimmermann, 2006). On the other hand, ATP can be synthesised by the enzymatic activity of the ecto-nucleoside diphosphate kinanes (E-NDPKs) or by the ecto-adenylate kinases (E-AKs) that mediate the *trans*-phosphorylation of extracellular ADP to ATP, using UTP, GTP and other nucleotide triphosphates as phosphate donors (Yegutkin, 2008). However, the catalytic action of E-NDPKs can be reverse and convert two extracellular ADPs into ATP and AMP. Overall, the mencioned enzymes contribute to a tight regulation of the extracellular levels of purines (Joseph *et al.*, 2004).

1.3 P2 RECEPTORS

The role of ATP as a neurotransmitter implied the existence of ATP receptors present at cell surface membranes. Since these receptors were found in the early nineties, several have already been cloned and characterized (Volonte *et al.*, 2006). The large number of ATP receptors and their ubiquitous distribution in the CNS accounts for the importance of this purine as a major mediator and neuromodulator of communication between cells (Khakh and Burnstock, 2009). The extracellular ATP and its breakdown products act on two types of receptors: the P2 receptors (P2R) selective to ATP and ADP, and the P1 receptors (P1R) selective to adenosine and antagonized by methylxanthines (Fields and Burnstock, 2006).

1. INTRODUCTION
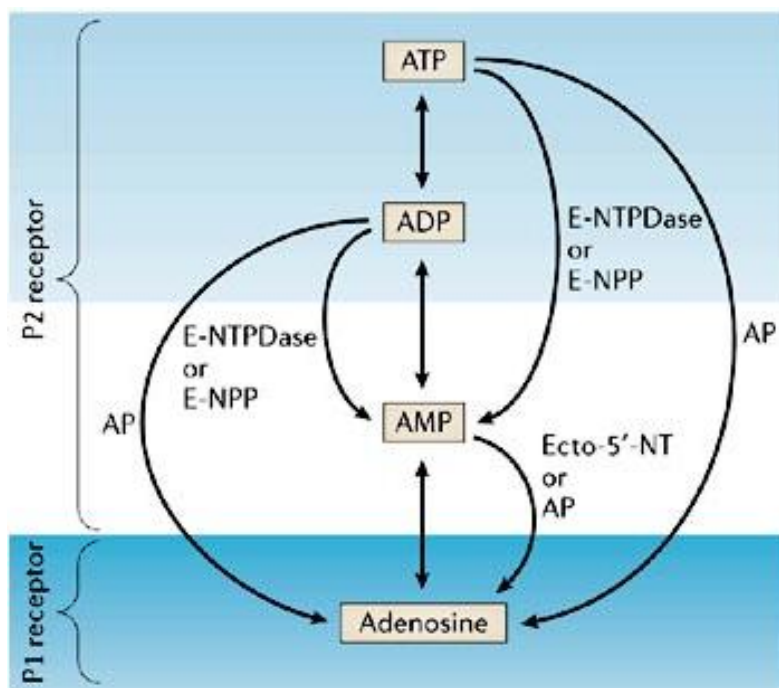
Figure 1. Extracellular catabolism of ATP by the ecto-nucleotidases. Once in the extracellular space, ATP is rapidly degraded into its metabolites by a group of enzymes denominated ecto-nucleotidases. Four major types of ecto-nucleotidases are involved in the catabolism of ATP: the E-NTPDases (ecto-nucleoside triphosphate diphosphohydrolases) and the E-NPP (ecto-nucleotide pyrophosphatases/phosphodiesterases) families, which convert ATP into ADP and ADP into AMP; the ecto-5'-nucleotidase (Ecto-5´-NT/CD73) family that hydrolyse AMP to adenosine and finally the AP (alkaline phosphatase) family which catalyse ATP to adenosine (Fields and Burnstock, 2006).

A pharmacological basis for distinguishing between different P2R was first proposed in 1985 (Burnstock and Kennedy, 1985) and when these receptors started being cloned it was confirmed the existence of two different subtypes, based on protein structure, physiology and signalling mechanisms: the P2X ion channel receptors (P2XR) and the P2Y G-protein coupled receptors (P2YR) (Lustig *et al.*, 1993; Webb *et al.*, 1993; Brake *et al.*, 1994; Valera *et al.*, 1994). One main criteria to distinguish between P2XR and P2YR is the affinity for ATP and analogue molecules such as the α,β-methyleneATP (αβmeATP) and the 2-methyltioATP (2-MeSATP). It was shown that P2XR are sensitive to low concentrations of αβmeATP and that 2-MeSATP is the most potent agonist at P2YR (Abbracchio and Burnstock, 1994). Later on, selective antagonists allowed a better distinction between the two classes of P2R.

1.3.1 P2X RECEPTORS

The P2XR are ATP-gated, non-selective cation channels (ionotropic receptors) with an equal permeability to sodium (Na^+) and potassium (K^+) ions and significant higher permeability to calcium ions (Ca^{2+}). The P2XR channels respond to ATP binding in the micromolar range and mediate ATP fast transmission (Fountain and Burnstock, 2009). These receptors are part of the Ligand-gated ion channels superfamily which comprise two other major groups: the glutamate-gated channels (kainate receptors, AMPA receptors and NMDA receptors) and the Cys-loop receptors that include nicotinic acetylcholine receptors, serotonin receptors, $GABA_A$ receptors and glycine receptors (Green *et al.*, 1998; Khakh, 2001). So far, seven mammalian P2XR subunits have been cloned ($P2X_{1-7}$) which contain from 384 ($P2X_4$) to 595 ($P2X_7$) aminoacid residues. Each subunit has two hydrophobic regions of sufficient length to cross the plasma membrane: the first transmembrane (TM)- spanning region is involved in the channel gating and the second lines the ion pore, placing both the $-NH_2$ and -COOH termini in the cytoplasm (Li *et al.*, 2008a). The -COOH terminal regions diverge considerably in sequence between subunits and bind the motifs for protein kinases responsible for the receptor kinetics, permeation and desensitization properties. The $-NH_2$ terminal is glycosylated and seems to be essential for the trafficking of receptors to the plasmatic membrane. On the other hand, the extracellular domain presents considerable aminoacid homology, with ten conserved cysteine residues forming a series of disulphide bridges where the ATP, the antagonists and other modulators have their binding sites (Khakh, 2001; Surprenant and North, 2009). Their simple ion channel architecture, illustrated in Figure 2, is shared only with the acid-sensing ion channels and the trimeric intracellular cation channels families (Li *et al.*, 2010).

1. INTRODUCTION
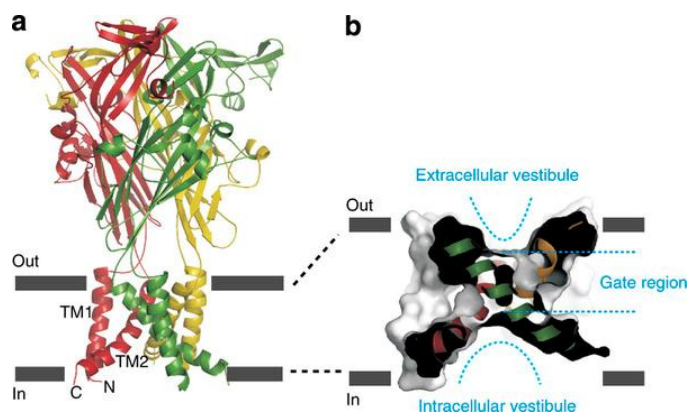
Figure 2. P2X receptor subunit topology. In the panel (a) is the representation of the receptor channel across the plasma membrane and in the panel (b) is a representation of the pore domain. The receptors are composed of a transmembrane (TM) domain containing three subunits, each with two TM helices and an extracellular domain with three ATP-binding sites (Li *et al.*, 2010).

Functional P2XR result from the assembly of three subunits in both homo- and heteromeric combinations. Both functional and structural data gathered from the heterologous expression of P2XR subunits point to the existence of trimeric or hexameric forms (Nicke *et al.*, 1998).

The P2X homomeric receptors can be divided into three different groups according to their kinetics and pharmacological properties: one composed of $P2X_1$ and $P2X_3$ receptors presenting a relative high sensitivity to αβmeATP (an ATP analogue resistant to enzymatic breakdown) and to ATP (both at an EC_{50} ~ 1 μM) and a rapid desensitization upon agonist application (Khakh *et al.*, 2001); a second group comprising $P2X_2$, $P2X_4$ and $P2X_5$ receptors, which present a lower sensitivity to ATP (EC_{50} ~ 10 μM) and a very low sensitivity to αβmeATP (EC_{50} >100 μM) together with a slow desensitization (1-2 s). The $P2X_2$ receptor can be further distinguished due to being more sensitive to ATP in an acidified medium, as well as in the presence of μM concentrations of zinc (Zn^{2+}) and copper (Cu^{2+}) ions. In addition, the $P2X_4$ receptors can be distinguished in this group for being little antagonized by the P2R antagonists suramin and PPADS (Khakh *et al.*, 2001). Finally, the third group is composed of $P2X_7$ receptors which present a lower sensitivity to ATP (EC_{50} >300 μM) and little to no desensitization (even displaying an increased permeability to organic cations and small peptides with prolonged activation). Moreover, unlike the other P2XR, the $P2X_7$ is more potently activated by the ATP analogue 2'-3'-O-(4-benzoyl)ATP (BzATP) than by ATP itself and its currents decrease in amplitude in the presence of μM concentrations of magnesium (Mg^{2+}) (Surprenant, 1996). In addition, there are at least 11 different and functional heteromeric P2XR with distinct characteristics from their

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homomeric counterparts (Khakh *et al.*, 2001). In fact, $P2X_6$ subunits are only found in heteromeric combinations (Barrera *et al.*, 2005) whereas $P2X_7$ subunits only form homomeric channels (Torres *et al.*, 1999). One of the best known heteromers are the $P2X_{2/3}$ combinations, studied at sensory neurons (Brederson and Jarvis, 2008). However, $P2X_{4/6}$ (Wong *et al.*, 2000), $P2X_{2/6}$ (Egan and Khakh, 2004) and $P2X_{1/5}$ (Surprenant *et al.*, 2000) combinations are also possible. In Table 1 is summarized the potential for assembly of the P2XR subunits.

Table 1. Potential assembly of P2XR subunits. The P2XR subunits carrying either one of two epitope tag units were expressed in pairs of HEK293 cells. The (+) signal means that the subunits immunoprecipitated with antibody to one epitope could be detected with an antibody to the second epitope (Torres *et al.*, 1999; North, 2002).

	P2X1	P2X2	P2X3	P2X4	P2X5	P2X6	P2X7
P2X1	+	+	+	-	+	+	-
P2X2		+	+	-	+	+	-
P2X3			+	-	+	-	-
P2X4				+	+	+	-
P2X5					+	+	-
P2X6						-	-
P2X7							+

One of the limitations to study the P2R is the lack of selective pharmacological tools to discriminate between them. PPADS and suramin are the most used general P2R antagonists as they block both P2XR and P2YR. However, suramin can block other ionotropic receptors together with P2XR (Nakazawa *et al.*, 1995; Peoples and Li, 1998) and therefore, PPADS is still the preferred general P2R antagonist. Nevertheless, more selective antagonists such as the TNP-ATP (Evans *et al.*, 1995) and the NF023 have been developed for $P2X_1$ and $P2X_3$ containing receptors, respectively (Soto *et al.*, 1999). In Table 2, are resumed the most used agonists and antagonists (and respective potencies) for the different P2XR.

Table 2. Principal agonists and antagonists (with the respective EC$_{50}$ and IC$_{50}$ values) of P2XR and their potency at functional homomeric and heteromeric receptors. The values of EC$_{50}$ and IC$_{50}$ are at μM unless otherwise stated (Khakh, 2001; Burnstock, 2007b).

		P2X$_1$	P2X$_{1/5}$	P2X$_2$	P2X$_{2/3}$	P2X$_3$	P2X$_4$	P2X$_{4/6}$	P2X$_5$	P2X$_6$	P2X$_7$
Agonists	ATP	1	1	10	1	1	10	10	10	12	>100
	αβmeATP	1-3	5	>100	1	1	>>100	30	>100	>100	>>300
	BzATP	3	x	30	x	x	x	x	>500	x	3
	2-MeSATP	x	x	x	x	x	x	x	x	x	x
Antagonists	Suramin	1	x	10	x	3	>300	x	4	>100	500
	PPADS	1	x	1	5	1	>300	x	3	>100	50
	NF023	200 nM	x	100	1	1	>100	x	x	x	x
	TNP-ATP	6 nM	200 nM	1	7 nM	1 nM	15	x	x	x	>30
	Brilliant blue G	>10	>10	>10	>10	>10	>10	x	x	x	10 nM
	NF449	0,3	0.7 nM	47	0.3	1.8	>300	x	x	x	x
	Calmidazolium	x	x	x	x	x	x	x	x	x	x
	IP$_5$I	3 nM	x	ineffective	x	2.8	potentiates	x	x	x	x

1.3.2 P2Y RECEPTORS

The P2YR are G-protein coupled receptors (GPCR) endogenously triggered by ATP, ADP and pyrimidine nucleotides (UTP, UDP and UDP-glucose) uncomplexed to Mg^{2+} or Ca^{2+} and, to date, eight different mammalian subtypes have been cloned (P2Y$_{1,2,4,6,11,12,13,14}$). The P2YR general structure comprises seven transmembrane regions with an extracellular −NH$_2$ terminal and an intracellular −COOH terminal as illustrated in Figure 3 (Abbracchio *et al.*, 2006; Burnstock, 2007b).
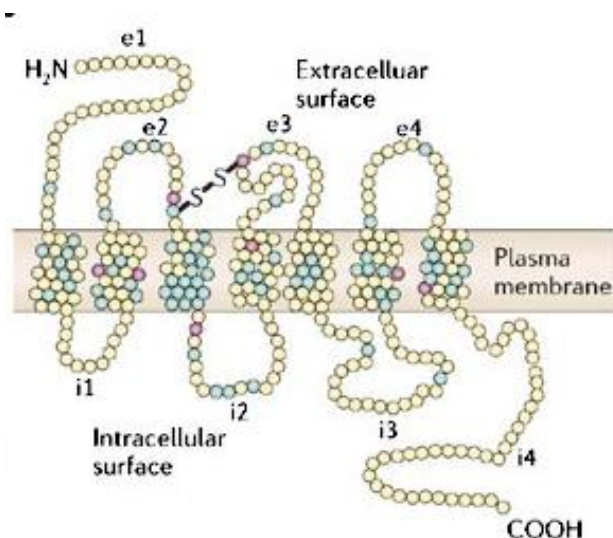
Figure 3. P2Y receptor subunit topology. S–S, disulphide bond; e1–e4, extracellular domain loops 1–4; i1–i4, intracellular domain loops 1–4. Figure taken from (Fields and Burnstock, 2006).

The missing numbers represent non-mammalian orthologs or receptors with some sequencing homology to P2YR but with no functional response to nucleotides. The hydrophobic transmembrane regions (especially TM3, TM6 and TM7), crucial for receptor activation by nucleotides, present a high homology between subtypes but the intracellular loop and -COOH terminus are quite diverse and influence the coupling to the G-protein types ($G_{q/11}$, G_S and G_i) that link the P2YR (Abbracchio *et al.*, 2006). The P2YR can be pharmacologically divided into four subgroups: those that mainly respond to ADP and ATP, comprised of the human and rodent $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors; those that respond mainly to UTP or UDP like the human $P2Y_4$ and $P2Y_6$ receptors; a group of mixed selectivity like the human and rodent $P2Y_2$ and the rodent $P2Y_4$ receptors and finally, those that respond only to sugar nucleotides (UDP-glucose and UDP-galactose) like the $P2Y_{14}$ receptor (Abbracchio *et al.*, 2006). On the other hand, P2YR can be divided into 2 main subgroups from the phylogenetic and structural point of view: one group comprising the $P2Y_{1,2,4,6,11}$ receptors and another comprising the $P2Y_{12,13,14}$ receptors (Abbracchio *et al.*, 2006). These two groups present a high level of sequence divergence and differences in specific amino acid motifs in the TM6 and TM7 regions and also differ in their primary coupling to G proteins: the $P2Y_{1,2,4,6,11}$ receptors use mainly G_q/G_{11} to activate the phospholipase Cβ (PLC_β)/ inositol-(1,4,5)-triphosphate (IP_3) pathway and trigger the release of intracellular Ca^{2+} whereas the $P2Y_{12,13,14}$ receptors couple almost exclusively to the $G_{i/o}$ family of G proteins (Abbracchio *et al.*, 2006). However, the P2YR subtypes and even the same subtype can link different G proteins and consequently trigger different signalling pathways. For instance, the $P2Y_{11}R$ when activated by ATP

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1. INTRODUCTION

leads to a rise in cAMP, IP$_3$ and in the cytosolic Ca^{2+} whereas activation by UTP can result in Ca^{2+} mobilization without cAMP or IP$_3$ increase (White *et al.*, 2003). Moreover, the P2Y$_{13}$R can simultaneously couple to G$_{16}$ and G$_i$ proteins and, at high ADP concentrations, to G$_S$ proteins in a way that suggests ligand-specific conformations of the receptor (Marteau *et al.*, 2003).

The activation of several P2YR can trigger mitogen-activated protein kinases (MAPK) and particularly of the extracellular signal-regulated protein kinase 1/2 (ERK$_{1/2}$ or p42/p44 MAPK) (Abbracchio *et al.*, 2006). Native P2YR have also been reported to activate the phospholipases A$_2$ (Ralevic and Burnstock, 1998; von Kugelgen and Wetter, 2000) and D (Martin and Michaelis, 1989; Benitez-Rajal *et al.*, 2006), receptor tyrosine kinases and the serine-threonine kinase Akt (Lazarowski *et al.*, 2003). In addition, P2YR were found to couple and modulate voltage-gated ion channels, including various classes of K$^+$ channels and voltage-gated Ca^{2+} channels (Lee and O'Grady, 2003). In particular, they modulate the N-type Ca^{2+} channels and the M-type K$^+$ currents, both critical in the neuronal response to stimulation (Abbracchio *et al.*, 2006; Filippov *et al.*, 2006; Hussl and Boehm, 2006). These channel interactions occur in a short timescale (about 100 ms) by a direct or almost direct pathway in the cell membrane (Abbracchio *et al.*, 2006). The N-type and P/Q-type of voltage-sensitive Ca^{2+} currents are typically involved in the pre-synaptic vesicular release of neurotransmitters upon neuronal excitation while the other type of channels are usually found at post-synaptic sites, in somatodendritic regions (Hussl and Boehm, 2006). Usually, the N-type and the P/Q-type are inhibited by P2YR via G-protein interaction (since it is abolished by the pertussis toxin) and independent of second messengers and protein kinases but dependent on voltage (as it is attenuated by large depolarizations), implying a direct interaction between the βγ subunits of the receptor-coupled G protein and the Ca^{2+} channel (Hussl and Boehm, 2006). In particular, both the G$_{i/o}$ coupled P2Y$_{12,13}$R and the G$_{q/11}$ linked P2Y$_{1,2}$R were found to inhibit the N-type Ca^{2+} currents (Hussl and Boehm, 2006). On the other hand, the L-type currents are either enhanced or inhibited by P2YR independently of voltage and involving diffusible second messengers, protein kinases, the α subunits of the G$_{q/11}$ protein and a PLC$_\beta$-dependent depletion of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) from the cell membrane (Hussl and Boehm, 2006). The M-type K$^+$ currents can be inhibited by G$_{q/11}$-linked P2YR and thus by the activation of most P2YR subtypes (P2Y$_{1,2,4,6,11,14}$) (Hussl and Boehm, 2006). Several neurotransmitters depolarize neurons by reducing the M-type K$^+$ currents (K$_M$), which open in the subthreshold voltage range for action potentials and are completely activated when neurons are further depolarized (Robbins, 2001). Consequently, the inhibition of these channels by P2YR increases neuronal excitability (Brown and Passmore, 2009). In addition, P2YR are known to control outwardly

rectifying K^+ currents in striatal (Ikeuchi and Nishizaki, 1995), cerebellar (Ikeuchi and Nishizaki, 1996) and hippocampal neurons (Ikeuchi *et al.*, 1996) and inward rectifier K^+ channels (Kir) through pertussis toxin (PTX)-sensitive G proteins (Hussl and Boehm, 2006). The $G_{i/o}$-linked P2YR can open Kir channels whereas the $G_{q/11}$-linked P2YR close them, as was shown for $P2Y_{1,2,4,6}$ subtypes (Filippov *et al.*, 1998; Filippov *et al.*, 1999; Brown *et al.*, 2000; Filippov *et al.*, 2003). Since the activation of Kir channels hyperpolarize neurons, their closure by P2YR may also contribute to increased neuronal excitability (Luscher and Slesinger, 2010).

Another type of ion channels modulated by P2YR are transmitter-gated ion channels such as the NMDA receptor of glutamate. Adenine and uridine nucleotides enhance NMDA currents in the rat prefrontal cortex, in the pyramidal neurons of the V layer, through activation of $P2Y_2R$ (Wirkner *et al.*, 2002) and inhibit these currents through $P2Y_1R$ (Luthardt *et al.*, 2003). However, ATP can also inhibit these currents by its direct binding to the glutamate binding site at the NR2B subunit of NMDA receptors (Ortinau *et al.*, 2003). The capsaicin vanilloid receptor 1 (VR1 OR TRPV1) is also a transmitter-gated ionotropic receptor modulated by nucleotides and its currents are enhanced by the activation of $P2Y_1R$ at nociceptive sensory nerves, through a protein kinase C (PKC)- dependent pathway (Tominaga *et al.*, 2001; Kennedy *et al.*, 2003). Finally, P2YR can modulate P2XR channels by either inhibiting (Gerevich *et al.*, 2005) or enhancing their currents (Vial *et al.*, 2004).

G-protein coupled receptors are found in functional monomers but many can form dimmers (homodimers or heterodimers) and oligomers. In fact, interaction between GPCRs can occur through the formation of oligomers or downstream, through the action of second messengers (Abbracchio *et al.*, 2006; Burnstock, 2007b). The most extensively characterized dimerisation involving P2YR and non-P2YR is the one between the $P2Y_1R$ and the A_1 receptor (A_1R) of adenosine (Yoshioka *et al.*, 2001; Yoshioka and Nakata, 2004; Tonazzini *et al.*, 2007; Tonazzini *et al.*, 2008). This heterodimeric complex can be co-immunoprecipitated from whole cell membrane lysates and displays mixed characteristics: the A_1R couples to G_i proteins resulting in decreased cAMP levels and the $P2Y_1R$ interacts with $G_{q/11}$ having no effect on cAMP levels; however, activation by ADPβS (a $P2Y_1R$ agonist) of the $P2Y_1/A_1$ complex inhibits the production of cAMP, an effect that is prevented by the A_1R antagonist DPCPX but not by the $P2Y_1R$ antagonist MRS2179 and is PTX-sensitive, characteristic of the $G_{i/o}$-coupled A_1R (Yoshioka *et al.*, 2001). This $A_1/P2Y_1$ complex was described in native tissue, including the rat cortex, hippocampus and the cerebellum (Yoshioka *et al.*, 2002) and even though its biological significance is still poorly understood, some studies suggest that this interaction may be relevant at elevated concentrations of agonists of both receptors, as occurs in pathological situations

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(Tonazzini *et al.*, 2007; Tonazzini *et al.*, 2008). Cross-talk between GPCRs has also been extensively described. For example, co-expression of native $P2Y_1R$ and $P2Y_2R$ in HEK293 cells with the recombinant human chemokine receptor CXCR2 (a G_i-coupled receptor with no effect on intracellular Ca^{2+}) results in significant increase of $[Ca^{2+}]_i$ upon the binding of interleukin-8 to the CXCR2, after prestimulation of cells with ATP or UTP (Werry *et al.*, 2002). On the other hand, P2YR can inhibit other non-P2Y GPCRs and an exemple of this is the P2YR-mediated inhibition of the signalling triggered by an agonist of the sphingosine-1-phosphate receptor to induce mitogenesis in renal mesangial cells (Xin *et al.*, 2004).

An important modulatory feature of P2YR is that as G-protein coupled receptors their signalling regulates nuclear gene transcription and protein synthesis. This has been mostly described for G_S protein coupled P2YR that induce an increase of cAMP levels and consequently the activation of cAMP response element-binding transcription factor (CREB), which in turn activates gene transcription. An example of this is the $P2Y_{11}R$-induced production of cytokines in dendritic cells (Wilkin *et al.*, 2001; Marteau *et al.*, 2004; Marteau *et al.*, 2005). Nevertheless, some studies showed a similar modulatory role for G_q-coupled P2YR: in 1321N1 astrocytoma cells, the agonism of $P2Y_2R$ resulted in the activation of genes like the anti-apoptotic bcl-2 and bcl-xl preceded by the phosphorylation of CREB and cell proliferation (Chorna *et al.*, 2004) and the activation of $P2Y_1R$ activated the transcription factor Elk-1 through the ERK1/2 MAPK pathway, resulting in apoptosis and inhibition of cell proliferation (Sellers *et al.*, 2001). Moreover, in rat astrocytes, the activation of native PTX-sensitive P2YR increased the binding to DNA of transcription factors such as the activator protein-1 and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) which may explain the increase in the production of cyclooxygenase-2 (COX-2) and the resultant astrogliosis (Brambilla and Abbracchio, 2001). Finally, P2YR can also control the synthesis and the insertion in the membrane of subunits of other receptors, as was shown for $P2Y_1$ and $P2Y_2$ receptors in relation to nicotinic receptors of acetylcholine in the neuromuscular junction (Choi *et al.*, 2001; Tung *et al.*, 2004).

The development of selective P2YR agonists and antagonists has made a slow progress due to cross-reactivity of the compounds with P2XR, interaction with other G-protein coupled receptors and interference with ecto-nucleotidases (Jacobson *et al.*, 2009). In this field, more selective agonists/antagonists were developed to the $P2Y_1$ and $P2Y_{12}$ receptors due to their importance in platelet aggregation (Gachet, 2006). Thus, ADPβS is a potent and commonly used $P2Y_1$ (EC_{50} of 96 nM), $P2Y_{12}$ (EC_{50} of 82 nM) and $P2Y_{13}$ (EC_{50} of 42 nM) agonist and the 2-methylthio derivative of ADP (2-MeSADP) is an even more potent agonist at these receptors (EC_{50} of 6, 1 and 1 nM, respectively). As

for the antagonists, MRS2179 is a commonly used selective $P2Y_1R$ antagonist but MRS2279 and MRS2500 are more recent and equally potent antagonists with a higher affinity for the $P2Y_1R$ in various species (Jacobson *et al.*, 2006; Jacobson and Boeynaems, 2010). In Table 3 are summarized the agonists/antagonists of P2YR and the coupled G-proteins.

Table 3. Pharmacological characteristics and transduction mechanisms operated by P2Y receptors. The information presented is reviewed in (Shaver *et al.*, 2005; Abbracchio *et al.*, 2006; Burnstock, 2007b; Jacobson and Boeynaems, 2010).

	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$	$P2Y_{13}$	$P2Y_{14}$
Agonists	2-MeSADP=ADPβS>2-MeSATP=ADP>ATP; MRS2365	UTP=ATP, Ins45973; MRS2698; MRS2768	UTP=ATP; Ins45973	UDP>UTP>>ATP; Ins415; PBS-0474; 5-iodo-UDP	BzATP=ATPγS>ATP; NF546	ADP=2-MeSADP>>ATP	ADP=2-MeSADP>>ATP	UDP-glucose=UDP-galactose
Antagonists	MRS2500; MRS2179	Suramin; RB2; PBS-716; AR-C1263;	RB2; Suramin; PPADS	MRS2578	Suramin; RB2; NF340	CT50547; Ins49266; AR-C69931MX; AZD6140; AR-C69931; PBS-0739	MRS2211	X
Transduction mechanisms	$G_{q/11}$; PLCβ activation	$G_{q/11}$ and possibly G_i; PLCβ activation	$G_{q/11}$ and possibly G_i; PLCβ activation	$G_{q/11}$; PLCβ activation	$G_{q/11}$ and G_S; PLCβ activation	$G_{i/0}$; inhibition of adenylate cyclase	$G_{i/0}$; inhibition of adenylate cyclase	$G_{i/0}$; inhibition of adenylate cyclase

1.3.3 DISTRIBUTION OF P2 RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

P2 receptors are ubiquitously expressed in the central nervous system, both in neuronal and glial cells (Illes and Alexandre Ribeiro, 2004; Abbracchio *et al.*, 2009). The $P2X_2$ receptor is widely expressed in several brain regions such as the cerebral cortex, the hippocampus, the habenula, the substantia nigra pars compacta and several hypothalamic nucclei (Norenberg and Illes, 2000; Illes and Alexandre Ribeiro, 2004). In addition, it is also expressed in the mesencephalic trigeminal nucleus, the ventrolateral medulla, the dorsal vagal complex and in the nucleus of the solitary tract (Norenberg and Illes, 2000; Illes and Alexandre Ribeiro, 2004). The $P2X_3$ receptor presents a high density in a subpopulation of small-diameter neurons of the dorsal root ganglia and in structures involved in pain transmission such as the nucleus tractus solitarius, the solitary tract and the spinal trigeminal nucleus (Norenberg and Illes, 2000; Illes and Alexandre Ribeiro, 2004). In addition, it was also found at pre-

synaptic sites in the hippocampus (Rodrigues *et al.*, 2005b) and in the midbrain (Diaz-Hernandez *et al.*, 2001). The $P2X_4R$ presents a strong expression in the rat cerebellum and spinal cord but it is also present in a relative high density in the cerebral cortex, the hippocampus, the thalamus and the brainstem (Norenberg and Illes, 2000; Illes and Alexandre Ribeiro, 2004). In particular, in the rat hippocampus, the $P2X_2R$, $P2X_4R$ and the $P2X_6R$ were found in the CA1 region, at post-synaptic membranes opposed to terminals of Schaffer collaterals, precisely where the density of glutamate ionotropic receptors decrease (Rubio and Soto, 2001). Despite being mostly found in activated glial and immunocompetent cells (Collo *et al.*, 1997; Butt, 2011), the $P2X_7$ receptor was targeted at pre-synaptic excitatory terminals in the spinal cord and in the rat hippocampus (Deuchars *et al.*, 2001; Sperlagh *et al.*, 2002) and also in the nuclear membrane of a subgroup of inhibitory hippocampal neurons (Atkinson *et al.*, 2002). In addition, it is present in synaptosomes from the midbrain and in cerebellar granule cells (Miras-Portugal *et al.*, 2003). As for the P2YR, the $P2Y_1R$ and the $P2Y_{11}R$ subtypes are particularly abundant in the brain in relation to their presence in other tissues (Moore *et al.*, 2001). The human $P2Y_1R$ mRNA is mostly found at neuronal structures in the basal ganglia (the striatum, nucleus accumbens, caudate putamen and globus pallidus) and also in the hippocampus, cerebellum and many regions of the cerebral cortex (Moore *et al.*, 2000a). A similar distribution was found for the $P2Y_1R$ in the rat brain (Moran-Jimenez and Matute, 2000). Finally, both the $P2Y_{12}$ and the $P2Y_{13}$ receptors have a high density in neurons of the cerebellum, cerebral cortex, hippocampus, caudate nucleus, substantia nigra and in the ventrolateral medulla (Laitinen *et al.*, 2001).

1.3.4 SYNAPTIC MODULATION OPERATED BY P2 RECEPTORS

In the central nervous system, ATP is the main adenine nucleotide released from nerve terminals upon stimulation (White, 1977) and, acting on P2 receptors, controls neurotransmission under both physiological and pathological conditions (Illes and Alexandre Ribeiro, 2004; Burnstock *et al.*, 2011). Synaptic transmission can be modulated by either controlling the pre-synaptic release of neurotransmitters or by controlling the excitability of post-synaptic membranes (Hussl and Boehm, 2006). The synaptic modulation operated by P2XR relies mostly on Ca^{2+} entry through their channel pores which leads to neurotransmitter release at the pre-synaptic sites and to membrane depolarization at the post-synaptic level (Surprenant and North, 2009). On the other hand, P2YR modulate ion channels that influence membrane excitability, as already described (Abbracchio *et al.*, 2006; Hussl and Boehm, 2006). However, the modulatory effects of ATP are difficult to clarify due to its rapid

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degradation by the ecto-enzymes and the consequent formation of another major neuromodulator, adenosine (Cunha and Ribeiro, 2000). The inhibitory effects of ATP can be confused with the activation of A_1 receptors by adenosine, which maintain an inhibitory tonus on synaptic transmission (Ribeiro, 1995; Cunha *et al.*, 1998). Likewise, the excitatory effects of ATP can be confused with the activation of adenosine A_{2A} receptors (Cunha and Ribeiro, 2000). Nonetheless, the hippocampus is one of the brain areas with the highest density of P2R accompanied by functional evidence of P2R-mediated effects (Inoue, 1998). In this brain region, it was found a facilitatory effect on glutamate release mediated by pre-synaptic P2XR (the $P2X_1$, $P2X_{2/3}$ and $P2X_3$ subtypes) and an inhibitory effect mediated by pre-synaptic P2YR (involving the $P2Y_1$, $P2Y_2$ or the $P2Y_4$ subtypes) (Mendoza-Fernandez *et al.*, 2000; Rodrigues *et al.*, 2005b). A pre-synaptic $P2X_2$R-mediated facilitation of transmission in the interneurons of the stratum radiatum, in the hippocampus CA1 region, was also described (Khakh *et al.*, 2003). In addition, ATP was found to cause inward currents at post-synaptic CA1 and CA3 hippocampal subregions through $P2X_2$-like receptors (Pankratov *et al.*, 1998; Mori *et al.*, 2001). Even though P2XR contribute to the excitatory post-synaptic current in CA1 pyramidal neurons (Pankratov *et al.*, 1998; Rubio and Soto, 2001), they may also facilitate the inactivation of NMDA receptors through a Ca^{2+}-dependent mechanism (Pankratov *et al.*, 2002). However, P2XR desensitize during fast synaptic frequency allowing the NMDA-mediated current to increase and cause long-term potentiation (LTP) (Pankratov *et al.*, 2002). In fact, ATP may directly induce LTP by phosphorylating the extracellular domain of NMDA receptors and increasing its permeability (Wieraszko *et al.*, 1989; Chen *et al.*, 1996; Fujii *et al.*, 2002). Nonetheless, P2XR are not always excitatory; for example, in hippocampal slices, the activation of $P2X_7$R was shown to induce the release of the major inhibitory neurotransmitter GABA (Sperlagh *et al.*, 2002) and in the CA3 pyramidal cells, at mossy fiber synapses, a potent $P2X_7$R agonist evoked a long-lasting inhibition of glutamate transmission (Armstrong *et al.*, 2002). P2YR can either potentiate the currents of NMDA receptors through the activation of $P2Y_2$R (Wirkner *et al.*, 2002) or inhibit those currents through the activation of $P2Y_1$R (Khakh *et al.*, 2000; Liu *et al.*, 2000a; Luthardt *et al.*, 2003). Thus, the outcome of P2R-mediated synaptic effects are complex and depend on the P2R subtype, the type of cells in which they are expressed, their membrane localization and interaction with other membrane receptors and/or ion channels (Volonte *et al.*, 2006; Volonte and D'Ambrosi, 2009).

1.3.5 P2 RECEPTORS IN GLIAL CELLS

In addition to the modulation of synaptic transmission, ATP is a major glial transmitter and modulator (Butt, 2011; Koles *et al.*, 2011). Glial cells make up over 70% of the total cell population of the central nervous system (Raghavendra and DeLeo, 2004) and are responsible for most of the extracellular ATP (Butt, 2011). In the brain there are three main types of glial cells: the astrocytes, the microglia and the oligodendrocytes, all endowed with different P2XR and P2YR (Ciccarelli *et al.*, 2001; Inoue, 2008; Butt, 2011).

The astrocytes participate in multiple brain functions including neuronal development, synaptic activity and homeostatic control of the extracellular environment (Ciccarelli *et al.*, 2001). Accordingly, astrocytes express neurotransmitter transporters and receptors and release classic neurotransmitters such as glutamate to activate neighbouring glial and neuronal cells (Parpura and Haydon, 2000; Newman, 2003; Panatier *et al.*, 2006; Jourdain *et al.*, 2007; Hamilton *et al.*, 2010). In addition, these cells participate in inflammatory reactions by releasing and responding to inflammatory mediators such as chemokines and cytokines, changing their morphology and proliferating under noxious conditions (Abbracchio and Ceruti, 2006). Astrocytes are the major source of adenine-based (ATP and adenosine) and guanine-based purines (guanosine and guanosine triphosphates) in the extracellular milieu, during both physiological and pathological conditions (Meghji *et al.*, 1989; Ciccarelli *et al.*, 1999). In fact, these cells express most of the characterized P2R which modulate and participate in many of the mechanisms underlying astrocytic functions (James and Butt, 2002; Koles *et al.*, 2011). Among the P2R known to be expressed in astrocytes are the subtypes $P2X_{1,2,4,7}$ and the heteromer $P2X_{1/5}$ (Lalo *et al.*, 2011) and also the subtypes $P2Y_{1,2,4,6,12,13}R$ (Verkhratsky *et al.*, 2009; Butt, 2011). Invariably, the CNS glial cells express P2YR linked to the IP_3-mediated Ca^{2+} signalling cascade while the expression of P2XR is more segregated (Abbracchio *et al.*, 2009). The subtypes $P2Y_1R$, $P2Y_2R$ and $P2Y_4R$ were shown to be primarily involved in the short-term Ca^{2+}-dependent signalling in astrocytes (Gallagher and Salter, 2003; Suadicani *et al.*, 2004; Bennett *et al.*, 2005), whereas multiple P2R subtypes mediate astrocytic long-term changes involving gene transcription (James and Butt, 2002; Neary *et al.*, 2006). Activation of P2YR is linked to features of reactive gliosis such as increased production of COX-2, in both human and rat astrocytes (Brambilla *et al.*, 1999; Brambilla *et al.*, 2000; Brambilla *et al.*, 2002). This is an important enzyme for prostaglandin synthesis (Hirst *et al.*, 1999b) which play a part on inflammatory reactions (Rossi and Volterra, 2009). Activation of P2YR was also shown to increase astrocyte proliferation and the expression of the glial fibrillary acidic protein (GFAP), both *in vitro*

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(Ciccarelli *et al.*, 1994) and *in vivo* (Hindley *et al.*, 1994; Franke *et al.*, 1999). On the contrary, the activation of P2XR was found to stop the proliferation of astrocytes (Neary *et al.*, 2005; Neary *et al.*, 2008). This opposite effect of P2YR and P2XR apparently involves the $P2Y_1$ and $P2X_7$ subtypes (Neary *et al.*, 2005; Franke *et al.*, 2009). The $P2Y_1$R-mediated Ca^{2+} release from the intracellular stores was also shown to induce the vesicular release of glutamate from astrocytes, a process also dependent on the $P2Y_1$R-induced release of TNF-α and prostaglandin from these cells (Domercq *et al.*, 2006). A posterior work elegantly showed that this control of glutamate release from astrocytes by the $P2Y_1$R has a prominent role in the synatic strength of proximal excitatory synapses in the hippocampus (Jourdain *et al.*, 2007), which can have important consequences during both physiological and pathological events.

Microglia are another main type of glial cells which account for 5 to 10% of the total CNS cell population (Parkhurst and Gan, 2010) and are considered the immune cells of the brain or the resident brain macrophages due to their functional and phylogenetic characteristics (Barron, 1995; Perry *et al.*, 2010). ATP has a determinant role in the activation, proliferation and attraction of microglia to the sites of injury, through the activation of different P2R (Inoue, 2008). In their quiescent or resting state, microglia cells have a small soma with thin, branched processes and undetectable levels of molecules belonging to the major class II histocompatibility complex (MHC) (Kreutzberg, 1996; O'Keefe *et al.*, 2002). However, when activated, these cells undergo hypertrophy, proliferate and acquire an amoeboid morphology with increased expression of MHC class II molecules, adhesion molecules and of stem cell antigens (Kreutzberg, 1996; O'Keefe *et al.*, 2002; Ladeby *et al.*, 2005). The activation of microglia by ATP was first shown in cultured cells from the rodent brain, where exogenously applied ATP triggered cationic currents and delayed outward K^+ currents, which resulted in a P2R-dependent increase of the intracellular calcium and was essencial for microglial-mediated responses (Walz *et al.*, 1993). Once activated, the microglial cells move and extend processes towards the sites of injury and this effect can be mimicked by local ATP injection and inhibited by the ATP-hydrolysing enzyme apyrase or blockers of P2YR (Davalos *et al.*, 2005). Moreover, both ATP and ADP were found to induce chemotaxis in microglia (Honda *et al.*, 2001) in a PTX-sensitive manner and through a mechanism independent of extracellular Ca^{2+} and blocked by antagonists of $P2Y_{12}$R and $P2Y_{13}$R (Hollopeter *et al.*, 2001; Fumagalli *et al.*, 2004). These results were corroborated by *in vivo* studies and provide evidence for the role of ATP as an 'alarm signal' and chemoattractant molecule for microglia, inducing directional branch extension in these cells (Haynes *et al.*, 2006; Ohsawa and Kohsaka, 2011).

An important characteristic of the late stages of microglia activation is phagocytosis (the uptake of particles into vacuoles) which is essential for tissue clearing of cellular debris and facilitation of tissue repair (Napoli and Neumann, 2009; Neumann *et al.*, 2009). This process is initiated by the activation of pattern recognition receptors present on the plasma membrane of microglia (such as Fc receptors, the complement system, scavenger receptors and Toll-like receptors) by correspondent extracellular ligands (Griffiths *et al.*, 2007), the designated "eat-me" signals present on the membrane of antigens (Lauber *et al.*, 2003). Nucleotides can also act as an "eat me" signal, as microglia phagocytosis can be triggered through activation of $P2Y_6R$ by the extracellular UDP (Koizumi *et al.*, 2007). Thus, it was proposed that a gradient of purines and pyrimidines was formed in the extracellular space from the site of injury, modulating the activation of microglia through the sequential and preferential activation of specific P2R (Davalos *et al.*, 2005; Inoue, 2008). In addition, nucleotides also contribute to microglia self-destruction, for example, the activation of $P2X_7R$ in these cells can trigger both the maturation and release of the pro-inflammatory cytokine IL-1 and the apoptotic pathway (Ferrari *et al.*, 1997; Bernardino *et al.*, 2008).

A third main type of glial cells are the oligodendrocytes, which main function is to improve the conduction of electric impulses by isolating the axons of neurons, wrapping around them a sheath of myelin that reduce ion leakage and decrease the capacitance of the cell membrane (Fields, 2006). This myelin layer also increases impulse speed through saltatory propagation of action potentials at the nodes of Ranvier. The ATP and adenosine released from axons during electrical impulse activity regulate the migration, proliferation and differentiation of oligodendrocyte precursor cells: adenosine via A_1 receptors (Othman *et al.*, 2003) and ATP via $P2Y_1$ and $P2X_7$ receptors (Agresti *et al.*, 2005). Low concentrations of ATP increase the intracellular Ca^{2+} of oligodendrocytes mainly through the activation of $P2Y_1$ receptors (IP_3-mediated release of Ca^{2+} from its intracellular stores) (Kirischuk *et al.*, 1995; Moran-Jimenez and Matute, 2000; James and Butt, 2001) whereas high concentrations of ATP preferentially activate the $P2X_7$ ion pore and trigger a sustained increase of the intracellular Ca^{2+} in these cells (James and Butt, 2002). In normal conditions, the ATP-mediated Ca^{2+} signalling stimulates the production of myelin by the oligodendrocytes (Stevens *et al.*, 2002) but activation of $P2X_7R$ during pathological and disease conditions (such as ischemia and multiple sclerosis) is known to cause demyelination and oligodendrocyte death (Matute *et al.*, 2007; Domercq *et al.*, 2010).

Overall, purines and purine receptors can orchestrate the communication between all cell types in the central nervous system, for example, ATP and adenosine released from non-synaptic sites of

firing neurons were shown to indirectly modulate the activity of Schwann cells and oligodendrocytes (Fields, 2004; Fields, 2006) through the activation of astrocytes (Ishibashi *et al.*, 2006).

1.3.6 P2 RECEPTORS IN DISORDERS OF THE CENTRAL NERVOUS SYSTEM

The key role of purines in the metabolism of cells and in the modulation of neuronal and glial function puts these molecules in the central stage of most neurodegenerative diseases. In the last years, the development of more potent and selective agonists and antagonists of P2R has allowed to unravel the involvement of specific receptor subtypes in the ethiopathology of several neurologic disorders (Franke and Illes, 2006; Burnstock, 2008). During pathological events, the extracellular levels of ATP raise significantly, not only because of increased neurotransmission but also due to membrane disruption of damaged or dying cells. Usually, brain damage results in an up-regulation of P2R in both neuronal and glial membranes, which contribute to the enhancement of purinergic transmission and sometimes to the aggravation of toxicity (Franke and Illes, 2006). In fact, direct application of ATP to brain primary neuronal and organotypic cultures is *per se* toxic (Amadio *et al.*, 2002). Often, in these situations, antagonists of P2R have been neuroprotective (Franke and Illes, 2006; Di Virgilio *et al.*, 2009; Burnstock *et al.*, 2011).

In neurodegenerative conditions the focus has been on the $P2X_7$ receptors since when activated for long periods by elevated concentrations of ATP, it forms large pores in the cell membranes, permeable to molecules up to 900 Da (Le Feuvre *et al.*, 2002; Takenouchi *et al.*, 2010). Moreover, the $P2X_7R$ elongated C-terminus is directly linked to the apoptotic pathway, activating specific protein kinases; caspases-1 and caspase-3 (Skaper *et al.*, 2010). Notwithstanding, the $P2Y_1R$ and the $P2Y_{12}R$ subtypes have a prominent role in platelet aggregation (Gachet, 2008; Houston *et al.*, 2008) which resulted in the clinical application of $P2Y_{12}R$ inhibitors to treat stroke and thrombosis (Hollopeter *et al.*, 2001). In addition, the $P2Y_1R$ was found to be highly expressed in ischaemia-sensitive areas of the hippocampus (Moran-Jimenez and Matute, 2000) and has been recently associated with ischaemic cell death (Coppi *et al.*, 2007; Olivecrona *et al.*, 2007; Kuboyama *et al.*, 2011). Both these and the $P2X_7R$ subtypes have been implicated in the ATP-mediated effects under *in vivo* ischaemic conditions, such as after permanent or transient occlusion of the middle cerebral artery in rats (Marrelli *et al.*, 1999; Marrelli, 2002; Franke *et al.*, 2004; Melani *et al.*, 2006a; Sun *et al.*, 2008; Yanagisawa *et al.*, 2008; Kuboyama *et al.*, 2011). In addition, the $P2X_{2,4}R$ subtypes were found to be up-regulated in neurons

and in microglia under ischaemic conditions (Cavaliere *et al.*, 2003). Thus, unsurprisingly, antagonists of P2R were neuroprotective in both *in vivo* (Lammer *et al.*, 2006; Kuboyama *et al.*, 2011; Lammer *et al.*, 2011) and *in vitro* models of ischaemia (Coppi *et al.*, 2007; Pedata *et al.*, 2007; Traini *et al.*, 2011).

As previously discussed, extracellular ATP plays a central role in the control of the reactivity, proliferation and survival of microglia (Davalos *et al.*, 2005; Inoue, 2008) and astrocytes (James and Butt, 2002; Fields and Burnstock, 2006; Di Virgilio *et al.*, 2009). Thus, P2R are regarded as promising pharmachological targets in brain diseases with a strong inflammatory component (Di Virgilio *et al.*, 2009). For example, the $P2X_7$ receptor is not only involved in the maturation and secretion of inflammatory mediators from glial cells (Narcisse *et al.*, 2005; Bernardino *et al.*, 2008) but also in the glia-mediated neurotoxicity by increasing reactive species in microglia (Parvathenani *et al.*, 2003) or decreasing the ability of astrocytes to clear extracellular glutamate (Liu *et al.*, 2010).

Under excitotoxic conditions in the brain as occurs in epilepsy models, the attention has once again been focused on P2XR, mainly because of their facilitation of excitatory transmission in the hippocampus. The expression of $P2X_2R$ and $P2X_4R$ was found to be downregulated in the hippocampus of seizure prone gerbils (Kang *et al.*, 2003) and during the chronic phase of pilocarpine-induced status epilepticus in rats (Dona *et al.*, 2009). Additionally, the expression of $P2X_7R$ was greatly up-regulated, in glial cells and in glutamatergic terminals, during the acute and chronic phase of seizures induced by both pilocarpine and kainate in the rat hippocampus (Vianna *et al.*, 2002; Dona *et al.*, 2009; Kim *et al.*, 2009). Recently, these receptors were shown to differentially affect astroglia during the status epilepticus in the rat brain (Kim *et al.*, 2010). Finally, the expression of different P2R was increased in seizure-induced activated microglia (Rappold *et al.*, 2006; Avignone *et al.*, 2008) where they were described to mediate microglia membrane currents during the status epilepticus (Avignone *et al.*, 2008).

The $P2X_7R$ and the $P2Y_1R$ have been implied in the pathophysiology of Alzheimer's disease (AD) (Moore *et al.*, 2000b; Parvathenani *et al.*, 2003; McLarnon *et al.*, 2006; Delarasse *et al.*, 2011). $P2X_7R$ were up-regulated around β-amyloid structures in a transgenic model of AD (Parvathenani *et al.*, 2003) and were shown to be involved in the activation of microglia in models of this disease (McLarnon *et al.*, 2006; Sanz *et al.*, 2009; Lee *et al.*, 2011). The $P2Y_1R$ were up-regulated in the hippocampus of AD patients and in characteristic AD structures such as neurofibrillary tangles, neuritic plaques and neuropil threads in the human entorhinal cortex and hippocampus (Moore *et al.*, 2000b). In line with these findings, ATP can promote the formation of β-amyloid plaques which can be blocked

by suramin, a general P2R antagonist (Exley and Korchazhkina, 2001), that can also block the astrocytic Ca^{2+} waves induced by the β-amyloid peptide (Haughey and Mattson, 2003).

In the rat brain, P2 receptors co-localize with dopamine receptors (Heine *et al.*, 2007) and modulate the release of dopamine into the nucleus accumbens (Krugel *et al.*, 1999; Kittner *et al.*, 2000; Krugel *et al.*, 2001b; Krugel *et al.*, 2003) and into the ventral tegmental area (Krugel *et al.*, 2001a). In addition, exogenously applied ATP stimulated the release of dopamine from the substantia nigra pars compacta to the striatum via P2YR (Zhang *et al.*, 1995). Thus, P2R can also be regarded as possible therapeutic targets in diseases where there is a loss of dopaminergic transmission, such as in Parkinson's disease (Amadio *et al.*, 2007).

The $P2X_7R$ are expressed in the Müller (glial) cells of the human and rat retinas (Jabs *et al.*, 2000; Pannicke *et al.*, 2000; Bringmann *et al.*, 2001) where they are up-regulated or their pathological activation enhanced during some retinopathies (Bringmann *et al.*, 2001; Sugiyama *et al.*, 2004). P2YR are also expressed in Müller cells (Ward and Fletcher, 2009; Wurm *et al.*, 2009) and were shown to mediate their proliferation *in vitro* (Moll *et al.*, 2002). Thus, pharmacological manipulation of P2R can be beneficial in different types of retinopathies (Fletcher, 2010; Ward *et al.*, 2010). Moreover, agonists and antagonists of P2R are also useful to either induce cytotoxicity in tumor cells or to arrest their cellular division (Rapaport, 1983; Agteresch *et al.*, 1999).

Not only in the case of diseases but also during acute and chronic alcohol and amphetamine consumpsion, the density and/or functioning of P2R is altered (Weight *et al.*, 1999; Koles *et al.*, 2008). Ethanol is known to modulate $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ subunits, desviating synaptic transmission from mostly excitatory to inhibitory or vice-versa, with an impact in the psico-motor and mnemonic effects of this drug (Asatryan *et al.*, 2008; Asatryan *et al.*, 2010; Popova *et al.*, 2010). On the other hand, Systemic administration of amphetamine to rodents enhaced the immunoreactivity of $P2Y_1R$ in astrocytes and neurons of the striatum and nucleus accumbens and treatment with PPADS prevented astrogliosis, inflammatory markers and drug sensitization (Franke *et al.*, 2003).

1.4 ADENOSINE AS A NEUROMODULATOR

The role of adenosine as a neuromodulator was first described in a study by Drury and Szent-Györgyi (1929) where the authors showed that injected adenosine caused a variety of biological effects: decrease of arterial blood pressure, dilatation of coronary arteries, relaxation of the small intestine muscles and promotion of sleep (Drury and Szent-Gyorgyi, 1929). Later on, Satin and Rall (1970) showed an accumulation of cAMP in cortical slices upon electrical stimulation which could be blocked by methylxanthines (Sattin and Rall, 1970). This finding together with the evidence that electrical stimulation induced adenosine release from neuronal preparations (Pull and McIlwain, 1972) and that exogenously applied adenosine depressed synaptic transmission in the neuromuscular junction (Ginsborg and Hirst, 1972) and in cortical neurons (Phillis *et al.*, 1974) supported a role for adenosine as a neuromodulator. Nowadays, it is well established that extracellular adenosine, either released *per se* or resulting from the catabolism of ATP, is a major neuromodulator of cell function and communication in the nervous system (Cunha, 2001).

1.5 ADENOSINE METABOLISM AND REGULATION OF EXTRACELLULAR LEVELS

Adenosine can be metabolized in both the intracellular and the extracellular space. Within cells, adenosine derives from adenosine monophosphate (AMP) by the enzymatic activity of the endo-5'-nucleotidase (5'-ribonucleotide phosphohydrolase, E.C. 3.1.3.5) (Phillips and Newsholme, 1979) and from S-adenosyl-L-homocysteine by the activity of the S-adenosyl-L-homocysteine hydrolase (E.C. 3.3.1.1) (Nagata *et al.*, 1984). On the other hand, it is phosphorylated to AMP by adenosine kinase (AK; ATP: adenosine 5'-phosphotransferase, E.C. 2.7.1.2.0) or deaminated to inosine by the adenosine deaminase (ADA; E.C. 3.5.4.4) (Fox and Kelley, 1978). At low levels of adenosine, AK has a prominent role converting the nucleoside to AMP and only when large amounts of adenosine have to be cleared it is catabolised to inosine by ADA (Fredholm *et al.*, 2005). The normal intracellular concentration of adenosine is around 10-50 nM however, in situations of metabolic imbalance this concentration increases considerably to 1-10 µM (Cunha, 2001). This is mainly due to the activity of adenylate kinase (E.C. 2.7.4.3) which converts small variations in ATP (normally in the millimolar range inside cells) into significant variations of AMP levels and consequently of adenosine levels, both of which are in the nanomolar range under physiological conditions (Veech *et al.*, 1979; Fell and

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Sauro, 1985). This interconversion of ATP-AMP-adenosine constitutes a highly sensible intracellular monitor of metabolic imbalance.

In the extracellular space, adenosine levels are usually around 25-250 nM (Ballarin *et al.*, 1991; Dunwiddie and Diao, 1994) and the maintenance of these concentrations depends on a balance between the release/removal of the nucleoside by membrane transporters and its formation from ATP due to the activity of ecto-nucleotidases (Dunwiddie *et al.*, 1997; Dunwiddie and Masino, 2001). In mammals there are two types of nucleoside transporters: the family of equilibrative nucleoside transporters (ENT1-4) and the family of the concentrative nucleoside transporters (CNT1-3) (Parkinson *et al.*, 2011). Equilibrative transporters are the predominant type in the CNS and carry both purine and pyrimidine nucleosides, being bidirectional and driven by chemical gradients. On the contrary, the concentrative transporters are unidirectional and mediate the influx of nucleosides driven by the force of Na^+ transmembrane electrochemical gradients (Parkinson *et al.*, 2011). Nucleoside transporters support a double function of either releasing adenosine or scavenging it from the extracellular space, promoting *de novo* synthesis of nucleotides in the cytosol (Baldwin *et al.*, 2004). They can be further divided into sensitive or insensitive, ENT1 and ENT2 being the predominant types in the brain, respectively, to the selective inhibitor nitrobenzylthioinosine (NBMPR). Both ENT1 and ENT2 are highly expressed in the rat brain (especially in the hippocampus, cerebellum, cortex and striatum) and in a variety of cell types such as neurons, astrocytes, vascular smooth cells and epithelial cells of the choroids plexus (Parkinson *et al.*, 2011). The CNTs comprise 5 subclasses, two of which were detected in the rat brain in strutures such as the posterior hypothalamus, the superior colliculus, the brainstem, the striatum, the hippocampus, the cerebellum and the cortex. When the Na^+ electrochemical gradient is reversed, these transporters can also contribute to the increase of adenosine extracellular levels (Parkinson *et al.*, 2011).

As previously mentioned, adenosine can derive from ATP through the enzymatic activity of ecto-nucleotidases which are ubiquitously distributed in the nervous system. In this process, the ecto-5'-nucleotidase responsible for the conversion of AMP into adenosine and primarily found in glial cells (Latini and Pedata, 2001; Zimmermann, 2006) is a rate-limiting step because of the inhibition of its activity exerted by both ATP and ADP (Dunwiddie *et al.*, 1997).

The origin of the extracellular adenosine depends on the neuronal preparations used as well as the method used to stimulate them. In hippocampal slices, for example, the application of a high-frequency stimulation burst leads to increased extracellular levels of adenosine mainly derived from the catabolism of ATP; however, after low frequency stimulation, adenosine is mainly released *per se*

(Cunha *et al.*, 1996c). Another source of extracellular adenosine is the released cAMP via a non-specific energy-dependent transporter (Henderson and Strauss, 1991). As illustrated in Figure 1, extracellular cAMP can be converted to adenosine by the ecto-5'-nucleotidase and the alkaline phosphatases. Thus, neurotransmitters that link G_s-protein coupled receptors that induce adenyl cyclase (E.C.4.6.1.1) increasing the production of cAMP can also contribute to the raise in the extracellular levels of adenosine (Brundege *et al.*, 1997).

1.6 ADENOSINE RECEPTORS

Adenosine receptors were suspected to exist from the early found effects of adenosine in the cardiac muscle (Degubareff and Sleator, 1965) and from its induction of cAMP in cortex slices (Sattin and Rall, 1970). Nowadays it is well established that adenosine signals through four types of cell surface receptors: A_1 (A_1R), A_{2A} ($A_{2A}R$), A_{2B} ($A_{2B}R$) and A_3 (A_3R) with 70 nM, 150 nM, 5.1 µM and 6.5 µM respective affinities for the ligand (Dunwiddie and Masino, 2001). All of them are metabotropic; G-protein coupled receptors with seven transmembrane domains (Furlong *et al.*, 1992). The classically first effector molecule of adenosine receptors is the enzyme adenyl cyclase which is either stimulated or inhibited (increasing or decreasing cAMP levels) according to the receptor triggered (van Calker *et al.*, 1979; Londos *et al.*, 1980). The A_1R and A_3R inhibit adenyl cyclase through the coupling to $G_{i1,2,3/o}$ and to $G_{i2,3}/Gq_{/11}$ proteins, respectively, whereas $A_{2A}R$ and $A_{2B}R$ stimulate adenyl cyclase through the respective coupling to G_S/G_{olf} and G_S proteins (Abbracchio *et al.*, 1995; Kull *et al.*, 2000; Fredholm *et al.*, 2001). The adenosine-induced signalling is intricate, involving different pathways that include the cAMP-dependent protein kinase A (PKA), the PLC- IP_3 and diacylglycerol pathways and also the MAPK pathway (Schulte and Fredholm, 2003). Both G_i- and G_q- coupled receptors are capable of activating different MAPKs, however, their signalling to ERK1/2 is better known than their signalling to the stress activated protein kinases (SAPKs) p38 and JNK. Still, all heterotrimeric G protein families can activate these SAPKs: Gs- coupled receptors activate p38 in a cAMP- and PKA- dependent manner and G_i- and G_q- coupled receptors can activate SAPK via hg subunits, PLC, PKC, Pyk2, focal adhesion kinases and diverse guanine nucleotide exchange factors (GEFs) for the Rho-family of GTPases, among other molecules (Schulte and Fredholm, 2003).

1.6.1 DISTRIBUTION OF ADENOSINE RECEPTORS IN THE CENTRAL NERVOUS SYSTEM

In the adult brain, the A_1R is one of the two predominant adenosine receptors that is present at a higher density in the cortex, cerebellum, hippocampus, spinal cord, eye and adrenal gland (Schindler *et al.*, 2001; Rebola *et al.*, 2003). Moreover, A_1R is found in all cell types: neurons, astrocytes, microglia and oligodendrocytes (Gebicke-Haerter *et al.*, 1996; Biber *et al.*, 1997; Othman *et al.*, 2003). In hippocampal neurons, A_1R is mainly present in nerve terminals and enriched in the post-synaptic density (Tetzlaff *et al.*, 1987; Rebola *et al.*, 2003) where it modulates glutamate receptors and potassium channels (Dunwiddie and Masino, 2001). In addition, the activation of pre-synaptic A_1R inhibit the release of neurotransmitters such as glutamate, acetylcholine and serotonin (Dunwiddie and Masino, 2001).

The second most expressed adenosine receptors in the CNS are $A_{2A}R$, mainly found in striatopallidal GABAergic neurons (in the caudate putamen and nucleus accumbens) and in the olfactory bulb. Much lower levels of these receptors are also found in the lateral septum, cerebellum, cortex and in the hippocampus (Cunha *et al.*, 1995; Svenningsson *et al.*, 1997; Fredholm *et al.*, 2005). The $A_{2A}R$ is present both in neurons and in glial cells, such as astrocytes (Nishizaki, 2004) and microglia (Saura *et al.*, 2005) and in the hippocampus, it is enriched at the pre-synaptic fraction of nerve terminals whereas in striatal synapses it predominates at the post-synaptic membranes (Rebola *et al.*, 2005a).

In the CNS, $A_{2B}R$ are mainly found in the eye and in the median eminence, being also present in neurons and glial cells of the pituitary gland, albeit in lower levels (Daly, 1977; Fredholm *et al.*, 2005). The A_3R is in a low density in all main types of brain cells, in regions such as the cortex, the striatum, the olfactory bulb, the nucleus accumbens, the hippocampus, the amygdala, the hypothalamus, the thalamus and the cerebellum (Fredholm *et al.*, 2005).

1.6.2 PHARMACOLOGY OF ADENOSINE RECEPTORS

Few compounds used to classify adenosine receptors meet the ideal criterion of differing in potency by at least two orders of magnitude for different receptors (Fredholm *et al.*, 2001) and usually agonists and antagonists are developed from modifications of adenosine itself (Jacobson and Gao, 2006). For example, xanthines such as caffeine and theophylline, typical non-selective antagonists of adenosine receptors with micromolar affinities for A_1R, $A_{2A}R$ and $A_{2B}R$ (Fredholm *et al.*, 2001), are often used as templates (Moro *et al.*, 2006). The CPA and the CCPA are commonly used agonists of the A_1R; however, *in vivo*, the first presents a very small half-life (of 6 minutes) in the rat blood (Mathot *et al.*, 1993) and the second displays a much higher affinity for these receptors (Fredholm *et al.*, 2001; Fredholm *et al.*, 2005). As for A_1R antagonists, DPCPX is a commonly used selective compound (Fredholm *et al.*, 2005). In the case of $A_{2A}R$, NECA, a non-selective agonist, and specially CGS21680, a more selective drug based in NECA, are usually used, even though the later is less potent and selective in humans than in rats (Hutchison *et al.*, 1989; Kull *et al.*, 1999). Nonetheless, CGS21680 has the advantage of binding to sites where the $A_{2A}R$ density is low (Johansson *et al.*, 1993; Cunha *et al.*, 1996b). One of the most commonly used $A_{2A}R$ selective antagonists is SCH58261, which displays a high affinity for the receptor in both humans and rats (Fredholm *et al.*, 2005; Yang *et al.*, 2007). Nowadays, KW6002 is a commonly used antagonist of $A_{2A}R$ for *in vivo* studies, mainly due to its characteristics of bioavailability, half-life and blood-brain barrier penetration (Yang *et al.*, 2007). In the case of $A_{2B}R$, LUF5835 is the most potent agonist known (Beukers *et al.*, 2004) while MRS1754 and MRE 2029-F20 are some of the usually used antagonists (Ji *et al.*, 2001; Gessi *et al.*, 2005; Jacobson and Gao, 2006). Finally, for the A_3R, Cl-IB-MECA is a well-known agonist (Fredholm *et al.*, 2001) despite the fact that this compound can also bind and activate A_1R which vastly outnumber A_3R in the brain (Klotz *et al.*, 1998; Lopes *et al.*, 2003). The most commonly used antagonists of A_3R are dihydropyridines, pyridines or flavonoids (Baraldi *et al.*, 2000). In the Table 4 bellow are listed some examples of agonists and antagonists of the four adenosine receptors.

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Table 4: Pharmacology of the adenosine receptors, taken from Jacobson and Gao (2006).

Adenosine receptor subtype	Compound	K_i value for AR (nM)			
		A_1AR^*	$A_{2A}AR^*$	$A_{2B}AR^*$	A_3AR^*
Agonists					
A_1	CPA	2.3	794	18,600[¶]	72
	CCPA	0.83	2,270	18,800[¶]	38
	S(–)-ENBA	0.38	>10,000	>10,000[¶]	915
	ADAC	0.85[‡]	210[‡]	N.D.	13.3
	AMP579	5.1[‡]	56[‡]	N.D.	N.D.
	NNC-21-0136	10[‡]	630[‡]	N.D.	N.D.
	GR79236	3.1[‡]	1,300[‡]	N.D.	N.D.
	CVT-510 (Tecadenoson)	6.5[§]	2,315[§]	N.D.	N.D.
	SDZ WAG 994	23[§]	25,000[§]	>10,000[§,¶]	N.D.
	Selodenoson	1.1[‡]	306[‡]	N.D.	N.D.
A_{2A}	NECA	14	20	140[¶]	25
	CGS21680	289	27	>10,000[¶]	67
	DPMA	168	153	>10,000[¶]	106
	Binodenoson	48,000	270	430,000[¶]	903
	ATL-146e	77	0.5	N.D.	45
	CV-3146	>10,000	290	>10,000[¶]	>10,000
A_{2B}	LUF5835	4.4	21	10[¶]	104
A_3	IB-MECA	51	2,900	11,000[¶]	1.8
	Cl-IB-MECA	220	5,360	>100,000[¶]	1.4
	LJ568	193	223	N.D.	0.38
	CP-608039	7,200	N.D.	N.D.	5.8
	MRS3558	260	2,300	>10,000[¶]	0.29
	MRS1898	136	784	N.D.	1.5
Antagonists					
A_1	DPCPX	3.9	129	56	3,980
	WRC-0571	1.7	105	N.D.	7,940
	BG 9719	0.43	1,051	172	3,870
	BG 9928	29	4,720	690	42,110
	FK453	18	1300	980	>10,000
	FR194921	2.9	>10,000	N.D.	>10,000
	KW3902	1.3[‡]	380[‡]	N.D.	N.D.
A_{2A}	KW6002	2,830	36	1,800	>3,000
	CSC	28,000[‡]	54[‡]	N.D.	N.D.
	SCH 58261	725	5.0	1,110	1,200
	SCH 442416	1,110	0.048	>10,000	>10,000
	ZM241,385	774	1.6	75	743
	VER 6947	17	1.1	112	1,470
	VER 7835	170	1.7	141	1,931
	'Schering compound'	82	0.8	N.D.	N.D.
A_{2B}	MRS1754	403	503	2.0	570
	MRE 2029-F20	245	>1,000	3.0	>1,000
	OSIP-339391	N.D.	N.D.	0.5	N.D.
A_3	OT-7999	>10,000	>10,000	>10,000	0.61
	MRS1292	12,100[‡]	29,800[‡]	N.D.	29.3
	PSB-11	1640	1,280	2,100[¶]	3.5
	MRS3777	>10,000	>10,000	>10,000[¶]	47
	MRS1334	>100,000	>100,000	N.D.	2.7
	MRE 3008-F20	1,200	141	2100	0.82
	MRS1220	305[‡]	52.0[‡]	N.D.	0.65
	MRS1523	15,600[‡]	2,050[‡]	N.D.	18.9
	'Novartis compound'	197	1,670	3.0[¶]	10.0

*Binding experiments at recombinant human A_1, A_{2A}, A_{2B} and A_3 adenosine receptors (ARs), unless noted. [‡]Binding experiments at rat ARs. [§]Binding or functional experiments at porcine ARs. [¶]Data are from a cyclic AMP functional assay. N.D., not determined or not disclosed.

1.6.3 ADENOSINE RECEPTORS: MODULATION OF SYNAPTIC TRANSMISSION, NEUROPROTECTION AND CONTROL OF NEUROINFLAMMATION

The neuromodulation exerted by adenosine in the brain seems to depend on the relative importance of A_1R and $A_{2A}R$ in a given time, local and environment since these are the two receptor types most expressed in the CNS (Fredholm *et al.*, 2005) and have the highest affinity for the ligand (Fredholm *et al.*, 2001). However, they mediate opposite effects of adenosine in most situations (Cunha, 2005; Fredholm *et al.*, 2005). The A_1R-mediated effects include inhibition of neurotransmitter release at pre-synaptic sites (Dunwiddie and Masino, 2001) and decrease of neuronal excitability at post-synaptic moieties through activation of potassium channels, decrease of NMDA receptor's currents and the inhibition of voltage-sensitive Ca^{2+} channels (de Mendonca *et al.*, 2000; Fredholm *et al.*, 2005). On the contrary, the activation of $A_{2A}R$ increases neuronal excitability through the facilitation of neurotransmitters release from nerve terminals (Cunha, 2001). However, at the post-synaptic level, the control of NMDA currents by $A_{2A}R$ is still not well comprehended and different authors revealed some contradictory data (Wirkner *et al.*, 2000; Gerevich *et al.*, 2002; Wirkner *et al.*, 2004; Tebano *et al.*, 2005; Rebola *et al.*, 2008; Ferguson and Stone, 2010). Paradoxically, in the hippocampus, $A_{2A}R$-mediated increase of excitatory transmission was found to depend on the activation of A_1R (Lopes *et al.*, 2002). In fact, A_1R and $A_{2A}R$ are frequently present at the same synapses (Rebola *et al.*, 2005c; Ciruela *et al.*, 2006a). This, however, contributes to the fine-tuning of neurotransmission by adenosine since $A_{2A}R$ can also control the activity of A_1R through intracellular transducing systems (Dixon *et al.*, 1997; Lopes *et al.*, 1999) or receptor dimerisation (Ciruela *et al.*, 2006a). In addition, adenosine modulates other neuromodulators (Sebastiao and Ribeiro, 2000) such as the calcitonin gene-related peptide (Correia-de-Sa and Ribeiro, 1994) and the brain derived neurotrophic factor (BDNF) (Diogenes *et al.*, 2004; Assaife-Lopes *et al.*, 2010; Sebastiao *et al.*, 2011). Sometimes this control by adenosine is due to receptor heteromerization as occurs between A_1R and dopamine D_1 receptors (Gines *et al.*, 2000), A_1R and ATP $P2Y_1$ receptors (Yoshioka *et al.*, 2001) and between A_1R and metabotropic glutamate type 1 receptors (mGluR1) (Ciruela *et al.*, 2001). Moreover, heteromerizations between $A_{2A}R$ and the metabotropic glutamate type 5 receptors (mGluR$_5$) (Ferre *et al.*, 2002) and between $A_{2A}R$ and the endocannabinoid CB1 receptors (Ciruela *et al.*, 2006b) have also been described. Finally, $A_{2A}R$ can also transactivate BDNF TrkB receptors (Lee and Chao, 2001).

In pathological situations in the brain, adenosine is known to be mainly neuroprotective (Andine *et al.*, 1990; Park and Rudolphi, 1994; Tatlisumak *et al.*, 1998; Pignataro *et al.*, 2007). This is

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attributed to the activation of A_1R, as demonstrated in models of ischaemia (Corradetti *et al.*, 1984), of methamphetamine- or 3-nitropropionic acid-induced toxicity in the striatum (Delle Donne and Sonsalla, 1994) and of epilepsy (O'Shaughnessy *et al.*, 1988; Li *et al.*, 2008b). In fact, the inhibition of adenosine kinase, the main enzyme responsible for the clearing of adenosine, is sufficient to prevent seizures in models of epilepsy (Kowaluk and Jarvis, 2000). Moreover, administration of A_1R antagonists was shown to aggravate brain damage (de Mendonca *et al.*, 2000). In certain situations, however, adenosine can exacerbate neurotoxicity and usually due to $A_{2A}R$-mediated effects (Stone *et al.*, 2009). The density of these receptors is frequently increased in noxious conditions (Rebola *et al.*, 2005b; Cunha *et al.*, 2006; Duarte *et al.*, 2006) and their blockade or genetic deletion is frequently neuroprotective (Cunha, 2005; Gomes *et al.*, 2011).

The neuroprotection afforded with the blockade of $A_{2A}R$ in so many different noxious conditions and especially in brain regions where the receptor is scarcely expressed is somewhat puzzling. However, the $A_{2A}R$-mediated effects seem to "gain protagonism" when adenosine levels increase significantly, as occurs at high frequency of neuronal firing (Cunha *et al.*, 1996a). Thus, $A_{2A}R$ are likely to be activated under stressful situations, when both ATP and adenosine reach high concentrations in the extracellular space (Cunha *et al.*, 1996a; Cunha *et al.*, 1996c; Pinto-Duarte *et al.*, 2005). Given that activation of $A_{2A}R$ facilitates glutamate release, one possible explanation for the afforded neuroprotection with the blockade of these receptors is that it prevents glutamate overflow (Lopes *et al.*, 2002; Marchi *et al.*, 2002; Rodrigues *et al.*, 2005a). Under pathological conditions, most of the extracellular glutamate comes from the astrocytes (Bezzi *et al.*, 2004; Lee and Haydon, 2007) due to a loss of efficiency or reversed transport of glutamate transporters (Rossi *et al.*, 2000) that result in increased efflux of this neurotrasmitter (Rossi and Volterra, 2009). Adenosine receptors are also present in glial cells (Boison *et al.*, 2010) where high extracellular concentrations of adenosine are likely formed (Pascual *et al.*, 2005; Studer *et al.*, 2006). Moreover, the density of the ATP degrading enzymes ecto-5'-nucleotidase and ecto-NTPDase are also increased in the activated microglia, contributing to the formation of adenosine (Kreutzberg and Barron, 1978). Thus, the control of neuronal damage by adenosine receptors may involve the control of glial transmission and of neuroinflammation, which is a common feature of all neurological disorders and of any noxious condition in the CNS (Skaper, 2007). Neuroinflammation is essential for host defence, clearance of pathogens/cell debris and tissue recovery. However, the cytotoxicity of some inflammatory mediators such as pro-inflammatory cytokines and reactive species can non-specifically cause damage to local tissues (Ohta and Sitkovsky, 2009). Thus, the balance between the beneficial *versus* detrimental effects

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of inflammation depends on a tight control of its "on" and "stop" signals. In the peripheral nervous system, the role of adenosine as a "stop" signal of inflammation is well established (Ohta and Sitkovsky, 2009). Early studies showed that cAMP inhibited the effector functions of T cells through a PKA-dependent pathway, which can be triggered by $A_{2A}R$ (Sitkovsky *et al.*, 1988; Huang *et al.*, 1997). Accordingly, elevated levels of adenosine are observed in complications of an inflammatory nature, such as asthma (Brown *et al.*, 2008) and sepsis (Sitkovsky, 2003). Moreover, the ablation of $A_{2A}R$ results in prolonged and severe inflammation, with sustained production of pro-inflammatory cytokines (Ohta and Sitkovsky, 2001; Lukashev *et al.*, 2004) and caffeine, a non-selective antagonist of receptors (Fredholm *et al.*, 1999), exacerbates inflammation (Ohta *et al.*, 2007). The same happens during induced ischemia (Day *et al.*, 2004) and sepsis (Nemeth *et al.*, 2006) in $A_{2A}R$ knock-out mice. On the other hand, activation of $A_{2A}R$ suppresses leukocyte infiltration in response to pro-inflammatory cytokines (Zhang *et al.*, 2006). However, in the brain, it seems to be the blockade of $A_{2A}R$ that reduces neuroinflammation as some studies have gathered evidence for the control of the activation of microglia and of the effects of neuroinflammatory mediators by the blockade of $A_{2A}R$ (Yu *et al.*, 2004; Stone and Behan, 2007; Yu *et al.*, 2008; Rebola *et al.*, 2011). Moreover, activation of these receptors inhibited glutamate uptake by astrocytes both *in vitro* (Li *et al.*, 2001; Nishizaki *et al.*, 2002) and *in vivo* (Pintor *et al.*, 2004). Notwithstanding, the control of neuroinflammation by $A_{2A}R$ is still controversial due to opposite results *in vivo* (Yu *et al.*, 2008; Dai *et al.*, 2010) and *in vitro* (Saura *et al.*, 2005, Orr, 2009 #3451; van der Putten *et al.*, 2009).

AIM

-       To determine the ability of adenosine A_{2A} receptors to control the neuroinflammation-induced biochemical and morphologic features in the hippocampus, using a rat model of sepsis.

-       To test the ability of A_{2A} receptors to control the direct effect of mediators of neuroinflammation such as pro-inflammatory cytokines on hippocampal neurons and to explore the underlying mechanism.

-       To test whether the ATP $P2Y_1$ receptors also control the effects of pro-inflammatory cytokines in hippocampal neurons.

-       To explore the neuroprotection afforded with the blockade of $P2Y_1$ receptors under excitotoxic conditions to hippocampal cultured neurons.

-       To investigate the long-term consequences of kainate-induced epileptic seizures in the density of P2 receptors in both synaptosomes and total membranes of the rat hippocampus.

-       To investigate, *in vivo*, the potential of the blockade of $P2Y_1$ receptors to prevent the seizure-induce loss of synaptic markers, excitotoxic cell death and neuroinflammation in the hippocampus, using a rat model of temporal lobe epilepsy.

-       To further explore the neuroprotection afforded with the blockade of $P2Y_1$ receptors against ischemic-induced lesion and mnemonic deficits, using a mouse model of middle cerebral artery occlusion.

MATERIALS AND METHODS

3.1 THE HIPPOCAMPUS

The hippocampus is one of the most studied brain structures and one that is particularly developed in rodents. It is part of the limbic system and is located inside the medial temporal lobe of the cerebral cortex, being therefore a part of the forebrain or telencephalon. The hippocampus and its associated medial temporal lobe structures have been described as essential for the formation, consolidation and retrieval of episodic memories (Morris *et al.*, 1982; Squire and Zola, 1997; Eichenbaum, 2000; Scoville and Milner, 2000). Animals with lesions in the hippocampus or adjacent structures (septum, fimbria/fornix or parahippocampus) have been described to present impaired spatial and working memory (O'Keefe and Conway, 1978).

The hippocampal formation is a bi-lateral limbic structure, which consists of two "C"-shaped interlocking cell layers leaning together at the top and spread apart at the base (Fig. 4). The top is known as the "dorsal hippocampus" and, since it is close to the septum (a structure at the midline of the brain) the dorsal tip of the hippocampus is called the "septal pole". The cross-section taken perpendicularly to the long axis (septal-temporal) reveals the two interlocking "C"-shapes. One of the "C"s is the Ammon's Horn of *Cornu Ammonis*, also known as the "Hippocampus proper". The principal cell layer of the Ammon's Horn is the *stratum pyramidale*, or the pyramidal cell layer. The other "C" is constituted by the dentate gyrus (DG), of which the *stratum granulosum* or granule cell layer is the main cell layer. This cross-section cut of the hippocampus also exhibits a strong afferent set of three connected pathways known as the "trisynaptic circuit" or loop (Andersen *et al.*, 1966; Swanson *et al.*, 1978; Swanson, 1982; Witter *et al.*, 1989) which is represented by three subdivisions of the hippocampus: CA1, CA2 and CA3 areas (represented in Fig. 4). These areas represent a trisynaptic excitatory circuit that process information throughout this brain structure (Amaral and Witter, 1989). The connections within the hippocampus generally follow a laminar format and, as a rule, are unidirectional. They form well-characterized closed loops from and to the adjacent entorhinal cortex (Teyler and DiScenna, 1984). First, layers II and III or "surface layers" of the entorhinal cortex project to the granule cells of the DG via the perforant path, the major input to the hippocampus, with minor contributions from the deeper layers IV and V. Second, the granule cells of the dentate gyrus project to the large pyramidal cells of *Cornu Amonnis* subfield 3 (CA3), via the mossy fibers system. Third, the CA3 pyramidal cells project to the pyramidal cells of the CA1 subfield, via the Schaffer collateral system (Blackstad, 1956; Blackstad, 1958; Amaral, 1978; Bayer, 1985; Amaral and Witter, 1989;

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Witter *et al.*, 1989). This network primarily results from a physiologic balance between inhibitory GABAergic and excitatory glutamatergic neurotransmissions; however, there are other neurotransmitters that can have a minor contribution and, therefore, a major contribution to fine-tune transmition in the hippocampus (Lopes da Silva *et al.*, 1990).

The hippocampus is an excellent structure for experiments on the role of purine receptors in neurodegenerative disorders and neuroinflammation since it is one of the primarily affected brain regions, specially in the cases of epilepsy and ischaemia-induced damage (Back *et al.*, 2004; Bertram, 2009; Chen *et al.*, 2010). Furthermore, the hippocampus has well defined circuits suited for biochemical studies and for the evaluation of memory processes dependent on the integrity of this area.
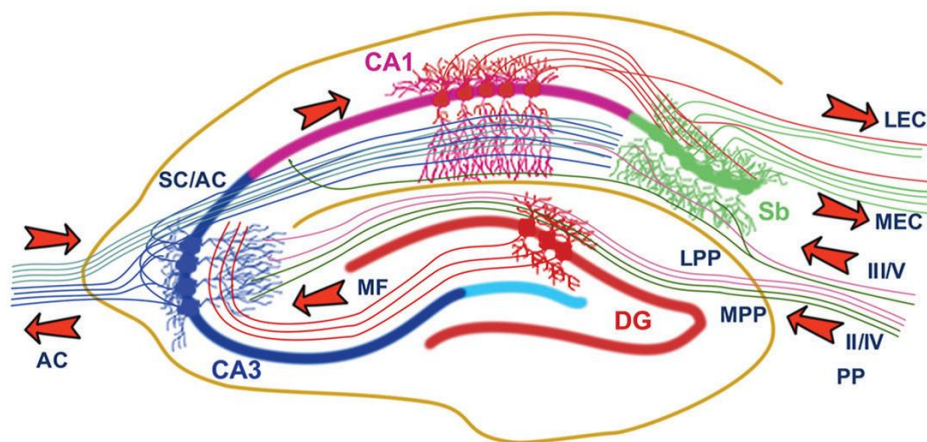
Figure 4. Hippocampal pathways. The main hippocampal input comes from the lateral or medial entorhinal cortex (LEC or MEC, respectively) which forms connections with the dentate gyrus (DG) and CA3 pyramidal neurons via the perforant path (PP-split into lateral and medial). The CA3 pyramidal neurons mainly receive inputs from the DG neurons via the mossy fibers (MF). The CA3 pyramidal neurons send axons to CA1 pyramidal neurons via the Schaffer collateral pathway (SC), as well as to other CA3 pyramidal neurons or to CA1 pyramidal neurons in the contralateral hippocampus via the associative comissural pathway (AC). The CA1 pyramidal neurons also receive inputs directly from the perforant path and send axons to the subiculum (Sb). Adapted from http://www.bristol.ac.uk/synaptic/info/pathway/hippocampal.htm.

3.2 BIOLOGICAL MODELS AND PREPARATIONS

3.2.1 ANIMALS

The animals used in all the experiments presented in this thesis were rodents: male rats of the lineage Wistar, 8-10 weeks old and weighting between 250-300 g; male C57BL/6 mice, 8-10 weeks old

and weighting between 20-40 g and finally, 8 weeks old Swiss male mice, weighting between 30-40 g, for the model of focal ischemia. All animals were obtained from Charles River (Barcelona, Spain).

In the preparation of hippocampal primary neuronal cultures, 18 days old embryos were used from pregnant female Wistar rats, subjected to caesarean in deep anaesthesia (using halothane). Animals were handled in accordance with the EU guidelines for the use of laboratory animals and were housed four per cage (unless otherwise stated) and maintained under a controlled environment: 23 ± 2 ºC of temperature, 55 ± 5 % humidity, 12 hours light/dark cycle with standard food pellets *ad libitum* and tap acidified water.

3.2.2 ANIMAL MODEL OF SEPSIS

Sepsis, also commonly named septicaemia or blood poisoning, is a medical condition found in association with a known or suspected infection, usually caused by bacteria (Remick *et al.*, 2000). This complication has been considered the main cause of death amongst hospitalized patients on intensive care worldwide (Remick *et al.*, 2000). Sepsis is characterized by a rapid and exacerbated systemic inflammatory reaction known as the systemic inflammatory response syndrome (SIRS) which can lead to organ failure and be, therefore, life-threatening. The treatment involves administration of antibiotics to kill the infectious agent and the control of SIRS (Remick *et al.*, 2000; Semmler *et al.*, 2005). The most commonly used animal model of sepsis is the intraperitoneal (ip) administration of lipopolysaccharide (LPS) (Semmler *et al.*, 2005; Dyson and Singer, 2009), an endotoxin that is a constituent of the cell wall of the Gram negative bacteria which is rapidly recognized by and activates the immune system (Dyson and Singer, 2009). Many studies focused on the peripheral most affected organs such as the liver, gut and kidney. However, the peripheral inflammation leads to the disruption of blood brain barrier which becomes permeable to peripheral pathological agents, activated immune cells as well as their released pro-inflammatory mediators (Banks and Erickson, 2010). In fact, septic encephalopathy occurs in the majority of septic patients and is the most common encephalopathy in intensive care units associated with a higher mortality rate (Pine *et al.*, 1983; Sprung *et al.*, 1990; Young *et al.*, 1990). In rodent models, the acute effects triggered by LPS have been well documented and involve the rapid production of cytokines, chemokines, prostaglandins and nitric oxide (NO) (Rietschel *et al.*, 1996). The neurotoxic effects of these pro-inflammatory mediators are also well known (Brown, 2007; Brown and Neher, 2010; Hu *et al.*, 2010) and the cell death within the CNS

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during sepsis has been described in both rodents and humans (Messaris *et al.*, 2004; Semmler *et al.*, 2005; Sharshar *et al.*, 2005).

Overall, this model is appropriated to study not only ATP and adenosine modulation and control of inflammation but also to evaluate long-term consequences and/or benefits of that modulation in terms of cell function and integrity. Moreover, since the hippocampus is one of the brain regions most affected by LPS-induced inflammation, it was decided to restrict the analysis to this structure.

3.2.2.1 EXPERIMENTAL PROCEDURES

The following experiments were carried in order to gain further insight into the mechanism through which the blockade of adenosine A_{2A} receptors control neuroinflammation:

Groups of two months old, male Wistar rats (weighting 300-350 g) were anaesthetised with an ip injection of urethane (1.5 g/kg) and placed in a stereotaxic frame to allow the intracerebroventricular (icv) administration of drugs through a 0.5 ml Hamilton syringe into the third ventricle (coordinates: - 0.8 mm posterior to Bregma, 0.0 mm lateral, i.e. in the midline, and -6,5 mm dorsal-ventral). The rats were divided into 4 groups: 1) control rats injected with 5 μl saline icv and 200 μl saline ip after 30 min; 2) LPS-treated rats injected with 5 μl saline icv and 200 μl LPS ip (1 μg/μl prepared in 0.9 % NaCl solution, saline) after 30 min; 3) A_{2A} receptor antagonist-treated rats injected with 5 μl SCH58261 (50 nM in saline) or with 5 μl MSX-3 (1 μM in saline) icv and 200 μl saline ip after 30 min; 4) LPS + A_{2A} receptor antagonist-treated rats injected with 5 μl SCH58261 (50 nM) or with 5 μl MSX-3 (1 μM) icv and 200 μl LPS (200 μg) ip after 30 min. Animals were sacrificed 4 hours after the intraperitoneal LPS or saline administration and the following studies were persued: quantification of interleukin-1β concentration; immunohistochemistry and Western blot analysis of mitogen-activated protein kinases (MAPKs); evaluation of caspase-3 activity; immunohistochemistry assessement of the profile of microglia and of the activated form of caspase-3 and analysis of both mRNA and density of protein markers of microglial activation through polymerase chain reaction (PCR) and Western blot analysis, respectively.

Drugs used: lipopolysaccharide (LPS) was isolated from Escherichia coli, serotype 055:B5 and acquired from Sigma-Aldrich, Portugal. the kit for ELISA quantification of interleukin 1β (Duoset) was acquired from Genzyme Diagnostics, SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) was a generous gift from Scott Weiss (Vernalis, UK) and MSX-3 (3,7-dihydro-8-[(1E)-2-(3-methoxyphenyl)ethenyl]-7-methyl-3-[3-(phosphonooxy)propyl-1-(2-propynyl)-1H purine-2,6-dione disodium salt hydrate) was a generous gift from Sergi Ferré (NIDA, USA).

The LPS dose was chosen based on previous studies that characterized the effect of peripheral administration on hippocampal neuronal functionality and viability (Vereker *et al.*, 2000a; Kelly *et al.*, 2003; Nolan *et al.*, 2003). The choice of SCH58261 and the administrated dose (0.00025 nmol) to block A_{2A} receptor relied on antagonist affinity studies, showing that it was in the sub-nanomolar range (Lopes *et al.*, 2004) and the selectivity towards the other adenosine receptors, evaluated by the disappearance of the selective binding of the antagonist in A_{2A} receptor knockout mice (Lopes *et al.*, 2004). The concentration of the antagonist administered, 50 nM, was chose based on its equivalent effect, applied through reverse-microdialysis and ip injection, of neuroprotective doses of 0.01-0.1 mg/kg (Cunha, 2005) on the evoked release of glutamate from the rat striatum (Pintor *et al.*, 2001; Corsi *et al.*, 2003). Likewise, the selected dose of MSX-3 was based on previous observation that this dose prevented the A_{2A} receptor-mediated phosphorylation of proteins in the striatum upon cortical stimulation (Quiroz *et al.*, 2006; Quiroz *et al.*, 2009).

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3.2.3 ANIMAL MODEL OF TEMPORAL LOBE EPILEPSY

One of the most accepted and best characterized animal models of mesial temporal lobe epilepsy (MTLE) is the systemic or local administration to animals of 2-carboxy-4(1-methylethenyl)-3-pirrolidiacetic acid (kainate), a cyclic analog of L-glutamate and about one hundred times more potent agonist of glutamate ionotropic receptors, which induces acute seizure episodes and subsequent development of spontaneous motor seizures and similar hippocampus lesions (such as specific loss of cell population in the Ammon's horn and aberrant mossy fiber sprouting) to those observed in humans (Nadler, 2003).

Seizures induced by kindling have been classified into five stages based on clinical signs and this scale was later adopted to describe seizures in other animal models of epilepsy (Racine, 1972). According to the Racine's scale to monitor rat behaviour immediately after chemoconvulsant-induced seizures, there are five stages to be considered: stage I- facial automatisms; stage II- head nodding; stage III- forelimb clonus and lordotic posture; stage IV- forelimb clonus as the animal rears; stage V- forelimb clonus and rearing with falling over or loss of the righting reflex. Stages III, IV and V, together with "wet-dog" shake movements, are considered motor or convulsive seizures and should happen until two hours after kainate injection to guarantee hippocampal neuronal degeneration.

3.2.3.1 EXPERIMENTAL PROCEDURE

This model was performed using adult, male, Wistar rats (weighting 250-300 g). Animals were separated into 4 different groups: the saline group, which received artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 10 mM glucose, 26 mM $NaHCO_3$, 1 mM $MgCl_2$ and 2 mM $CaCl_2$) icv and saline (SAL, 0.9 % NaCl) ip; the kainate group, which received aCSF icv and 10 mg/kg kainate (KA) ip; the PPADS + KA group, that received 1 nmol/4 µl PPADS icv and 10 mg/kg kainate ip; the MRS + KA group, that received 1 nmol/4 µl MRS2500 icv and 10 mg/kg kainate ip and, finally, the PPADS and MRS groups that received 1 nmol/4 µl PPADS or MRS2500 icv and SAL

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ip. Three days before the ip administration of kainate or saline, the animals were anaesthetized with sodium thiopental 50 mg/kg/ml ip and placed in a stereotaxic apparatus. The skull was opened and a guide cannula (CMA11 guide cannula) was introduced into the lateral ventricle and fixed to the skull with the help of two drills and cement, at the following coordinates relative to Bregma: -0,8 mm antero-posterior; -1,5 mm lateral and -3,5 mm dorso-ventral. On the icv injection day, animals were taken into a needle (4.2 mm length, 26-guage) connected to a PE 50 polyethylene tube. After unscrewing the cap, the needle was gently inserted through the guide cannula, 1 mm bellow, into the ventricle and drugs or saline were slowly manually infused. Administration was done at the rate of 0.5 µl/ min and, at the end of the injection, the needle remained in place for 3 min before being slowly removed from the cannula, to avoid reflux.

3.2.4 ANIMAL MODEL OF FOCAL CEREBRAL ISCHEMIA

Stroke is the third leading cause of death in the world and 80 % are of an ischemic nature caused by the occlusion of the middle cerebral artery (MCA) (Howells *et al.*, 2010). Ischemia is defined by a reduction of cerebral blood flow which prevents the affected tissue of normal supply of oxygen and nutrients, leading to cell death in the affected area (Braeuninger and Kleinschnitz, 2009). There are two types of *in vivo* experimental ischemia: global ischemia (achieved through cardiac arrest) and focal ischemia models. In focal ischemia models, the MCA is occluded either permanently or temporarily to allow reperfusion (Braeuninger and Kleinschnitz, 2009). The affected area or the area of stroke is designated the infarction area and is highlighted as a white region on brain slices stained with 2,3,5-triphenyltetrazolium chloride (TTC) (Boyko *et al.*, 2010). Microscopic visualization of this area shows edema and cellular swelling. The volume of stroke is calculated integrating the areas of infarction of a series of brain slices (Tureyen *et al.*, 2004). Blockade of the MCA origin in rodents results in infarcts which incorporate the grey matter of the motor and somatosensory cortex, the underlying white matter tracts and the basal ganglia (caudate-putamen and thalamus) which have blood supplied by small perforating arteries that branch from the MCA or from adjacent segments of the circle of Willis (Howells *et al.*, 2010). In accordance to its ability to affect the mencioned brain areas, MCA occlusion leads to motor and mnemonic impairments (Hunter *et al.*, 2000).

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Despite similarities, there are important differences between species and strains of animals that can alter the ischemic outcome. The Swiss Webster mice used in the present work have good sensitivity to ischemic MCA occlusion and reproducible outcome, which is dependent on the frequency of the sequence of posterior communicating arteries (Barone *et al.*, 1993).

3.2.4.1 EXPERIMENTAL PROCEDURE

The animals were subjected to transcranial surgical, distal occlusion of MCA through cautery using a transorbital approach. Animals were first anesthetized with avertine (12 µL/g in mice): 10 g of the tribromoethyl alcohol (2, 2, 2 tribromoethanol) and 10 ml of the tertiary amyl alcohol (2 methyl-2-butanol), prepared according to the institutional animal care and use committee- IACUC. The animal's skull was opened, the dura was breached and a hole was drilled exposing the segment of the artery to be blocked through electrocauterization (cut just distal to the inferior cerebral vein) which results in permanent MCA occlusion (MCAO) (Tamura *et al.*, 1981; Marks *et al.*, 2001). The wound was sutured and the animals were allowed to recover for 24 hours. The orbital route approach used is the least traumatic and, compared to procedures requiring craniotomy, results in less blood loss and artefacts (Sicard and Fisher, 2009).

Ten minutes before MCA occlusion, animals were placed in a stereotaxic apparatus, and administered either artificial cerebrospinal fluid (vehicle, V), PPADS (0.5 nmol/µl or 1 nmol/µl) or MRS2500 (1 nmol/µl), intracerebroventricularly (icv) according to the following coordinates, taken from the Franklin and Paxinos, 1997 (The Mouse Brain in Stereotaxic Coordenates): anterior-posterior (AP), -1.0mm, dorso-ventral (DV), -2.0mm, and lateral (L), -0.5mm, relative to Bregma, as used in (Yu *et al.*, 2008). Six groups of animals were organized as follows: the group receiving PPADS at the lower dose (0.5 nmol/µL); the group receiving PPADS at the higher dose (1 nmol/µl); the group receiving MRS2500; the group receiving vehicle; the group receiving vehicle and where the MCA was exposed but not occluded (sham-operated) and also a sham-operated group that received PPADS at the highest dose.

Deficits in the motor function are common features of the ischemic-induced neurological status (Hunter *et al.*, 2000). The neurological evaluation of animals was performed before and 24 hours after MCAO, according to the table 5 as described in (Hunter *et al.*, 2000) and based on SHIRPA (SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment) protocol. Only animals scoring significantly different from the sham-operated mice in terms of

locomotion, body posture and muscle strength were further evaluated. Twenty-four hours after ichemia, animals were sacrificed by decapitation, brains dissected in a sucrose solution and sectioned on a mouse brain slicer matrix (0.5 mm coronal slice intervals, Zivic Instruments). The Infarct area was revealed by submersing the brain sections in a 2 % in normal saline 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Aldrich, Portugal) solution at 37 ºC during 20 minutes, in the dark. The slices were washed twice in saline solution and fixed in 4 % paraformaldehyde for 30 min at room temperature. The slices were again passed twice by saline solution and, afterwards, both sides of sections were scanned on a flatbed colour scanner. TTC staining allows the evaluation of infarct area macroscopically. The colorless TTC is reduced to red formazan product by dehydrogenases that exist most abundantly in the mitochondria and therefore stain intensity correlates positively with the number and functionality of mitochondria. The infarcted volume is presented as a white region among the redness of the rest of the slice and was calculated using the image J software and as a percentage of the total volume of the contralateral hemisphere, to evaluate the extent of ischemic damage (Tureyen *et al.*, 2004).

Three days after ischemia, another group of animals were tested for motor skills in an open field and for mnemonic skills in a Y-maze; a Morris water maze and a passive avoidance apparatus.

Table 5. Neurological scoring in mice models of middle cerebral artery occlusion (Hatcher *et al*. 2002)

0	NO DEFICIT
1	Flexion of contralateral torso and forelimb upon lifting of the whole animal by the tail
2	Circling to the contralateral side when held by tail with paws on the floor
3	Spontaneous circling to the contralateral side
4	No spontaneous motor activity

3.3 BEHAVIORAL TESTS

The evaluation of locomotor function was done using an open field and the assessment of mnemonic aspects was performed using the simple and modified Y-maze, the passive avoidance test, the object recognition test and the Morris water maze. The analysis of anxiety was performed on an elevated plus maze.

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The behavioural tests were carried in a sound-isolated room with red light and unchanged environment, taking special care of spatial and odor cues. The apparatus were always carefully cleaned and removed of any smell before and after each animal tested and at the end of experiments. To avoid influences of circadian rhythms, animals were tested between 9:00 am and 4:00 pm.

3.3.1 ORDER OF BEHAVIORAL TESTS

To avoid stressing the animals and to minimize the interference between tests, the following order was used: first the animals were tested for locomotor proficiency in the open field; next, the animals were tested in the Y-maze, subjected to the object recognition test or tested in the Morris water maze for the evaluation of working memory. The evaluation of aversive memory was performed next, using a passive avoidance apparatus, and in the end, animals were tested for anxiety-like behaviour in an elevated plus maze.

3.3.2 OPEN FIELD

The open field test to evaluate locomotor activity was performed according to (Walsh and Cummins, 1976). The test takes advantage of the rodent's natural tendency to explore a novel environment. The rat open field consists in a square arena, 50 x 50 cm, which is divided into 4 squares of 25 cm. The mice open field has, naturally, smaller dimensions: 30 x 30 cm divided into 9 squares. The space is build of acrylic material painted in black to minimize the animal´s anxiety. The exploratory behaviour of animals was evaluated by counting the total number of line crossings (horizontal exploration) and the total number of rearings (vertical exploration) during 5 minutes.

3.3.3 OBJECT RECOGNITION

Object recognition is a useful test to study declarative memory in the rodents, taking advantage of their preference in exploring novel objects over familiar ones (Bevins and Besheer, 2006). Moreover, this type of memory test requires mechanisms dependent on hippocampal integrity. Long-term potentiation in the hippocampus, a mechanism underlying memory consolidation and characterized by an activity-dependent enhancement of synaptic strength, is stimulated during this type of test(Clarke *et al.*, 2010). As explained in (Dere *et al.*, 2007) for "one trial object recognition task",

the assay consists in letting the animals explore two equal objects for 5 minutes. These will be the familiar objects; placed equidistant from the open field walls, so that the animals have enough space to move around them. The objects were carefully chosen (colour, shape, dimensions, texture and odor), being neutral for the animals, i.e., not attractive nor repulsive. Exploration was defined as sniffing or touching the object with the nose and/or forepaws. Sitting on or going around the objects was not considered exploratory behaviour. One hour and a half later, having changed one of the objects for a new different one, the animals are left to explore for another 5 minutes. In both sessions, the time the animals spent exploring each object is recorded. It is expected that, animals without memory impairment will have a higher recognition index, i.e., spend more time exploring the new object. The recognition index is calculated as follows:

Recognition index = (Time exploring the new object – Time exploring the familiar object) / Total time

3.3.4 Y-MAZE

The Y-maze test was used to evaluate spatial working memory based on the tendency of rodents to alternate between equal arms of a maze in successive opportunities. The animal must remember which arm it entered on a previous occasion in order to alternate its choice on a following trial, therefore allowing evaluation of cognitive searching behaviour (Hughes, 2004). The classical Y-maze test was performed as described in (Canas *et al.*, 2009), i.e., each animal, new to the maze, was placed in a "start" arm and left to explore between the three equal arms during 8 minutes. It was considered that an animal entered one arm if it got the four paws inside. To be considered a valid test, animals must have accomplished a minimal of 10 entries. One alternation is the consecutive entry in the three arms of the maze. The total percentage of alternation is calculated as follows: **Total of alternations / (Total arm entries – 2) X 100**. The Y maze was made of black painted acrylic; in that rat Y-maze, each arm was 36 cm long, 16 cm high and 9 cm wide and converged at equal angles of 120º and in the mice Y-maze, the dimensions are 36 cm long, 16 cm high and 5 cm wide (PanLab, S.L., Power Reagent 5, Porto, Portugal). The modified Y-maze was performed as described in (Choy *et al.*, 2010) in which one of the maze's arm is closed (the "novel" arm) during the first trial of the test. In the first trial, each animal, new to the maze, was allowed to explore the two arms (the "start" arm and the "other" arm) of the maze, while the novel arm was closed by a blocker made of the same material of

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the rest of the maze, during 10 minutes. Two hours after this first trial, animals were placed in the "start" arm and allowed to explore all of the maze's arms during 5 minutes. The number of times each arm was visited and the duration of time spent in each arm were recorded and calculated as a percentage relative to the sum of the time and number of entries in all arms. It was expected increased number of visits and time spent (> 33%) in the novel arm compared to the other, familiar arms. This type of Y-maze is more robust for spatial memory evaluation since it doesn't depend so much on the randomness of chance.

3.3.5 MORRIS WATER MAZE

The Morris water maze, described in (Morris, 1984), is another useful test to assess spatial memory that depends on the correct function of the hippocampus, as this brain region, together with other temporal lobe structures, is one of the major structures involved in declarative memory (Squire and Zola, 1996; Sharma *et al.*, 2010). The maze consists of a circular, black-painted tank filled with water at 22 °C to a depth of 30 cm. The tank has a diameter of 150 cm and is 60 cm deep and has a circular escape platform of 12 cm, made of black plexiglass, placed in the pool 1 cm bellow the surface of the water, halfway between the centre and the edge of the pool and halfway between the north and the east. The room contained several visual cues, since animals learn the platform's place based on these. Animals were tested in four trials per day over three consecutive days, in each trial the animal was placed into the water facing the wall and always varying the starting location. Then, the animals were allowed to swim until they reached the escape platform for a maximum of 120 s, after which they were placed on the platform. Once on the platform, the animals remained there for 30 s and then removed for an interval of 30 min between trials. Spatial memory retention was evaluated 48 hours after the last trial by hiding the platform and allowing the mice swim for 30 s and recording the time spent on the area where the platform once was.

3.3.6 PASSIVE AVOIDANCE

Passive avoidance, as described by (Crine, 1984), is used to evaluate aversive memory in rodents. The test was conducted as described in (Cho *et al.*, 2010) with few differences. First the animals are habituated to the passive avoidance box, which is 48 cm long, 22 cm wide and 22 cm high; divided into two compartments separated by a guillotine door. One of the compartments is illuminated

and has a thin platform, while the other is dark and made of stainless steel rods 1 cm apart, able to conduct electricity. Animals are placed in the illuminated compartment (which is a step-down to the dark, steel rods compartment) and allowed to explore the box for one minute. Thirty seconds after the animal is taken off the box, it is placed there again, on the illuminated side, for the test. As soon as the animal enters the dark side of the box, the dividing door closed and the animal receives a shock of 0.5 mA during 1 s. Fifteen minutes later, the animal was placed again in the illuminated compartment and the latency time to entry the dark compartment was recorded (recent memory). Twenty four hours later, the animals were tested again to evaluate long-term memory or retention. This time, the animals did not receive a shock and the maximum latency time allowed was 300 s.

3.3.7 ELEVATED PLUS MAZE

The elevated plus maze is the most popular test to evaluate anxiety-like behaviour of rodents (Carobrez and Bertoglio, 2005). The maze consists in an acrylic, black cross made of two opposite closed arms (50 cm long, 10 cm wide and 40 cm tall for rats; 31 cm long, 5 cm wide and 15 cm tall for mice) and two opposite open arms (50cm long and 10 cm wide for rats; 31 cm long and 5 cm wide for mice) set up 50 cm above the floor, for rats, and 40 cm above the floor, for mice. The central platform, the junction area of the four arms is 10 cm long and wide, for rats and 5 cm long and wide, for mice. The test was performed essentially as described in (Gazarini *et al.*, 2011) and (Sampath *et al.*). Since the maze is elevated, the open arms are aversive per se to rodents, but given the exploratory nature of these animals, the less anxious they are the longer they will spend adventuring outside of the closed arms. Animals were placed ate the central platform of the maze facing the one of the closed arms and left to explore during a total of 5 minutes. During the test, the time spent in the open and closed arms, as well as the number of entries, was recorded. Only when the animal was with the four paws inside, was it considered an entry. Afterwards, the percentage of time spent in the open *versus* in the closed arms, as well as the percentage of time spent exploring was calculated as follows: **time spent on open-arms / total time X 100**; **time spent on closed arms / total time X 100**; **total time – (time spent on open arms + time spent on closed arms) X 100**.

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3.4 RAT HIPPOCAMPAL PRIMARY NEURONAL CULTURES

Rat hippocampal neuronal cultures were prepared as optimized by (Brewer *et al.*, 1993) which allows glia-free neuronal cultures. Briefly, Wistar, female, pregnant rats were anaesthetised with halothane and 18 day old embryos were collected through caesarean for a container with sterile Hank's balanced salt solution without calcium and magnesium (NaCl 137 mM, KCl 5.36 mM, KH_2PO_4 0.44 mM, $NaHCO_3$ 4.16 mM, $Na_2HPO_4.2H_2O$ 0.34 mM, Glucose 5 mM supplied with phenol red 0.001 % and with pH 7.2, sterilized in a 0.2 μm filter). The still anaesthetised female rats were immediately sacrificed by decapitation using a guillotine. All the surgical material was sterilized and kept in 75 % ethanol and all the procedures were executed in a flow chamber. The hippocampi were dissected in the already described Hank's solution and digested for 10 minutes in a bath at 37 ℃ in 2 mg/ml of trypsin (from bovine pancreas, Sigma-Aldrich, Portugal) prepared in the Hank's solution and filtered. The trypsin reaction was quickly stopped with 1.5 mg/ml of trypsin inhibitor (from bovine pancreas, Sigma-Aldrich, Portugal) prepared in the Hank's solution and filtered and then hippocampi were washed in Hank's solution. The Hank's solution was carefully removed and 1 ml of Neurobasal medium supplemented with B27 (1:50, GIBCO, Invitrogen, Portugal), L-glutamine (0.5 mg/ml), L-glutamate (25 μM) and penicillin-streptomycin antibiotic (1:100, GIBCO, Invitrogen, Portugal). The tissue was further dissociated mechanically with a P1000 Gilson pipetter until it was homogeneous. The cells were counted in a 1:3 dilution in three equal parts of cell medium and trypan blue solution (0.4% in PBS; Sigma-Aldrich), using a hemocytometer and a light microscope. Further dilutions until the final, desired concentration, were done by adding the supplemented Neurobasal medium. Finally, cells were plated on previously coated with poly-D-lysine (0.1 mg/ml prepared in borate buffer 166 mM, pH 8.2) coverslips and/or multiwells. For the viability and immunocytochemistry assays, 16 mm diameter coverslips were used, in 12-well dishes, and cells were plated at a 50 000/coverslip density. For the single cell calcium image assays, cells were plated at a density of 37 000/ coverslip in 12 mm diameter coverslips. For Western blot assays, cells were plated at a density of 800 000/ well in 6-well dishes. Neurons grew for 7 days *in vitro* in a 37 ℃ incubator in a 95 % O_2-5 % CO_2 and humidity controlled atmosphere.

For Western blot analysis, the cultured neurons were lysated in RIPA buffer [Radio-immunoprecipitation assay buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet-P40 (IGEPAL), 0.5 % sodium-deoxycholate, 1mM EDTA and 0.1 % sodium dodecyl sulphate (SDS)]

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supplemented with 1mM phenylmethanesulfonylfluoride (PMSF, Sigma Aldrich, Portugal), 1µg/ ml CLAP (protease inhibitor cocktail containing leupeptin, pepstatin A, chymostatin and aprotinin all at 1mg/ ml; Sigma Aldrich, Portugal), 1 mM dithiothreitol (DTT), 1 mM sodium orthovanadate (Na_3VO_4) and 1 mM sodium fluoride (NaF).

3.5 PREPARATION OF SYNAPTOSOMES AND TOTAL MEMBRANES

Membrane preparations are a very useful tool to determine the presence and relative abundance of receptors. The specific localization of receptors at synaptic membranes and their subsynaptic distribution helps understanding their physiological role, allowing the probing of alterations in the density of the receptors in pathophysiological conditions. Total membrane and synaptosomal preparations have been extensively used in our and other groups to study the presynaptic modulatory roles of adenosine and ATP, as well as the localization and relative density of their respective receptors and of other molecules under the control of purinergic signalling.

When homogenized, the neural tissue, which is composed of neurons and glial cells, is broken into fragments. The cell bodies are separated from long and/or branched processes, which also brake into discrete fragments. The plasma membranes of cell fragments may reseal to form osmotically active particles and when those particles contain the organelles of the synapse they are known as synaptosomes (Turner and Backelard, 1987).

Initially, sucrose gradients were used to isolate synaptosomes; however, the concentration used is hypertonic making the synaptosomes shrunk. The fact that the synaptosomes had to return to a physiological medium and reswollen membranes are more susceptible to osmotic shock, led to introducing material such as Percoll or Ficoll in the isolation protocols. Moreover, sucrose gradients took longer times of centrifugation (Turner and Backelard, 1987).

3.5.1 SIMPLE SYNAPTOSOME ISOLATION:

A simple and rapid isolation of viable synaptosomes was performed as follows: animals were anaesthetised under halothane atmosphere before being sacrificed by decapitation. Brains were isolated; the hippocampi dissected and homogenized in a 0,32M sucrose solution containing 1mM EDTA, 10 mM HEPES and bovine serum albumin (BSA) 1 mg/ml, pH7.4 at 4ºC. Then, the homogenates were centrifuged at 3000x g for 10 minutes, at 4ºC, the pellet (P1) was discarded and the

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supernatant (S1) was further centrifuged at 14000x g for 12 minutes at 4ºC. The supernatant (S2) was discarded and the resulting pellet (P2) was resuspended in 1ml of 45% (v/v) percoll solution prepared in a Krebs solution (KHR, Krebs HEPES Ringer: 140 mM NaCl, 1mM EDTA, 10mM HEPES, 5mM KCl, 5mM glucose, pH 7.4 at 4ºC). Then, the percoll homogenates were transferred to eppendorfs and centrifuged for 2 minutes at the maximum velocity of the microcentrifuge, 14000 rpm, 4ºC. The resulting, white, top layer (synaptosomal fraction) was removed to new eppendorfs, washed in KHR solution and resuspended in 1 ml of KHR for a new centrifugation of 14000 rpm, 4ºC. The pellets (synaptosomes) were then resuspended in different buffers according to the purpose they were intended for. For protein density evaluation, through Western Blot analysis, synaptosomes were homogenized in a solution of 5 % sodium dodecyl sulphate (SDS from Sigma-Aldrich) with 0.1 mM PMSF.

These isolated synaptosomes are not free of contaminating particles, such as axonal fragments, free mitochondria, segments of axon and dendrites, myelin and glial cell fragments. The purity of the preparation can be assessed by probing biochemical markers expected to be enriched in the preparation and comparing it to other markers not expected to be there, or to be relatively low concentrated. Unlike synaptosomes, subcellular components derived from synaptosomes such as pre, post and extrasynaptic densities are sufficiently pure for biochemical analysis (Turner and Backelard, 1987).

3.5.2 SYNAPTOSOME ISOLATION IN A DISCONTINUOUS GRADIENT OF PERCOLL

For a more purified preparation of synaptosomes the isolation can be achieved using a discontinuous Percoll gradient, as described in (Dunkley *et al.*, 1988). The procedure used was the following:

Animals were sacrificed and the hippocampi dissected as described above. The tissue was homogenised in a sucrose-HEPES medium (0.25 M of sucrose and 10 mM of HEPES, pH 7.4) and centrifuged at 2000 x *g* for 3 minutes at 4ºC. The resulting pellet was discarded (P1) and the supernatant (S1) was centrifuged at 9500 x *g* for 13 minutes at 4 ºC. The supernatant (S2) was discarded and the pellet (P2) was resuspended in 2 ml of sucrose-HEPES medium and laid on top of a Percoll gradient. For each sample, i.e., centrifuge tube, the gradient was built as follows:

2 ml homogenate
3 ml Percoll 3 %
4 or 5 ml Percoll 10 %
3 ml Percoll 23 %

The Percoll solutions were prepared in a 0.32 M sucrose solution with 1 mM EDTA and 0.25 mM dithiothreitol (DTT), pH 7.4 at 4 ℃.

The gradients were then subjected to a 25 000 x g centrifugation for 11 minutes, at 4 ℃, and without deceleration, to preserve the gradient. The synaptosome layer was collected from the interface 23-10 % of Percoll and diluted in 15 ml of HEPES buffered medium without calcium (140 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.2 mM $MgCl_2$, 10 mM glucose and 10 mM HEPES, pH 7.4). Then, a new centrifugation of 22000 x g of 11 minutes at 4 ℃ was performed and the resulting freely-moving pellet was captured with a P1000 pipetter, transferred to eppendorfs and resuspended in 2 ml of HEPES buffered medium for washing. The synaptosomes were finally collected through a centrifugation of 11000 rpm during 11 minutes at 4 ℃, using a microcentrifuge.

3.5.3 FRACTIONATION OF SYNAPTIC MEMBRANES

To isolate the subcellular components of synaptosomes, such as the presynaptic, postsynaptic and extrasynaptic fractions, from rat hippocampal synaptosomes, the method used was the described by (Phillips *et al.*, 2001) and adapted as in (Pinheiro *et al.*, 2003) and (Rebola *et al.*, 2003). This subsynaptic fractionation method allows an over 90 % effective separation of the active zone (enriched in SNAP-25 protein), postsynaptic density (enriched in PSD-95 protein) and non-active zone fraction (enriched in synaptophysin protein) markers and can be used to assess the subsynaptic distribution of receptors (Pinheiro *et al*., 2003; Rebola *et al*., 2003).

For synaptosomes preparation, the hippocampi were homogenised in 2.5 ml of isolation buffer (0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 µg/ml CLAP and 1 mM PMSF), keeping 100 µl of the homogenate at -80 ℃, for latter control analysis. The homogenate was transferred to 50 ml centrifuge tubes and 12 ml of 2 M sucrose solution was added as well as 5 ml of 0.1 mM $CaCl_2$. The mixture was agitated at 4 ℃ giving a solution of 1.25 M sucrose. This solution was divided into 2 tubes Ultraclear^{TM} and covered carefully with 2.5 ml/tube with 1 M sucrose solution (containing 0.1 mM $CaCl_2$). The tubes were filled and equilibrated with isolation buffer and then centrifuged at 100000 x g, 4 ℃, for 3 hours. The isolation buffer and the myelin layer present at the interface isolation buffer/ 1M sucrose were aspirated. The synaptosomes were captured at the interface 1.25/ 1M sucrose and then were diluted 1/10 in isolation buffer and centrifuged at 15000 x g during 30 minutes. The pellet was

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resuspended in 1.1 ml isolation buffer and 100 μl of the supernatant (synaptosomal fraction) was kept at -80 ℃ for control analysis.

The fractionation of the synaptic membranes was then pursued: the synaptosomes were diluted 10 x their volume in cooled 0.1 mM CaCl$_2$ in 50 ml beakers, and a similar (10 ml) volume of 2x solubilization buffer pH 6.0 (40 mM Tris, 2 % Triton X-100, pH 6.0 precisely adjusted at 4 ℃) was added. The mixture was softly stirred during 30 minutes on ice and divided into 2 Ultraclear^{TM} tubes for a centrifugation at 40000 x g for 30 minutes, 4 ℃. The pellet corresponds to synaptic junctions and the supernatant to extrasynaptic proteins. The supernatants were kept on ice while the pellet was washed in once in solubilization buffer pH 6.0 (20 mM Tris, 1 % Triton X-100, pH 6.0 precisely adjusted at 4 ℃) and resuspended in 5 ml of solubilization buffer pH 8.0 (20 mM Tris, 1 % Triton X-100, pH 8.0 precisely adjusted at 4 ℃). This mixture was stirred softly for 30 minutes on ice and centrifuged at 40000 x g for 30 minutes at 4 ℃. The pellet corresponds to the postsynaptic density and the supernatant to presynaptic proteins. The supernatant was transferred to centrifuge tubes and the pellet resuspended in 5 ml of solubilization buffer pH 8.0 and again stirred moderately on ice for 30 minutes and centrifuged 40 000 x g for 30 minutes at 4 ℃. The supernatant was added to the presynaptic fraction and the pellet, correspondent to the re-extracted postsynaptic fraction, was resuspended in a minimal volume of 5 % SDS solution with 0.1 mM PMSF. To concentrate the extrasynaptic and presynaptic proteins, a maximum of cold acetone (-18 ℃) was added to the supernatants and kept for a minimum of 30 minutes at -20 ℃. Both fractions were pelleted by centrifuging at 18000 x g for 30 minutes at -15 ℃. Both pellets were resuspended in a minimal of 5 % SDS solution with 0.1 mM PMSF.

3.5.4 ISOLATION OF TOTAL MEMBRANES

Purification of total membranes was used for assessing protein density in hippocampal cytoplasmic membranes. Given the higher proportion of glial cells to neurons in the brain, this preparation is enriched in glial membranes as compared with synaptosomes.

Animals were sacrificed and hippocampi isolated as already described. The tissue was homogenised in a 0.32 M sucrose solution containing 1 mM EDTA, 10 mM HEPES and 1 mg/ml BSA, pH7.4 at 4 ℃. Then, the homogenates were centrifuged at 3000 x g for 10 minutes, at 4 ℃. The pellet (P1) was discarded and the supernatant (S1) was further centrifuged at 100000 x g for 30 minutes at 4 ℃, in an ultracentrifuge. The supernatants (S2) were discarded and the pellet (P2), correspondent

essentially to total cytoplasmic membranes, was resuspended in a 5 % SDS solution with 0.1 mM PMSF.

3.6 PROTEIN QUANTIFICATION BY THE BICINCHONINIC ACID METHOD

Protein quantification was carried using the bicinchoninic acid (BCA) protein assay reagent kit (Thermo Scientific, Pierce Biotechnology, USA), a colorimetric method compatible with high concentrations of most components of lysis buffers. Briefly, a standard curve was prepared in milliQ water, using 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 µg/µl of BSA. All the samples were diluted 1/10 in milliQ water, the reagent A was mixed with reagent B of the kit (BCA reagent) in a 50:1 proportion and protected from light. The buffer used to lyse the samples was also diluted 1/10. In a 96 well dish, the standard curve was prepared by pipetting 25 µl of each concentration of BSA, in triplicates, for different wells. To each well, 25 µl of the diluted lysis buffer was added, as well as 200 µl of the BCA reagent. Triplicates of the diluted samples were prepared in the same way, but 25 µl of milliQ water were added to each well instead of the diluted lysis buffer. The dish was protected from light and placed in a 37 ºC incubator for 30 minutes. Finally, the protein was read at 570 nm in a spectrophotometer.

For Western blot analysis, the samples were normalized to a maximum of 2.5 µg/µl, by adding 1/6 volume of 6x SDS-PAGE sample buffer and correcting with milliQ water. The SDS-PAGE buffer was used to further denaturate protein to a primary conformation, give it an overall negative charge and correct the pH for an optimum run in the electrophoresis gel. It is composed of 4x Tris.Cl/SDS solution (0.5 M Tris and 0.4 % SDS, pH 6.8 corrected with HCl and filtered with 0.45 µm pore filters), 30 % glycerol, 10 % SDS, 0.6 M DTT and 0.012 % of 3',3",5',5"-tetrabromophenolsulfonphthalein (bromophenol blue). The samples were finally boiled at 95 ºC during 5 minutes.

3.7 WESTERN BLOT

Western blot analysis was performed using the Bio-Rad system. The samples diluted in SDS-PAGE buffer and the prestained molecular weight markers (dual-colour standards from BioRad, Portugal) were loaded and separated by SDS-PAGE electrophoresis (in 7.5 % polyacrylamide resolving gels with 4 % polyacrylamide stacking gels) under denaturating, reducing conditions and using a bicine buffered solution (20 mM Tris, 192 mM bicine and 0.1 % SDS, pH 8.3). The velocity of the running

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was determined by applying a voltage of 80-100 mV. After separation in the electrophoresis gel, the proteins were electro-transferred (with 1A current, for 1.5 hours at 4 ℃ and constant agitation) to previously activated polyvinylidene difluoride (PVDF) membranes (pore of 0.45 μm from GE Healthcare, UK), using a CAPS [3-(cyclohexylamino)-1-propane-sulfonic acid] buffered solution with methanol [10mM CAPS, 10 % (v/v) methanol, pH 11.0]. The PVDF membranes were activated during 5-15 seconds in 100 % methanol, hydrated during 5 minutes in distilled water and equilibrated for 30 minutes in the electrotransference buffer. Membranes were then blocked for 1 hour at room temperature (RT) with 5 % low fat and Ca^{2+}-free milk in Tris-buffered saline (20 mM Tris, 140 mM NaCl, pH7.6) with 0.1 % Tween 20 (TBS-T). Afterwards, membranes were incubated with the primary antibodies diluted in TBS-T with 5 % milk, overnight, at 4 ℃. After being washed three times, 15 minutes each, in TBS-T with 0.5 % milk, the membranes were incubated with the phosphatase-linked secondary antibodies, also diluted in TBS-T with 5 % milk for 1 hour at RT. Again, membranes were washed three times, 15 minutes each, in TBS-T with 0.5 % milk and then incubated with Enhanced Chemi-Fluorescence substrate (ECF, GE Healthcare, UK) for different times in a maximum of 5 minutes. Finally, proteins were detected and analysed with Molecular Imager VersaDoc 3000 and Quantity One software (BioRad, Portugal). The milk used for blocking unspecific binding of antibodies to the membrane was substituted for 3 % BSA (and 0.5 % BSA in the washing TBS-T solution) when the target were phosphorylated proteins and the antibodies were phospho-specific, since milk contains casein, a phosphoprotein, that can react with the antibody and increase the background.

Western blot gels were made of a polyacrylamide matrix (30 % Acrylamide/Bis solution, BioRad, Portugal) and were 1.5 mm thick. The stacking gel was prepared in a 0.5 M Tris-HCl at pH 6.8 buffer (same pH of the sample buffer) and the resolving gel was prepared in a 1.5 M Tris-HCl buffer, pH 8.8, creating a pH gradient optimum for the separation of negatively-charged proteins. According to the Bio-Rad table, the gel formulations are as follows:

Table 6. Gel formulations in Western blot. The monomer solutions were prepared by mixing all reagents except the N,N,N′,N′-Tetramethylethylenediamine (TEMED from Sigma-Aldrich, a tertiary amine that catalyze the formation of free radicals from ammonium persulfate, which, in turn, leads to the polymerization of acrylamide and bis-acrylamide) and 10 % ammonium persulfate (APS from Sigma-Aldrich), added at the end.

GEL (10 ml)	4 %	7.5 %
1.5M Tris-HCl pH 8.8 (resolving gel)		3.0 ml
0.5M Tris-HCl pH 6.8 (stacking gel)	2.5 ml	
Acrylamide 30 %	1.3 ml	2,3 ml
Ultrapure water	6.1 ml	3,5 ml
10 % SDS	100 µl	195µl
TEMED	10 µl	6 µl
10 % APS (freshly prepared in water)	50 µl	90 µl

Re-probing of the same membrane with a different antibody was achieved by washing the ECF in 40 % methanol for 30 minutes and stripping the previous antibodies in a mild stripping solution of 0.2 M glycine (Sigma-Aldrich, Portugal) with 0.1 % SDS and 1 % (v/v) Tween 20, pH 2.2, for 1 hour. The membranes were washed 3 times, 20 minutes each, in TBS-T with 0.5 % milk or BSA, between different solutions. Finally, before incubation with new antibodies, membranes were again blocked with either TBS-T with 5 % milk or TBS-T with 3 % BSA.

Western blot analysis of the activated (phosphorylated) form of MAPKs was performed after incubation of cultured hippocampal neurons, 7 *days in vitro*, during different times of exposure and concentrations of IL-1β (5, 10, 15, 20, 25, 30 or 60 minutes and 3 hours of incubation with 10 ng/ml IL-1β and 15 minutes of exposure to 1, 10 and 100 ng/ml IL-1β; R&D Systems, USA) and of TNF-α (1 and 5 ng/ml of TNF-α were incubated for 10, 15, 20 and 30 minutes; TNF-α was purchased to R&D Systems, USA). At the mentioned endpoints, the cell medium was aspirated and the cells collected in a minimum volume of RIPA's lysis buffer and the protein quantification was performed using the BCA method.

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Table 7. Primary antibodies used in the Western blot analysis.

Antibodies	Supplier	Host	Type	Dilution
A_{2A}	Santa Cruz Biotechnology	goat	polyclonal	1:500
$P2X_1$	Alomone labs	rabbit	polyclonal	1:500
$P2X_2$	Alomone labs	rabbit	polyclonal	1.500
$P2X_3$	Alomone labs	rabbit	polyclonal	1:1000
$P2X_4$	Alomone labs	rabbit	polyclonal	1:500
$P2X_5$	Santa Cruz Biotechnology	goat	polyclonal	1:200
$P2X_6$	Santa Cruz Biotechnology	goat	polyclonal	1:200
$P2X_7$	Alomone labs	rabbit	polyclonal	1:5000
$P2Y_1$	Alomone labs	rabbit	polyclonal	1:500
$P2Y_2$	Alomone labs	rabbit	polyclonal	1:500
$P2Y_4$	Alomone labs	rabbit	polyclonal	1:1000
$P2Y_6$	Santa Cruz Biotechnology	goat	polyclonal	1:500
$P2Y_{12}$	Alomone labs	rabbit	polyclonal	1:500
α-tubulin	Sigma-Aldrich	mouse	monoclonal	1:20000
β-actin	Sigma-Aldrich	mouse	monoclonal	1:20000
Synaptophysin	Sigma-Aldrich	mouse	monoclonal	1:20000
SNAP-25	Sigma-Aldrich	mouse	monoclonal	1:5000
PSD-95	Sigma-Aldrich	mouse	monoclonal	1:20000
$vGLUT_1$	Millipore	guinea-pig	polyclonal	1:10000
$vGLUT_2$	Millipore	guinea-pig	polyclonal	1:10000
vGAT	Calbiochem	guinea-pig	polyclonal	1:1000
GFAP (Glial Fibrillary Acidic Protein)	Sigma-Aldrich	rabbit	polyclonal	1:5000
OX-6	Abcam	mouse	monoclonal	1:500
Phospho-p38 MAPK	Cell Signaling Technology	mouse	monoclonal	1:1000
Total p38 MAPK	Cell Signaling Technology	rabbit	monoclonal	1:1000
Phospho-SAPK/JNK	Cell Signaling Technology	mouse	monoclonal	1:1000
Total SAPK/JNK	Cell Signaling Technology	rabbit	monoclonal	1:1000
Phospho-p44/p42 MAPK (Erk1/2)	Cell Signaling Technology	mouse	monoclonal	1:1000
Total p44/p42 MAPK (Erk1/2)	Cell Signaling Technology	rabbit	monoclonal	1:1000

Table 8. Secondary antibodies used in the Western blot analysis.

Antibodies	Supplier	Host	Type	Dilution
Rabbit-alkaline phosphatase conjugated	Amersham Biosciences	goat	IgG	1:20000
Mouse-AP	Santa Cruz Biotechnology	goat	IgG+IgM	1:10000
Goat-AP	Santa Cruz Biotechnology	rabbit	IgG	1:2500
Guinea-pig-AP	Sigma-Aldrich	goat	IgG	1:5000

3.8 IMMUNOCYTOCHEMISTRY IN HIPPOCAMPAL NEURONAL CULTURES

Immunocytochemistry in hippocampal neuronal cultures, 7 *days in vitro*, was used to evaluate the density and localization of the activated MAPKs induced by the pro-inflammatory cytokines IL-1β (100 ng/ml) and TNF-α (5 ng/ml), at 15 and 10 minutes of exposure, respectively. At the studied endpoints, cells were rapidly washed, once with Neurobasal medium and once with phosphate buffered saline (PBS, in mM: 140 NaCl, KCl, 26 NaH_2PO_4, 15 KH_2PO_4, pH 7.4), before being fixed with 4 % paraformaldehyde, prepared in a 0.9 % NaCl and 4 % sucrose solution, for 30 minutes. After fixation, cells were washed three times with PBS and permeabilized with PBS + 0.2 % Triton X-100 for 5 minutes. After washing twice with PBS, cells were incubated with 3 % BSA in PBS during 1 hour at room temperature (RT), for blocking unspecific binding of antibodies. Cells were then incubated with primary antibodies, individually, prepared in the 3 % BSA solution, overnight, at 4 ºC. After washing three times with PBS, cells were incubated with secondary antibodies conjugated with a fluorophore (Alexa Fluor), 1 hour at RT. Vectashield mounting medium H-1500 with nucleus fluorescent marker 4,6-diamidino-2-phenylindole (DAPI), from Vector Laboratories, UK, was used to maintain fluorescence. Finally, the preparations were observed with the transmission and fluorescence Zeiss Axiovert 200 microscope (Axiovision software 4.6, PG-Hitec, Portugal), with Zeiss/P.A.L.M. Laser Dissecting Microscope coupled to Axiovert 200M microscope or with a laser scanning confocal microscope with spectral analysis (LSM 510 Meta, Zeiss).

Table 9. Primary antibodies used in immunocytochemistry.

Antibodies	Supplier	Host	Type	Dilution
Synaptophysin	Sigma-Aldrich	rabbit	polyclonal	1:500
β-III-tubulin	Sigma-Aldrich	rabbit	polyclonal	1:600
GFAP	Sigma-Aldrich	rabbit	polyclonal	1:600
Phospho-p38 MAPK	Cell Signalling	mouse	monoclonal	1:100
Phospho-SAPK/JNK	Cell Signalling	mouse	monoclonal	1:100
Anti-IL-1RI	R & D systems	rabbit	polyclonal	1:200
Anti-caspase 3	Cell Signalling	rabbit	polyclonal	1:300

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Table 10. Secondary antibodies used in immunocytochemistry.

Antibodies	Supplier	Host	Type	Dilution
Anti-rabbit Alexa Fluor 594	Invitrogen	Donkey	IgG (H+L)	1:200
Anti-mouse Alexa Fluor 488	Invitrogen	Donkey	IgG (H+L)	1:200

3.9 PREPARATION OF FIXED BRAIN SLICES

For immunohistochemistry analysis, brains were fixed as described in (Cunha *et al.*, 2006) and (Rebola *et al.*, 2011). The heart of the anaesthetised animal was exposed, the descending aorta clamped and a catheter was inserted in the ascending aorta. The right atrium was opened to allow the outflow of the perfusate. The animal was then perfused, first with a cold saline solution of 0.9 % NaCl and 4 % sucrose (200 ml) and then with cold 4 % paraformaldehyde prepared in the saline solution (200 ml) (all reagents were from Sigma-Aldrich, Portugal). At the end of this procedure, the brains were removed and kept overnight in the same paraformaldehyde solution. Subsequently, brains were transferred to PBS with 30 % sucrose. Once descended in this solution, brains were embedded in Tissue-Tek (Sakura-Americas, USA), frozen at -21 ℃ and cut into 30 μm coronal sections using a cryostat (CM3050 S from Leica Microsystems, Portugal). Each series of brain sections comprised slices 300 μm apart. In this way, there were sections representative of different areas of the brain structures, such as the hippocampus.The slices were stored in PBS with 0.01 % NaN_3 (sodium azide, powder from Sigma-Aldrich, Portugal) and, when utilized, were mounted on slides coated with 2 % gelatine with 0.08 % $CrK(SO_4)_2$ (chromium and potassium sulphate from Sigma-Aldrich, Portugal).

3.9.1 IMMUNOHISTOCHEMISTRY FOR CD11b, GFAP AND NeuN

The sections were first rinsed in PBS for 5 minutes and then three times for 5 min with Tris buffered saline, TBS (0.05 M Trizma base buffer containing 150 mM of NaCl, pH 7.2) at RT. Slices, were simultaneously permeabilized and blocked with TBS containing 0.2 % Triton X-100 and 10 % of normal goat serum (NGS), for 1 hour at RT. Afterwards, slices were incubated, free-floating, with the primary antibodies prepared in the blocking solution (anti-CD11b, 1:100 dilution, mouse or rat IgG1, AbD Serotec, Portugal; anti-OX-6, 1:200 dilution, mouse IgG1, abcam, USA; anti-GFAP, 1:500 dilution, rabbit polyclonal, Sigma-Aldrich, Portugal; anti-NeuN, mouse IgG1, Chemicon/Millipore, Spain) overnight at RT. Sections were then rinsed 3x, 10 minutes each, in TBS and subsequently incubated 2 hours at RT, with goat anti-mouse, goat anti-rabbit or goat anti-rat secondary antibodies

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(depending on the animal in which the primary antibody was produced) conjugated with a fluorophore (Alexa Fluor 488 or 594, diluted 1:200 in the blocking solution, Invitrogen, Portugal). After rising 3x for 10 minutes in TBS the brain sections were mounted on the gelatine coated slides, using Vectashield mounting medium H-1500 with DAPI (Vector Laboratories, UK) and allowed to dry protected from light before being kept at -20ºC until analysis. Sections were visualized under a transmission and fluorescence Zeiss Axiovert 200 microscope (Axiovision software 4.6, PG-Hitec, Portugal), with Zeiss/P.A.L.M. Laser Dissecting Microscope coupled to Axiovert 200M microscope or with a laser scanning confocal microscope with spectral analysis (LSM 510 Meta, Zeiss).

3.9.2 IMMUNOHISTOCHEMISTRY FOR THE PHOSPHO-p38 MITOGEN-ACTIVATED KINASE AND FOR THE CLEAVED CASPASE-3

Immunohistochemistry for the activated (cleaved) caspase-3 was carried out as previously described in (Egeland *et al.*, 2010). Four hours after receiving an intraperitoneal injection of lipopolysaccharide (LPS) (200 µg from a 1 µg/ µl solution) or saline, rats (Wistar males, 10 weeks old) were sacrificed and the brains were fixed by trans-cardiac perfusion of a 4 % paraformaldehyde solution and sliced as described above. Each labelling was performed using a complete series of brain sections and the analysis was focused on the hippocampus. Slices were permeated and blocked in 3 % BSA with 3 % Triton-X-100, prepared in PBS, for 1 hour at RT, before being incubated with the primary antibodies (anti-phospho-p38, 1:100 dilution, rabbit, from Cell Signaling or anti-activated caspase-3, 1:300 dilution, rabbit, from Cell Signalling) prepared and incubated individually in the blocking solution. Excess of antibody was rinsed three times, 10 minutes each, with PBS before incubation, for 1 hour at RT, with the secondary antibody (1:200 dilution, donkey anti-rabbit Alexa Fluor 594, Invitrogen) prepared in PBS. After rising three times with PBS, 10 minutes each, slices were mounted onto 2 % gelatine-subbed microscope slides dried at RT and covered with Vectashield Hard Set H-1500 Mounting Medium with DAPI. For double-labelling with either anti-CD11b (1:100 dilution, mouse anti-rat IgG1 from Serotec) or anti-NeuN (1:600 dilution, mouse from Chemicon, Millipore) the primary antibodies were incubated together as well as the secondary antibodies (1:200 dilution, donkey anti-rabbit Alexa Fluor 594 and donkey anti-mouse Alexa Fluor 488, Invitrogen). However, the double-labelling with the phopho-p38 and the NeuN antibodies was carried out sequentially: first it was incubated the primary antibody for phospho-p38 followed by the respective secondary antibody and then it was incubated the primary antibody for NeuN followed by its secondary

antibody. The negative controls were performed by labelling in the absence of each or both primary antibodies, to check for non-specific labelling or cross-reactivity of the secondary antibodies. Images were acquired using a Zeiss Axiovert 200 microscope and some images were also acquired with a Zeiss LSM510 META confocal laser-scanning microscope. In the studies attempting to define co-localization of phospho-p38 or cleaved caspase-3 with either NeuN or CD11b, we only carried out a qualitative evaluation of the co-localization rather than a quantification of the actual relative co-localization of both proteins, which would require a considerably larger number of animals.

The detection of microglia-like profiles was carried out as previously described (Cunha *et al.*, 2006), using an immunohistochemical detection of OX-42 and CD11b, epitopes which are up-regulated in activated microglia and to a lesser extent in macrophages (Jensen *et al.*, 1997). Four hours (the same time period used for electrophysiological analysis) after the administration of lipopolysaccharide (200 µg in 200 µl, ip) under urethane anaesthesia, the heart was exposed and after clamping the descending aorta, a catheter was inserted in the ascending aorta and the brain was fixed and sliced as described above. For the immunohistochemistry analysis, the sections were first rinsed for 5 min in PBS, then three times for 5 min with TBS (0.05 M Trizma base buffer containing 150 mM of NaCl, pH 7.2) and blocked with TBS containing 0.2 % Triton X-100 and 10 % goat serum during 45 min at room temperature. Sections were incubated in the presence of the mouse anti-CD11b antibody (1:200 dilution in TBS containing 0.2 % Triton X-100 and 10 % normal goat serum, from Serotec) for 72 hours at 4 ºC, rinsed three times for 10 min in TBS and subsequently incubated with goat anti-mouse secondary antibody conjugated with a fluorophore (Alexa Fluor 488, 1:50 dilution in 0.1 M phosphate buffer containing 0.2 % Triton X-100 and 10 % normal serum), for 2 hours at room temperature. The sections were then rinsed twice for 10 min in TBS and once for 10 min in distilled water and, finally, dehydrated and passed through xylol before mounting on slides, using Vectashield mounting medium (Vector Laboratories). Images were acquired using a Zeiss Axiovert 200 microscope and some images were also acquired with a Zeiss LSM510 META confocal laser-scanning microscope.

3.9.3 CRESYL VIOLET STAINING

Cresyl violet staining of Nissl bodies, which disappears under pathological conditions leading to cell degeneration, was used to evaluate neuronal morphology in hippocampal sections, as previously described in (Lopes *et al.*, 2003). Sections were incubated for 10 minutes in 0.5 % cresyl violet solution (Sigma-Aldrich, Portugal) prepared in acetate buffer (20 % sodium acetate at 2.7 % and 80 % glacial

acetic acid at 1.2 %, both reagents from Merck, Portugal, pH 3.8-4.0). The staining was fixated through two rinses in the acetate buffer and dehydrated twice, 20 seconds each, in 100 % ethanol. Finally, sections are cleared in xylene (Merck, Portugal) for 5 minutes and mounted with DPX non-aqueous mounting medium (Merck, Portugal).

3.9.4 FLUORO-JADE C STAINING

Fluoro-Jade C staining was used to evaluate general neurodegeneration in brain sections, regardless of specific insult or mechanism of cell death (Schmued *et al.*, 2005). Brain sections on slides were defrost, dried and immersed for 5 minutes in 0.01 % sodium hydroxide (NaOH pellets from Sigma-Aldrich, Portugal) prepared in an 80 % ethanol solution. After rising for 2 minutes in 70 % ethanol and for 2 minutes in distilled water, slides were transferred to a 0.06 % potassium permanganate solution during 10 minutes, under agitation and protected from light. Sections were again rinsed in distilled water, for 2 minutes, and immersed in 0.0001 % Fluoro-Jade C (Histo-Chem Inc., Jefferson, AR, USA) prepared by diluting a 0.01 % stock solution (in ultrapure water) in 0.1 % of acetic acid vehicle. Sections remained in this solution for 10 minutes, protected from light and under agitation, before being rinsed three times, 1 minute each, in distilled water. Slices were dried on a slide warmer, dehydrated in an ethanol gradient (50 %, 70 %, 100 %) and cleared in xylene. Finally, slices were coversliped with DPX non-aqueous mounting medium (Merck, Portugal). Images were acquired with Zeiss Axiovert 200 microscope.

3.10 QUANTIFICATION OF mRNA AND PROTEIN MARKERS OF MICROGLIAL ACTIVATION

Quantitative determination of microglial activation in the dentate gyrus of the hippocampus was carried out by Western blot of two different markers of microglial activation, namely the CR3/43 protein of the major histocompatibility complex class II (MHC-II) and inducible nitric oxide synthase (iNOS). For the 4 groups of rats (control; treated with LPS; treated with $A_{2A}R$ antagonists or treated with both LPS and $A_{2A}R$ antagonists), animals were sacrificed by decapitation 4 hours after the LPS administration and their hippocampi dissected and sliced (1 mm) to isolate the dentate gyrus. The dentate gyrus slices from one hippocampus were homogenised and solubilized in 5% SDS, diluted in sample buffer and boiled for 5 min, as described above. These samples (50 μg of protein for CR3/43 or

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iNOS analysis) and the pre-stained molecular weight markers (from Amersham) were analysed by SDS-PAGE as described above, using antibodies against CR3/43 (1:200 raised in mouse from Santa Cruz Biotechnology) or iNOS/NOS type II (1:500, rabbit, BD Transduction Laboratories). The membranes were then re-probed and tested for tubulin immunoreactivity to confirm that similar amounts of protein were applied to the gels, as described above. The dentate gyrus slices from the other hippocampus were used to extract total RNA with a MagNA Lyser Instrument and MagNA Pure Compact RNA Isolation kit (Roche, Portugal), according to the manufacturer's instructions. The integrity, quantity and purity of the RNA yields were checked by electrophoresis and spectrophotometry. Reverse transcription for first-strand cDNA synthesis from each sample was performed using random hexamer primer with the Transcriptor First Strand cDNA Synthesis kit (Roche) according to manufacturer's instructions. Resulting cDNAs were used as template for real-time polymerase chain reaction (PCR), which was carried out on LightCycler instrument (Roche) using the FastStart DNA Master SYBR Green I kit (Roche). The $A_{2A}R$ and CD11b mRNA expression was calculated relative to β-actin mRNA expression. The following primers (obtained from Tib MolBiol, Germany) were used: $A_{2A}R$ (forward: 5'-AGT CAG AAA GAC GGG AAC-3'; reverse: 5'- CAG TAA CAC GAA CGC AA-3'), CD11b (forward: 5'-GAT GCT TAC TTG GGT TAT GCT T-3'; reverse: 5'-CGA GGT GCC CCT AAA ACC A-3') and β-actin (forward: 5'-AAG TCC CTC ACC CTC CCA AAA G-3'; reverse: 5'-AAG CAA TGC TGT CAC CTT CCC-3'). Quantification was carried out based on standard curves run simultaneously with the test samples, with $A_{2A}R$, CD11b and β-actin standards being generated by conventional PCR amplification, as previously described (Duarte *et al.*, 2007). The PCR products were run in a 3 % agarose gel electrophoresis to verify fragment size and the absence of other contaminating fragments, samples were quantified by 260 nm absorbance, and serially diluted to produce the standard curve (100 to 108 copies/ μl). Each real-time PCR reaction was run in triplicate and contained 2 μl of cDNA template, 0.3 μM of each primer, and 3.5 mM $MgCl_2$ in a reaction volume of 20 μl. Cycling parameters were: 95 °C for 10 min to activate DNA polymerase, followed by 40-45 cycles at 95 °C for 10 s, annealing temperature of 60 °C for 10 s, and a final extension step at 72 °C for 10 s, in which fluorescence was acquired. The purity and specificity of the resulting PCR products were assessed by melting curve analysis and electrophoresis. Control reactions were performed to verify that no amplification occurred without cDNA.

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3.11 QUANTIFICATION OF CASPASE-3 ACTIVITY

Cleavage of the caspase-3 substrate (Ac-DEVD-AFC peptide, from Alexis) to its fluorescent product was used as a measure of caspase-3 activity, as previously described (Nolan *et al.*, 2003). Briefly, slices of hippocampal tissue were washed, homogenized in ice-cold lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsufonyl fluoride, 6.25 µg/ml pepstatin A, 6.25 µg/ml aprotinin, pH 7.4) and lysed by cycles of freezing and thawing. Aliquots of these samples (50 µl) were mixed with 50 µl of reaction buffer (50 mM HEPES, 2 mM EDTA, 20% glycerol, 10 mM dithiothreitol, pH 7.4) and 4 µl of caspase-3 substrate (final concentration 10 µM from a stock solution of 250 µM in reaction buffer) and added to 96-well plates. Samples were incubated at 37ºC for 60 min in the dark, fluorescence was assessed (excitation 400 nm; emission 505 nm) and enzyme activity was calculated with reference to a standard curve of 7-amino-4-trifluoromethylcoumarin (AFC; 0-10 µM) *versus* absorbance. The protein concentration of each sample was determined using BSA as a standard (Bradford, 1976) and values expressed as nmol AFC/ mg protein/ min.

3.12 QUANTIFICATION OF INTERLEUKIN-1β CONCENTRATION

The concentration of interleukin-1β in hippocampal homogenates was assessed by enzyme-linked immunosorbent assay, as previously described (Vereker *et al.*, 2000a). Antibody-coated (2,0 µg/ml final concentration, diluted in 0.1 M sodium carbonate buffer, pH 9.5; monoclonal hamster anti-mouse interleukin-1β antibody) 96-well plates were incubated overnight at 4 ºC, then were washed four times with PBS containing 0.05 % Tween 20, blocked for 2 h at 37 ºC with 250 µl of blocking buffer (PBS, pH 7.3, 0.1 M with 4 % BSA) and incubated with interleukin-1β standards (100 µl; 0-1000 pg/ml) or samples (supernatants of hippocampal samples homogenized in Krebs solution containing 2 mM $CaCl_2$) for 1 h at 37 ºC. Samples were incubated with secondary antibody (100 µl; final concentration 0.8 µg/ml in PBS containing 0.05 % Tween 20 and 1 % BSA; biotinylated polyclonal rabbit anti-mouse antibody) for 1 h at 37 ºC, washed and incubated in detection agent (100 µl; horseradish peroxidase-conjugated streptavidin; 1:1000 dilution in PBS containing 0.05 % Tween 20 and 1 % BSA) and incubated for 15 min at 37 ºC. The chromogenic substract for the enzyme, 3,3',5,5'-tetramethylbenzidine (100 µl; Sigma-Aldritch), was added, samples were incubated at room

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temperature for 30 min and absorbance was read at 450 nm. Values are expressed as pg interleukin-1β / mg of protein, quantified as described (Bradford, 1976).

3.13 VIABILITY ASSAYS:

3.13.1 ALAMAR BLUE

Alamar Blue (resazurin, from Sigma-Aldrich, Portugal) is a non-toxic, cell permeable compound, blue in colour and non-fluorescent. Upon entering cells, resazurin is reduced to resorufin, which is red in colour and highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and colour of the media surrounding the cells. In cultured cells that do not divide, such as neurons, the Alamar Blue assay allows evaluation of cell viability along the time. This method is advantageous because it is simple to execute, inexpensive and non-toxic to the cells or the user (Nakayama *et al.*, 1997). The Alamar Blue assay was used to evaluate the toxicity of long-term stimuli with agonists of glutamate ionotropic receptors, such as the kainic acid, the N-methyl-D-aspartic acid (NMDA) and the quinolinic acid, to hippocampal neurons (all purchased from Sigma-Aldrich, Portugal). Once found the optimal incubation time and concentration in which these toxic agents induced significant decrease of cellular reducing capacity, it was used the MTT assay for cell viability, where the risk of colour saturation does not occur. Hippocampal neuronal cultures, 7 *days in vitro*, were incubated with 10, 50, 100, 250 μM of kainate; 10, 50, 100 μM of NMDA; 50, 100, 250 and 3000 μM of quinolinic acid, all prepared and incubated in Krebs-Ringer's saline (in mM: 117 NaCl, 3 KCl, 10 glucose, 26 $NaHCO_3$, 1,25 NaH_2PO_4, 10 HEPES, 2 $CaCl_2$ and 1 $MgCl_2$). The toxic agents and the Alamar Blue (10 times concentrated and added in a 10 %, v/v, of the total volume) were incubated for 3, 4, 6, 8 and 12 hours. At every endpoint, 100 μl of the medium from each well was pipetted in triplicates to a 96-well plate and the absorbance was read on a spectrophotometer, at the maximum absorbance wave length of 570 nm and at the reference wave length of 600 nm. The plates were returned to the incubator every time, until the next endpoint. Saturation curves for Alamar Blue were carried out under the same experimental conditions, without the toxic agents. To minimize differences due to uneven number of plated cells *per* well, each plaque had triplicates of the same condition and the results for each triplicate were averaged. The percentage of reduction was calculated by the quotient between the difference of the absorbance at 570 nm and the absorbance at 600 nm of the samples and

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the difference of absorbance at 570 nm and at 600 nm of the negative control, in which no toxic agent was added. Results were analysed as percentage of control (wells incubated with vehicle).

3.13.2 MTT ASSAY

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide from Sigma-Aldrich, Portugal) assay uses de same principle of Alamar Blue, i.e, evaluates the metabolic state of cells. The tetrazolium ring of MTT is cleaved by dehydrogenases present in the cytosol and in active mithocondria and precipitates as a purple formazan product. The intensity of the purple colour is directly proportional to the absorbance at 570 nm (600 nm is used as reference). Thus, higher absorbance values are related to a greater number of viable cells (Luscher and Slesinger, 2010). As the formazan product is toxic, this test was used for analysis of cell viability at a single, well defined endpoint, upon which the cell medium was aspirated and a new one with the MTT was added.

Hippocampal neurons, 7 *days in vitro*, were stimulated for different times with ionotropic glutamate receptor agonists: 100 μM of kainate during 6 hours; 100 μM of NMDA during 12 hours and 3 mM quinolinic acid during 24 hours prepared and incubated in Kreb-Ringer's saline. Upon each endpoint, cells were washed twice with Krebs and incubated with MTT (0.5 mg/ml), also in Krebs, for 1 hour at 37 ℃ and in a 5 % O_2- 95 % CO_2 atmosphere. Then, MTT was aspirated and the formazan formed was diluted with the same volume of dimethyl sulfoxide (DMSO, Sigma-Aldrich, Portugal), for 1 hour at RT, under agitation and protected from light. Finally, 100 μl of each well were pipetted to a 96-well plate in triplicates and the absorbance was read at 570 nm and 600 nm. The percentage of reduction was calculated as explained for the Alamar Blue assay and expressed as percentage of control. In order to evaluate the contribution of P2 receptors to the excitotoxicity induced by overactivation of glutamate ionotropic receptors, PPADS (20 μM, a non-selective P2 receptor antagonist, from Tocris Bioscience, Spain) was incubated 30 minutes before the stimuli and prevailed throughout. To evaluate the specific contribution of the subtype $P2Y_1$ receptors, cells were pre-incubated with MRS2500 (10 μM, a potent and selective $P2Y_1$ receptor antagonist, Tocris Bioscience, Spain). The effect of a short-term exposure (25 minutes) of hippocampal neurons to the same stimuli and to glutamate itself (100 μM, L-glutamic acid from Sigma-Aldrich, Portugal) was also evaluated using the MTT assay. In this case, after 25 minutes of stimuli, neurons were washed three times with Krebs and fresh Neurobasal medium was added. Upon 24 hours, the medium was aspirated and the Krebs with MTT was added. When used, the antagonists were incubated 30 minutes before the stimuli

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and were again added to the fresh Neurobasal medium after washing the stimuli. SCH58261 (50 nM, from Vernalis, UK).

The MTT assay was also used to evaluate pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α (purchased to R & D Systems) conditioning of glutamate-induced excitotoxicity to hippocampal neurons. Cells were incubated with either 100 ng/ml interleukin-1β or 5 ng/ml tumor necrosis factor-α, 5 minutes prior to incubation of 100 μM glutamate, for a total of 25 minutes. The cell viability assay was conducted as already described for the short-term stimuli, with analysis at 24 hours. The effect of blocking adenosine $A_{2A}R$ in the modulation of glutamate-induced neurotoxicity and on interleukin-1β exacerbation of excitotoxicity was evaluated by adding SCH58261 (50 nM, from Vernalis, UK), a selective antagonist of $A_{2A}R$, 30 minutes before the toxic agents. Likewise, the effect of the blockade of ATP $P2Y_1R$ was evaluated by adding a selective antagonist of these receptors, MRS2179 (10 μM, Tocris Bioscience, Spain).

3.13.3 SYTO-13 AND PROPIDIUM IODIDE STAINING

The significant results obtained with the MTT assay were further analysed through the Syto-13 *plus* propidium iodide (PI) staining (both reagents from Molecular Probes, Leiden, Netherlands), as previously described in (Rebola *et al.*, 2005d). Syto-13 is a cell-permeant nucleic acid stain that shows a large (green) fluorescence enhancement upon biding nucleic acids. Therefore, the Syto-13 staining allows the visualization of viable cells (darker, homogeneous and larger green fluorescent nuclei) and of apoptotic cells in which the plasmatic membrane is still intact (brighter, fragmented and smaller green fluorescent nuclei). The propidium iodide also binds nucleic acids with resulting strong red fluorescent. However, this stain is not permeable to cytoplasmic membranes. Therefore, the propidium iodide staining labels cells in which the membrane integrity is compromised, i.e., necrotic cells (darker, homogeneous and larger red fluorescent nuclei) and cells undergoing secondary apoptosis (small, very bright red spots of fragmented nuclei). After incubation with the toxic agents, cells were washed 3x in Krebs and incubated with a mixture of Syto-13 (4 μM) and PI (4 μg/ml) prepared in Krebs, for 3 minutes. After mounting the coverslips on microscope slides, cells were observed and counted using a fluorescence microscope. At least six fields *per* coverslip were analysed with a total of 300 cells, in average.

3.13.4 LACTATE DEHYDROGENASE ASSAY

Lactate Dehydrogenase (LDH) is an oxidoreductase (EC 1.1.1.27) that catalyses the interconversion of pyruvate and lactate with concomitant interconversion of NADH and NAD^+. When any form of injury damages tissues, cells release LDH into the extracellular space and, being a fairly stable enzyme, it has been widely used to evaluate the degree of damage induced by toxic events to cells (Tajeddine *et al.*, 2008). Since LDH leakage depends on plasmatic membrane integrity, this assay is particularly useful when cell death occurs mainly through necrosis (Tajeddine *et al.*, 2008). Cell viability assays are not consensual among the scientific community and all present some degree of accuracy problems depending on the experimental conditions (Galluzzi *et al.*, 2009). Therefore, we also used this method to assess cell viability and, possibly, confirm the significant results previously obtained relative to glutamate-induced cell death, exacerbation of this effect by interleukin-1β and neuroprotection by blocking adenosine A_{2A} receptors. LDH activity was measured spectrophotometrically according to the method developed by Bergmeyer and Brent (1974), by following the rate of conversion of NADH to NAD^+, at 340 nm. Thus, 24 hours after the stimuli, the cell medium was aspirated (extracellular fractions) and kept in eppendorfs at 4ºC until analysis. Simultaneously, the plated cells were lysated on ice with 1 ml of 0.02 % Triton X-100 in 10 mM HEPES buffer (pH 7.4) and 3 freezing/thawing cycles. The lysates (intracellular fractions) were also kept at 4 ºC in eppendorfs until analysis. Before the assay, both intracellular and extracellular fractions were centrifuged at 14000 rpm, 10 minutes at 4 ºC. The pellets (cellular organelles and membrane fractions) were discarded and the supernatants were used to measure LDH activity. Fresh solutions of 0.244 mM NADH and of 9.76 mM pyruvate were prepared in 81.3 mM Tris / 203.3 mM NaCl buffer, pH 7.2 at 30 ºC. Reactions were carried in cuvettes, kept at 30ºC, to which 0.5 ml of pyruvate and 100 µl of the sample was added and, immediately before the reading, 2.5 ml of NADH was also added, which triggers the convertion of pyruvate to lactate by LDH. The loss of absorbance at 340 nm was followed with readings at 0.5 seconds intervals, during 2 minutes. The curve slope, the reaction's velocity, is directly proportional to the quantity of LDH present in the sample. Triplicates were carried for each sample and the results averaged. The percentage of LDH leakage was calculated using the ratio between extracellular LDH activity and the sum of intracellular and extracellular LDH activity. The blank of these experiment consisted of adding NADH to the cuvette in the absence of sample (LDH). Results were expressed as percentage of control values.

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3.14 MONITORING INTRACELLULAR CALCIUM WITH SINGLE CELL CALCIUM IMAGING

To investigate the effect of blocking adenosine A_{2A} receptors or ATP $P2Y_1$ receptors in the glutamate-induced increase of intracellular calcium ($[Ca^{2+}]_i$) and also if the interleukin-1β exacerbation of glutamate excitotoxic effects was associated with a deregulation of $[Ca^{2+}]_i$ homeostasis, it was used the single cell calcium imaging as previously described in (Bernardino *et al.*, 2008). This method takes advantage of Fura-2-acetoxymethyl ester (Fura-2AM) properties, a membrane-permeable, selective calcium radiometric dye, to measure cellular calcium concentrations by fluorescence. The probe crosses the plasmatic membrane and, once inside, unspecific cellular esterases remove the acetoxymethyl (lipophilic) groups making it membrane-impermeable. The regenerated Fura-2 ($C_{44}H_{47}N_3O_{24}$), which is an analogous of the Ca^{2+}-chelating molecule EGTA with an additional chemical group with fluorescent properties, is then free to bind Ca^{2+} through its four carboxyl groups (Grynkiewicz *et al.*, 1985). The great advantage of Fura-2 is that, once bound to Ca^{2+} it has an absorption peak shift from 380 nm to 340 nm excitation wavelengths. Cytosolic Ca^{2+} transients can be followed by monitoring the 510 nm emission (maximum Fura-2 fluorescence) and measuring 340/380 nm ratio of Fura-2 absorbance along the time (Hirst *et al.*, 1999a). The use of the ratio eliminates variables such as uneven dye loading, cell thickness, leakage of fura-2 and bleaching. Fura-2 dye provides the possibility to perform measurements for about 1 hour without significant bleaching and given its high molar extinction coefficient ($\varepsilon = 30,800$ cm^{-1}M^{-1}), meaning a high fluorescence intensity *per* Fura-2 molecule, allows measurements of intracellular concentrations of the dye that do not cause significant Ca^{2+} buffering or altering of Ca^{2+} transients.

Hippocampal neurons, 7 *days in vitro*, plated on coverslips, were loaded with 5 μM Fura-2AM (Molecular Probes, Leiden, Netherlands) and 0.02 % pluronic acid F-127 (Molecular Probes, Leiden, Netherlands) for 30 minutes in Krebs-Ringer saline buffer (in mM: 150 NaCl, 5 KCl, 10 glucose, 10 HEPES, 2 $CaCl_2$ and 1 $MgCl_2$, pH 7.4) supplemented with 0.1 % fatty-acid free BSA, in an incubator with 95 % CO_2- 5 % O_2 atmosphere, at 37ºC. After 3 passages through Krebs buffer to wash excess of probe, coverslips were placed in a RC-20 perfusion chamber in a PH3 platform (Warner Instruments, Harvard, UK), on the stage of an inverted fluorescence microscope (Axiovert 200, Carl Zeiss, Germany). Hippocampal neurons were alternately excited at 340 and 380 nm using a Lambda DG4 apparatus (Sutter Instruments Company, Novato, CA, USA) and the emitted fluorescence was collected

with a 40× oil objective and driven to a CoolSNAP digital camera (Roper Scientific, Trenton, NJ, USA). Acquired values were processed using the MetaFluor software (Universal Imaging Corporation, Buckinghamshire, UK). Areas of the cell bodies were drawn and the average value of pixel intensities was evaluated at each time point. Image acquisition was done every second during a total of 35 minutes. Results were expressed by plotting the ratio of fluorescence intensity emitted at 510 nm (using a 510 nm band-pass filter from Carl Zeiss), following alternate excitation (750 milliseconds, using a Lambda DG4 apparatus from Sutter Instrument, Novato, CA,USA) at 340 and 380 nm, *versus* time (Resende *et al.*, 2007). Solutions were added to the cells by perfusion using a fast-pressurized (95 % air, 5 % CO_2 atmosphere) system (AutoMate Scientific, Inc., Berkeley, CA, USA). Drugs were prepared in normal Krebs solution. Basal ratio was measured during the first 2 minutes of the experiments. Interleukin-1β, (IL-1β, 100ng/ml), when present, was added for 5 minutes prior to glutamate (100 μM). Cells were left in the presence of the stimuli for the next 15 minutes after which it was washed through superfusion of normal Krebs. Whenever A_{2A} receptors or $P2Y_1$ receptors selective antagonists, SCH58261 (50 nM) or MRS2179 (10 μM), respectively, were present, they were incubated for 15 minutes prior to the beginning of the experiment and prevailed throughout. To assure that the selected cell bodies belonged to neurons, response to 50 mM KCl was tested before calibration, at the end of each experiment. The calibration was made using 10 μM ionomycin prepared in normal Krebs, for maximum ratio, and 10 mM $MnCl_2$ prepared in calcium-free Krebs, to assess the background fluorescence. The experiments were carried as exemplified in the scheme bellow:
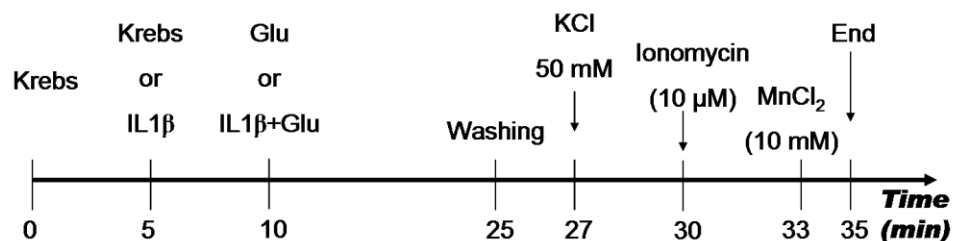
Statistical analysis: values are presented as mean ± SEM of n experiments. Either a Student's t test for independent means or a one-way analysis of variance (ANOVA) followed by post hoc Bonferroni's test, was used to define statistical differences between values, which were considered significant at $P<0.05$, unless otherwise specified.

RESULTS

4.1 ADENOSINE A$_{2A}$ RECEPTORS CONTROL NEUROINFLAMMATION

4.1.1 INTRODUCTION

The brain was considered an immunopriviledge organ for a long time, mainly due to the belief in a physical separation between this organ and the immune system (Medawar, 1948; Barker and Billingham, 1977) through what became known as the blood brain barrier (BBB) (Carson *et al.*, 2006). However, in the late years, that concept changed dramatically as the BBB was found to be a dynamic structure in constant "cross-talk" with the peripheral nervous system (PNS) (Carson *et al.*, 2006). Nowadays, it is well known that neuroinflammation is a common feature of most neurological disorders and pathological situations in the central nervous system (CNS) (Skaper, 2007; Glass *et al.*, 2010; Wee Yong, 2010). Neuroinflammation is initiated by microglia, the immuno-competent cells and injury/pathogen "sensors" of the CNS (Stence *et al.*, 2001; Ransohoff and Perry, 2009; Streit and Xue, 2009) and mainly orchestrated by these and the astrocytes (Norton *et al.*, 1992; Skaper, 2007; Zhang *et al.*, 2010a), which have a central role in the control of synaptic transmission (Perea *et al.*, 2009), brain repair (Sofroniew, 2005) and in the maintenance homeostasis (Sofroniew, 2005; Zhang *et al.*, 2010a). Both microglia and astrocytes rapidly respond to an adverse situation by changing their morphology, multiplying and migrating to the site of injury (Streit *et al.*, 1999; Streit, 2000; Zhang *et al.*, 2010a). This reaction is termed reactive gliosis (microgliosis and astrogliosis, respectively) and involves the expression of specific structural proteins and cell surface receptors (O'Keefe *et al.*, 2002; Ladeby *et al.*, 2005; Zhang *et al.*, 2010a), accompanied by the expression and release of several inflammatory mediators, including pro-inflammatory cytokines (Chakraborty *et al.*, 2010). Uncontrolled or chronic neuroinflammation is involved in the etiology and/or aggravation of several noxious conditions and pathologies in the CNS (Marchetti and Abbracchio, 2005; Skaper, 2007; Glass *et al.*, 2010; Wee Yong, 2010). Accordingly, the control of neuroinflammation is associated with a general improvement in different neurodegenerative diseases (Allan *et al.*, 2005; Marchetti and Abbracchio, 2005).

Inflammation results in elevated extracellular levels of purines which in turn influence the inflammatory response (Abbracchio and Verderio, 2006; Abbracchio and Ceruti, 2007; Hasko and Pacher, 2008; Di Virgilio *et al.*, 2009; Orr *et al.*, 2009). In the PNS, the activation of adenosine A$_{2A}$Rs is well established as a "stop" signal of inflammation (Sitkovsky and Ohta, 2005). On the other hand, in the brain it is the blockade of A$_{2A}$Rs that is associated with the prevention or the improvement of

different pathological conditions (Cunha, 2005; Chen *et al.*, 2007; Gomes *et al.*, 2011). This was shown in models of Alzheimer's disease (Dall'Igna *et al.*, 2003; Dall'Igna *et al.*, 2007; Canas *et al.*, 2009), ischaemia (Chen *et al.*, 1999; Higashi *et al.*, 2002; Yu *et al.*, 2004), diabetes (Duarte *et al.*, 2009), epilepsy (Zeraati *et al.*, 2006; El Yacoubi *et al.*, 2009) and in particular, in models of Parkinson's disease where A_{2A}R antagonists have the ability to both normalize the motor function and afford a marked neuroprotection, being currently in phase IIb studies as anti-parkinsonian drugs (Xu *et al.*, 2005). Since A_{2A}Rs are expressed in brain glial cells and modulate its activity (Dare *et al.*, 2007; Orr *et al.*, 2009; Boison *et al.*, 2010), the control of neuroinflammation may in part explain the broad spectrum of deleterious situations in which the blockade of A_{2A}Rs confers neuroprotection (Cunha, 2005; Chen *et al.*, 2007; Boison *et al.*, 2010). However, the role of A_{2A}R in the control of neuroinflammation is currently unclear given that in animal models of Parkinson's disease (Pierri *et al.*, 2005; Yu *et al.*, 2008) and of traumatic brain injury (Dai *et al.*, 2010) it was suggested that A_{2A}R blockade control microglia activation whereas studies using primary cultures of microglia have reached opposite conclusions related to the control of function by A_{2A}R (Saura *et al.*, 2005; Orr *et al.*, 2009; van der Putten *et al.*, 2009), questioning a direct role of A_{2A}R in the control of neuroinflammation in an *in vivo* setting.

The present work was designed to test if the blockade of A_{2A}Rs can control specific features of neuroinflammation in the hippocampus, such as biochemical and morphological changes resulting from peripheral (intraperitoneal, ip) administration of lipopolysaccharide (LPS). This toxin from Gram negative bacteria is a prototypical trigger of inflammation and is also known to be a potent trigger of neuroinflammation (Kim *et al.*, 2000; Chakravarty and Herkenham, 2005); furthermore considerable work has already been made to understand the relation between the genesis of neuroinflammation caused by LPS and the consequent neuronal dysfunction in the hippocampus (Vereker *et al.*, 2000b; Kelly *et al.*, 2003; Nolan *et al.*, 2003).

4.1.2 RESULTS

4.1.2.1 A$_{2A}$R BLOCKADE PREVENTS THE LPS-INDUCED BIOCHEMICAL MODIFICATIONS IN THE RAT HIPPOCAMPUS

Previous studies by our collaborators have suggested that the LPS-induced increase in hippocampal IL-1β and neuronal downstream signalling events (i.e. activation of kinases of the stress pathway) mediate the neuroinflamation-induced depression of synaptic plasticity in the dentate gyrus (DG) (Vereker *et al.*, 2000b; Kelly *et al.*, 2003; Nolan *et al.*, 2003). Here it was asked whether the ability of SCH58261, an A$_{2A}$R selective antagonist, to reverse the LPS induced impairment in LTP, as was shown in a recent work from our group (Rebola *et al.*, 2011), might be paralleled by a similar ability to attenuate the LPS-induced increases in the phosphorylation of p38, JNK and in the activation of caspase-3. The LPS-induced activation of JNK and p38 was assessed by Western blot analysis of the phosphorylated forms of the kinases. Analysis of caspase-3 activity in hippocampal slices was assessed using an enzymatic kit in which the cleavage of caspase-3 substrate (Ac-DEVD-AFC peptide, from Alexis) gave origin to a fluorescent product, measurable in a spectrophotometer (at 400 nm excitation and emission at 505 nm), as previously described by (Nolan *et al.*, 2003). As shown in Figure 1A-D, LPS increased the density of the phosphorylated forms of p38 and of JNK in hippocampal tissue to 131±6% and 129±9% of control values, respectively (n=5, P<0.05). Administration of SCH58261 did not significantly change the density of the phosphorylated forms of p38 or JNK (103±8% and 98±9% of control, respectively; P>0.05 versus control, n=5), but prevented the LPS-induced increases in both p38 and JNK (100±5% and 98±9% of control; P<0.05 *versus* LPS, n=5). Activated MAPKs play different roles in different cell types in the central nervous system. Immunohistochemistry analysis was used to evaluate whether LPS-induced (4 h after LPS ip administration) p38 was on neurons or on microglia cells. This was achieved through double-labelling with antibodies directed against the phosphorylated form of p38 and against the nuclear neuronal marker (NeuN) or against the microglia marker CD11b. As shown in Figure 1E, LPS triggered an enhancement of the immunoreactivity of the phosphorylated form of p38 throughout the dentate gyrus (n=3), which was co-localised both with neuronal markers (NeuN, left panel of Fig. 1F) and with a microglia marker (CD11b, right panel of Fig. 1F).
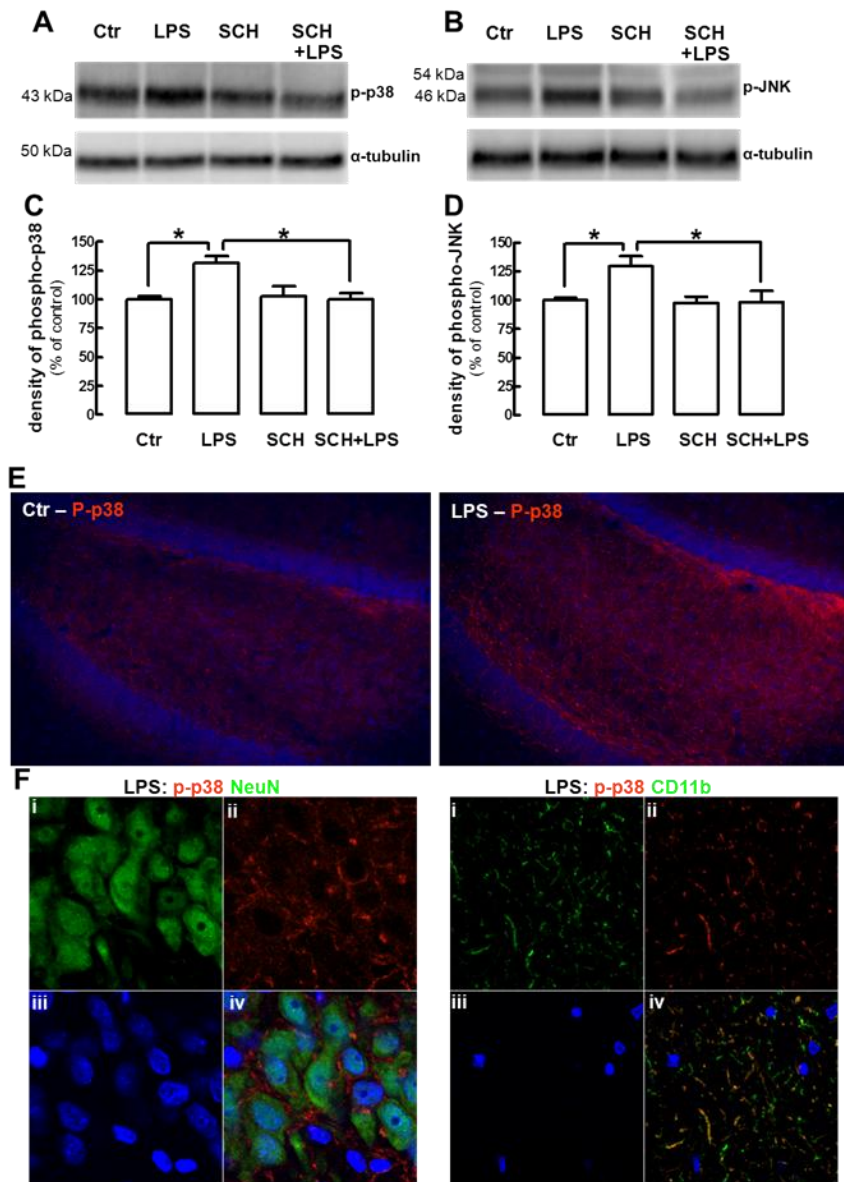
FIGURE 1: Effect of LPS administration (200 μg in 200 μl, ip) and/or the selective antagonist of A$_{2A}$R, SCH58261 (5 μl of a 50 nM solution, icv) on the density of the phosphorylated forms of two stress activated kinases, p38 (panels A and C) and JNK (panels B and D) in the rat dentate gyrus of the hippocampus. The rats were separated into 4 groups: 1) control (icv and ip administration of saline); 2) SCH58261 (SCH58261 icv and saline ip); 3) LPS (saline icv and LPS ip); 4) LPS + SCH58261 (SCH58261 icv and LPS ip). Four hours after LPS injection, the animals were sacrificed, the hippocampus dissected and homogenized for Western blot analysis. Panels A and B show a Western blot membrane comparing the density of immunoreactivity of the phosphorylated form of p38 (panel A) and of JNK (panel B) in the hippocampus of each group of rats. The re-probing of the same membranes against α-tubulin is displayed below. Panels C and D present the average results (mean ± SEM), obtained in 3-5 experiments, comparing the density of the immunoreactivity of the phosphorylated form of p38 (panel C) and of JNK (panel D) in the hippocampus of each group of rats. *P<0.05 between the indicated bars. Note that SCH58261 did not modify the density of phosphorylation of these two activated stress proteins but prevented the ability of LPS to increase their density. Immunohistochemical analysis confirmed an enhanced density of the phosphorylated p38 immunoreactivity in the dentate gyrus of LPS-treated rats (right photograph of panel E) compared to control (left photograph of panel E). This LPS-induced enhancement of phosphorylated p38 immunoreactivity was co-localised with NeuN (neuronal nuclei), a marker of neurons (left set of photograph of panel F: i-NeuN; ii-phosphorylated p38; iii-Hoescht 33342, which stains DNA; iv-merging of the previous 3 photographs), as well as with CD11b, a marker of microglia (right set of photograph of panel F: i-CD11b; ii-phosphorylated p38; iii-Hoescht 33342, which stains DNA; iv-merging of the previous 3 photographs). These photographs are representative of immunohistochemical analysis carried out in 3 LPS-treated rats.

The location of the LPS-induced activation of caspase-3 was also assessed in this way, in the dentate gyrus. In parallel with the effect of LPS on phosphorylation of JNK and p38, we report a significant increase in the activity of caspase-3 in hippocampal tissue prepared from LPS-treated rats comparing to control animals (n=5, P<0.05; Fig. 2A). Administration of SCH58261 did not significantly change the activity of caspase-3 (P>0.05, n=3), but prevented the LPS-induced increase of caspase-3 activity so that there was a significant difference (P<0.05) in the enzyme activity in hippocampal tissue prepared from LPS-treated rats and in tissue prepared from rats which were treated with LPS and SCH58261 (Fig. 2A). Immunohistochemical analysis revealed that LPS caused a scattered increase of activated (cleaved) caspase-3 in the dentate gyrus (Fig. 2B), which was not co-localised with neuronal markers (NeuN, Fig. 2C) but rather with a microglia marker (CD11b, Fig. 2D).
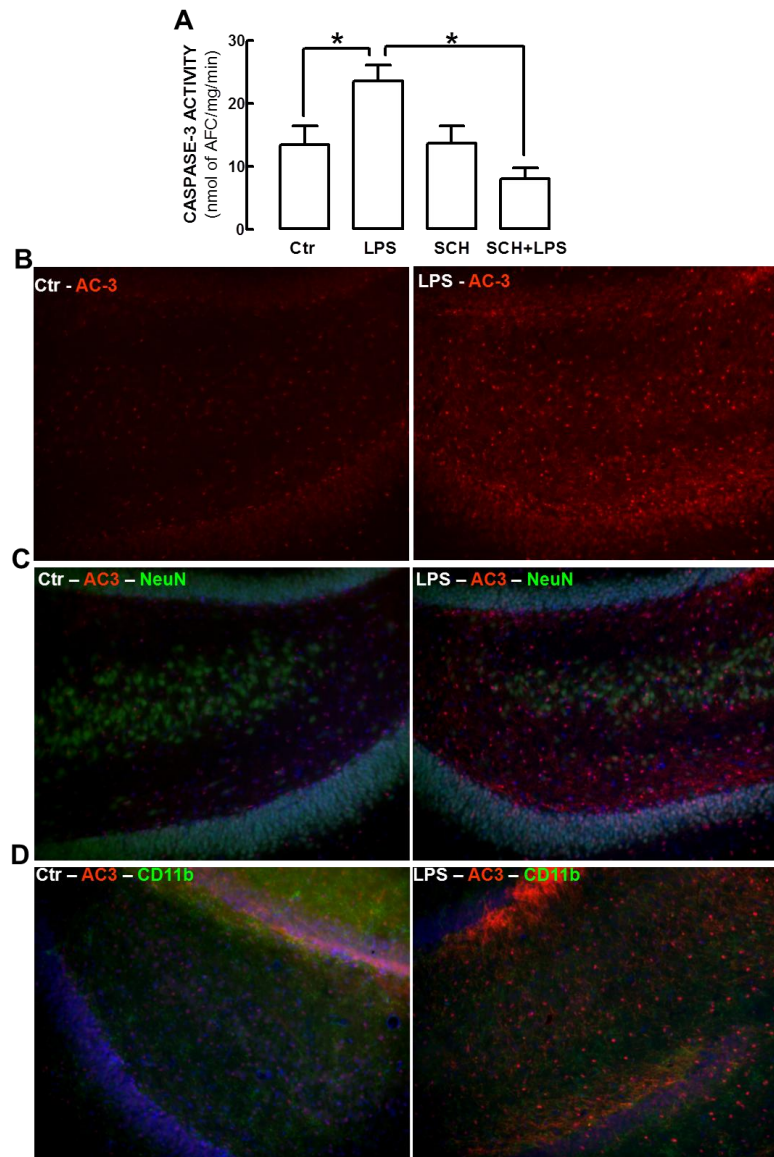
FIGURE 2: Effect of LPS administration (200 μg in 200 μl, ip) and/or the selective A$_{2A}$R antagonist, SCH58261 (5 μl of a 50 nM solution, icv) on the activity of caspase-3 in the rat hippocampus. In panel A, the rats were separated into 4 groups: 1) control (icv and ip administration of saline); 2) SCH58261 (SCH58261 icv and saline ip); 3) LPS (saline icv and LPS ip); 4) LPS + SCH58261 (SCH58261 icv and LPS ip); four hours after LPS injection, the rats were sacrificed, the hippocampus dissected and homogenized for the fluorimetric analysis of caspase-3 activity, quantified by the hydrolysis of its selective substrate, Ac-DEVD-AFC peptide, which produces a fluorescent product (emission at 505 nm after excitation at 400 nm). In panel A, the ordinates represent the activity of caspase-3 expressed in nmol of 7-amino-4-trifluoromethylcoumarine (AFC), normalized by the concentration of protein in each sample. Each bar represents the mean ± SEM of 5 experiments. *P<0.05 between the indicated bars. Note that SCH58261 did not modify the activity of caspase-3 but prevented the LPS-induced increase in caspase-3 activity. Immunohistochemical analysis confirmed an enhanced density of activated (cleaved) caspase-3 immunoreactivity in the dentate gyrus of LPS-treated rats (right photograph of panel B) compared to control (left photograph of panel B). Panel C displays triple-labelling immunohistochemical analysis of cleaved caspase-3 (red), the neuronal marker (NeuN, green) and Hoescht 33342-labelled nuclei (blue) in control (left photograph) and LPS-treated rats (right photograph) showing that cleaved caspase-3 was not co-localised with NeuN immunoreactivity. Panel D displays triple labelling immunohistocehmical analysis of cleaved caspase-3 (red), the microglia marker (CD11b, green) and Hoescht 33342-labelled nuclei (blue) in control (left photograph) and LPS-treated rats (right photograph) showing that cleaved caspase-3 was instead co-localised with CD11b immunoreactivity. These photographs are representative of immunohistochemical analysis carried out in 3 LPS-treated rats.

4.1.2.2 A$_{2A}$ RECEPTORS BLOCKADE PREVENTS THE LPS-INDUCED ACTIVATION OF MICROGLIA

The protective effect of the blockade of A$_{2A}$R against LPS-induced inhibition of LTP might either result from the ability of neuronal A$_{2A}$R to control the effects of inflammatory mediators on neurons or result from the ability of A$_{2A}$R to control the reactivity of microglia and the consequent generation of inflammatory mediators. Thus, it was tested if the blockade of A$_{2A}$R could control the activation of microglia and the previously-described LPS-induced increase in IL-1β (Vereker *et al.*, 2000a). The identification of microglial cells was carried out by immunohistochemical detection of the CD11b antibody, a validated marker of microglia cells (Jensen *et al.*, 1997). As presented in Figure 3, it can be concluded that 4 hours after administration of LPS, there is an increase in the number of elements labelled with the anti-CD11b antibody (compare panels A and C from Fig. 3). Furthermore, the labelled profiles displayed a more intense immunoreactivity and a morphology characteristic of early activated microglia *in situ*, i.e., enlarged cell body with short and thick processes (Fig. 3C) (Jensen *et al.*, 1997). Administration of SCH58261 did not modify the profile of CD11b immunoreactivity compared to control (n=4; Fig. 3B), but prevented the LPS-induced changes in CD11b immunoreactivity (n=4; Fig. 3D).
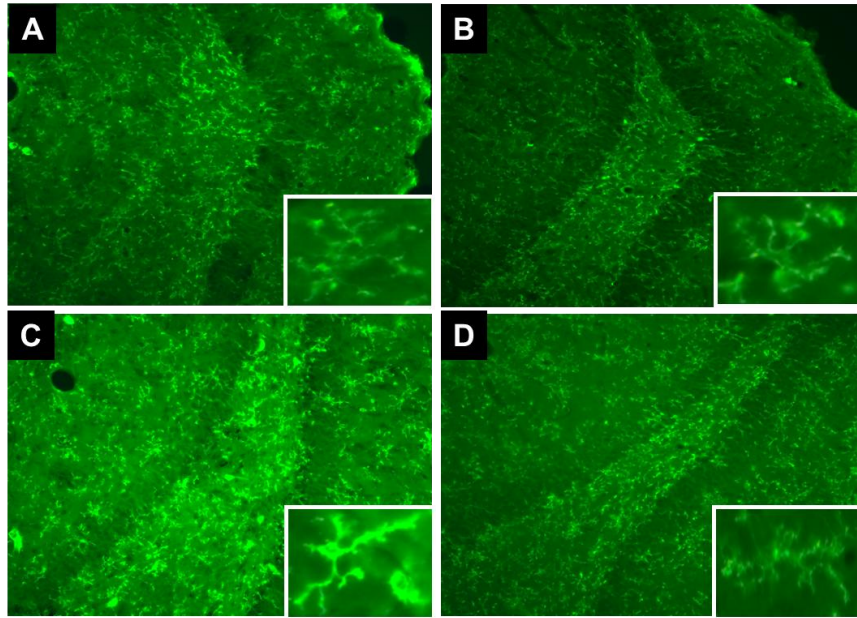
FIGURE 3: Effect of LPS administration (LPS, 200 µg in 200 µl, ip) and/or the selective A$_{2A}$R antagonist, SCH58261 (5 µl of a 50 nM solution, icv) on the appearance of reactive microglia in the rat dentate gyrus. The animals were separated into 4 groups: 1) control (icv and ip administration of saline; panel A); 2) SCH58261 (SCH58261 icv and saline ip; panel B); 3) LPS (saline icv and LPS ip; panel C); 4) LPS + SCH58261 (SCH58261 icv and LPS ip; panel D). Four hours after the drug treatments, the rats were perfused with paraformaldehyde for fixation of the brain, which was sliced into 20 µm sections. The coronal sections were labelled by immunohistochemistry using a mouse anti-CD11b antibody (a marker of microglial cells) and a goat anti-mouse secondary antibody labelled with Alexa Fluor 488 (green fluorescence). The inserted figures in each panels display the pattern of labelling and morphology of a single microglia cell, obtained from the presented picture at higher magnification. The pictures presented are representative of 3-4 experiments with qualitatively similar results. Note that LPS increase the general density of CD11b positive elements (panel C) displaying a morphology characteristic of activated microglial cells (see inserted Figure in panel C) and these LPS-induced modifications were prevented by SCH58261 (panel D), which by itself was devoid of effects (panel B).

Accordingly, 4 hours after the administration of LPS, there was an increase in the density of markers of microglia activation, such as the MHC-II protein CR3/43 (n=7, Fig. 4A) and the iNOS (n=7, Fig. 4B). The administration of either SCH58261 or of another selective A$_{2A}$R antagonist, MSX-3, did not modify the density of each of these microglia markers, but prevented the LPS-induced changes in both CR3/43 (n=3-4, Fig. 4A) and iNOS (n=3-4, Fig. 4B).
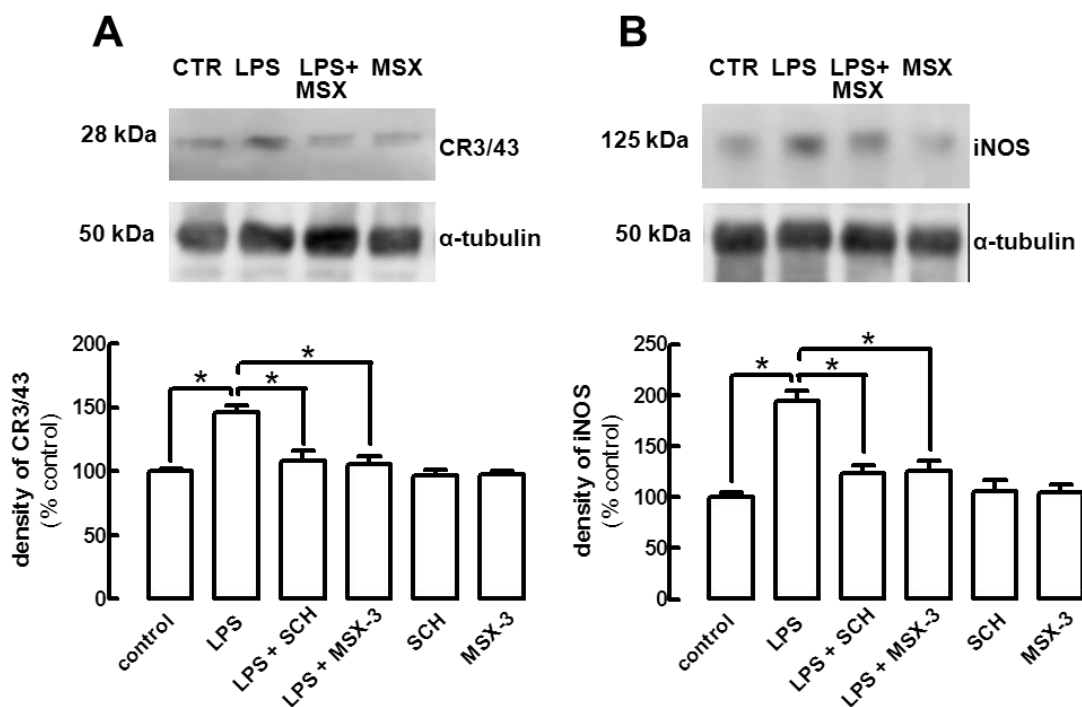
FIGURE 4: Effect of LPS administration (200 μg in 200 μl, ip) and/or the A$_{2A}$R selective antagonists, SCH58261 (5 μl of a 50 nM solution, icv) or MSX-3 (5 μl of a 1 μM solution, icv), on the density of markers of activated microglia (CR3/43 and iNOS) in the rat dentate gyrus. Rats were separated into 4 groups: 1) control (icv and ip administration of saline); 2) A$_{2A}$R antagonist-treated (SCH58261 or MSX-3 icv and saline ip); 3) LPS-treated (saline icv and LPS ip); 4) LPS + A$_{2A}$R antagonist-treated (SCH58261 or MSX-3 icv and LPS ip). Four hours after LPS injection, the animals were sacrificed and one hippocampus was used for Western blot analysis whereas the other was used for real-time PCR analysis. Panels A and B present a Western blot result comparing the immunoreactivity density of the MHC-II protein CR3/43 (panel A) and of iNOS (panel B) in the hippocampus of each group of rats. The re-probing of the same membranes against α-tubulin is displayed below. The graph bars below show the average results (mean ± SEM), obtained in 3-4 experiments, comparing the immunoreactivity density of the 2 markers of microglia activation; *P<0.05 between the indicated bars. Note that neither SCH58261 nor MSX-3 modified the density of each of these proteins but prevented the ability of LPS to increase their density.

Further linking the control of LPS-induced effects by the blockade of A$_{2A}$R with the control of the activation of microglia, it is observed a parallel increase in the expression of both CD11b (n=7; Fig. 5C) and of A$_{2A}$R mRNA (n=7, Fig. 5D) in the hippocampus, measured 4 h after the administration of LPS.
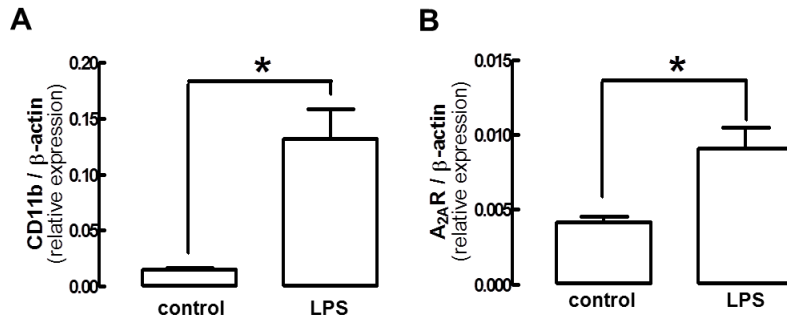
FIGURE 5: Effect of LPS administration (200 μg in 200 μl, ip) on the expression of CD11b and of A2ARs in the rat dentate gyrus. Rats were separated into 2 groups: 1) control (icv and ip administration of saline); 2) LPS-treated (saline icv and LPS ip). Four hours after the LPS injection, the animals were sacrificed; one hippocampus was used for Western blot analysis (results presented in Fig. 4) and the other for real-time PCR analysis. Panels A and B display the expression of CD11b (panel A) and of A2AR (panel B) 4 h and 45 min after LPS administration. CD11b and A2AR mRNA levels (copies/μl) were determined with quantitative real time PCR (QRT-PCR) and normalized to the level of β-actin mRNA. Bars represent mean ± SEM from 7 different rats, run in triplicate; *P<0.05 between bars.

In accordance with a direct ability of A$_{2A}$R to control microglia activation, it was found that LPS treatment caused a significant increase in hippocampal concentration of IL-1β (n=5, P<0.05; Fig. 6). Administration of SCH58261 alone did not significantly change (P>0.05, n=3) IL-1β concentration, but prevented the LPS-induced increase in this pro-inflammatory cytokine (n=3, P<0.05; Fig. 6).
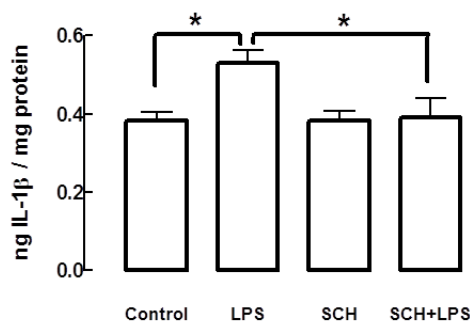

FIGURE 6: Effect of the administration of LPS (200 μg in 200 μl, ip) and/or the selective antagonist of adenosine A$_{2A}$R, SCH 58261 (5 μl of a 50 nM solution, icv) on the activity levels of interleukin-1β in the rat hippocampus. The rats were separated into 4 groups: 1) control (administration icv and ip of saline); 2) SCH 58261 (SCH 58261 icv and saline ip); 3) LPS (saline icv and LPS ip); 4) LPS + SCH 58261 (SCH 58261 icv and LPS ip). Four hours after LPS injection, the rats were sacrificed, the hippocampus dissected and homogenized for analysis by ELISA of IL-1β levels. Each bar represents the mean±SEM of 5 experiments. *P<0.05 between the indicated bars. Note that SCH 58261 did not modify the levels of IL-1β but prevented the LPS-induced increase in IL-1β levels.

4.1.3 DISCUSSION

From the present study we conclude that the blockade of adenosine A$_{2A}$R prevents the induction of a neuroinflammation triggered by LPS and the consequent biochemical modifications in the hippocampus. As it is well established in the literature, LPS triggers an inflammatory response that results in elevated levels of the pro-inflammatory cytokine IL- 1β in the brain (Buttini and Boddeke, 1995; Quan *et al.*, 1998; Tonelli and Postolache, 2005). This cytokine, in turn, mediates most of the consequences of LPS administration, such as neuronal dysfunction (Vereker *et al.*, 2000a; Allan *et al.*, 2005). The mechanism by which peripherally administered LPS leads to neuroinflammation is known to involve the activation of CNS microglia (Kim *et al.*, 2000), independent of systemic inflammation (Chakravarty and Herkenham, 2005). The mitogen-activated protein kinases (MAPKs) pathway is triggered both in neurons and glia in response to inflammation. In particular, activation of stress kinases like p38 and c-Jun N-terminal kinase (JNK) has been shown to occur in neurons, which negatively impact synaptic function leading to depression of LTP in dentate gyrus (Vereker *et al.*, 2000b; Kelly *et al.*, 2003; Nolan *et al.*, 2003; Srinivasan *et al.*, 2004; Barry *et al.*, 2005). In parallel, poly-ADP ribose polymerase (PARP) cleavage and activation of caspase-3 are up-regulated leading to a delayed LPS-induced neuronal apoptosis (Hauss-Wegrzyniak *et al.*, 2002; Nolan *et al.*, 2003). In the present study, it was observed that the blockade of A$_{2A}$Rs, using its selective antagonist SCH58261 see (Fredholm *et al.*, 2005) for a review, prevented the principal modifications caused by LPS that are associated with the LPS-induced neuroinflammation and neuronal dysfunction. Administration of SCH58261 prevented the ability of LPS to activate p38, JNK and the apoptotic-mediator caspase-3. In another part of the presented work not discussed here, it was shown that SCH58261 was also able to prevent LPS-induced depression of LTP (Rebola *et al.*, 2011) which is unlikely to be a direct synaptic effect of the A$_{2A}$R antagonist (although this cannot be excluded), since this A$_{2A}$R antagonist has previously been shown to inhibit rather than to increase LTP at other hippocampal synapses (Rebola *et al.*, 2008; Costenla *et al.*, 2011).

It is therefore concluded that the blockade of A$_{2A}$R conferred a robust protection in this model of neuroinflammation, as observed in other *in vivo* models of brain neurotoxicity, reviewed in (Cunha, 2005; Chen *et al.*, 2007). In the presented study, we also showed that antagonism of A$_{2A}$R also decreased the extent of neuroinflammation caused by LPS. This was concluded by simultaneous ability of A$_{2A}$R blockade to prevent the LPS-induced recruitment and activation of microglia and also the increase in IL-1β levels, a master regulator of neuroinflammation that contributes to neurodegeneration

(Allan *et al.*, 2005). Furthermore, the observed ability of A$_{2A}$R to prevent biochemical changes, such as increased phosphorylation/activation of p38 and increased cleaved caspase-3, found to be associated with microglia dynamics, see (Wirenfeldt *et al.*, 2007; Ohnishi *et al.*, 2010), further argues for a direct ability of A$_{2A}$R on microglia cells to control LPS-induced neuroinflammation. However, it remains to be determined if the A$_{2A}$R mainly control the chemotaxis or the activation of microglial cells. This might require analysis *in vivo*, since there is a notable difference between the impact of A$_{2A}$R on microglia reactivity *in vivo,* the presented results shown in (Rebola *et al.*, 2011) and also (Pierri *et al.*, 2005; Melani *et al.*, 2006b; Minghetti *et al.*, 2007; Yu *et al.*, 2008), and in purified microglial cells in culture (Orr *et al.*, 2009; van der Putten *et al.*, 2009; Dai *et al.*, 2010). It is likely that other factors or cell types in the brain parenchyma may play a crucial role in formatting the pattern of A$_{2A}$R-mediated control of microglial reactivity (Saura *et al.*, 2005; Dai *et al.*, 2010). Furthermore, the role of A$_{2A}$R in controlling neuroinflammation may also depend on the cell types involved in supporting brain inflammation. In fact, it is notable that in experimental situations associated with disruption of the blood brain barrier and invasion of the brain by peripheral inflammatory cells, such as brain hemorrhage (Mayne *et al.*, 2001) or prolonged stenosis (Duan *et al.*, 2009), it is the activation rather than the blockade of A$_{2A}$R that control neuroinflammation. However, when resident microglia triggers and sustains neuroinflammation, such as in early periods after LPS administration (Chakravarty and Herkenham, 2005), the presented results indicate that it is the blockade of A$_{2A}$R that controls neuroinflammation. These results are in agreement with a recent report describing the ability of chronic caffeine consumption (a non-selective antagonist of adenosine receptors) to control neuroinflammation (Brothers *et al.*, 2010) and experimental autoimmune encephalomyelitis (Chen *et al.*, 2010). A question that still remains to be resolved is the identity of the cell type in which A$_{2A}$Rs are located that mount the neuroinflammatory response. A previous study concluded that it were the A$_{2A}$Rs located in bone-marrow cells infiltrating in the brain parenchyma that were responsible for the control of cortical infarct after middle cerebral artery occlusion, while blockade of A$_{2A}$Rs on brain resident cells only accounted for 20% of observed neuroprotection (Yu *et al.*, 2004). However, ischemic brain injury causes an extensive disruption of the blood-brain barrier and consequent massive invasion of peripheral lymphoid cells, far greater than that occurring in other neurodegenerative conditions, reviewed in (Ballabh *et al.*, 2004). Accordingly, in another similar study using a model of Parkinson's disease based on MPTP intoxication, we found that it was non-neuronal brain resident A$_{2A}$R that contributed for neuroprotection, rather than infiltrating bone marrow-derived cells; this was based on the combined use of forebrain neuronal-selective A$_{2A}$R knockout mice and bone marrow transplants after bone

marrow destruction by γ-irradiation (Yu *et al.*, 2008). Given that the neuroinflammation triggered by intraperitoneal administration of LPS requires a response from CNS-resident cells independent of systemic cytokine effects (Chakravarty and Herkenham, 2005), it is most likely that it is A$_{2A}$R present in microglia (Saura *et al.*, 2005) or astrocytes (Nishizaki *et al.*, 2002) that play a role in the control of LPS-induced neuroinflammation. The currently observed parallel increase in the expression of A$_{2A}$R, together with markers of microglial activation in brain tissue, also observed by others in cultured cells (van der Putten *et al.*, 2009) and upon different brain insults (Trincavelli *et al.*, 2008; Yu *et al.*, 2008), further strengthens the hypothesis that it may be A$_{2A}$R in resident microglia cells that control neuroinflammation in conditions where the invasion of the brain parenchyma by peripheral inflammatory cells is limited. This observed ability of A$_{2A}$R to control microglia recruitment and activation and the increase in brain levels of IL-1β prompts the hypothesis that the A$_{2A}$R-mediated control of the genesis of neuroinflammatory processes in pathological conditions may play an important hitherto unrecognised role in the neuroprotective effect afforded by A$_{2A}$R blockade. This does not rule out the possibility that A$_{2A}$R located in neurons (Rebola *et al.*, 2005a) may also contribute to the control of neurodegeneration, see (Dall'Igna *et al.*, 2003; Mojsilovic-Petrovic *et al.*, 2006; Silva *et al.*, 2007; Stone and Behan, 2007; Canas *et al.*, 2009). However, it is important to note that the blockade of A$_{2A}$R is particularly effective when tested *in vivo*. In fact, blockade of A$_{2A}$R affords a more robust neuroprotection in hippocampal regions upon *in vivo* ischemia (Chen *et al.*, 1999) when compared with the effect found upon chemical ischemia in hippocampal slices *in vitro* (Latini *et al.*, 1999; Higashi *et al.*, 2002). This argues that mechanisms other than a direct neuronal protection, such as the control of neuroinflammation, might be prominent in the neuroprotection afforded by A$_{2A}$R blockade. Thus, the present study provides evidence to support the hypothesis that blockade of A$_{2A}$R abrogates the LPS-induced neuroinflammation and the consequent neuronal dysfunction in the hippocampus. These observations support the hypothesis that the control of neuroinflammation by A$_{2A}$R might be a common mechanism underlying the robust neuroprotection afforded by antagonists of these receptors against a diversity of neurodegenerative conditions which involve neuroinflammation, such as epilepsy, Alzheimer's or Parkinson's disease.

4.2 BLOCKADE OF A$_{2A}$ RECEPTORS PREVENTS INTERLEUKIN-1β ASSOCIATED NEURONAL TOXICITY

4.2.1 INTRODUCTION

Neuroinflammation is a common feature of most neurological disorders and pathological situations in the brain (Marchetti and Abbracchio, 2005; Skaper, 2007). The CNS has specific immuno-competent cells (microglia) (Graeber, 2010) and mediators of the inflammatory response (astrocytes) (Zhang *et al.*, 2010a) that when activated release a large number of inflammatory mediators, including cytotoxic reactive species and pro-inflammatory cytokines (Chakraborty *et al.*, 2010). Pro-inflammatory cytokines are usually expressed in low levels in the healthy brain where they modulate several physiological functions including responsiveness to different stressors (Vitkovic *et al.*, 2000; Goshen and Yirmiya, 2009), sleep (Krueger *et al.*, 2001; Opp, 2005 2003 #2943) and synaptic plasticity (Schneider *et al.*, 1998; Liu *et al.*, 2007; McAfoose and Baune, 2009; Yirmiya and Goshen, 2011). However, during neuroinflammation the expression and release of pro-inflammatory cytokines greatly increases which is responsible for some of the inflammation-induced deleterious effects (Allan and Rothwell, 2001). In this respect, two of the best studied pro-inflammatory cytokines are the tumor necrosis factor alpha (TNF-α) and the interleukin-1 beta (IL-1β) (Gosselin and Rivest, 2007). During neuroinflammation, TNF-α can either trigger cell death or promote cell survival (McCoy and Tansey, 2008; Kraft *et al.*, 2009) whereas the IL-1β has been consistently associated with the increased susceptibility of neurons to excitotoxicity and the inhibition of synaptic plasticity (McAfoose and Baune, 2009; Yirmiya and Goshen, 2011).

Despite being mainly released from microglia and astrocytes, both TNF-α and IL-1β as well as their receptors are expressed in neurons (Wolvers *et al.*, 1993; Wong and Licinio, 1994; Yabuuchi *et al.*, 1994) and some of their deleterious effects, such as the priming of neurons to excitotoxicity or the depression of long-term potentiation (LTP), arises from a direct interaction with the neuronal membranes, as gauged by the parallel *in vivo* (Vereker *et al.*, 2000a; Ma *et al.*, 2002; Kelly *et al.*, 2003; Chaparro-Huerta *et al.*, 2005; Bernardino *et al.*, 2008; Chapman *et al.*, 2010; Rebola *et al.*, 2011) and *in vitro* results (Curran *et al.*, 2003; Viviani *et al.*, 2003; Leonoudakis *et al.*, 2004; Zhang *et al.*, 2008; Gardoni *et al.*, 2011). The activation of mitogen-activated protein kinases (MAPKs) by these pro-inflammatory cytokines on hippocampal neurons seems to underlie both the IL-1β-mediated and the

TNF-α-mediated inhibition of long-term potentiation (LTP) (Vereker *et al.*, 2000b; Curran *et al.*, 2003; Kelly *et al.*, 2003; Butler *et al.*, 2004; Pickering *et al.*, 2005). Moreover, the mechanism through which these cytokines prime neurons to excitotoxic death have also been shown to involve the activation of MAPKs and in particular of the p38 MAPK and of the c-jun-N-terminal kinase (JNK) (Nolan *et al.*, 2003; Srinivasan *et al.*, 2004; Barry *et al.*, 2005; Chaparro-Huerta *et al.*, 2005; Moore *et al.*, 2007; Zhang *et al.*, 2010b). Accordingly, the targeting of these pathways has been shown to control neurodegeneration (Borsello and Forloni, 2007; Munoz and Ammit, 2010).

We have previously developed the concept that adenosine A_{2A} receptors (A_{2A}Rs) control synaptic plasticity, reviewed in (Cunha, 2008), and neurodegeneration, reviewed in (Cunha, 2005; Chen *et al.*, 2007). In addition, we have already tackled the question of whether A_{2A}Rs can control neuroinflammation and found that the blockade of A_{2A}Rs prevented the lipopolysaccharide (LPS)-induced effects on the hippocampus, including the recruitment of microglia, the rise in IL-1β levels and the IL-1β-induced depression of LTP (Rebola *et al.*, 2011). Accordingly, it is known that the adenosinergic system is tightly coupled to that of pro-inflammatory cytokines (Sperlagh *et al.*, 2004; Zhu *et al.*, 2006; Abbracchio and Ceruti, 2007) and that the activation of adenosine A_{2A}Rs is involved in the inflammation-induced damage (Stone and Behan, 2007; Dai *et al.*, 2010; Rebola *et al.*, 2011). In line with these findings, in the following work, it was tested whether A_{2A}Rs could also control the direct impact of IL-1β and TNF-α on neurons. This was tested in hippocampal neuronal cultures since hippocampal neurons express both of these pro-inflammatory cytokines and their receptors (Wong and Licinio, 1994; Yabuuchi *et al.*, 1994; Ogoshi *et al.*, 2005; Figiel and Dzwonek, 2007; Harry *et al.*, 2008; Gardoni *et al.*, 2011) and their physio-pathological impact is well characterised in this brain region (Kelly *et al.*, 2003; Pickering and O'Connor, 2007).

4.2.2 RESULTS

4.2.2.1 EFFECT OF INTERLEUKIN-1β AND TUMOR NECROSIS FACTOR-α ON NEURONAL MITOGEN-ACTIVATED PROTEIN KINASES

As previously stated, most of the cellular functions regulated by pro-inflammatory cytokines and in particular some of their deleterious effects, are triggered by the activation of MAPKs (Allan and Rothwell, 2001; Pickering *et al.*, 2005). The MAPK protein family comprises three main groups: the extracellular signal-regulated protein kinases (ERK) and the stress-activated protein kinases (SAPK) p38 and c-jun N-terminal kinases (JNK) (Schulte and Fredholm, 2003). The phosphorylation of these proteins and the activation of its enzymatic activity are closed linked events and can be used as a measure of enzyme activation (Schulte and Fredholm, 2003). Thus, to begin studying the effects of pro-inflammatory cytokines directly on neurons without the interference of glial cells, rat hippocampal cultured neurons, grown during 7 days *in vitro* (DIV), were exposed to IL-1β (1-100 ng/ml) and to TNF-α (1-5 ng/ml) and the impact of this exposure on the activation (i.e. phosphorylation) of different MAPKs was evaluated through Western blot analysis. It was first observed that IL-1β (10 ng/ml) rapidly activates JNK in cultured neurons in a transient way as shown in Figure 1A, reaching significance only at 15 min of incubation (131.2 ± 9.4 % of control; n= 8; p<0.01) and decreasing to basal levels thereafter until 3 hours of exposure. The activation of JNK also depended on the concentration of IL-1β, being significant at 10 and 100 ng/ml (p<0.001, n=8) (Fig. 1B).

Since the highest concentration of IL-1β produced more robust results, we tested the impact of incubation for 5-15 minutes with 100 ng/ml of IL-1β on the activation (phosphorylation) of p38. As shown in Figure 2A, phosphorylated p38 levels were significantly (P<0.05) increased (192.8 ± 18.8 %, n= 5) in cultured neurons after 15 minutes of incubation with IL-1β. However, IL-1β (100 ng/ml) failed to activate ERK in hippocampal cultured neurons in the same period of incubation, 10-15 minutes (73.1 ± 16.6 % and 113.0 ± 13.6 % of control, respectively; n= 4), as shown in Figure 2C. Immunocytochemical analysis of hippocampal cultured neurons confirmed that the exposure to 100 ng/ml of IL-1β for 15 minutes triggered an evident increase in the immunoreactivity of phosphorylated JNK throughout the neurons (Fig. 1C) and of phosphorylated p38, mainly in neuronal cell bodies (Fig. 2B).
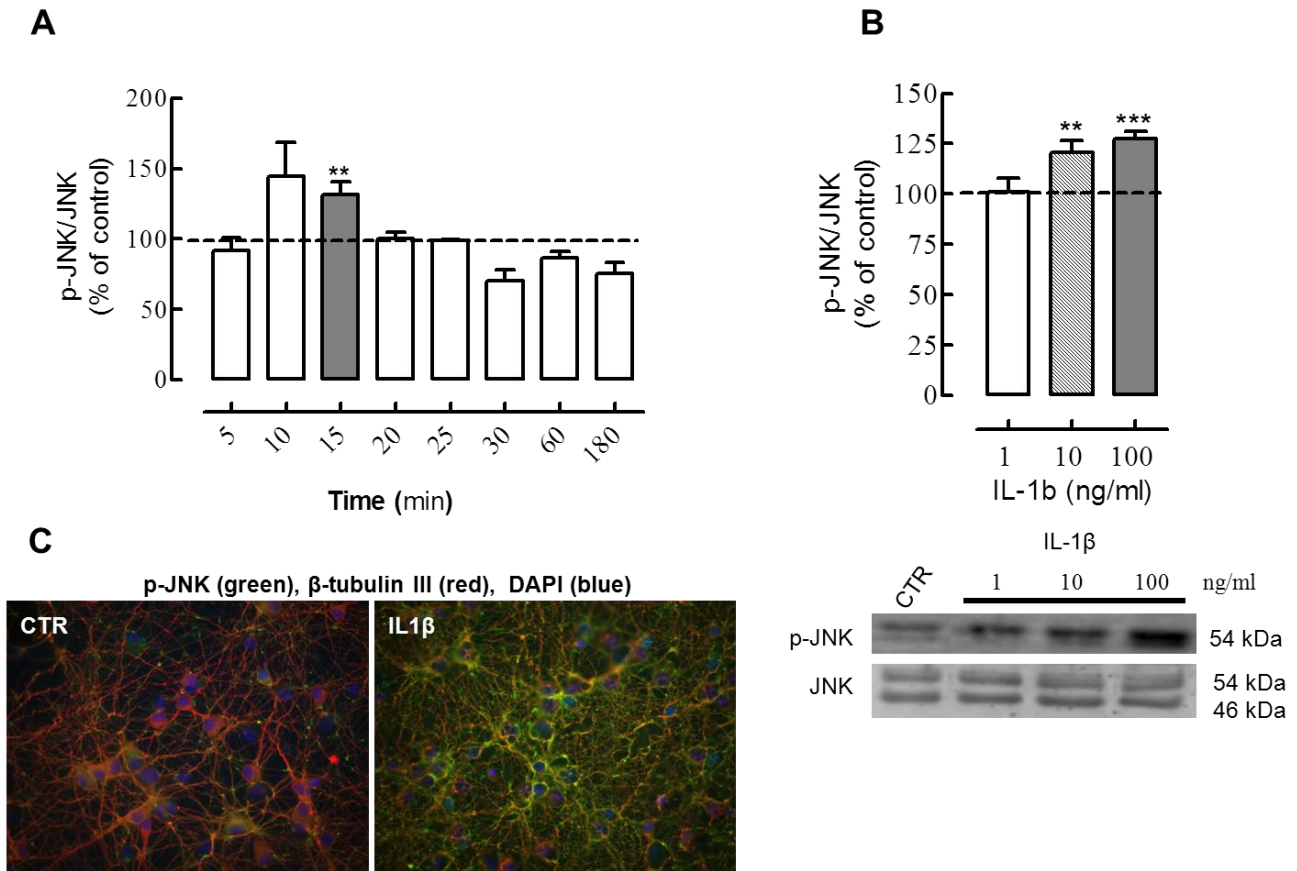
FIGURE 1: IL-1β activates JNK in hippocampal neurons in a time and concentration dependent manner. Hippocampal neuronal cultures, 7 DIV, were exposed to a concentration of 10 ng/ml of IL-1β (prepared in Krebs buffer, pH7.4) for different times (5, 10, 15, 20, 25, 30, 60 and 180 min) (A) and to different concentrations of IL-1β (1, 10 and 100 ng/ml prepared in Krebs buffer, pH 7.4) during 15 min (B) after which cells were immediately lysed on ice in a minimum volume of RIPA's buffer. The immunoreactivity of the activated (phosphorylated) form of c-jun N-terminal kinases (p-JNK) was quantified by Western blot analysis. Membranes were re-probed for the total form of JNK and the activity of JNK was calculated as the ratio between the immunoreactivity of the phosphorylated form and the immunoreactivity of the total form of the protein (p-JNK/JNK). The results are presented as percentage of control (neurons exposed to vehicle only). Data corresponds to cultures from 8 pregnant Wistar female rats (n=8) and is presented as mean ± SEM; **p<0.01 and ***p<0.001. In the panel C are representative immunocytochemistry images of hippocampal neurons probed for the activated (phosphorylated) form of JNK (green fluorescence) which colocalizes with the class III β-tubulin, a constitutive protein exclusively of neuronal microtubules (red fluorescence). In blue are the neuronal nuclei stained with DAPI.
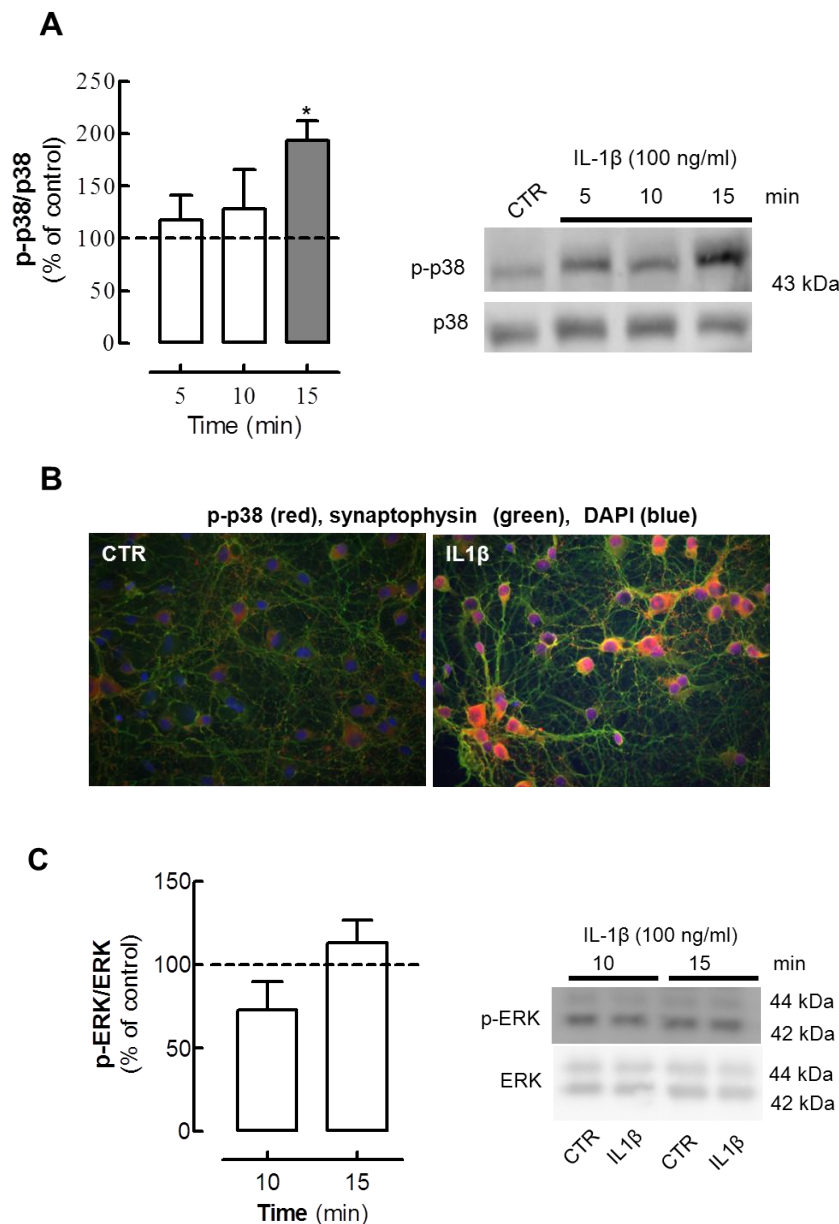
A

B

C

FIGURE 2: IL-1β activates p38 (but not ERK) in hippocampal neurons in a time dependent manner. Hippocampal neuronal cultures, 7 DIV, were exposed to 100 ng/ml of IL-1β (prepared in Krebs buffer, pH7.4) for different times (corresponding to the optimal conditions in which it was observed the activation of JNK by IL-1β, showed in Figure 1) after which cells were immediately lysed on ice in a minimum volume of RIPA's buffer. The immunoreactivity of the activated (phosphorylated) form of p38 (p-p38) was quantified by Western blot analysis. Membranes were re-probed for the total form of p38 (p38) and the activity of p38 was calculated as the ratio between the immunoreactivity of the p-p38 and the immunoreactivity of the p38 (p-p38/p38) (A). The same procedure was done for the activity of ERK (C). The results are presented as percentage of control (neurons exposed to vehicle only). Data corresponds to cultures from 5 pregnant Wistar female rats (n=5) and is presented as mean ± SEM; *p<0.05. In the panel B are representative immunocytochemistry images of hippocampal neurons probed for the activated (phosphorylated) form of p38 (red fluorescence) which colocalizes with synaptophysin (green fluorescence). In blue are the neuronal nuclei stained with DAPI. Note that p38 is mainly activated in the cell body of neurons.

On the other hand, TNF-α (5 ng/ml) strongly induced the activation (phosphorylation) of the p38 MAPK on hippocampal neurons in a transient way and peaking at 10 min of incubation (384.7 ± 51.1 % of control; $p < 0.01$; n= 8) (Fig. 3B) but failed to activate both JNK (Fig. 3A) and ERK (Fig. 3C) at the same times. The values of the ratio between the phosphorylated form of p38 (p-p38) and the total form of the protein (p38) were still higher than the control values at 15 min and 20 min of incubation with TNF-α (277.7 ± 56.1 % of control; $p < 0.05$ and 190.1 ± 71.1 % of control, respectively; n= 6-7) decreasing rapidly to control values (129.0 ± 16.5 % of control; n= 6) at 25 min of exposure of the neurons to the cytokine (Fig. 3B). At the lowest concentration, TNF-α did not activate any of the MAPKs at any of the incubation periods tested (Fig. 3A-C).

Immunocytochemical analysis of hippocampal cultured neurons confirmed that the exposure to 5 ng/ml of TNF-α for 10 min triggered an evident increase in the immunoreactivity of phosphorylated p38 throughout the neurons (Fig. 3D).
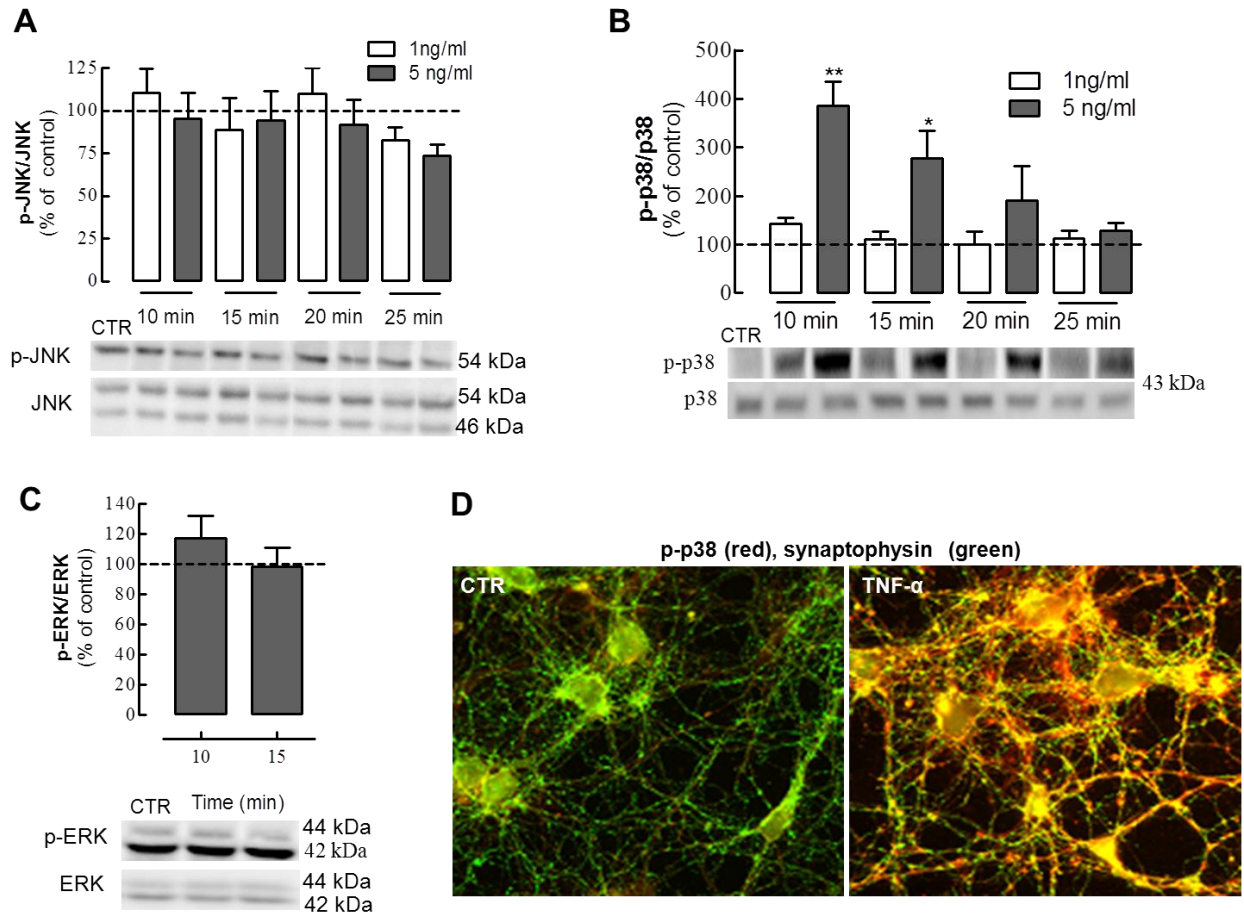
Figure 3: TNF-α activates p38 (but not JNK or ERK) in hippocampal neurons in a time and concentration dependent manner. Hippocampal neuronal cultures, 7 DIV, were exposed to TNF-α (1 or 5 ng/ml) (prepared in Krebs buffer, pH 7.4) for different times after which cells were immediately lysed on ice in a minimum volume of RIPA's buffer. The immunoreactivity of the activated (phosphorylated) forms of the mitogen-activated protein kinases (MAPKs), p-JNK, p-p38 and p-ERK, was quantified by Western blot analysis. Membranes were re-probed for the total form of the MAPKs (JNK, p38 and ERK) and their activity was calculated as the ratio between the immunoreactivity for the phosphorylated forms and the immunoreactivity for the respective total form of the proteins. The exposure of neurons to TNF-α (1 and 5 ng/ml) did not activate JNK (panel A) or ERK (panel C) but activated p38 in a transient and concentration dependent manner (panel B). In the panel D are representative immunocytochemistry images of hippocampal neurons probed for the activated (phosphorylated) form of p38 (red fluorescence) which colocalizes with synaptophysin (green fluorescence).

4.2.2.2 BLOCKADE OF A_{2A} RECEPTORS PREVENTS THE INTERLEUKIN-1β-INDUCED ACTIVATION OF NEURONAL MITOGEN-ACTIVATED PROTEIN KINASES

The key question directing this study was to determine if A_{2A} receptors control the impact of the pro-inflammatory cytokines IL-1β and TNF-α on neurons. For this purpose, it was tested the ability of a previously validated A_{2A}R antagonist, SCH58261, to prevent both the IL-1β-induced activation of p38 and of JNK and the TNF-α induced activation of p38 in cultured hippocampal neurons. As shown in Figure 4, the addition of SCH58261 (50 nM), 20 min before exposing neurons to IL-1β (100 ng/ml) for 15 minutes prevented the IL-1β-induced phosphorylation of p38 (108.0 ± 20.2 % of control in the presence of IL-1β and SCH58261 comparing with 195.5 ± 18.8 % of control in the presence of IL-1β alone; n= 6; $p < 0.05$ between IL-1β and control and between SCH58261 + IL-1β and IL-1β) (Fig. 4A) and of JNK (86.6 ± 14.6 % of control in the presence of IL-1β and SCH58261 comparing with 142.3 ± 9.1% of control in the presence of IL-1β alone; n= 7; $p < 0.05$ between IL-1β and control and $p < 0.01$ between IL-1β and SCH58261 + IL-1β) (Fig. 4B) but the addition of SCH58261 (50 nM) 20 min before exposing neurons to TNF-α (5 ng/ml) for 10 minutes did not prevent the TNF-α-induced phosphorylation of p38 (435.4 ± 65.7 % of control in the presence of SCH58261 + TNF-α comparing with 436.4 ± 60.7 % of control in the presence of TNF-α alone; n= 7; $p < 0.01$ between the two treated groups and the control group) (Fig. 4C), whereas SCH58261 failed to affect p38 (112.8 ± 14.2 % of control, Fig. 4A and 112.4 ± 8.7 % of control, Fig. 4C; n= 6-7) or JNK phosphorylation (89,1 ± 8,5% of control, Fig. 4B; n= 7) per se (n= 6-7).
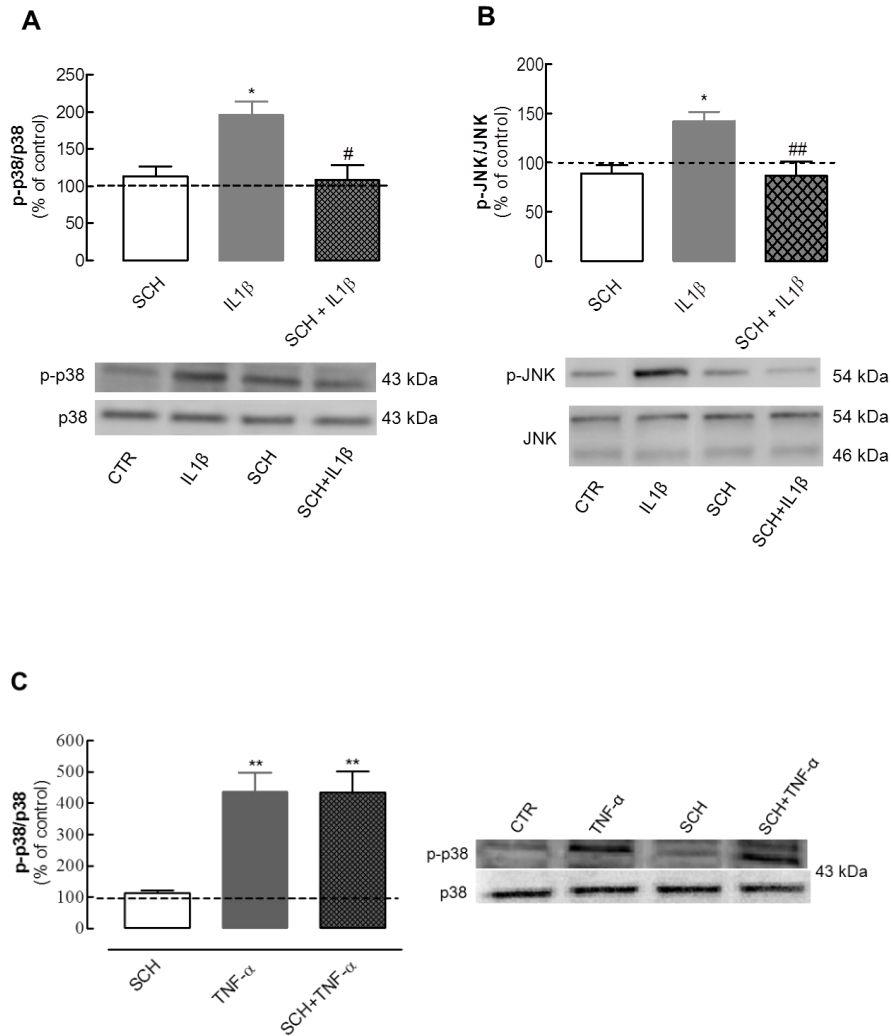
Figure 4: Blockade of adenosine A$_{2A}$R prevents IL-1β-induced activation of p38 and JNK, but not TNF-α-induced activation of p38, in hippocampal neurons. Hippocampal neuronal cultures, 7 DIV, were exposed to vehicle and/or A$_{2A}$R selective antagonist, SCH582615 (50 nM) 20 min before adding 100 ng/ml IL-1β or TNF-α (5 ng/ml) (or vehicle for the control groups), for 15 and 10 min, respectively. After the incubations cells were immediately lysed on ice in a minimum volume of RIPA's buffer. The immunoreactivity of the activated (phosphorylated) form of p38 (p-p38), panels A and C, and of the activated form of JNK (p-JNK), panel B, was quantified by Western blot analysis. Membranes were re-probed for the total form of p38 and JNK, respectively, and the activity of both MAPKs was calculated as the ratio between the immunoreactivities of the phosphorylated and total forms of the proteins. The results are presented as percentage of control (neurons exposed to vehicle only). Data corresponds to cultures from 6 to 7 pregnant Wistar female rats (n=6-7) and is presented as mean ± SEM, *p<0.05 and **p<0.01 compared to control; #p<0.05 and ##p<.0.01 compared with neurons exposed to IL-1β only.

4.2.2.3 THE EFFECT OF INTERLEUKIN-1β ON NEURONAL MITOGEN-ACTIVATED PROTEIN KINASES IS OPERATED BY INTERLEUKIN-1 TYPE I RECEPTORS

Since the blockade of A$_{2A}$Rs was able to prevent the IL-1β-induced signalling on neurons, it was tested the involvement of IL-1 type I receptors (IL-1RI) through which IL-1β signals. Thus, neurons were exposed for 30 min to the endogenous antagonist of IL-1RI, IL1Ra (5 μg/ml), which prevents the docking of the IL1β receptor accessory protein to form the hetero-trimeric complex that is necessary for signal transduction. Afterwards neurons were exposed to IL-1β for 15 min. As shown in the Figure 5, IL-1β (100 ng/ml) induced the phosphorylation of p38 (532.3 ± 85.5 % of control; n= 4; $p<0.01$ versus control) and of JNK (151.0 ± 22.1 % of control, n= 4; $p<0.05$ versus control) and IL1Ra prevented this IL-1β-induced phosphorylation of p38 (261.3 ± 50.2 % of control, n= 4, $p<0.05$ versus the absence of IL1Ra) (Fig. 5A) and attenuated the activation (phosphorylation) of JNK (123.0 ± 20.0 % of control, n= 3) (Fig. 5B).
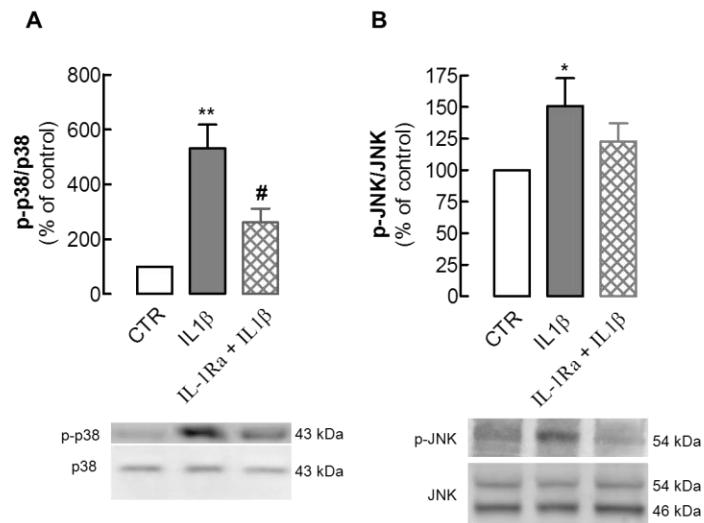

FIGURE 5: IL-1β type I receptor (IL-1RI) mediates the IL-1β-induced phosphorylation of JNK and p38 in hippocampal cultured neurons. Rat hippocampal neuronal cultures with 7 days in vitro (DIV) were exposed to interleukin-1β (IL-1β, 100 ng/ml) for 15 minutes in the absence or in the presence of the antagonist of interlukin 1β type I receptor, IL-1Ra (5 μg/ml), added 30 min before IL-1β. Cells were then lysed on ice in a minimum volume of RIPA buffer for Western blot analysis to quantify the immunoreactivity of the activated (phosphorylated) form of p38 (p-p38, panel A) or of the activated form of JNK (p-JNK, panel B). In both panels, the bar graphs display the mean ± SEM of 3-4 experiments and the blots below illustrate a representative experiment showing that IL-1Ra prevented the IL1β-induced phosphorylation of p38 (panel A) and JNK (panel B). In each experiment, membranes were first used to detect the phospho-MAPK and then re-probed for the total amount of MAPK, so that the activation of each MAPK was calculated as a ratio between the phosphorylated and total immunoreactivities, which were expressed as percentage of control (CTR) values (i.e. in the absence of any added drug). *$p<0.05$ and **$p<0.01$ compared to control; #$p<0.05$ compared to the effect of IL1β in the absence of IL1Ra.

4.2.2.4 SYNAPTIC AND SUB-SYNAPTIC LOCALIZATION OF THE INTERLEUKIN-1β TYPE I RECEPTORS

Albeit several effects operated by the IL-1β type I receptor (IL-1RI) have been reported to occur in brain cells (Allan *et al.*, 2005; Lynch, 2010), little is known about the localization of the IL-1β type I receptor in neurons (Gardoni *et al.*, 2011). Thus, it was investigated if IL-1RI is indeed located in native brain neurons, taking particular attention to its putative synaptic and sub-synaptic localization. For this purpose, first it was compared the density of IL-1RI immunoreactivity in total membranes and in synaptic membranes prepared from the hippocampus of adult rats. Total membranes, which are mostly comprised of glial and endothelial cell membranes, are enriched with IL-1RI relative to synaptic membranes (Fig. 6A). For instance, when using 30 μg of protein in the Western blot analysis, total membranes displayed significantly higher (P<0.05) IL-1RI immunoreactivity than synaptosomal membranes (43.7 ± 3.2 % compared to 31.1 ± 3.8 % of total immunoreactivity, respectively, n= 4). This difference was also observed when loading 60 μg of protein (Fig. 6A), but disappears as the signal becomes saturated at 90 μg of protein (Fig. 6A). Albeit mainly located outside synaptic regions, we further detailed the sub-synaptic distribution of IL-1RI. As shown in Figure 6B, IL-1RI was almost exclusively located at post-synaptic (46.9 ± 4.8 %) and pre-synaptic sites (40.0 ± 4.3 %) with a low density at peri-synaptic sites (13.1 ± 1.3 %, n= 3).
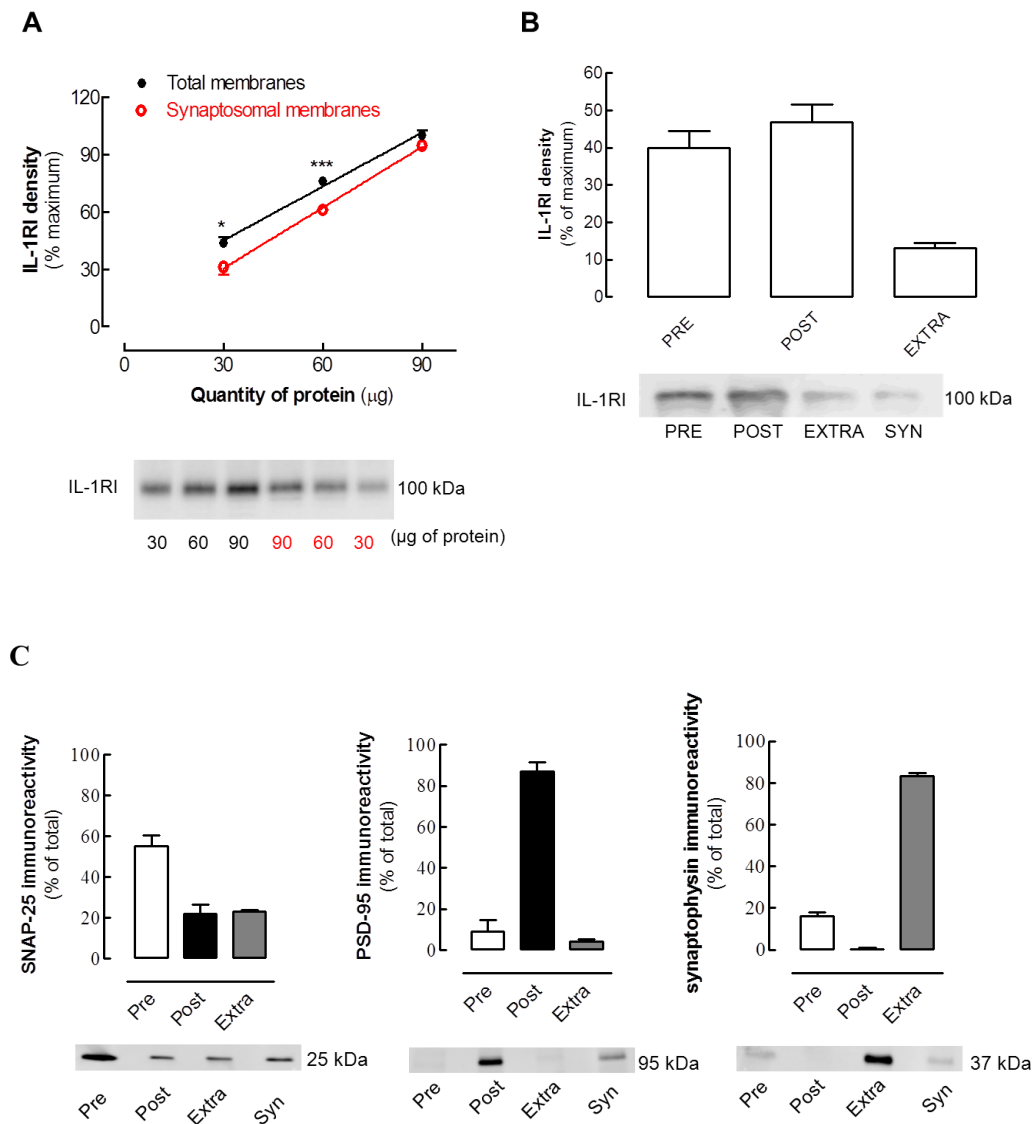
FIGURE 6: IL-1β type 1 receptor is present in membranes from nerve terminals, both presynaptically and postsynaptically. Panel A compares the immunoreactivity of interleukin 1β type 1 receptor (IL-1RI) in total membranes (comprising neuronal, glial and endothelial, membranes) and in membranes from purified nerve terminals (synaptosomes) prepared from the hippocampus of adult male Wistar rats. The graph display the mean ± SEM of 4 experiments and the blot below illustrates a representative experiment where it was always used 3 different protein quantities (30, 60, 90 μg) of both total and synaptic membranes of the same hippocampus in the same electrophoresis gel. The percentage of IL-1RI immunoreactivity for each amount of protein was calculated relative to the maximum immunoreactivity obtained for each Western blot membrane. *$p < 0.05$ and ***$p < 0.0001$ comparing the same quantities of total and synaptosomal membranes. Panel B shows the subsynaptic distribution of IL-1RI upon comparison of IL-1RI immunoreactivity in synaptosomal membranes (SYN) and in its fractionated fractions corresponding to pre- post- and extra-synaptic membranes (PRE, POST and EXTRA, respectively), which purity was gauged by the segregation of the active zone marker (SNAP-25), of the postsynaptic density marker (PSD-95) and of the extrasynaptic (vesicular) marker (synaptophysin), as show in panel C. The percentage of IL-1RI immunoreactivity was calculated relative to the maximum reactivity of each membrane. The data in the bar graphs are mean ± SEM of n=4.

4.2.2.5 BLOCKADE OF A$_{2A}$Rs PREVENTS THE INTERLEUKIN-1β-INDUCED EXACERBATION OF NEUROTOXICITY

Pro-inflammatory cytokines, in particular IL-1β and TNF-α, can worsen the outcome of excitotoxic conditions (Pickering *et al.*, 2005; Fogal and Hewett, 2008). After investigating the role of A$_{2A}$Rs on the neuronal transduction pathways recruited by IL-1β and TNF-α, next it was explored whether A$_{2A}$Rs can control the impact of IL-1β and TNF-α on neurotoxicity. First, it was investigated the effect of IL-1β on glutamate-induced neurodegeneration. This was achieved by incubating hippocampal neurons with IL1β (100 ng/ml) for 5 minutes before adding glutamate (100 μM) for a period of 25 min. After this time, the stimuli were washed and new neurobasal medium (without the antibiotic, glutamate and the B27 supplements) was added to the neurons which were kept at 37ºC in a 95% O$_2$-5% CO$_2$ atmosphere until analysis, 24 h later. The evaluation of cell viability was performed using a syto-13 and propidium iodide (PI) staining, which are nucleic acid dyes that emit fluorescence at different wave lengths (green in the case of syto-13 and red in the case of PI). The nucleic acid dye syto-13 permeates the plasma membrane while the PI dye does not. Therefore, the cells with red fluorescence have disrupted plasma membranes and are unviable, while the cells with green fluorescence may either be viable (dark green fluorescence and bigger cell bodies) or dying (small, bright and fragmented green fluorescent nuclei). As shown in Figure 7A, this short time exposure to glutamate decreased neuronal viability by 21.0 ± 4.8 % (n= 5; p<0.05 compared to control) and IL-1β exacerbated this glutamate-induced neurotoxicity to 51.1 ± 13.3 % (n= 7; p<0.05 compared to the effect of glutamate alone), whereas IL-1β was devoid of effects per se on neuronal viability (98.5 ± 2.6 % of viable cells, n= 6). Interestingly, SCH58261 (50 nM) prevented this exacerbation of glutamate-induced neurotoxicity caused by IL-1β (20.0 ± 4.0 % loss of neuronal viability, n= 5; p<0.01 compared to the effect of glutamate in the presence of IL-1β), whereas SCH58261 failed to significantly affect neurotoxicity in the presence of glutamate alone (31.8 ± 17.1 % loss of neuronal viability; n= 5). Finally, SCH58261 had no effect per se on neuronal viability (100.0 ± 1.6 % of viable cells, respectively; n= 5).

Next, it was confirmed this particular ability of A$_{2A}$Rs to control the exacerbation of glutamate-induced neurotoxicity by IL-1β using another assay of neuronal damage, the release of lactate dehydrogenase (LDH). As shown in Figure 7B, the short term exposure to glutamate (100 μM, 25 minutes) tended to increase LDH release by 24.8 ± 8.8 % relative to the control (n= 6, P>0.05), which

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A$_{2A}$ receptor control of the effects of pro-inflammatory cytokines was significantly (P<0.05) potentiated in the presence of 100 ng/ml of IL-1β (LDH release was 62.2 ± 14.5 % above the control, n= 6). As previously observed with propidium iodide and Syto-13, the blockade of A$_{2A}$Rs with SCH58261 (50 nM) abrogated the exacerbation of glutamate-induced neurotoxicity in the presence of IL-1β (LDH release was 21.1 ± 15.6 % above control; n= 6; p<0.05 comparing to the effect of glutamate in the presence of IL-1β) (Fig. 7B).
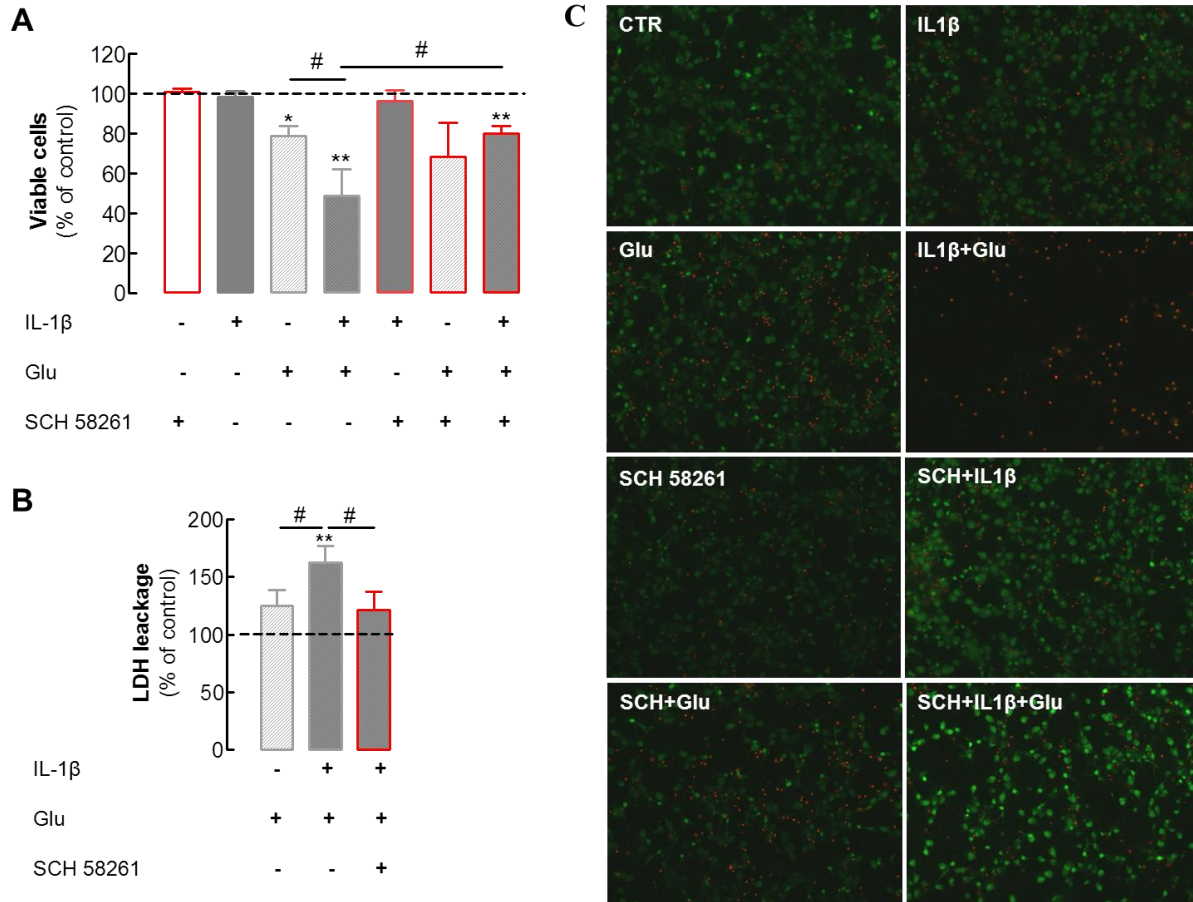

FIGURE 7: Blockade of adenosine A$_{2A}$ receptors prevents the IL-1β-induced exacerbation of glutamate-mediated excitotoxicity in hippocampal cultured neurons. Rat hippocampal neuronal cultures, 7 days *in vitro* (DIV) were exposed to vehicle or to interleukin-1β (IL-1β, 100 ng/ml), added 5 minutes prior to vehicle or glutamate (Glu, 100 μM), for a total of 25 min, in the absence or in the presence (added 20 min prior to the stimuli) of the selective A$_{2A}$ receptor antagonist, SCH58261 (50 nM), as indicated below each bar. Neurons were washed twice with Krebs buffer (pH 7.4) and fresh Neurobasal medium (with or without SCH58261) was added. Neurons were kept for 24 hours in the incubator (37ºC, 95% O2 and 5% CO2) until analysis of neuronal viability using Syto-13 plus propidium iodide (panel A) and the significant findings were further confirmed using the lactate dehydrogenase (LDH) assay (panel B). Results are mean ± SEM of n= 5-7 experiments (corresponding to different cultures); *p<0.05 and ** p<0.01 comparing to control (absence of any added drug), #p<0.05 comparing between treated groups. Panel C displays representative images of Syto-13 (green fluorescence) plus propidium iodide (red fluorescence) staining of nucleic acids for the indicated experimental conditions. Note that neither IL-1β nor SCH58261 had any effect in cell viability per se but IL-1β exacerbated glutamate-induced neurotoxicity, which was abrogated by SCH58261.

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The effect of TNF-α on the glutamate-induced neurodegeneration was tested by exposing the cultured hippocampal neurons to TNF-α (5 ng/ml) 5 minutes before adding glutamate (100 μM) for a total period of 25 min. The stimuli were washed and the evaluation of cell viability was performed 24 h later using the syto-13 and propidium iodide staining. As shown in Figure 8A, in these series of experiences glutamate decreased neuronal viability by 26.8 ± 4.0 % (n= 4; $p < 0.05$ compared to control) but TNF-α had no effect on the glutamate-induced neurotoxicity (32.0 ± 3.5 % of decreased neuronal viability; n= 4; $p < 0.01$ compared to control) and had no effect per se (90.8 ± 6.9 % of viable cells; n= 4). Moreover, the blockade of A$_{2A}$Rs with the selective antagonist SCH58261 (50 nM) did not alter the lack of effect of TNF-α on glutamate-induced neurotoxicity (35.0 ± 1.6 % of decreased neuronal viability; n= 4; $p < 0.001$ compared to control).
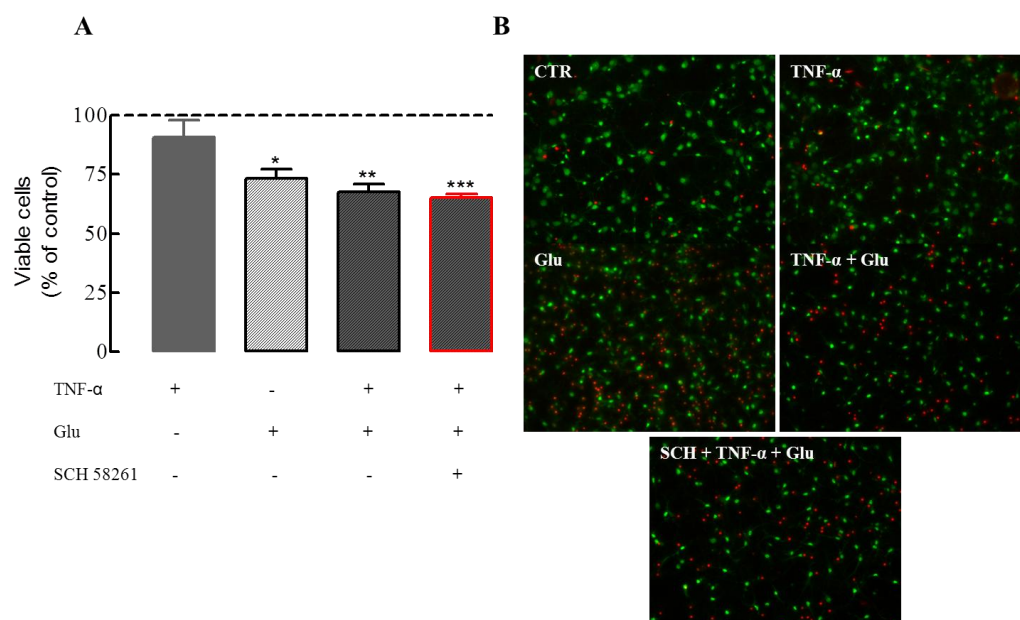

FIGURE 8: TNF-α, in the absence or presence of an A$_{2A}$R selective antagonist, does not alter the glutamate-induced excitotoxicity in hippocampal neurons. Rat hippocampal neuronal cultures, 7 days *in vitro* (DIV), were exposed to TNF-α (5 ng/ml) 5 minutes before adding glutamate (Glu, 100 μM) for a total of 25 min, in the absence or in the presence (added 20 min before the stimuli) of the selective A$_{2A}$R antagonist, SCH58261 (50 nM), as indicated below each bar. Neurons were washed twice with Krebs buffer (pH 7.4) and fresh Neurobasal medium (with or without SCH58261) was added. Neurons were kept in the incubator (37 ºC in a 95 % O$_2$-5 % CO$_2$ atmosphere) until analysis, 24 h later. The evaluation of neuronal viability was performed using the Syto-13 (green fluorescence) and propidium iodide (PI, red fluorescence) staining (panel A). In the panel B are representative images of Syto-13 + PI staining for the experimental groups. Results are presented as mean $\pm$ SEM of 4 different neuronal cultures (n= 4); **$p < 0.01$ and ***$p < 0.001$ comparing to the control (exposed to vehicle only).

4.2.2.6 BLOCKADE OF A$_{2A}$ RECEPTORS PREVENTS THE EXACERBATION CAUSED BY INTERLEUKIN-1β OF GLUTAMATE-INDUCED CALCIUM ENTRY AND CALCIUM DEREGULATION IN HIPPOCAMPAL NEURONS

Previous studies have suggested that the impact of IL-1β on the priming of neuronal viability involves an abnormal activation of NMDA receptor-mediated calcium influx (Ma *et al.*, 2002; Zhang *et al.*, 2010b; Gardoni *et al.*, 2011). Thus, it was tested if IL-1β could bolster the glutamate-induced calcium entry and calcium deregulation in neurons and investigated the impact of A$_{2A}$Rs blockade thereupon. This was achieved using a single cell calcium imaging approach upon loading hippocampal cultured neurons with the selective ratiometric calcium dye, Fura-2. As shown in Figure 9B, glutamate (100 μM) caused an immediate rise of intracellular free calcium concentration ($[Ca^{2+}]_i$) gauged by an increase of Fura-2 fluorescence ratio of 0.38 ± 0.03 above control (n= 6 cultures, 3-4 coverslips per culture and 30-50 neurons analysed per coverslip; P<0.05). The presence of IL-1β (100 ng/ml) consistently increased this effect of glutamate (ratio of 0.47 ± 0.03, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0,05) (Fig. 9B), whereas IL-1β per se failed to trigger any modification in Fura-2 signal. Pre-incubation of cells with SCH58261 (50 nM) attenuated this effect of IL-1β on the glutamate-induced increase of $[Ca^{2+}]_i$ (Fura-2 ratio of 0.39 ± 0.03, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.05 compared to the effect of glutamate in the presence of IL-1β) (Fig. 9B); in contrast, it was observed that SCH58261 actually tended to amplify the effect of glutamate alone (Fura-2 ratio of 0.50 ± 0.07, n= 4 cultures, 4 coverslips per culture and an average of 30-50 neurons analysed per coverslip) (Fig. 9B), whereas SCH58261 alone was devoid of effects on $[Ca^{2+}]_i$.

Apart from this initial effect of glutamate on calcium transients, we also evaluated how IL-1β and A$_{2A}$R blockade affected the ability of neurons to adapt to the continuous presence of glutamate. Thus, we evaluated the variation of the Fura-2 fluorescence ratio from its peak value shortly after the addition of glutamate until the end of the incubation period with glutamate (*i.e.* during 15 minutes). As shown in the Figure 9C and is represented in the Figure 9A, most neurons are able to adapt to the continuous presence of glutamate and decrease their $[Ca^{2+}]_i$ over time (slope of -0.0022 ± 0.0008, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip); in contrast, in the presence of IL-1β (100 ng/ml), neurons lost their adaptive capacity to the continuous

presence of glutamate, as testified by their tendency to keep increasing their [Ca^{2+}]$_i$ (slope of +0.0019 ± 0.0009, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.01 compared with glutamate alone) (Fig. 9C). Notably, blockade of A$_{2A}$R with SCH58261 (50 nM) inverted this effect of IL-1β (slope of -0.0015 ± 0.0008, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.05 comparing to the simultaneous presence of IL-1β and glutamate) (Fig. 9C). Again, SCH58261 selectively prevented the exacerbation by IL-1β of glutamate-induced responses, since SCH58261 actually enhanced the response to glutamate alone (slope of +0.0016 ± 0.001, n= 4 cultures, 4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.05 comparing with glutamate alone) (Fig. 9C).
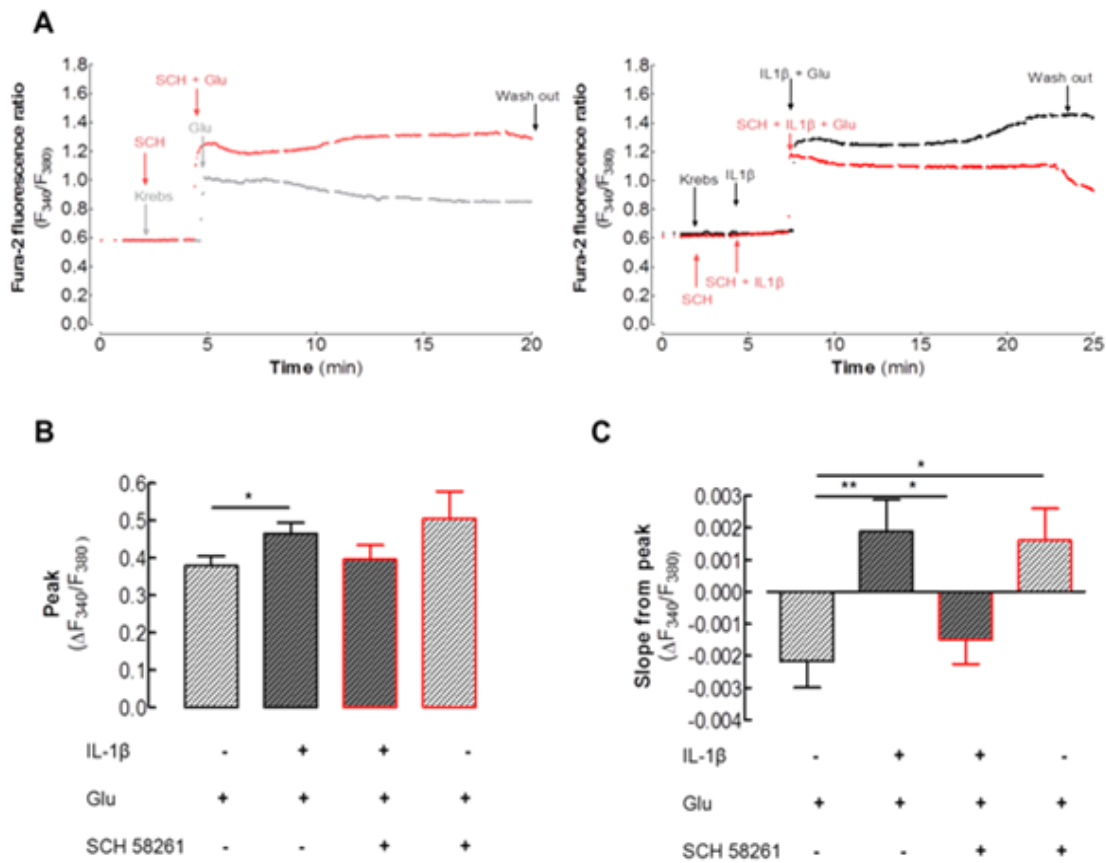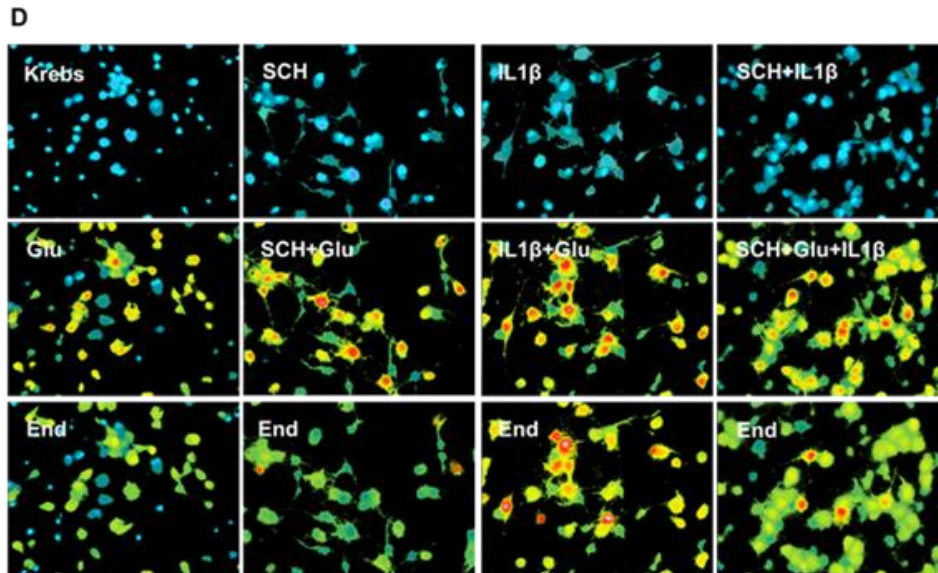
FIGURE 9: Blockade of adenosine A_{2A} receptors prevents IL-1β-induced exacerbation of glutamate-mediated calcium deregulation in hippocampal neurons. Hippocampal neurons were loaded with the calcium probe Fura-2-AM and the fluorescence emitted at 510 nm upon alternate excitation at 340 and 380 nm (F_{340} and F_{380}, respectively) was followed on cell bodies every second during 35 minutes. Basal fluorescence ratio was measured during the first 2 minutes and drugs, prepared in normal Krebs solution, were added to the superfusion using a fast-pressurized system, as indicated by the arrows in the representative graphics of the panel A. Interleukin-1β (IL1β, 100 ng/ml), when present, was added for 5 minutes prior to glutamate (Glu, 100 μM). Cells were left in the presence of the stimuli for the next 15 minutes and then washed through superfusion of normal Krebs. The A_{2A} receptor selective antagonist, SCH58261 (50 nM), was incubated for 15 minutes prior to the beginning of the experiment and prevailed throughout. The results are presented as the ratio (F_{340}/F_{380}) of recordings done in healthy neurons (accessed by the response to 50 mM KCl, after washing the stimuli). IL-1β increased both the glutamate-induced calcium entry (peak value of the difference between the basal ratio and the stimuli-induced ratio) (panel B) and the glutamate-induced calcium deregulation, calculated as the slope from peak values until the wash out of the stimuli (panel C). SCH58261 attenuated the exacerbation by IL-1β of glutamate-induced calcium (B) and prevented IL-1β plus glutamate-induced calcium deregulation (C); however, SCH58261 seemed to aggravate the impact of glutamate alone. In the panel D are representative fluorescence images of the neuronal cell bodies in basal conditions (first row), in the presence of stimuli (middle row) and immediately before washing the stimuli (last row). Colour scale is composed of blue, green, yellow, red and white corresponding to increasing calcium concentrations. Data are mean ± SEM of n= 4-6 different cell cultures (480-1200 cells analysed per condition for each n), *p<0.05, **p<0.01.

4.2.3 DISCUSSION

In the present work, the interleukin-1β (IL-1β) receptor associated with signal transduction, the IL-1 type I receptor (IL-1RI) was shown to be present at synaptic regions in the hippocampus of adult rats. The comparison of total membranes, which have a high content of glial and endothelial membranes, with membranes from purified nerve terminals (synaptosomes), revealed that IL-1RI are indeed located in synapses, albeit they are more abundant in total membranes, in agreement with the well established predominant expression and localization of these receptors in endothelial cells in the brain parenchyma (Ericsson *et al.*, 1995; Konsman *et al.*, 2004). However, IL-1RI is also expressed in

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neurons (Gardoni *et al.*, 2011) and especially in conditions of brain diseases (Ravizza *et al.*, 2006). Our results are in agreement with the previously reported localization of IL-1RI at the post-synaptic density (Gardoni *et al.*, 2011), as expected from the ability of IL-1β to control NMDA receptor-mediated currents *in vitro* (Ma *et al.*, 2002; Viviani *et al.*, 2003; Zhang *et al.*, 2010b) and *in vivo* (Balosso *et al.*, 2008). Additionally, we now report that IL-1RI are also present at the presynaptic active zone, as would be expected to justify the ability of IL-1β to control the release of glutamate from nerve terminals (Murray *et al.*, 1997). This neuronal localization of IL-1RI in neurons, which has also been confirmed to occur in cultured hippocampal neurons (Zhang *et al.*, 2010b), supports our observation that IL-1β can recruit different mitogen-activated protein kinases (MAPKs) in cultured neurons, in a manner sensitive to the antagonist of the IL-1RI (IL1Ra). Actually, both the IL-1β and the tumor necrosis factor-α (TNF-α) activated MAPKs on hippocampal neurons, as already described in the literature (Coogan *et al.*, 1999; Yang *et al.*, 2002; Curran *et al.*, 2003; Kelly *et al.*, 2003; Srinivasan *et al.*, 2004; Pickering *et al.*, 2005; Moore *et al.*, 2007). Despite the fact that the activation of MAPKs induced by these pro-inflammatory cytokines has been related to their deleterious effects on neurons (Curran *et al.*, 2003; Butler *et al.*, 2004; Barry *et al.*, 2005; Moore *et al.*, 2007; Chaparro-Huerta *et al.*, 2008; Zhang *et al.*, 2010b), neither IL-1β nor the TNF-α had an effect on neuronal viability per se, which is in line with what has been reported using *in vivo* or *ex vivo* models (Bernardino *et al.*, 2005; Hailer *et al.*, 2005; Stone and Behan, 2007; Bernardino *et al.*, 2008). However, the exposure of neurons to IL-1β immediately before exposing them to glutamate significantly increased the glutamate-induced neurotoxicity. These priming of neurons to excitotoxicity by IL-1β has also been previously observed *in vivo* and in organotypical cultures of the hippocampus (Bernardino *et al.*, 2005; Hailer *et al.*, 2005; Stone and Behan, 2007; Bernardino *et al.*, 2008). In addition, TNF-α was also described to increase the susceptibility of neurons to an excitotoxic stimuli (Bernardino *et al.*, 2005; Stellwagen *et al.*, 2005; Zou and Crews, 2005), which has been related to its modulation of the glutamate AMPA receptors at the synaptic level (Beattie *et al.*, 2002; Ogoshi *et al.*, 2005; Stellwagen *et al.*, 2005). However, in the present work, TNF-α did not have an effect on the glutamate-induced neurotoxicity. This may not be surprising when considering that TNF-α can signal through two different receptors (the TNF-α type I receptor, TNFRI or p55, and the TNF-α type II receptor, TNFRII or p75) (Beutler and van Huffel, 1994), both found on neuronal membranes (Yang *et al.*, 2002; Pickering *et al.*, 2005; Figiel and Dzwonek, 2007), which mediate different cellular responses using distinct pathways (Hsu *et al.*, 1995; Yang *et al.*, 2002). In fact, the TNF-α activation of the p38 pathway on hippocampal cultured neurons has been related to the activation of the TNFRII (Yang *et al.*, 2002) and thus to the TNF-α-induced

promotion of survival rather than neurodegeneration (Guo *et al.*, 2001; Yang *et al.*, 2002; Watters *et al.*, 2011). Nevertheless, a higher concentration of this cytokine may be needed in order to result in the exacerbation of the glutamate-induced neurotoxicity (Yang *et al.*, 2002; Bernardino *et al.*, 2005; Stone and Behan, 2007).

In addition to the direct priming of neurons to excitotoxicity by IL-1β, the present study adds a new mechanistic insight by showing that IL-1β causes a larger glutamate-induced calcium entry into neurons and a late calcium deregulation upon exposure of cultured hippocampal neurons to glutamate. The later is of particular interest in view of the tight association between late calcium deregulation and the irreversible loss of cellular, especially of neuronal viability. This opens new avenues of research to explore the underlying mechanisms of this IL-1β-induced late calcium deregulation, which may be of key importance in the control of the inflammatory-mediated amplificatory loop mediating the propagation of brain damage (Giacomello *et al.*, 2007).

As important as defining the mechanisms of inflammation-associated amplification of excitotoxic neuronal damage, is the identification of novel strategies to control this mechanism associated with the evolution of brain damage. We now show that the blockade of adenosine A₂ₐ receptors (A₂ₐR) blunts the negative impact of IL-1β on neurons. This is of particular relevance in view of the ability of A₂ₐR antagonists to prevent neuronal damage caused by different noxious brain insults (Cunha, 2005; Chen *et al.*, 2007; Gomes *et al.*, 2011). Notably, A₂ₐR blockade is effective both prophylactically as well as therapeutically (Gomes *et al.*, 2011). Given that A₂ₐR are enriched in glutamatergic synapses (Rebola *et al.*, 2005a), the prophylactic effect of A₂ₐR antagonists is most probably related to the ability of A₂ₐR to prevent synaptic dysfunction and damage, one of the early features of different brain disorders {Coleman, 2004 #4061;Waites, 2011 #3986}. In contrast, the therapeutic beneficial effect of A₂ₐR antagonists should depend on their ability to control a general feature associated with the amplification of brain damage and neuroinflammation emerges as a potentially relevant candidate mechanism (Cunha, 2005; Chen *et al.*, 2007; Yu *et al.*, 2008; Gomes *et al.*, 2011). Accordingly, we previously reported that A₂ₐR antagonists prevent the induction of neuroinflammation (Dai *et al.*, 2010; Rebola *et al.*, 2011). This is now complemented by the demonstration that A₂ₐR also controls the impact of a main pro-inflammatory cytokine on neuronal viability. Thus, A₂ₐR blockade displayed a particular ability to control the exacerbation of glutamate-induced neurodegeneration caused by IL-1β, extending the previous observation that A₂ₐR blockade prevented the combined neurotoxicity of IL-1β and quinolinic acid (Stone and Behan, 2007).

Furthermore, the present study further argues for a prime importance of p38 MAPK as the transduction pathway associated with A$_{2A}$R neuroprotection, as previously reported to occur upon different noxious brain conditions (Melani *et al.*, 2006b; Canas *et al.*, 2009; Rebola *et al.*, 2011). Finally, the present results also pave the way to explore the potential role of A$_{2A}$R in the control of glutamate-induced calcium deregulation; this is of particular interest since we have previously found that A$_{2A}$R control mitochondria function (Silva *et al.*, 2007; Canas *et al.*, 2009), which plays a key role in the occurrence of calcium deregulation leading to neuronal damage (Giacomello *et al.*, 2007) and is known to be involved in the etiology of diverse neurodegenerative disorders (Lin and Beal, 2006).

In conclusion, the present study demonstrates that A$_{2A}$Rs can control the signalling of IL-1β in neurons and the priming by IL-1β of glutamate-induced calcium entry and late calcium deregulation that results in the exacerbation of neuronal damage. Therefore, the present results prompt the hypothesis that the A$_{2A}$R-mediated control of the priming effects of IL-1β might be a possible mechanism underlying the striking ability of A$_{2A}$R antagonists to curtail neuronal damage caused by a variety of brain insults involving glutamate-induced neurotoxicity and neuroinflammation.

4.3 THE BLOCKADE OF P2Y$_1$ RECEPTORS PREVENTS BOTH GLUTAMATE AND INTERLEUKIN-1β-INDUCED EFFECTS ON HIPPOCAMPAL NEURONS

4.3.1 INTRODUCTION

In addition to adenosine, ATP is also a major modulator of cell-to-cell communication and a glial transmitter *par excellence* (Butt, 2011). The activation of P2 receptors by purines is involved in the different stages of microglia activation (Inoue, 2008) and also in the patho-physiology of astrocytes and of oligodendrocytes (Ciccarelli *et al.*, 2001; Fields and Burnstock, 2006). Thus, ATP can control neuroinflammation not only by generating adenosine and activating P1 receptors, but also by acting on its ubiquitously expressed P2 receptors (Di Virgilio *et al.*, 2009).

The coupling of the purinergic system to the release of pro-inflammatory cytokines from different immunocompetent cells is well established (Ferrari *et al.*, 2000; Liu *et al.*, 2000b; Inoue, 2002; Narcisse *et al.*, 2005; Bernardino *et al.*, 2008) and both P2R and pro-inflammatory cytokines can modulate cell fate through the activation of MAPKs (Allan and Rothwell, 2001; Pickering *et al.*, 2005; Abbracchio *et al.*, 2006). The activation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2) has been implied in the P2R-induced activation of glial cells (Neary and Kang, 2005) and in the production of inflammatory mediators (Brambilla *et al.*, 2002; Gendron *et al.*, 2003). Also, the activation of the p38 pathway was shown to correlate with the ATP-induced secretion of interleukin-1β (IL-1β) by microglia (Clark *et al.*, 2010).

The long-term effects of ATP on glial cells are thought to involve mainly the activation of P2Y receptors (Abbracchio *et al.*, 2009; Verkhratsky *et al.*, 2009; Butt, 2011). In particular, the P2Y$_1$ receptors (P2Y$_1$R) were shown to be central for the initiation and propagation of the astrocytic Ca^{2+} waves (Gallagher and Salter, 2003; Suadicani *et al.*, 2004; Bennett *et al.*, 2005), which are altered under pathological situations (Tsurusawa *et al.*, 2010), and were found to influence K^{+} currents in microglia (Walz *et al.*, 1993), of importance in the physiology and pathophysiology of these cells (Skaper, 2011). In addition to their fundamental role in the glia, P2Y$_1$R modulate synaptic events in hippocampal neurons, such as the release of glutamate from pre-synaptic sites (Rodrigues *et al.*, 2005b) and the inhibition of K^{+} currents at post-synaptic sites (Filippov *et al.*, 2006). Moreover, an alteration of the expression and/or function of P2Y$_1$R has been associated with pathological situations in the brain

　　　　　　　　　　　　P2Y$_1$ receptor control of the effects of pro-inflammatory cytokines

such as in Alzheimer's disease (Moore *et al.*, 2000b) or in ischaemic/hypoxic conditions in the hippocampus (Coppi *et al.*, 2007; Kuboyama *et al.*, 2011; Traini *et al.*, 2011). Thus, as previously done in relation to adenosine A$_{2A}$ receptors, here it was investigated whether the blockade of P2Y$_1$R could control the direct impact of the pro-inflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α) on cultured hippocampal neurons, including the priming of neurons to excitotoxic death by IL-1β.

4.3.2 RESULTS

4.3.2.1 BLOCKADE OF P2Y$_1$ RECEPTORS PARTIALLY PREVENTS THE INTERLEUKIN-1β-INDUCED ACTIVATION OF MITOGEN-ACTIVATED PROTEIN KINASES

To beging answering the question of whether the blockade of P2Y$_1$Rs can control the impact of pro-inflammatory cytokines on neurons, it was tested the ability of a potent and selective antagonist of P2Y$_1$Rs (MRS2179) to prevent the already established (see Chapter 4.2) IL-1β- and TNF-α- induced activation of p38 and the IL-1β- induced activation of c-jun-N-terminal kinases (JNK) in cultured hippocampal neurons.

As shown in Figure 1A, the presence of MRS2179 (10 μM), 20 min before exposing neurons to IL-1β (100 ng/ml) for another 15 minutes, prevented the IL-1β-induced phosphorylation of JNK (86.0 ± 11.0 % of control comparing with 134.8 ± 7.0 %; n= 8; p<0.01) (Fig. 1A) and attenuated the IL-1β-induced phosphorylation of p38 (136.0 ± 20.1 % of control comparing with 180.4 ± 17.3%; n= 8) (Fig. 1B) while not altering the phosphorylation of JNK (101.7 ± 4.7 % of control; n= 8) (Fig. 1A) or the phosphorylation of the p38 (117.0 ± 4.7 % of control; n= 8) (Fig. 1B) per se. On the other hand, MRS2179 did not alter the TNF-α-induced (exposure to 5 ng/ml for 10 minutes) phosphorylation of p38 (350.3 ± 92.8 % of control comparing with 436.4 ± 60.7 %; n= 8) (Fig. 1C) and once again failed to affect the phosphorylation of p38 per se (112.4 ± 8.7 % of control; n= 8) (Fig. 1C).
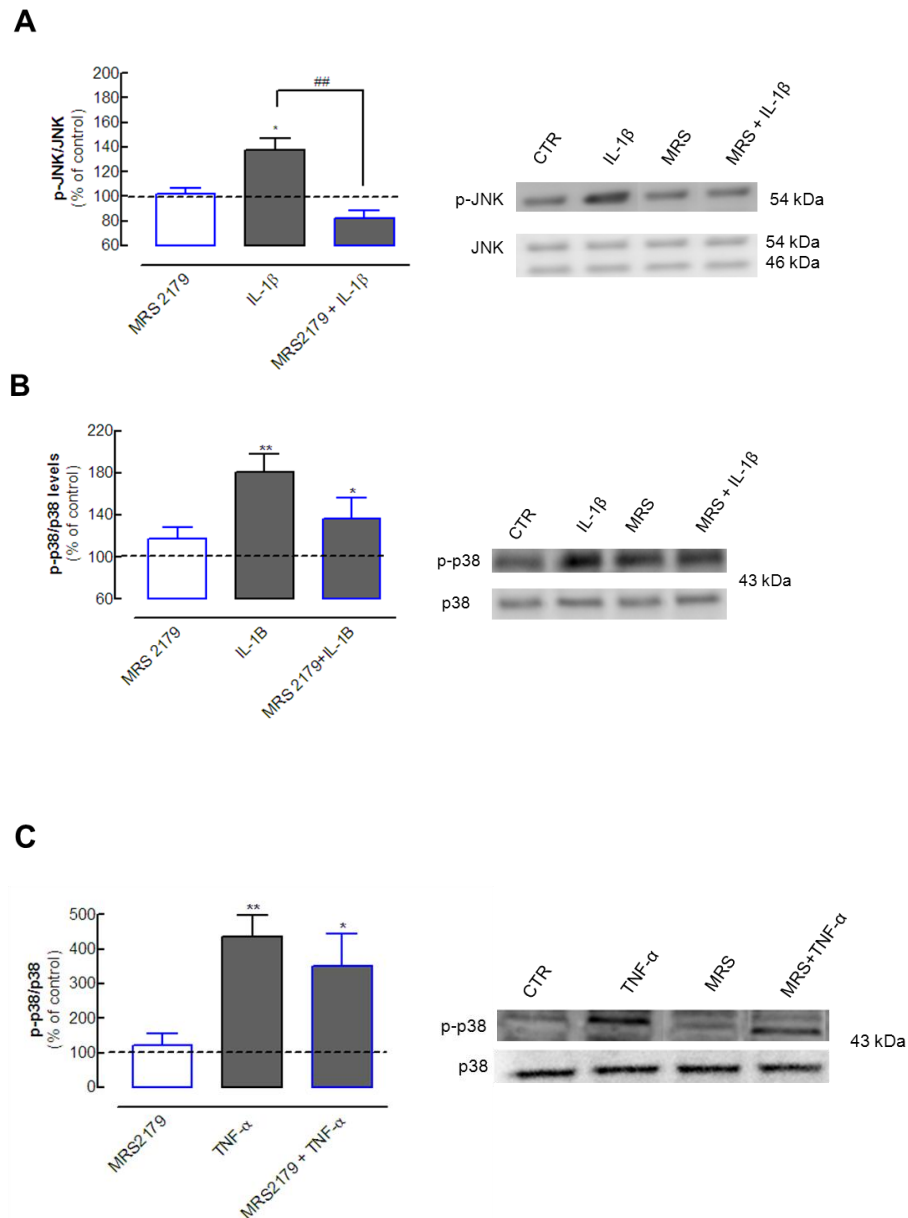
Figure 1: Blockade of the ATP P2Y$_1$ receptors partially prevents the IL-1β-induced signaling, but not the TNF-α-induced signaling, in hippocampal neurons. Hippocampal neuronal cultures, 7 DIV, were exposed to vehicle and/or P2Y$_1$R selective antagonist, MRS2179 (10 µM) 20 min before adding IL-1β (100 ng/ml) or TNF-α (5 ng/ml) (or vehicle for the control groups), for 15 and 10 min, respectively. After the stimuli cells were immediately lysed on ice in a minimum volume of RIPA's buffer. The immunoreactivity of the activated (phosphorylated) form of JNK (p-JNK) (panel A) and of the activated (phosphorylated) form of p38 (p-p38), panels B and C, was quantified by Western blot analysis. Membranes were re-probed for the total form of JNK and p38, respectively, and the activity of both mitogen-activated protein kinases (MAPKs) was calculated as the ratio between the immunoreactivities of the phosphorylated and total forms of the proteins. The results are presented as percentage of control (neurons exposed to vehicle only). Data corresponds to cultures from 8 pregnant Wistar female rats (n= 8) and is presented as mean ± SEM, *p<0.05 and **p<0.01 compared to control; ##p<0.01 compared with neurons exposed to IL-1β only.

4.3.2.2 BLOCKADE OF P2Y$_1$ RECEPTORS PREVENTS BOTH GLUTAMATE-INDUCED NEUROTOXICITY AND THE EXACERBATION OF THE EXCITOTOXICITY BY INTERLEUKIN-1β

Since the blockade of P2Y$_1$ receptors partially prevented the signalling induced by IL-1β on neurons, in the next experiment it was tested whether it could also control the impact of IL-1β on the glutamate-induced neurotoxicity. As previously described (see Chapter 4.2), the exposure of cultured hippocampal neurons to IL-1β (100 ng/ml) for 5 minutes before adding glutamate (100 µM) for a total of 25 minutes, resulted in an exacerbation of the glutamate-induced excitotoxicity (21.0 ± 4.8 % decrease in neuronal viability with glutamate alone comparing with 51.1 ± 13.3 % decrease in neuronal viability with glutamate in the presence of IL-1β; n= 5-7; $p<0.05$), as evaluated using the Syto-13 and propidium iodide staining, 24 hours after the end of the stimuli (Fig. 2A). The presence of IL-1β or of the antagonist of P2Y$_1$R (MRS2179, 10 µM) were devoid of effects per se on the neuronal viability (98.5 ± 2.6 % and 97.0 ± 2.9 % of viable cells, respectively; n= 6) (Fig. 2A). The blockade of P2Y$_1$Rs with MRS2179 (10 µM), added 20 minutes before the stimuli, prevented both the glutamate-induced toxicity (98.3 ± 4.0 % of viable cells; n= 5; $p<0.05$ comparing with glutamate alone) (Fig.2A) and the exacerbation of the glutamate-induced toxicity by IL-1β (81 ± 4.1% of viable cells; n= 4; $p<0.05$ comparing with glutamate in the presence of IL-1β) (Fig. 2A).

Next, it was confirmed this ability of P2Y$_1$Rs to control both the glutamate-induced neurotoxicity and the exacerbation of it by IL-1β using the lactate dehydrogenase (LDH) assay. As shown in Figure 2B, the short term exposure to glutamate (100 µM, 25 minutes) tended to increase LDH release by 24.8 ± 8.8 % relative to the control (n= 6, $P>0.05$), which was significantly ($P<0.05$) potentiated in the presence of 100 ng/ml of IL-1β (LDH release of 62.2 ± 14.5 % above the control, n= 6). Again, the blockade of P2Y$_1$Rs with MRS2179 (10 µM) abrogated the exacerbation of glutamate-induced neurotoxicity by IL-1β (LDH release of 25.0 ± 8.5 % above the control; n= 6; $p<0.05$ comparing to the effect of glutamate in the presence of IL-1β) (Fig. 2B).
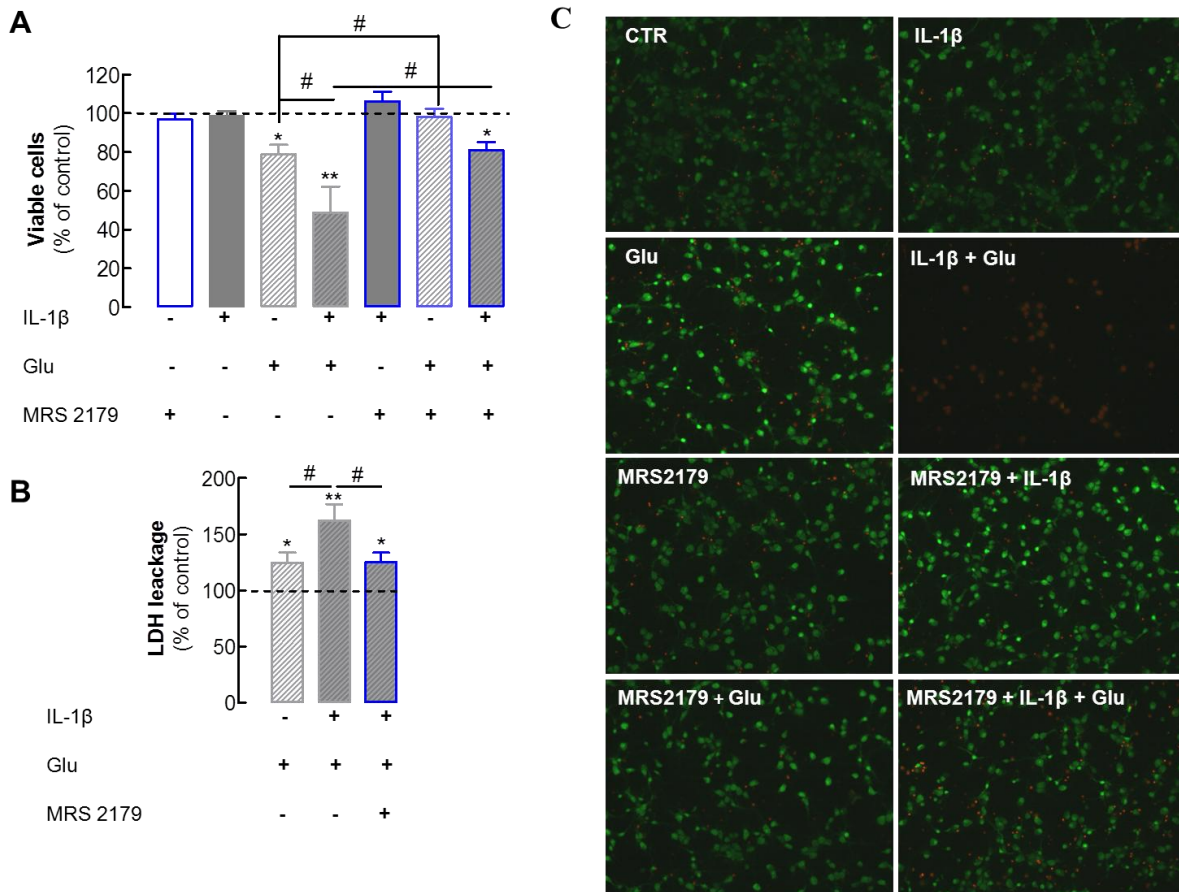
FIGURE 2: Blockade of the ATP P2Y$_1$ receptors prevents both the glutamate-induced neurotoxicity and the exacerbation of it by IL-1β in hippocampal cultured neurons. Rat hippocampal neuronal cultures, 7 DIV, were exposed to vehicle or to interleukin-1β (IL-1β, 100 ng/ml), added 5 minutes prior to vehicle or glutamate (Glu, 100 μM), for a total of 25 min, in the absence or in the presence (added 20 min prior to the stimuli) of the selective P2Y$_1$ receptor antagonist, MRS2179 (10 μM), as indicated below each bar. Neurons were washed twice with Krebs buffer (pH 7.4) and then fresh Neurobasal medium (with or without MRS2179) was added. Neurons were kept for 24 hours in the incubator (37ºC, 95% O2 and 5% CO2) until analysis of neuronal viability using the Syto-13 plus propidium iodide staining (panel A) and the significant findings were further confirmed using the lactate dehydrogenase (LDH) assay (panel B). Results are mean ± SEM of n= 5-7 experiments (corresponding to different cultures); *p<0.05 and ** p<0.01 comparing to control (absence of any added drug), #p<0.05 comparing between treated groups. Panel C displays representative images of Syto-13 (green fluorescence) plus propidium iodide (red fluorescence) staining of nucleic acids for the indicated experimental conditions. Note that neither IL-1β nor MRS2179 had any effect in cell viability per se but IL-1β exacerbated glutamate-induced neurotoxicity. The presence of MRS2179 abrogated both the glutamate-induced neurotoxicity and the exacerbation of it by IL-1β.

4.3.2.3 BLOCKADE OF P2Y$_1$ RECEPTORS PREVENTS THE GLUTAMATE-INDUCED CALCIUM ENTRY AND CALCIUM DEREGULATION AND ATTENUATES THE INTERLEUKIN-1β EFFECTS

In the following experiments it was investigated the effect of the blockade of P2Y$_1$ receptors on the glutamate-induced increase of the intracellular calcium and in the bolstering of the glutamate effects by IL-1β. This was achieved using the single cell calcium imaging approach upon loading hippocampal cultured neurons with the selective ratiometric calcium dye, Fura-2. As shown in Figure 3B, glutamate (100 μM) caused an immediate rise of intracellular free calcium concentration ($[Ca^{2+}]_i$) gauged by an increase of Fura-2 fluorescence ratio of 0.38 ± 0.02 above control (n= 6 cultures, 3-4 coverslips per culture and 30-50 neurons analysed per coverslip; P<0.05). The presence of IL-1β (100 ng/ml) consistently increased this effect of glutamate (ratio of 0.45 ± 0.03, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.05) (Fig. 3B), whereas IL-1β per se failed to trigger any modification in Fura-2 signal. Pre-incubation of cells with MRS2179 (10 μM) attenuated this effect of IL-1β on the glutamate-induced increase of $[Ca^{2+}]_i$ (Fura-2 ratio of 0.38 ± 0.03, n= 4 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.05 compared to the effect of glutamate in the presence of IL-1β) (Fig. 3B). However, MRS2179 also significantly prevented the glutamate-induced increase in the $[Ca^{2+}]_i$ (Fura-2 ratio of 0.32 ± 0.01, n= 4 cultures, 4 coverslips per culture and an average of 30-50 neurons analysed per coverslip) whereas MRS2179 alone was devoid of effects (Fig. 3B).

As can be observed in the graph of the Figure 3C and in the representative curves of the Figure 3A, the analysis of the variation of the Fura-2 fluorescence ratio from its peak value, shortly after the addition of the stimuli, until the end of the incubation period (i.e. during 15 minutes) showed that neurons continuously adapt to the presence of glutamate and decrease their $[Ca^{2+}]_i$ over time (slope of -0.0020 ± 0.0010, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip); in contrast, in the presence of IL-1β (100 ng/ml), neurons lost their adaptive capacity to the continuous presence of glutamate, as testified by their tendency to keep increasing their $[Ca^{2+}]_i$ (slope of + 0.0011 ± 0.0009, n= 6 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.01 compared with glutamate alone). The blockade of P2Y$_1$ receptors with MRS2179 (10 μM) attenuated this effect of IL-1β albeit not significantly (slope of -0.0013 ± 0.0017, n= 4 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip) (Fig.

3C). On the other hand, the presence of MRS2179 resulted in a greater ability of neurons to adapt to the continuous presence of glutamate, resulting in a faster recovery of the $[Ca^{2+}]_i$ towards basal values (slope of -0.0071 ± 0.0003; n= 4 cultures, 3-4 coverslips per culture and an average of 30-50 neurons analysed per coverslip; P<0.05 compared with glutamate alone) (Fig. 3C).
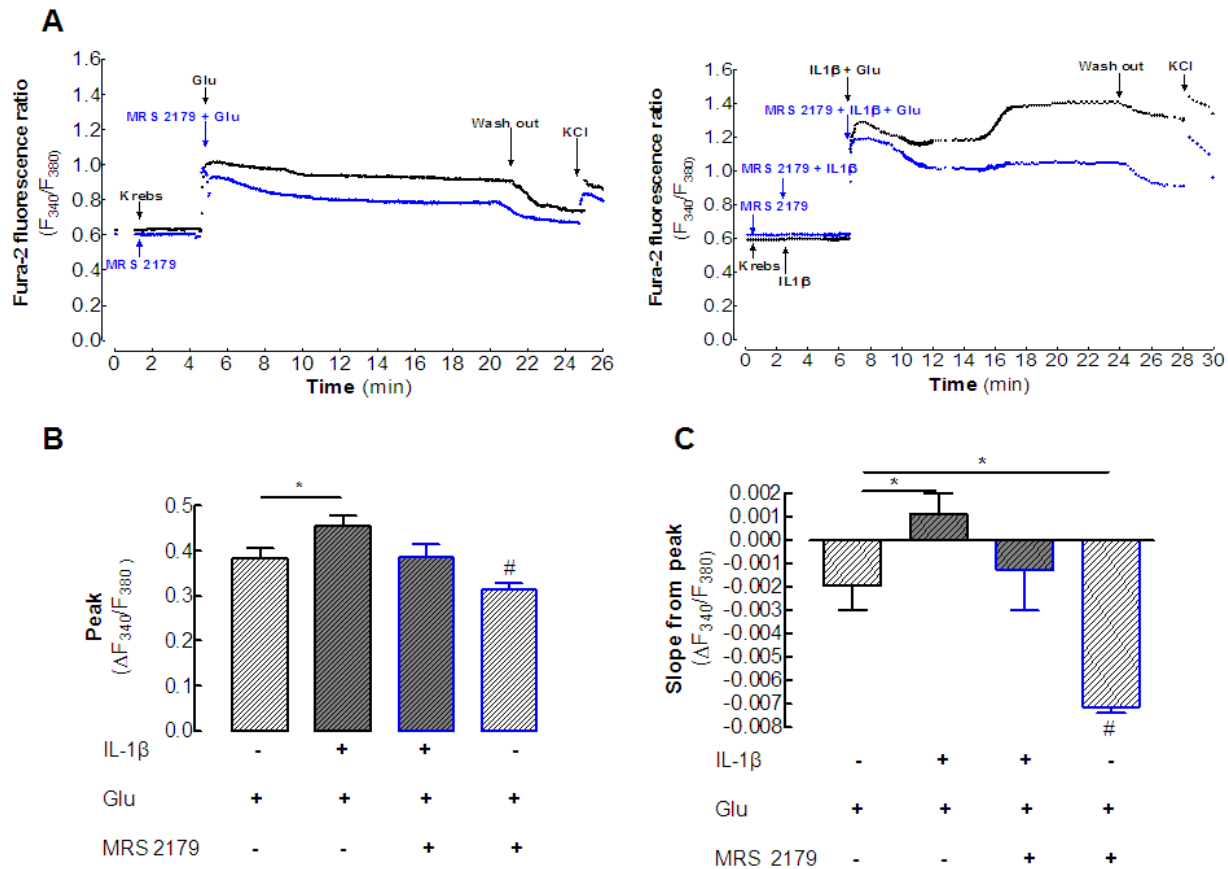

FIGURE 3: Blockade of P2Y$_1$ receptors prevents the glutamate-induced calcium deregulation and attenuates the IL-1β-induced exacerbation of the glutamate effects in hippocampal neurons. Hippocampal neurons were loaded with the calcium probe Fura-2-AM and the fluorescence emitted at 510 nm upon alternate excitation at 340 and 380 nm (F_{340} and F_{380}, respectively) was followed on cell bodies every second during 35 minutes. Basal fluorescence ratio was measured during the first 2 minutes and drugs, prepared in normal Krebs solution, were added to the superfusion using a fast-pressurized system. Interleukin-1β (IL1β, 100 ng/ml), when present, was added for 5 minutes prior to glutamate (Glu, 100 μM). Cells were left in the presence of the stimuli for the next 15 minutes and then washed through superfusion of normal Krebs. The P2Y$_1$ receptor selective antagonist, MRS2179 (10 μM), was incubated for 15 minutes prior to the beginning of the experiment and prevailed throughout. The results are presented as the ratio (F_{340}/F_{380}) of recordings done in healthy neurons (accessed by the response to 50 mM KCl, after washing the stimuli). In the panel A are representative curves of all experimental conditions. IL-1β increased both the glutamate-induced calcium entry (peak value of the difference between the basal ratio and the stimuli-induced ratio) (panel B) and the glutamate-induced calcium deregulation, calculated as the slope from peak values until the wash out of the stimuli (panel C). MRS2179 attenuated the exacerbation by IL-1β of glutamate-induced calcium (B) the IL-1β plus glutamate-induced calcium deregulation (C); however, MRS2179 significantly prevented the impact of glutamate alone (panels B and C). Data are mean ± SEM of n= 4-6 different cell cultures (480-1200 cells analysed per condition for each n); *p<0.05 comparing between the indicated groups; #p<0.05 comparing between the glutamate in the presence of MRS2179 and glutamate alone.

4.3.3 DISCUSSION

The main conclusion of this work is that the blockade of P2Y$_1$ receptors (P2Y$_1$Rs) partially prevents the direct effects of the interleukin-1β (IL-1β) on hippocampal neurons, by preventing the IL-1β-induced activation of the JNK pathway and attenuating the IL-1β-induced activation of the p38 pathway and the IL-1β-induced exacerbation of the glutamate-evoked calcium entry and calcium deregulation. On the other hand and in line with previous findings in the group (Cunha *et al*., 2007), the blockade of P2Y$_1$Rs abrogated the glutamate-induced effects on hippocampal cultured neurons. This is at odd with the inhibitory role of these receptors at glutamatergic synapses where they were shown to either inhibit glutamate release (Rodrigues *et al.*, 2005b) or to inhibit the glutamate NMDA receptor currents (Wirkner *et al.*, 2002; Luthardt *et al.*, 2003; Guzman *et al.*, 2005). Notwithstanding, P2Y$_1$Rs can also increase the $[Ca^{2+}]_i$ by promoting the leakage from intracellular Ca^{2+} stores (Leon *et al.*, 2006; Rubini *et al.*, 2006) and control neuronal excitability by triggering Ca^{2+}-dependent K^+ currents (Schicker *et al.*, 2010) or inhibiting the M-type K^+ currents, as was shown for native P2Y$_1$Rs in hippocampal neuronal cultures (Filippov *et al.*, 2006). Thus, it remains to be investigated whether the effects of the blockade of P2Y$_1$Rs on glutamate-induced calcium are a direct effect on glutamate receptors or an effect on other ion channels and/or intracellular Ca^{2+} reservoirs.

In the present work, the effect of the blockade of P2Y$_1$Rs in the IL-1β-induced exacerbation of the glutamate-mediated excitotoxicity is partially masked by the neuroprotection afforded against glutamate-induced effects. However, it is clear that the antagonism of P2Y$_1$ receptors prevents some of the IL-1β direct actions on neurons. Thus, given their widespread distribution in the hippocampus (Moore *et al.*, 2000a; Moore *et al.*, 2001) and their importance in noxious conditions involving both excitotoxic and inflammatory-mediated cell death, such as in ischaemia/hypoxia (Coppi *et al.*, 2007; Kuboyama *et al.*, 2011; Traini *et al.*, 2011), the P2Y$_1$ receptors can be important targets to afford neuroprotection in different neurodegenerative conditions in the brain.

4.4 BLOCKADE OF P2 RECEPTORS DIFERENTIALLY AFFECTS KAINATE- AND NMDA-INDUCED NEUROTOXICITY IN CULTURED HIPPOCAMPAL NEURONS

4.4.1 INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the mammalian brain and plays a fundamental role in higher functions such as body movements, learning and memory, emotions and sensory perception (Hudspith, 1997). However, overactivation of glutamate receptors result in excitotoxic neuronal degeneration which contributes to the pathogenesis of many different disorders (Sattler and Tymianski, 2000), including ischemic stroke (Paschen, 1996), Alzheimer's disease (Hynd *et al.*, 2004) and epilepsy (Meldrum, 1994). Excitotoxicity is mediated by glutamate-induced Ca^{2+} influx which, if not rapidly removed or buffered, initiates a cascade of events involving oxygen radical production, mitochondrial dysfunction and protease activation, ultimately leading to cell death (Sattler and Tymianski, 2000). There are two types of glutamate receptors: ligand-gated ion channels (ionotropic receptors) and GTP-binding protein-coupled receptors (metabotropic receptors) (Nakanishi, 1992). Glutamate-mediated excitotoxicity occurs primarily through overactivation of ionotropic receptors (Sattler and Tymianski, 2000), which can be divided into those selectively activated by N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors, i.e., those responsive to the agonists 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid (AMPA), kainate and quisqualate. The NMDA receptors are heteromeric complexes formed by an obligatory NR1 subunit and one or more NR2 subunits termed NR2A-D and their channel opening is dependent upon glutamate binding and prior membrane depolarization, resulting in Ca^{2+} influx (Michaelis, 1998). The AMPA receptors are heterodimers composed of subunits GluR1-GluR4 and are permeable to both Na^+ and Ca^{2+} ions (Michaelis, 1998).

Upon any type of brain injury or toxic stimuli, the extracellular concentration of ATP raises significantly through the leakage from damaged cells and as a consequence of increased neuronal and glial transmission (Abbracchio and Verderio, 2006). High extracellular concentrations of ATP are toxic to cell cultures (Amadio *et al.*, 2002) and *in vivo* (Ryu *et al.*, 2002)**,** mediating degeneration of fully differentiated neurons and comprising both apoptotic and necrotic features such as cellular swelling, lactic acid dehydrogenase (LDH) release, increase of intracellular reactive oxygen species and nuclear fragmentation (Volonte *et al.*, 2003). In cerebellar granule neurons, agonists of P2 receptors had toxic

effects which could be prevented both by P2 receptor antagonists and by competitive and non-competitive NMDA receptor antagonists (Amadio *et al.*, 2002), demonstrating an interplay between P2 receptors and glutamate ionotropic receptors in the mediation of toxic events. In agreement, P2 receptors can modulate glutamate release from hippocampal terminals, facilitating or inhibiting it through presynaptic P2X (P2X$_1$, P2X$_{2/3}$, P2X$_3$) and P2Y (P2Y$_1$, P2Y$_2$, P2Y$_4$) receptors, respectively (Rodrigues *et al.*, 2005b) and a post-synaptic co-operativity between extracellular ATP and NMDA receptors in the induction of long-term potentiation in the hippocampus has also been described (Fujii *et al.*, 1999; Fujii *et al.*, 2002). In face of the tight control of the glutamatargic system operated by P2 receptors in the hippocampus and of the previously observed neuroprotection afforded with the blockade of P2Y$_1$ receptors under pathological conditions (Coppi *et al.*, 2007; Kuboyama *et al.*, 2011; Traini *et al.*, 2011), the following experiments were designed to test the neuroprotective potential of P2 receptors and, in particular, of P2Y$_1$ receptors against different excitotoxic stimuli to hippocampal neurons.

4.4.2 RESULTS

4.4.2.1 P2 RECEPTORS AND IN PARTICULAR P2Y$_1$ RECEPTORS CONTROL NMDA-MEDIATED EXCITOTOXICITY IN HIPPOCAMPAL CULTURED NEURONS

To tackle the question of whether P2 receptors can control different excitotoxic stimuli in hippocampal neurons, hippocampal neuronal cultures (grown for 7 days *in vitro*) were exposed to different agonists of glutamate ionotropic receptors: N-methyl-D-aspartic acid (NMDA, 100 μM), kainate (KA, 100 μM) and quinolinic acid (QA, 3 mM), for different times and in the presence or absence of phosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium salt (PPADS, 20μM), a non-selective P2 receptor antagonist, or of MRS2500 (10 μM), a selective and potent P2Y$_1$ receptor antagonist, both added 20 min before the excitotoxic stimuli and prevailing throughout. Neuronal damage was estimated by modifications of the ability of neurons to reduce MTT and by Syto-13 and propidium iodide nucleic acid staining. Within 25 min, only NMDA was able to decrease the ability of cells to reduce the MTT (68.9 ± 3.9 % of control; n= 6; $p<0.01$) and the blockade of P2 receptors before and during the stimulus diminished the NMDA effect (86.5 ± 9.4 % of control; n= 6). Likewise, though to a lesser extent, the blockade of P2Y$_1$ receptors also decreased the NMDA-induced toxicity (77.24 ± 3.6% of control; n= 6); however these results were only significant when the antagonists were

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also present after withdrawing the stimulus (PPADS: 94.6 ± 3.1 %; p<0.01; MRS2500: 85.8 ± 6.3 %; p<0.05 comparing to NMDA; n= 6) (Fig. 1).
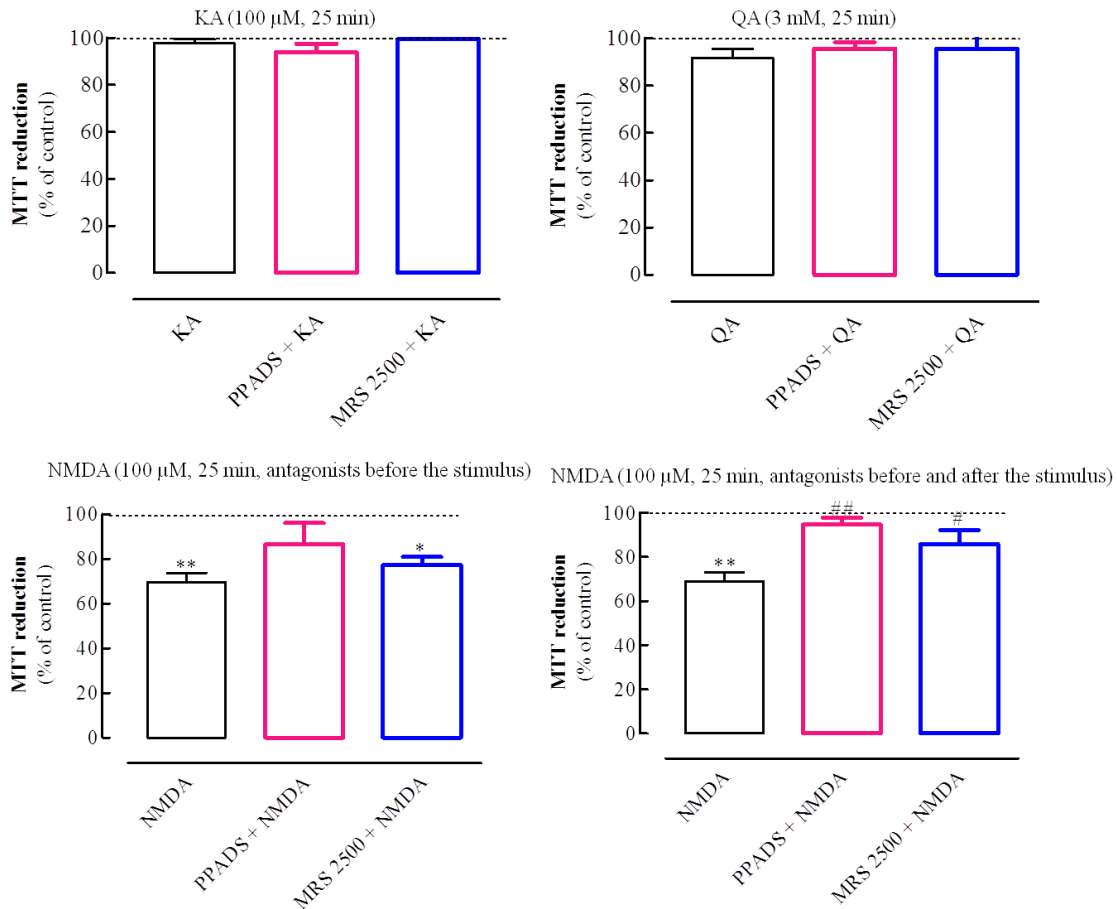

FIGURE 1: Blockade of P2 receptors and of the P2Y$_1$ receptors subtype protects hippocampal neurons against excitotoxicity induced by short-term exposure to NMDA. Hipocampal neuronal cultures, 7 DIV, were exposed for 25 min in Krebs buffer (pH 7.4) to vehicle or agonists of glutamate ionotropic receptors: kainate, KA (100 µM), quinolinic acid, QA (3 mM) and N-Methyl-D-aspartic acid, NMDA (100 µM). When present, the general antagonist of P2 receptors, PPPADS (20 µM) and the selective antagonist of P2Y$_1$ receptors, MRS 2500 (10 µM), were added to the medium 20 min before the stimuli and prevailed throughout the experiment until analysis, unless otherwise stated. After incubation, cells were washed twice with Krebs buffer and fresh Neurobasal medium was added. Neurons were kept in the incubator (37ºC, 95% O$_2$-5% CO$_2$) until analysis of cell ability to reduce the MTT, 24 h after the stimuli. Results are presented as percentage of control (neurons incubated with vehicle only); mean ± SEM; n= 6 (each n corresponds to different cultures); *p<0.05 and **p<0.01 comparing to control, #p<0.05 and ##p<0.01 comparing to NMDA. Note that only NMDA decreased cell ability to reduce MTT and that the blockade of P2 receptors or of the P2Y$_1$ receptors alone prevented this effect when the antagonists were present before and after the stimuli.

The results obtained with the MTT assay were further confirmed through Syto-13 (green fluorescence) and propidium iodide (red fluorescence) staining of the neuronal nuclei. The exposure of hippocampal neuronal cultures to NMDA for 25 minutes reduced cell viability, 24 hours after, in 22.0 $\pm$ 1.5 % bellow the control (n= 4; p<0.01) and the effect was prevented by both P2 receptors blockade (99.0 $\pm$ 4.2 %; n= 4; p<0.01, comparing to NMDA) and by the blockade of $P2Y_1$ receptors (89.5 $\pm$ 3.9 % of control; n= 4; p<0.05, comparing to NMDA) (Fig. 2).
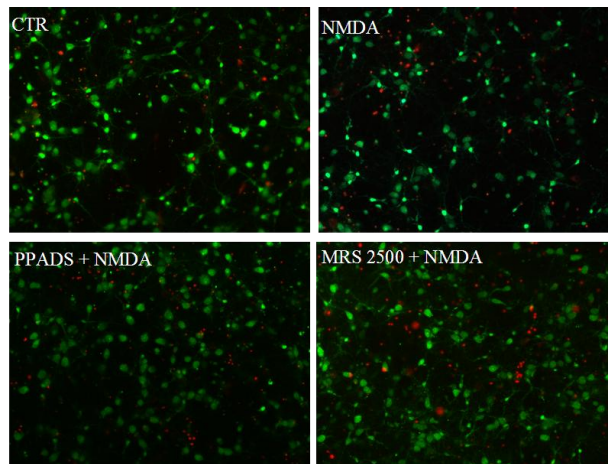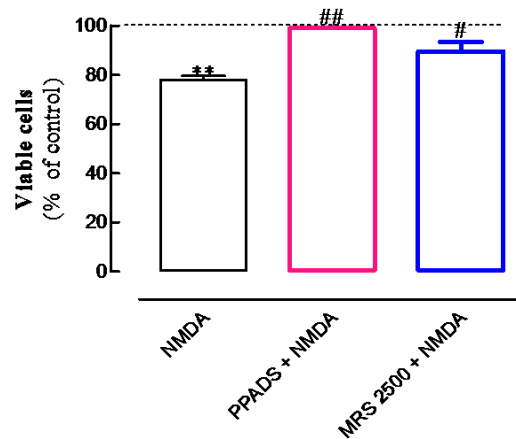

FIGURE 2: Blockade of P2 receptors and of $P2Y_1$ receptors prevents NMDA-induced degeneration of hippocampal neurons. Hipocampal neuronal cultures, 7 DIV, were exposed for 25 min in Krebs buffer (pH 7.4) to vehicle or to NMDA (100 µM). When present, the P2 receptors antagonist, PPADS (20 µM), and the P2Y1 receptors selective antagonist, MRS2500 (10 µM), were added to the medium 20 min before NMDA and prevailed throughout the experiment until analysis. After incubation, cells were washed twice with Krebs buffer and fresh Neurobasal medium was added. Neurons were kept in the incubator (37ºC, in a 95% O_2-5% CO_2 atmosphere) until evaluation of cell viability, 24 h after the stimulus, using the Syto-13 (green fluorescence) *plus* propidium iodide (red fluorescence) staining of the nuclei. Results are presented as mean $\pm$ SEM; n=4 (each n corresponds to different cultures); **p<0.01 comparing to control; #p<0.05 and ##p<0.01 comparing to NMDA. Note that NMDA reduced neuron viability and that this was prevented by the blockade of both P2 receptors and of $P2Y_1$ receptors.

Since a short-term exposure to different agonists of glutamate ionotropic receptors, in our experimental conditions, resulted in neurodegeneration of hippocampal neurons, evaluated 24 hours later, mediated only by NMDA receptors, next it was confirmed that the neuronal dysfunction caused by the exposure of neurons to glutamate for 25 minutes could be prevented by the pre-incubation (20 minutes before glutamate) with a non-competitive and selective antagonist of the NMDA receptors, MK-801 (10 µM). As shown in Figure 3, glutamate (Glu, 100 µM) diminished the ability of cells to reduce the MTT (60.3 ± 10.0 % of control; n= 5; $p<0.01$) that was completely prevented by the pre-incubation of neurons with MK-801 (98.0 ± 2.6 % of control; n= 5).
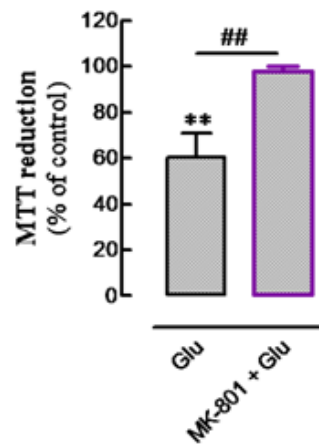
FIGURE 3: The neuronal dysfunction resulting from a short-time exposure of hippocampal cultured neurons to glutamate is mediated by the activation of NMDA receptors. Hippocampal neuronal cultures, 7 DIV, were exposed to glutamate (100 µM) in Krebs buffer (pH 7.4) for 25 minutes in the presence or absence of the selective antagonist of NMDA receptors, MK-801 (10 µM), after which the stimulus was washed twice and fresh Neurobasal medium was added to the cells with or without MK-801. Neurons were kept in the incubator (37ºC, in 95% O_2-5% CO_2 atmosphere) until evaluation of their ability to reduce the MTT, 24 hours after, by reading the absorbance at 570 nm (using 600 nm as a reference). Results are presented as percentage of control (wells incubated with vehicle only); mean ± SEM; n=5-7 (each n corresponds to different cultures); **$p<0.01$ comparing to control, #$p<0.01$ comparison between the indicated groups.

Longer exposures of the hippocampal neurons to the same agonists of glutamate receptors reduced cell viability through activation of both NMDA and AMPA receptors (Fig. 4). The exposure of neurons to kainate (100 µM) for 6 hours decreased neuronal viability in circa 43 % (57.6 ± 3.7 % of control; n= 6; $p<0.001$); incubation with quinolinic acid (3 mM) for 24 hours diminished cell viability in circa 33 % ($67.0 \pm 1,9$ % of control; n= 6; $p<0.001$) and exposure of neurons to NMDA (100 µM) for 12 hours lead to a decrease in MTT reduction of approximately 53 % (47.8 ± 1.1% of control; n= 6; $p<0.001$) (Fig. 4). Blockade of P2 receptors with the general antagonist PPADS (20 µM), added 20 minutes before the stimuli and prevailing throughout, did not prevent the kainate-induced dysfunction of the metabolic state of neurons (56.7 ± 6.7 % of control; n= 6). The same happened with the selective blockade of $P2Y_1$ receptors (62.3 ± 5.7 % of control; n= 6) (Fig. 4). Accordingly, elimination of the putative kainate-induced extracellular ATP by incubating neurons with apyrase (20 U/ml), an enzyme (EC 3.6.1.5) that catalyses the hydrolysis of ATP to AMP and inorganic phosphate, added 5 minutes before the stimulus, did not prevent the effect of kainate (62.8 ± 1.6% of control; n= 6) (Fig. 4). On the other hand, decreased MTT reduction due to stimulation of neurons with quinolinic acid, an endogenous agonist of NMDA receptors, was prevented by the blockade of P2 receptors (82.6 ± 3.5 % of control; n= 6; $p<0.01$ comparing with quinolinic acid) but not by the selective blockade of $P2Y_1$ receptors (74.6 ± 4.7% of control, n= 6) (Fig. 4). As expected, the elimination of the putative extracellular ATP induced by the quinolinic acid, through the incubation of neurons with apyrase just before the stimuli, prevented the effect of quinolinic acid (92.3 ± 4.5 %; n= 6; $p<0.01$) (Fig. 4). Likewise, the excitotoxicity induced by NMDA was prevented by the blockade of P2 receptors (63.9 ± 2.5 % of control; n= 6; $p<0.01$ comparing to NMDA) but not by the selective blockade of $P2Y_1$ receptors (50.1 ± 2.4 % of control; n= 6) (Fig. 3). Again, the catabolism of the extracellular ATP upon the NMDA stimulus prevented the effect of NMDA (74.7 ± 9.7 % of control; n= 6; $p<0.001$ comparing to NMDA) (Fig. 4). In order to know if the blockade of P2 receptors or the selective blockade of $P2Y_1$ receptors could specifically prevent apoptotic cell death, neurons were exposed to staurosporine (STS; 30 nM), a prototypical apoptotic inducer, for 24 hours in the presence or absence of PPADS, MRS2500 or apyrase. The results, in the last graphic of Figure 4, show that staurosporine caused a decrease in the ability of neurons to reduce the MTT of approximately 30 % (69.4 ± 4.0 % of control; n= 6; $p<0.001$) which was prevented by the blockade of P2 receptors with PPADS (88.9 ± 4.9 % of control; n= 6; $p<0.01$ comparing to staurosporine) and also by the blockade of $P2Y_1$ receptors with MRS2500 (83.1 ± 4.1 % of control; n= 6; $p<0.05$ comparing to staurosporine) and by apyrase (87.8 ± 3.5 % of control; n= 6; $p<0.01$ comparing to staurosporine).
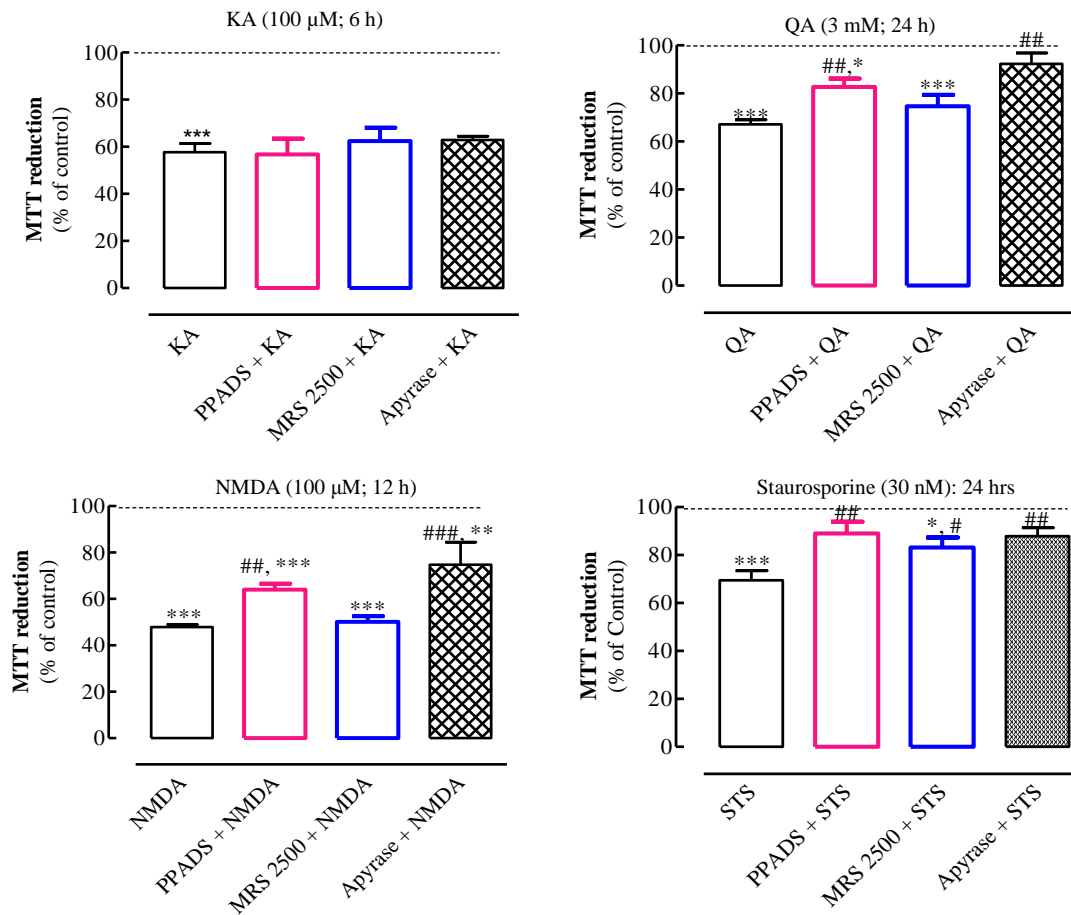
FIGURE 4: The general blockade of P2 receptors, but not the selective blockade of P2Y$_1$ receptors, protects hippocampal neurons against the excitotoxicity induced by long-term exposures to agonists of the NMDA receptors. Hipocampal neuronal cultures, 7 DIV, were exposed for different periods, in Krebs buffer (pH 7.4), to vehicle or agonists of Glu ionotropic receptors: kainate, KA (100 µM; 6h), quinolinic acid, QA (3 mM; 24 h) and N-methyl-D-aspartic acid, NMDA (100 µM; 12 h). In addition, neurons were exposed to staurosporine (STS, 30 nM; 24h), a prototypic apoptotic inducer. When present, the general anatagonist of P2 receptors, PPADS (20 µM) and the selective antagonist of P2Y$_1$ receptors, MRS2500 (10 µM) were added to the medium 20 minutes before the stimuli and prevailed throughout the experiment until analysis. The enzyme that catalyses the hydrolysis of ATP, apyrase (20 U/mL), when present, was added 5 minutes before the stimuli. Evaluation of cell viability was performed through the MTT assay after the indicated incubation times. Results are presented as percentage of control (wells incubated with vehicle only); mean ± SEM; n= 6 (each n corresponds to different cultures); *p<0.05, **p<0.01, ***p<0.001 in comparison to control; #p<0.05 and ##p<0.01 in comparison to the respective excitotoxic stimuli. Note that all Glu receptor agonists decreased cell ability to reduce the MTT and that the blockade of P2 receptors but not the selective blockade of P2Y$_1$ receptors prevented the effect mediated by the quinolinic acid and by the NMDA. Also, the blockade P2 receptors or the selective blockade of P2Y$_1$ receptors alone prevented the staurosporine-induced dysfunction.

The prevention of the effect of a long-term exposure of hippocampal neurons to NMDA by the blockade of P2 receptors was confirmed by the Syto-13 and propidium iodide staining of the neuronal nuclei. As shown in Figure 5, the exposure of cells to quinolinic acid for 24 hours induced approximately 25 % of cell death (74.5 ± 2.3 % of control; n= 4; p<0.001) which was prevented by the

blockade of P2 receptors with PPADS ($87.3 \pm 4.4\%$ of control; n= 4; $p<0.05$ comparing to quinolinic acid) but not by the selective blockade of $P2Y_1$ receptors with MRS2500 (77.5 ± 3.2 % of control; n= 4). The exposure of neurons to NMDA for 12 hours resulted in approximately 36 % of loss of viable cells ($63.5 \pm 3.8\%$ of control; n= 4; $p<0.01$) which was again prevented by the blockade of P2 receptors with PPADS (90.8 ± 7.8 % of control; n= 4; $p<0.05$ comparing to NMDA) but not by the selective blockade of $P2Y_1$ receptors with MRS2500 (73.8 ± 8.5 % of control; n= 5).
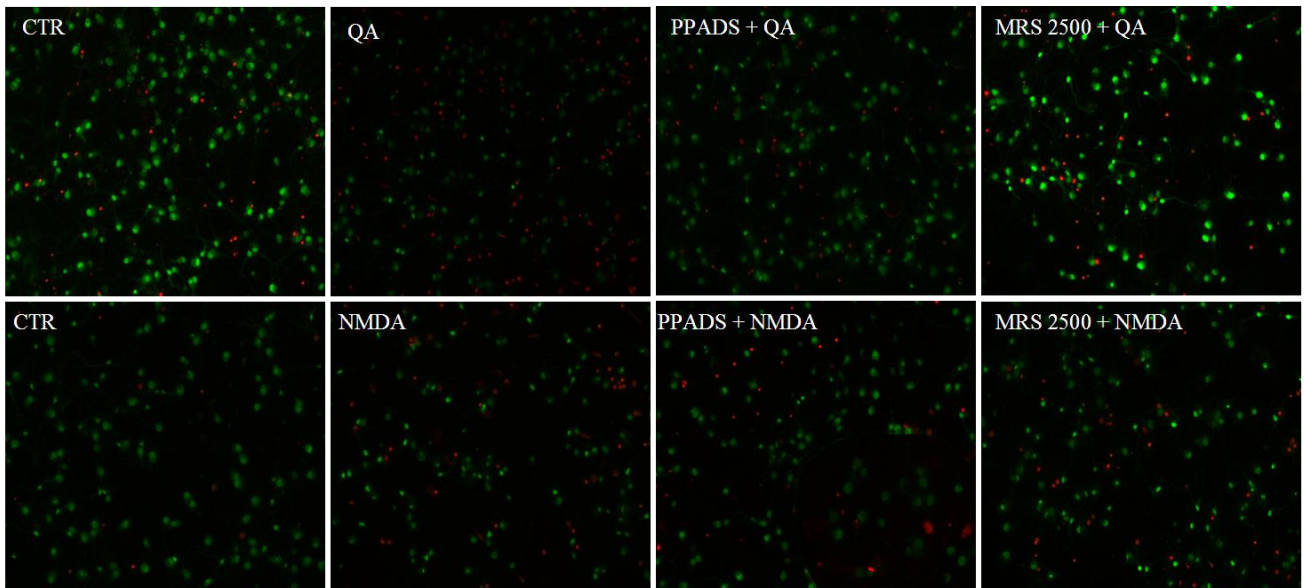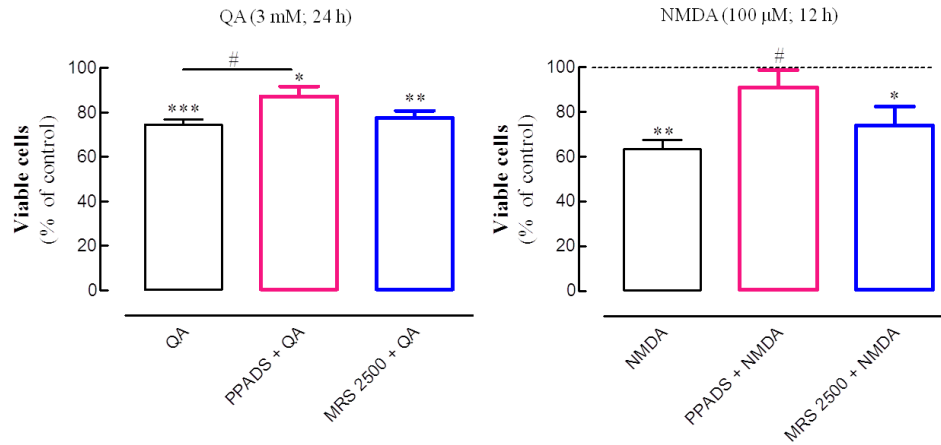
FIGURE 5: Blockade of P2 receptors, but not of the P2Y$_1$ receptors, prevents the effects of long-term exposure of hippocampal neurons to quinolinic acid and to NMDA. Hippocampal neuronal cultures, 7 DIV, were exposed to quinolinic acid (QA; 100 µM; 24 h) or to N-methyl-D-aspartic acid (NMDA; 100 µM; 12 h) in Krebs buffer (pH 7.4). When present, the antagonist of P2 receptors (PPADS; 20 µM) and the selective antagonist of P2Y$_1$ receptors (MRS2500; 10 µM) were added to the medium 20 min before the stimuli and prevailed throughout the experiment until analysis. Evaluation of cell viability was performed through Syto-13 (green fluorescence) *plus* propidium iodide (red fluorescence) staining of the neuronal nuclei nucleic after the indicated periods of incubation with the stimuli. Results are presented as percentage of control (wells incubated with vehicle only); mean $\pm$ SEM; n= 4-5 (each n corresponds to different cultures); *p<0.05, **p<0.01, ***p<0.001 in comparison to control; #p<0.05 in comparison to the respective excitotoxic stimuli. Note that the general blockade of P2 receptors blockade, but not the selective antagonism of P2Y$_1$ receptors, prevented the effect mediated by the quinolinic acid and by the NMDA.

4.4.3 DISCUSSION

The present results show that hippocampal neurons are differently affected by short and long-term exposure to the same agonists of glutamate receptors. Long-term exposures (hours) to these agonists, result in neurodegeneration mainly mediated by the activation of AMPA/kainate receptors while short-term exposure (25 minutes) results in cell death mediated exclusively by NMDA receptors. Accordingly, incubation of hippocampal neurons with glutamate for 25 minutes resulted in approximately 20 % of cell death 24 hours later, which was completely prevented by previously adding an NMDA receptor selective and non-competitive antagonist (MK-801). These results are in agreement with the rapid desensitization of AMPA/kainate receptors in hippocampal neurons (Ambrosio *et al.*, 2000; Silva *et al.*, 2001) and the absence of neurodegeneration mediated by these receptors during short-term activation which can be reverted by the presence of cyclothiazide, a positive allosteric modulator of the AMPA receptors that eliminates their rapid desensitization and potentiates their currents (Ambrosio *et al.*, 2000). In the extracellular space, ATP induces cell death in fully differentiated neurons of the CNS, which express both P2X and P2Y receptors, causing cell degeneration with necrotic and apoptotic features (Amadio *et al.*, 2002). Thus, unsurprisingly, agonists of P2 receptors were shown to aggravate toxicity whereas antagonists were neuroprotective (Amadio *et al.*, 2002; Ryu *et al.*, 2002; Volonte *et al.*, 2003). However, the modulation by distinct P2 receptors of the neuronal response to glutamate is still controverse, for example, P2Y receptors were found to increase the conductance NMDA receptors in the pyramidal neurons of the V layer of the rat prefrontal cortex (Wirkner *et al.*, 2002) whereas others found the opposite (Luthardt *et al.*, 2003). Curiously, both competitive and non-competitive antagonists of NMDA receptors are able to prevent the noxious effects of ATP (Amadio *et al.*, 2002) and in turn, some P2 receptor antagonists prevent glutamate-evoked Ca^{2+} influx, neurotransmitter release and excitotoxicity (Volonte *et al.*, 1999; Volonte *et al.*, 2003). In the present work, long-term exposures to the glutamate receptor agonists resulted in cell death mediated by both AMPA/kainate receptors and NMDA receptors, but the blockade of P2 receptors only prevented the neurodegeneration mediated by the last, confirming a close interaction between P2 and NMDA receptors. In agreement with these results, in cortical and hippocampal neuronal cultures, PPADS was ineffective in preventing AMPA/kainate receptor currents, though more selective P2Y receptor antagonists reversibly decreased their amplitude (Zona *et al.*, 2000).

Presynaptic activation of P2X receptors in the hippocampus elicits glutamate release (Rodrigues *et al.*, 2005b) thus potentiating glutamate-mediated excitotoxicity. At the post-synaptic level, immediate Ca^{2+} entry through P2X receptors, which co-localize with glutamate ionotropic receptors at post-synaptic membranes (Rubio and Soto, 2001), can also increase excitotoxicity (Volonte *et al.*, 2003). Thus, excitotoxicity can be prevented by the blockade of P2X receptors (Volonte *et al.*, 1999). Nevertheless, the $G_{q/11}$-linked P2Y receptors can also mediate excitotoxic events through phospholipase C (PKC)-dependent mobilization of Ca^{2+} from its intracellular stores (von Kugelgen and Harden, 2011). In addition, P2Y receptors can activate mitogen-activated protein kinases resulting in caspase-3 activation and apoptosis (Sellers *et al.*, 2001). In the present work, the neuroprotection afforded with the blockade of P2 receptors and in particular, with the selective blockade of $P2Y_1$ receptors was associated with the prevention of the NMDA receptor-mediated neurodegeneration only; thus there seems to be a particular interaction between these two types of receptors. On the other hand, $P2Y_1$ can also control a large variety of neuronal ion channels, such as voltage-activated Ca^{2+} channels and K^+ channels involved in the excitability of post-synaptic membranes, as was recently shown in primary cultures of hippocampal neurons (Filippov *et al.*, 2006; Schicker *et al.*, 2010), therefore this interaction may result from an indirect mechanism. The present results also show that when the general antagonist of P2 receptors (PPADS) or the selective antagonist of $P2Y_1$ receptors (MRS2500) are present after the excitotoxic stimuli rather than just before and during, the prevention of excitotoxicity is more robust, indicating that ATP released after the initial damage continues to play an important part in neurodegenerative processes. In conclusion, the blockade of P2 receptors might be particularly effective for long-term insults that mainly involve the activation of NMDA receptors for expression of neurotoxicity whereas the $P2Y_1$ receptor subtype might be important in the expression of early NMDA-induced neurodegenerative events.

4.5 P2 RECEPTORS CONTROL NEUROINFLAMMATION AND NEURODEGENERATION IN A RAT MODEL OF TEMPORAL LOBE EPILEPSY

4.5.1 INTRODUCTION

Epilepsy is a multietiological disease with a high incidence worldwide and its treatment consists basically in alleviating symptoms (DeLorenzo *et al.*, 1992). One of the most common adult forms of treatment-resistant epilepsy is the temporal lobe epilepsy (TLE), characterized by a state of chronic neuronal hyperexcitability and hypersynchrony manifested as recurrent partial seizures (Bernasconi and Bernhardt, 2010). Seizures occur as the result of an imbalance between the inhibitory and excitatory transmission, involving an impairment of GABAergic and glutamatergic systems, respectively, that is perperctuated by the abnormal expression of glutamate and GABA receptors and their acquired pathophysiology (Sharma *et al.*, 2007). In fact, epilepsy leaves a long-term impact in the brain characterized by synaptic rearrangements and aberrant mossy fiber sprouting underlying the phenomenon known as "epileptic long-term potentiation" (Ben-Ari, 2001).

The hippocampus is the brain region most affected by epileptic seizures since glutamatergic transmission is prominent is this area (de Lanerolle and Lee, 2005). Accordingly, the most frequent types of TLE are the mesial temporal lobe epilepsies (MTLE), characterized by seizure generation from the mesial temporal lobe with prominent neuronal loss and gliosis in the hippocampus (Yang *et al.*, 2010). About 70% of patients with MTLE-induced sclerosis of the hippocampus present neuronal degeneration, astrogliosis and aberrant mossy fiber sprouting in the inner molecular layer of the dentate gyrus (Sharma *et al.*, 2007).

One of the best characterized animal models of MTLE is the systemic or local administration to animals of 2-carboxy-4(1-methylethenyl)-3-pirrolidiacetic acid (kainate), a potent agonist of AMPA and kainate receptors which induces acute convulsive episodes followed by the development of spontaneous motor seizures (Ben-Ari and Cossart, 2000). The hippocampus CA3 subregion is especially sensitive since it is enriched in kainate receptors (Fisahn, 2005); however, in this animal model, apoptotic cell death and reactive gliosis (both astrogliosis and microgliosis) can be detected in all hippocampal subregions from 24 hours to 4 weeks after the administration of kainate (Ben-Ari and Cossart, 2000). The aberrant neuronal firing and excitotoxicity characteristic of the epileptic

hippocampus result in elevated concentrations of ATP in the extracellular melieu (Kumaria *et al.*, 2008). Accordingly, ATP-mediated pathophysiology and altered P2 receptor expression has been described in the hippocampus of animal models of TLE (Vianna *et al.*, 2002; Rappold *et al.*, 2006; Avignone *et al.*, 2008; Dona *et al.*, 2009). The prominent role for P2 receptors in the physiology of glial cells in the brain (Inoue, 2008; Butt, 2011) and therefore in neuroinflammation (Di Virgilio *et al.*, 2009) has been driving attention to these receptors as possible targets in neurological disorders with an important inflammatory component as is the case of epilepsy (Kumaria *et al.*, 2008; Vezzani *et al.*, 2011). Considering the modulation of the glutamatergic system operated by P2 receptors and in particular by $P2Y_1$ receptors, in both neurons (Fujii *et al.*, 2002; Rodrigues *et al.*, 2005b; Franke *et al.*, 2006) and glial cells (Domercq *et al.*, 2006) and the previously found neuroprotection afforded with the blockade of P2 receptors under excitotoxic conditions, next it was tested if the same neuroprotection could be observed *in vivo*, in a rodent model of TLE.

4.5.2 RESULTS

4.5.2.1 KAINATE-INDUCED CONVULSIONS RESULT IN LONG-TERM CHANGES IN THE DENSITY OF P2 RECEPTORS IN THE RAT HIPPOCAMPUS

To investigate the seizure-induced impact on the purinergic system, adult rats were injected intraperitoneally (ip) with saline or with 10 mg/kg of kainate and sacrificed 1, 7 and 30 days after the kainate-induced convulsions. Only the animals reaching at least the level IV of the Racine's scale for motor seizures (Racine, 1972) were further analysed in order to assure kainate-induced hippocampal damage. Upon sacrifice, the hippocampi were isolated and the density of the different P2 receptors was evaluated through western blot analysis in both total and synaptic membranes.

As shown in Figure 1, most of the P2X receptor subtypes are altered after the kainate-induced seizures in either total membranes or synaptosomes, comparing to the saline-injected animals (control group). Moreover, the density of some P2X receptors is significantly altered up until 1 month after the kainate administration. In particular, in both synaptic and total membranes, the density of $P2X_1$ receptors is significantly increased (125.2 ± 5.2 % of control; n= 4; p<0.05 and 163.0 ± 2.6 % of control; n= 4; p<0.01, respectively) 24 hours after the kainate stimulus. The $P2X_2$ receptors are down-regulated in synaptosomes 30 days after the kainate injection (67.2 ± 5.7 % of control; n= 4; p<0.05) and up-regulated in total membranes both 24 hours (138.2 ± 6.0 % of average of control; n= 4; p<0.05)

and 30 days after the kainate administration (166.9 ± 12.7 % of control; n= 4; p<0.01). The $P2X_3$ receptors density is only different from control in synaptosomes, 30 days after the kainate injection (130.1 ± 5.7 % of control; n= 4; p<0.01). Likewise, the density of $P2X_4$ receptors is only up-regulated 30 days after the kainate-induced seizures but in total membranes (247.3 ± 30.6 % of; n= 4; p<0.05). The $P2X_5$ receptors are increased in both synaptosomes and total membranes 1 day after the toxic insult (144.2 ± 12.9 % and 235.1 ± 30.3 % of control, respectively; n=4; p<0.05) and the $P2X_6$ receptors are increased in synaptosomes both 1 and 30 days after the kainate administration (244.7 ± 18.3 % of control; n=4; p<0.01 and 191.8 ± 14.9 % of control; n=4; p<0.01, respectively). Finally, the $P2X_7$ receptors are significantly up-regulated in total membranes, 7 days upon the kainate-induced seizures (134.3 ± 7.1 %; n= 4; p<0.05) (Fig. 1).
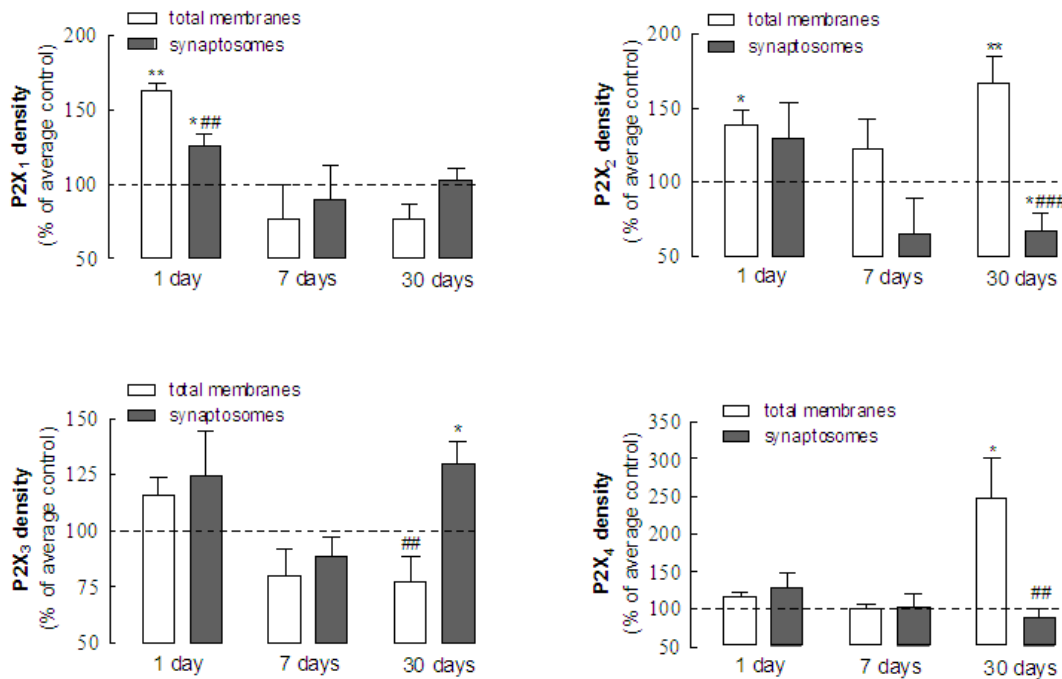
FIGURE 1: The density of P2X receptors is altered in the rat hippocampal membranes at different periods after the convulsions induced by the systemic administration of kainate. Male Wistar rats (2 months old and weighting between 250-300g) were injected intraperitoneally with 10 mg/kg of kainate or with saline (control). The animals that convulsed, i.e., reached at least the stage IV of Racine's scale for motor seizures, were sacrificed 1, 7 and 30 days later. Hippocampal total membranes and synaptosomes were prepared and the samples were run in electrophoresis gels for Western blot analysis. The results are presented as percentage of the averaged control ± SEM (n= 4-5); *p<0.05 and **p<0.01 comparing with the control; #p<0.05 and ##p<0.01 comparing between total and synaptic membranes.

The Figure 2 shows the density profiles for the P2Y receptors in synaptosomal and total membranes of the hippocampus, at different times after the systemic administration of kainate to rats. As can be observed, the density of $P2Y_1$ receptors significantly increases 1 day after the kainate-induced convulsions in both synaptosomes and total membranes (157.5 ± 5.3 % and 143.2 ± 3.6 % of control; n= 4; p<0.01) and remains increased 7 days later, in total membranes (158.3 ± 11.8 % of control; n= 4; p<0.05). The $P2Y_2$ receptor's density is also up-regulated in total membranes 1 and 7 days after the kainate injection (126.9 ± 4.6 % and 139.7 ± 11.4 % of control, respectively; n= 4; p<0.05) as are the $P2Y_4$ receptors, 1 day after kainate, in total membranes (157.0 ± 13.5 % of control; n= 4; p<0.05), 7 days after, in synaptosomes (193.1 ± 15.6 % of control; n= 4; p<0.01) and 30 days after, in total membranes again (216.3 ± 32.9 % of control; n= 5; p<0.05). Finally, the $P2Y_6$ receptor levels are significantly increased in both synaptic and total hippocampal membranes, 1 day upon kainate-induced seizures (175.5 ± 14.7 % and 317.2 ± 12.3 % of control; n= 4; p<0.05 and p<0.001,

respectively); however, it is significantly decreased in total membranes, 7 days upon kainate-induced seizures (81.6 ± 2.1 % of control; n= 4; p<0.01) but increased again, this time in synaptosomes, 30 days after (157.6 ± 11.3 % of control; n= 4; p<0.05).
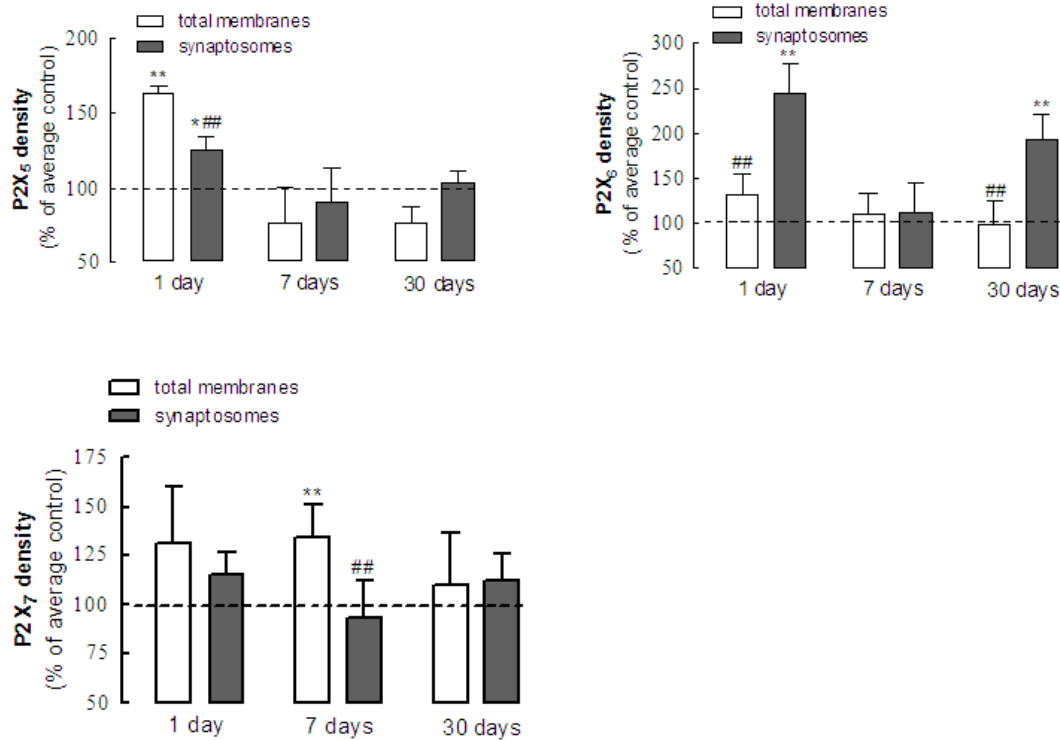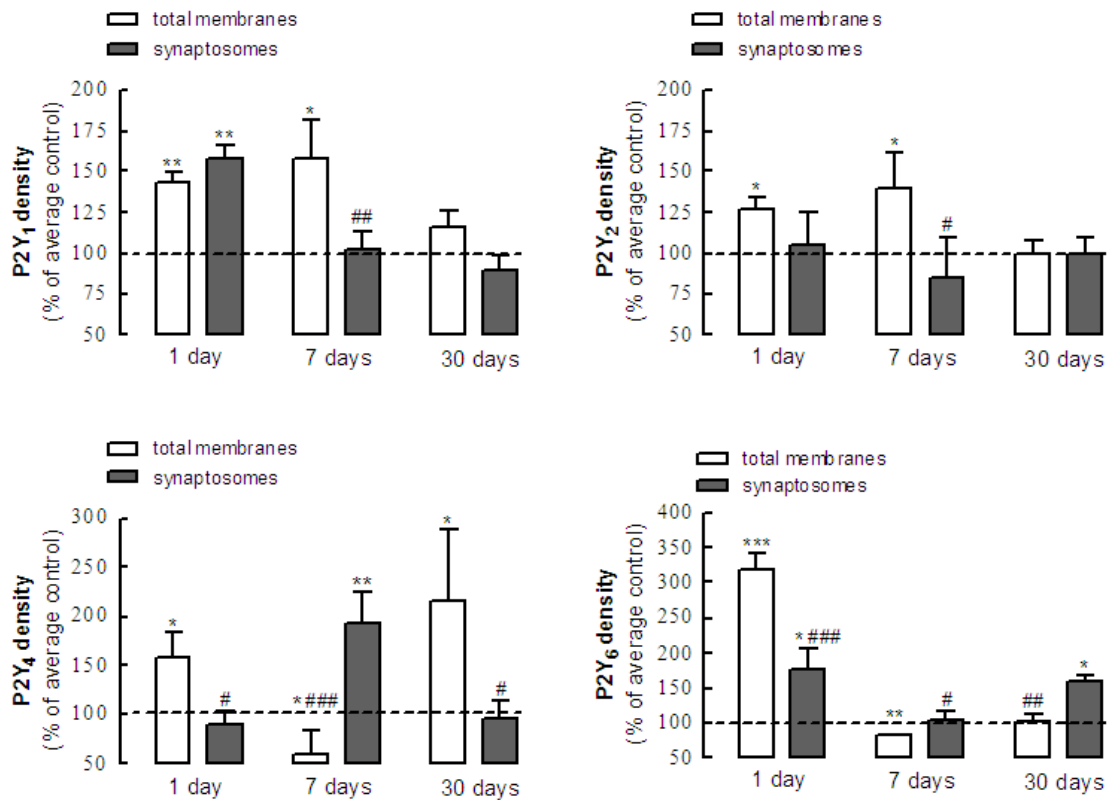

FIGURE 2: The density of P2Y receptors is altered in the rat hippocampal membranes at different periods after the convulsions induced by the systemic administration of kainate. Male Wistar rats (2 months old and weighting between 250-300g) were injected intraperitoneally with 10 mg/kg of kainate or with saline (control). The animals that convulsed, i.e., reached at least the stage IV of Racine's scale for motor seizures, were sacrificed 1, 7 and 30 days later. Hippocampal total membranes and synaptosomes were prepared and the samples run in electrophoresis gels for Western blot analysis. The results are presented as percentage of averaged control $\pm$ SEM (n= 4-5); *p<0.05, **p<0.01 and ***p<0.001 comparing with the control; #p<0.05, ##p<0.01 and ###p<0.001 comparing between total and synaptic membranes.

4.5.2.2 PHARMACOLOGICAL BLOCKADE OF P2Y$_1$ RECEPTORS PREVENTS SEIZURE-INDUCED LOSS OF SYNAPTIC MARKERS

The previous results show that the synaptic and non-synaptic density of P2 receptors is altered in the hippocampus as a result of kainate-induced seizures, in agreement with the central role of ATP in the pathophysiology of epilepsy. Many neurodegenerative diseases are now recognized to start with an early synaptic toxicity before the onset of neurodegeneration, as is the case of Alzheimer's disease (Coleman *et al.*, 2004; Canas *et al.*, 2009). Nowadays, the targeting of the synapse as a strategy to prevent neurodegeneration is being considered for other brain disorders (Coleman *et al.*, 2004). A synaptotoxicity also occurs as a consequence of epileptic seizures (Cognato *et al.*, 2010) thus, next it was investigated whether the general blockade of P2 receptors with the antagonist PPADS or the selective blockade of P2Y$_1$ receptors with the selective antagonist MRS2500, were able to prevent the seizure-induced loss of synaptic markers. First the animals were cannulated at the lateral ventricle in order to allow the intracerebroventricular (icv) administration of drugs. After recovering from the surgery during 3 to 4 days, the rats received a 10 mg/kg intraperitoneal (ip) injection of kainate (KA) or saline, 15 minutes after receiving PPADS/MRS2500 or ACSF (artificial cerebrospinal fluid) icv. The experimental groups were as follows: the control group or ACSF/SAL (ip and icv administration of ACSF); the KA group, ACSF/KA (icv administration of ACSF and ip injection of KA); the PPADS/SAL group (icv administration of 1 nmol/4 μL PPADS and ip injection of ACSF); the MRS/SAL group (icv administration of 1 nmol/4 μL MRS2500 and ip injection of ACSF); the PPADS/KA group (icv administration of 1 nmol/4μL PPADS and ip injection of 10 mg/kg KA) and finally the MRS/KA group (1 nmol/ 4μL MRS2500 icv and ip injection of 10 mg/kg KA). The animals were sacrificed 24 hours after the kainate-induced seizures and total membranes were prepared from the ipsilateral hippocampi for Western blot analysis.

In Figure 3 it can be observed that the kainate injection induced a dramatic decrease of the membrane content in the synaptosomal-associated protein 25 (SNAP-25), a pre-synaptic terminal marker, in 94.4 ± 2.8 % bellow the average of the group that received only saline (ACSF/SAL or control), which was only rescued by the blockade of P2Y$_1$ receptors, through administration of MRS2500 (1 nmol/4 μl, icv) before the kainate injection (65.9 ± 5.6 % of the average control; n= 4; p<0.001) (Fig. 3A). The membrane content in the post-synaptic density protein (PSD-95) is also decreased by seizures (44.9 ± 15.2 % of the average control; n= 4; p<0.05), albeit not significantly

prevented by PPADS or MRS2500 (Fig. 3B). No significant differences were found in the density of the major synaptic vesicle protein, synaptophysin (Fig. 3C) but, on the other hand, there was significant loss of the membrane content in syntaxin (41.0 ± 5.4 % of the average control; n= 4; $p<0.01$), a family of proteins that are part of the Q-SNARE complex (a complex of proteins involved in the vesicular exocytosis), which was completely prevented by the selective blockade of $P2Y_1$ receptors (101.5 ± 12.4 % of control; n= 4; $p<0.01$ comparing to the ACSF/KA group) (Fig. 3D). Again, the kainate injection resulted in a decrease of the glutamate vesicular transporter 1 ($vGLUT_1$) indicative of a loss of glutamatergic terminals (24.3 ± 10.9 % of average control; n= 4; $p<0.01$) which was rescued only by the previous administration of MRS2500 (77.6 ± 8.7 % of average control; n= 4; $p<0.05$ comparing to the ACSF/KA group) (Fig. 3E). Moreover, the blockade of $P2Y_1$ receptors also prevented the loss of the GABAergic terminal marker vGAT (77.0 ± 10.0 % of average control of the MRS/KA group comparing with 31.7 ± 5.5 % of average control of the ACSF/KA group; n= 4; $p<0.01$) (Fig. 3F). Finally, the blockade of $P2Y_1$ receptors prevented the loss of a marker of the GABAergic post-synaptic density, gephyrin (90.4 ± 8.7 % of control of the MRS/KA group comparing with 41.8 ± 10.7 % of control of the ACSF/KA group; n= 4; $p<0.05$) (Fig. 3G).
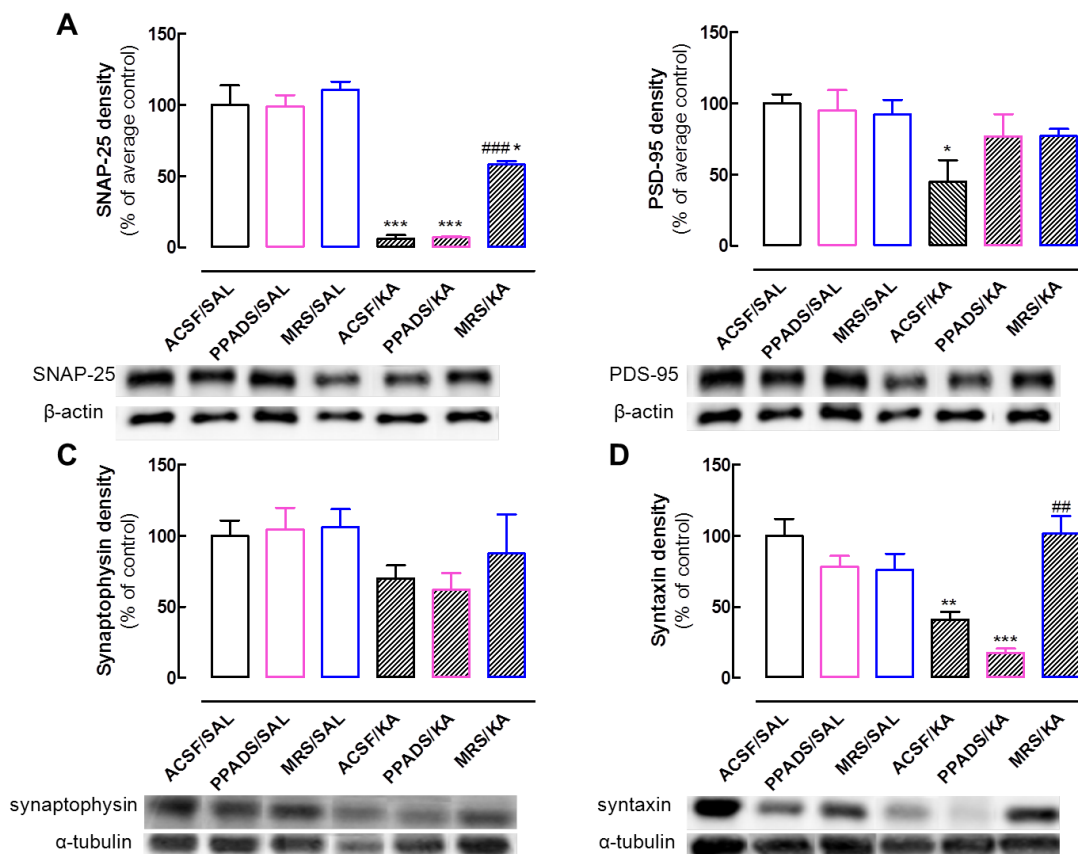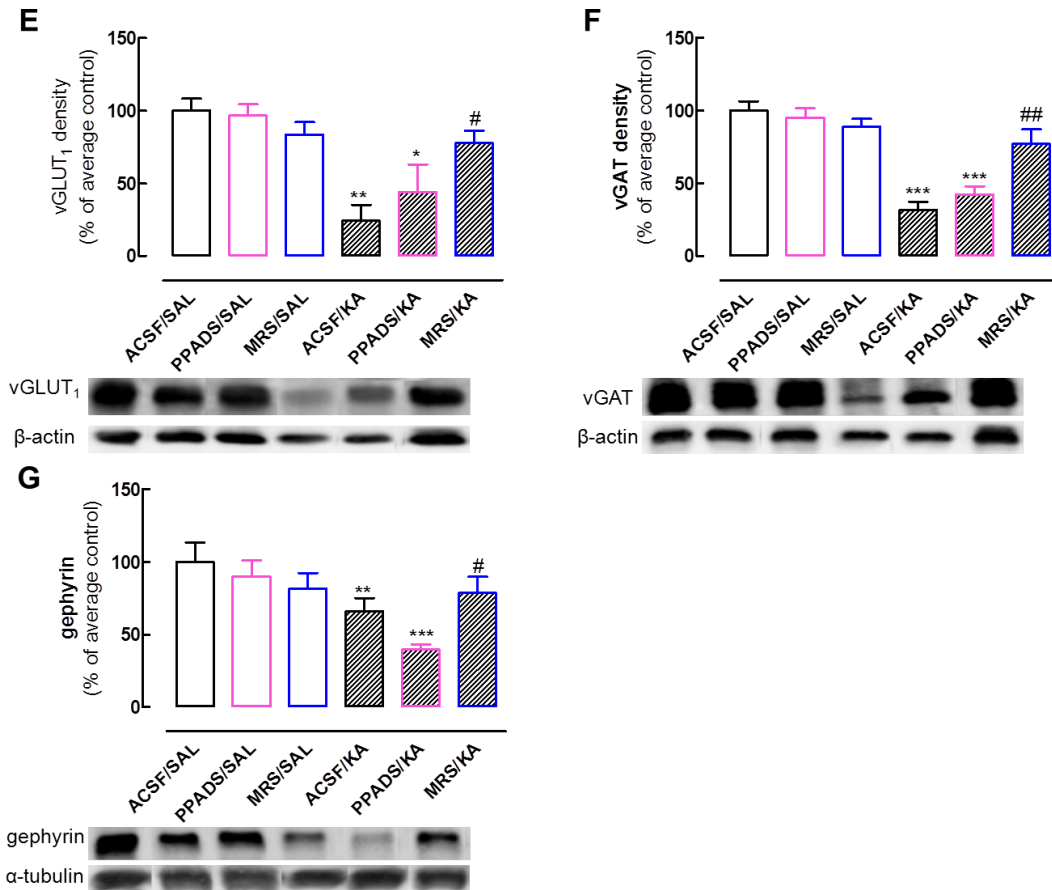
FIGURE 3: Blockade of P2Y1 receptors prevents the loss of synaptic markers induced by seizures in the rat hippocampus. Wistar male rats were cannulated at the lateral ventricle to allow the intracerebroventricular (icv) administration of drugs. After recovering from the surgery, the animals received a 10 mg/kg intraperitoneal (ip) injection of kainate (KA) or of saline, 15 minutes upon receiving PPADS, MRS2500 or ACSF (artificial cerebrospinal fluid) icv. Thus, the animals were grouped as follows: the control group or ACSF/SAL (ip and icv administration of ACSF); the kainate (KA) group, ACSF/KA (icv administration of ACSF and ip injection of KA); the PPADS/SAL group (icv administration of 1 nmol/4 μL PPADS and ip injection of ACSF); the MRS/SAL group (icv administration of 1 nmol/4 μL MRS2500 and ip injection of ACSF); the PPADS/KA group (icv administration of 1 nmol/4μL PPADS and ip injection of 10 mg/kg KA) and finally the MRS/KA group (1 nmol/ 4μL MRS2500 icv and ip injection of 10 mg/kg KA). Animals were sacrificed 24 hours after kainate-induced seizures and total membranes were prepared from the ipsilateral hippocampus for Western blot analysis. The results are presented as percentage of averaged control ± SEM (n= 4); *p<0.05, **p<0.01 and ***p<0.001 comparing with the control; #p<0.05, ##p<0.01 and ###p<0.001 comparing between the ACSF/KA group and the indicated group.

4.5.2.3 BLOCKADE OF P2 RECEPTORS OR OF P2Y$_1$ RECEPTORS DECREASES THE SEIZURE-INDUCED MICROGLIOSIS AND NEURODEGENERATION IN THE RAT HIPPOCAMPUS

The same experimental procedure was performed in a second group of rats but this time the ipsilateral hippocampus was fixed through transcardiacal perfusion, 24 hours after the kainate administration, for immunohistochemistry analysis. The activation of microglia was evaluated through immunohistochemistry for CD11b, a constitutive protein of microglia that is up-regulated upon the activation of these cells, being a marker of microgliosis (Rotshenker, 2009). The immunoreactivity of CD11b was quantified in a series of 8 coronal sections (30 μm thick and 300 μm apart) representative of the ipsilateral hippocampus, in 4 animals of each of the 6 experimental groups. The mean fluorescence intensity (MFI) was used as a measure of the CD11b immunoreactivity and was quantified using the program Image J. The values of MFI obtained for the control group (ACSF/SAL) were averaged and all of the MFI values were calculated as a percentage of that value.

As shown in the panels A-C of the Figure 4 and in the graphs of the panel D, 24 hours after the systemic administration of kainate there is an obvious microgliosis in all hippocampal subregions (CA1; CA3 and dentate gyrus, DG), which is significantly prevented in the CA1 subregion by the previous icv administration of MRS2500 (137.9 ± 32.3 % of control for the MRS/KA group comparing with 281.9 ± 32.6 % of control for the ACSF/KA group; n=4; p<0.01), in the CA3 subregion by the administration of both MRS2500 and PPADS (108.1 ± 34.6 % of control for the MRS/KA group and 138.0 ± 38.1 % of control for the PPADS/KA group comparing with 291.8 ± 65.5 % of control for the ACSF/KA group; n= 4; p<0.01 and p<0.05, respectively) and in the DG subregion by the MRS2500 alone (109.2 ± 23.2 % of control for the MRS/KA group comparing with 217.8 ± 23.7 % of control for the ACSF/KA group; n=4; p<0.05).

A

CD11b (green) ; DAPI (blue)

B

CD11b (green) ; DAPI (blue)

C

CD11b (green) ; DAPI (blue)
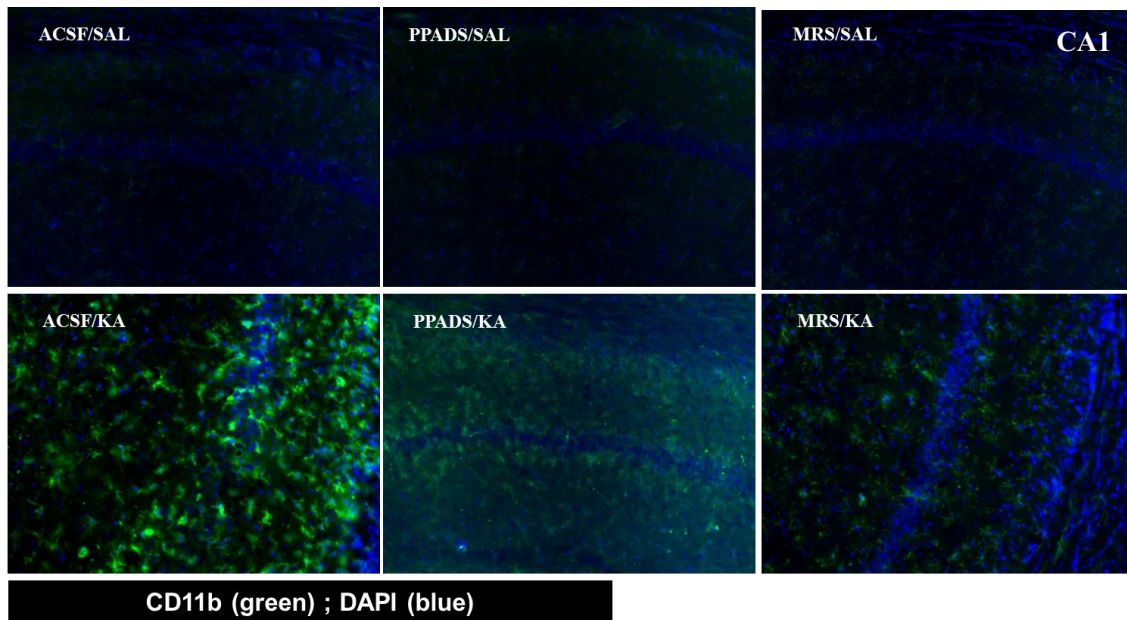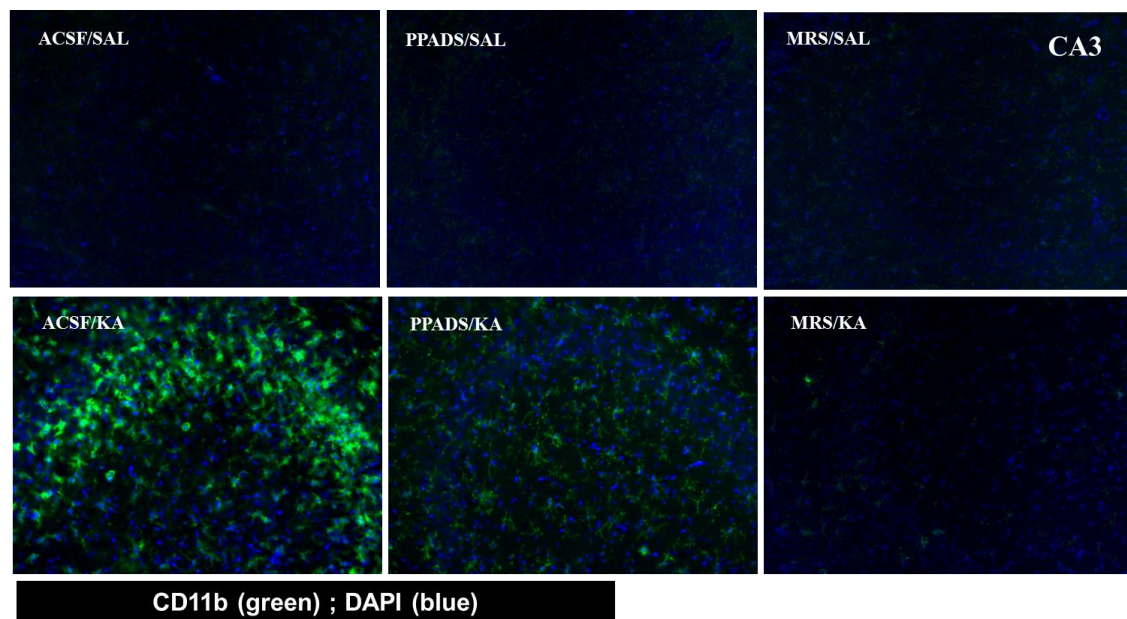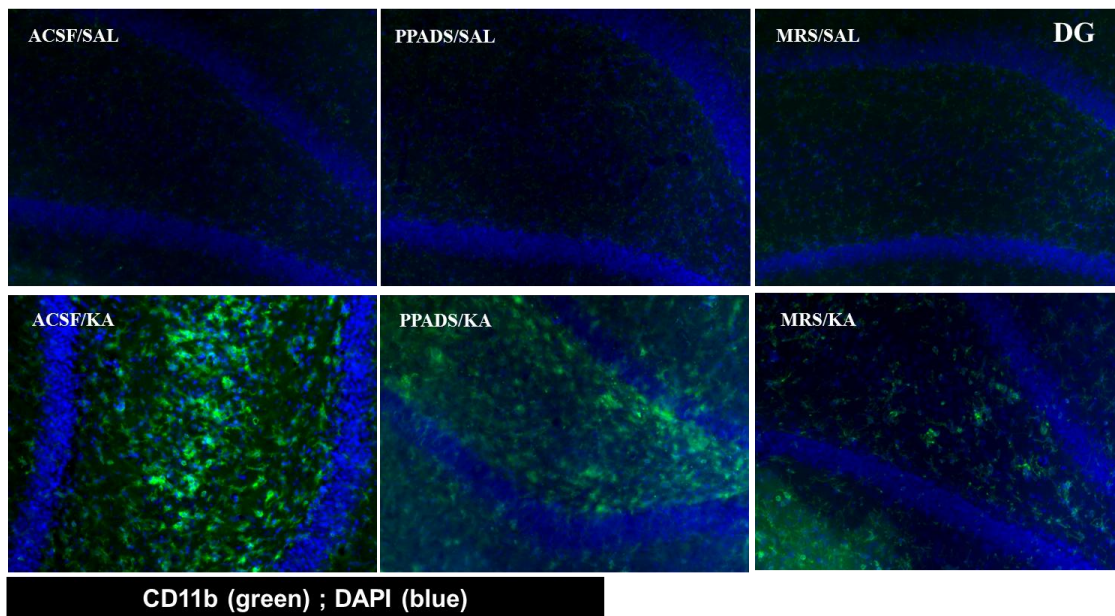
D
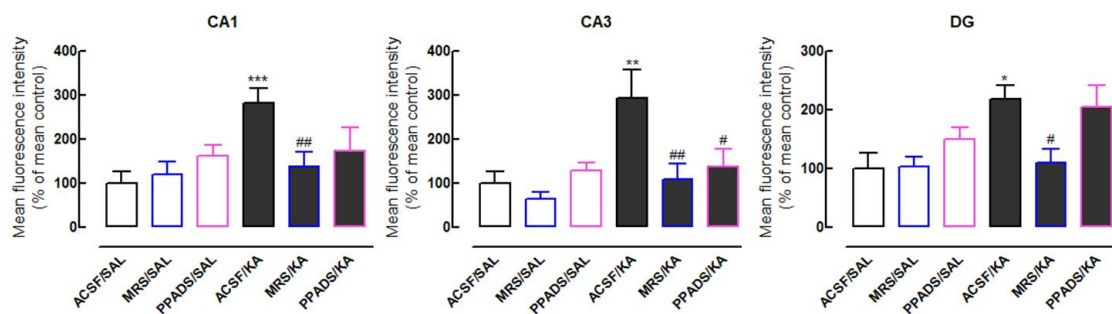
FIGURE 4: Blockade of P2Y$_1$ receptors prevents the microgliosis induced by seizures in all subregions of the rat hippocampus. Wistar male rats were cannulated at the lateral ventricle to allow the intracerebroventricular (icv) administration of drugs. After recovering from the surgery, the animals received a 10 mg/kg intraperitoneal (ip) injection of kainate (KA) or of saline (SAL), 15 minutes upon receiving PPADS, MRS2500 or artificial cerebrospinal fluid (ACSF) icv. Thus, the animals were grouped as follows: the control group or ACSF/SAL (ip and icv administration of ACSF); the kainate group, ACSF/KA (icv administration of ACSF and ip injection of KA); the PPADS/SAL group (icv administration of 1 nmol/4 μL PPADS and ip injection of ACSF); the MRS/SAL group (icv administration of 1 nmol/4 μL MRS2500 and ip injection of ACSF); the PPADS/KA group (icv administration of 1 nmol/4μL PPADS and ip injection of 10 mg/kg KA) and finally the MRS/KA group (1 nmol/ 4μL MRS2500 icv and ip injection of 10 mg/kg KA). Animals were sacrificed 24 hours after the KA administration through transcardiacal perfusion of 4% paraformaldehyde (prepared in saline) to fixe the brain. Coronal hippocampal sections of the hippocampus were then obtained and used for immunohistochemistry analysis of the ipsilateral hippocampus probed for the microglia marker, CD11b (green fluorescence). The immunoreactivity of CD11b was quantified in a series of 8 coronal sections (30 μm thick and 300 μm apart) representative of the ipsilateral hippocampus in 4 animals of each of the 6 experimental groups. The panel D shows the quantification of the mean fluorescence intensity (MFI), used as a measure of the CD11b immunoreactivity, that was calculated using the program Image J. The values of MFI obtained for the control group (ACSF/SAL) were averaged and all of the MFI values were calculated as a percentage of that mean. The results are presented as percentage of

averaged control $\pm$ SEM (n= 4); *$p<0.05$, **$p<0.01$ and ***$p<0.001$ comparing with the control; #$p<0.05$ and ##$p<0.01$ comparing with the ACSF/KA group.

Next, it was investigated whether the blockade of P2 or of P2Y$_1$ receptors could prevent the seizure-induced neurodegeneration through the staining of dead cells with FluoroJade C. As shown in the representative pictures of Figure 5 (A-C), while neither PPADS nor MRS2500 completely prevented the the kainate-induced neurodegeneration in the hippocampus, 24 hours after the ip injection of kainate, both attenuated it and especially in the CA1 subregion (Fig. 5A).
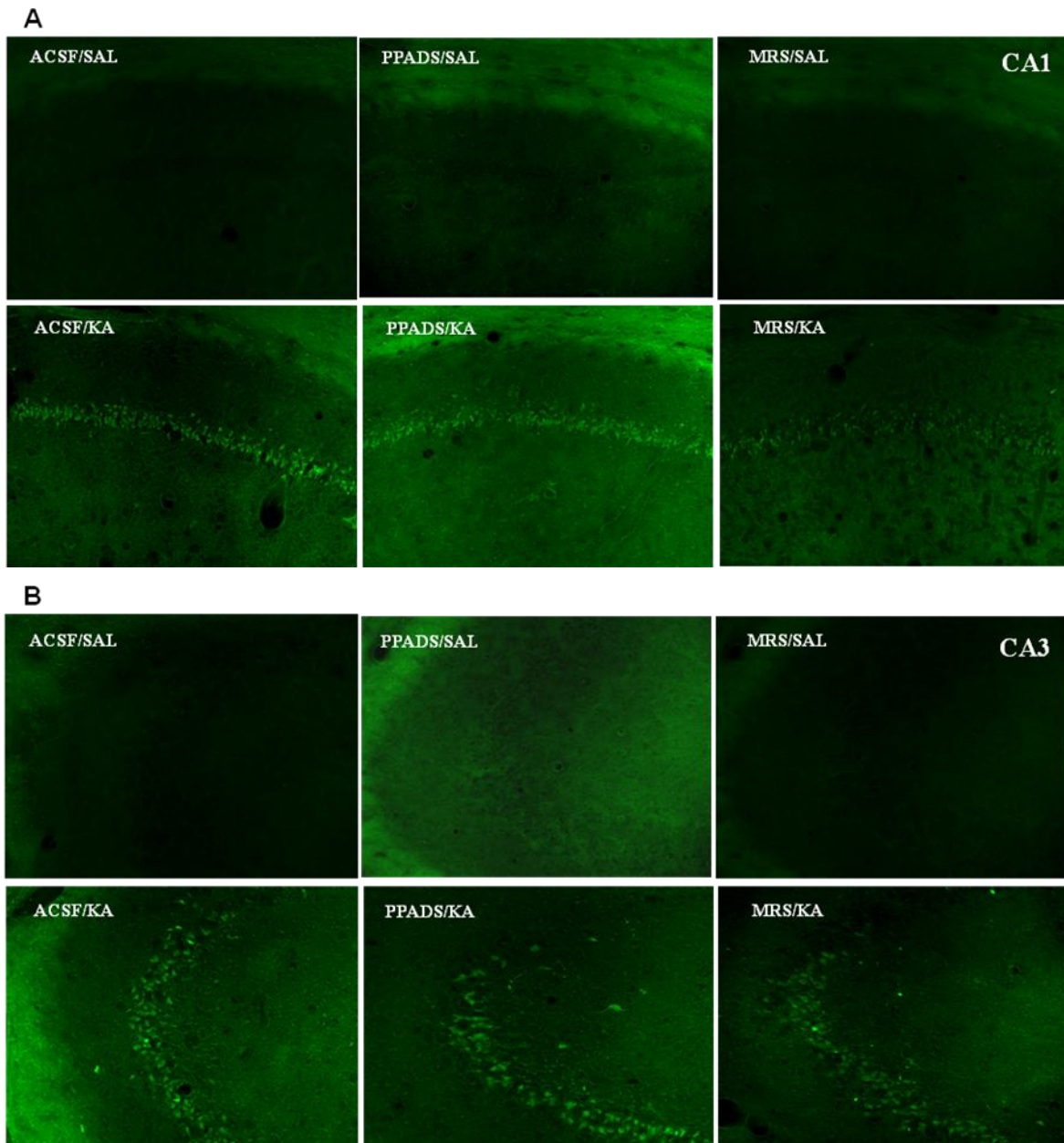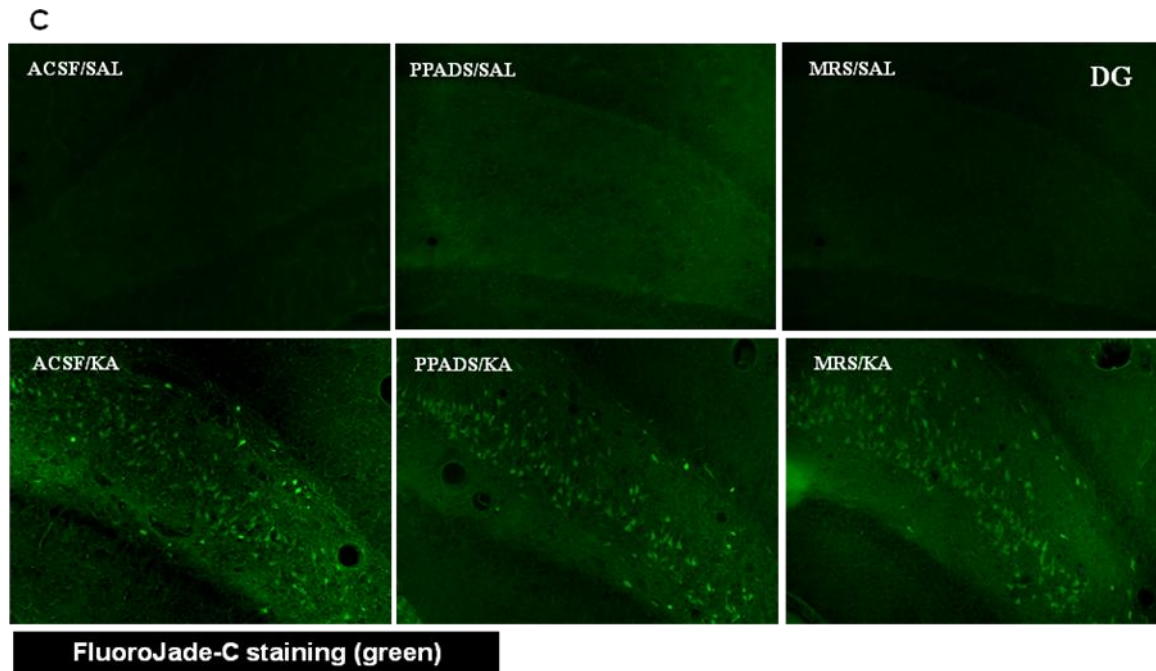
FIGURE 5: Blockade of P2 and of P2Y$_1$ receptors attenuates the neurodegeneration induced by seizures in the rat hippocampus. Wistar male rats were cannulated at the lateral ventricle to allow the intracerebroventricular (icv) administration of drugs. After recovering from the surgery, the animals received a 10 mg/kg intraperitoneal (ip) injection of kainate (KA) or of saline (SAL), 15 minutes upon receiving PPADS, MRS2500 or artificial cerebrospinal fluid (ACSF) icv. Thus, the animals were grouped as follows: the control group or ACSF/SAL (ip and icv administration of ACSF); the kainate group, ACSF/KA (icv administration of ACSF and ip injection of KA); the PPADS/SAL group (icv administration of 1 nmol/4 μL PPADS and ip injection of ACSF); the MRS/SAL group (icv administration of 1 nmol/4 μL MRS2500 and ip injection of ACSF); the PPADS/KA group (icv administration of 1 nmol/4μL PPADS and ip injection of 10 mg/kg KA) and finally the MRS/KA group (1 nmol/ 4μL MRS2500 icv and ip injection of 10 mg/kg KA). The animals were sacrificed 24 hours after the KA injection through transcardiacal perfusion of 4% paraformaldehyde (prepared in saline) to fix the brain. Coronal hippocampal sections were then obtained and used for FluoroJade C staining of dead cells in the ipsilateral hippocampi (bright, green fluorescence). The FluoroJade C staining was performed in a series of 8 coronal sections (30 μm thick and 300 μm apart), representative of the hippocampus, for each of a total of 4 animals from all of the experimental groups. The panels show representative images obtained for the indicated experimental groups, of the CA1 (A), CA3 (B) and DG (C) subregions.

4.5.3 DISCUSSION

In the present work, it was shown that the density of P2 receptors at hippocampal membranes (glial and synaptic) was greatly affected by the seizures resultant from the systemic administration of kainate. Previous works have already hinted at the involvement of ATP and P2 receptors in epilepsy: an early study showed that seizure prone mice had increased extracellular ATP levels, owing in part to increased neuronal firing and to decreased activity of the brain ectonucleotidases (Wieraszko *et al.*, 1989) and more recent works supported the involvement of P2 receptors in the pathophysiology of seizures (Vianna *et al.*, 2002; Rappold *et al.*, 2006; Avignone *et al.*, 2008; Dona *et al.*, 2009; Kim *et al.*, 2009). The present results showed that the altered density of P2 receptors lasted, in some cases, until one month after the kainate injection but was mainly evident at 24 hours, where most of them were up-regulated both in synaptosomes and in total membranes. Moreover, at this endpoint there is an evident microgliosis and neurodegeneration in all of the hippocampal subregions which were prevented and attenuated, respectively, by the blockade of P2 receptors and, especially, by the blockade of $P2Y_1$ receptors alone. The role of $P2Y_1$ receptors in microglia is still largely unknown even though recent studies have suggested an involvement of these receptors in the migration of microglia (De Simone *et al.*, 2010; Lambert *et al.*, 2010). Despite the fact that glial cells are endowed with both P2X and P2Y receptors (James and Butt, 2002; Inoue, 2008; Di Virgilio *et al.*, 2009) the last ones seem to have a prominent role in these cells, as was shown in the optic nerve glia where high levels of extracellular ATP induced large and prolonged P2Y-mediated increases in the $[Ca^{2+}]_i$ (James and Butt, 2001). Additionally, a crutial role for P2 receptors in the pathophysiology of microglia has been recently unravelled (Davalos *et al.*, 2005) and the $P2Y_6$ and the $P2Y_{12}$ receptors were shown to modulate microglia phagocytosis and directional motility (chemotaxis), respectively (Haynes *et al.*, 2006; Koizumi *et al.*, 2007). Notwithstanding, the $P2X_7$ receptors have a well defined role in the maturation and production of the pro-inflammatory cytokine interleukin-1β from microglia (Narcisse *et al.*, 2005) and were shown to induce the production of the superoxide radical, associated with the cytotoxic phenotype of these cells (Parvathenani *et al.*, 2003). The $P2X_4$ receptors were also shown to be up-regulated in the activated microglia and to play a part in the inflammation-mediated neuropathic pain (Inoue, 2006), contributing to the rapid ATP-mediated Ca^{2+} response in these cells (Light *et al.*, 2006).

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Thus, the prevention of kainate-induced microgliosis with the general blockade of P2 receptors is not surprising. Nevertheless, in the present work, a previously unknown role for $P2Y_1$ receptors in the control of neuroinflammation is suggested since the blockade of these receptors was even more effective at preventing the kainate-induced effects in microglia. However, this control operated by $P2Y_1$ receptors may also be indirect since these receptors hold important functions both at glutamatergic (Luthardt *et al.*, 2003; Guzman *et al.*, 2005; Rodrigues *et al.*, 2005b) and GABAergic synapses (Kawamura *et al.*, 2004; Safiulina *et al.*, 2006), which are highly compromised during epileptic activity. In fact, the present results also show a prevention of the kainate-induced loss of synaptic markers in hippocampal membranes, including markers of GABAergic and glutamatergic terminals, with the pharmacological ablation of $P2Y_1$-mediated signalling. This is particularly importante when considering the growing evidence that many neurological disorders begin with a synaptotoxicity before progressing to neurodegeneration (Coleman *et al.*, 2004; Canas *et al.*, 2009). Additionally, $P2Y_1$ receptors have a prominent role in the physiology of astrocytes, namely controlling the astrocytic calcium waves (Fam *et al.*, 2003; Gallagher and Salter, 2003) and the glutamate release from these cells (Domercq *et al.*, 2006). This is especially relevant under pathological conditions since the astrocytic Ca^{2+} waves increase with neuronal firing (Araque *et al.*, 2002) and the glutamate released from astrocytes modulates synaptic transmission (Parpura and Haydon, 2000). In fact, an impairment in the astrocytic Ca^{2+} wave is regarded as a putative initial event in epileptic seizures, leading to the characteristic hypersynchronous neuronal firing (Tian *et al.*, 2005; Kumaria *et al.*, 2008).

In conclusion, the results obtained support a role for $P2Y_1$ receptors in the deleterious effects induced by epileptic seizures in the hippocampus and the view of these receptors as possible targets for the development of new neuroprotective drugs.

4.6 NEUROPROTECTION AFFORDED BY P2Y$_1$ RECEPTORS BLOCKADE DURING *IN VIVO* ISCHAEMIA

4.6.1 INTRODUCTION

Ischaemia is defined by a reduction of cerebral blood flow which prevents the affected tissue of the normal supply of oxygen and nutrients, leading to cell death in the affected area (Braeuninger and Kleinschnitz, 2009). The area of stroke is designated the infarction area and can be highlighted as a white region on brain slices stained with 2,3,5-Triphenyltetrazolium chloride (TTC) (Sicard and Fisher, 2009). The central area immediately affected is called the core of the infarct and the ischemic region around the core is called the ischemic penumbra (Braeuninger and Kleinschnitz, 2009). The process of cell degeneration within the affected area is a snow ball of deleterious events: deprivation of oxygen and glucose results in accumulation of acidic byproducts of the metabolism. The loss of substrate and decrease in pH levels leads to cessation of the electron transport chain activity within the mitochondria, resulting in a rapid decline in ATP concentration (Smith, 2004). This, in turn, leads to failure of the K^+/Na^+-ATPase, increase in Na^+ concentration and cell depolarization, resulting in increased Ca^{2+} entry and overexcitation (Smith, 2004; Sicard and Fisher, 2009). Neurons inside the core infarct area fire repeatedly, releasing their transmitters locally and at distant targets. At the core of the infarct area there is a greater depletion of ATP and cells die essentially by necrosis, whilst in the penumbra region apoptotic cell death is more common (Smith, 2004). Thus, the ATP accumulated in the extracellular milieu plays a part in the pathophysiologic events resultant from an ischemic insult (Burnstock and Kennedy, 2011). The outflow of ATP during ischemic conditions has been observed both in hippocampal slices (Juranyi *et al.*, 1999; Frenguelli *et al.*, 2007) and *in vivo* (Melani *et al.*, 2005). In addition, the involvement of P2 receptors in the deleterious consequences of ischemia was demonstrated in brain slices, organotypical cultures (Cavaliere *et al.*, 2003; Runden-Pran *et al.*, 2005) and in animal models of ischemia (Kharlamov *et al.*, 2002). A time-dependent up-regulation of P2X$_7$ receptors was found in both glia and neurons (Franke *et al.*, 2004) and P2 receptor antagonists like reactive blue 2 (Melani *et al.*, 2006a) and PPADS (Lammer *et al.*, 2006) were shown to improve the neurological deficit and to reduce the damage induced by focal ischemia. However, in most works the attention has relayed on P2X receptors (Cavaliere *et al.*, 2007; Sperlagh *et al.*, 2007; Milius *et al.*, 2008) while the involvement of P2Y receptors in ischemic damage is still poorly understood.

Therefore, in the following work, it was tested the neuroprotective potential of P2Y$_1$ receptors blockade in a mouse model of permanent focal ischemia as compared to the general antagonism of P2 receptors.

4.6.2 RESULTS

4.6.2.1 BLOCKADE OF P2 RECEPTORS OR OF P2Y$_1$ RECEPTORS PROTECTS AGAINST ISCHAEMIA-INDUCED INJURY

In the present work, distal occlusion of the medial cerebral artery (MCAO) was performed in Swiss male, adult mice via a transorbital approach. The dura was removed and a segment of the artery was exposed and electrocoagulated, cut just distal to the inferior cerebral vein as described in (Marks *et al.*, 2001). Previous to MCAO (10 minutes before) the animals were placed in the stereotaxic under anesthesia for the intracerebroventricular administration of drugs or saline. The mice were organized in the following experimental groups: the Sham-operated group (SO + V, in which the animals received saline icv and then received the same procedure as the operated animals except for the cauterizing of the MCA); the MCAO group (MCAO + V, in which mice received saline icv and underwent MCAO 10 minutes after); the PPADS group (PPADS + MCAO, mice that received different doses of PPADS, 0.5 nmol/ μl and 1 nmol/μl, icv, 10 minutes before MCAO) and the MRS2500 group (MRS2500 + MCAO, mice that received 1nmol/ μl MRS2500, icv, 10 minutes before MCAO). The highest dose of PPADS (1 nmol/ μl, icv) was also tested in the Sham-operated animals (SO + PPADS). The ischaemia outcome was investigated 24 hours after MCAO, through the evaluation of motor and mnemonic skills and quantification of the ischaemia-induced infarct volume.

As shown in the image of Figure 1, the ischemia induced by MCAO resulted in tissue damage involving the cortical peri-infarct area of coronal brain sections affecting the frontoparietal cortex and the hippocampus as was previously described (Franke *et al.*, 2004; Gunther *et al.*, 2005). Also in Figure 1, it can be observed that the sham-operated (SO) animals that received saline (V) icv or the non-selective antagonist of P2 receptors, PPADS (1 nmol in 1 μl, icv) before the surgical procedures did not present any ischemic-like lesion. The treatment with either PPADS (0.5 or 1 nmol/ 1μl) or with the selective P2Y$_1$ receptor antagonist MRS2500 (1 nmol/1μl, icv), 15 min before MCAO, greatly reduced the ischemic-induced lesion, revealed as a white region in the TTC coloured brain slices shown in the picture of Figure 1. The infarct volume induced by MCAO was of 6.8 ± 1.0 mm^3 (MCAO + V group) which was significantly different (p<0.001) from the values obtained with the SO + V group (0.9 ± 0.1

mm^3) and with the SO + PPADS 1.0 group (1.2 ± 0.1 mm^3) (Fig. 1). Previous icv administration of 0.5 nmol of PPADS (MCAO + PPADS 0.5 group) significantly reduced the lesion induced by MCAO to 2.2 ± 0.3 mm^3 (p<0.001) as well as the higher dose of 1 nmol PPADS (MCAO + PPADS 1.0 group) which reduced the infarct volume to 1.9 ± 0.2 mm^3 (p<0.001) (Fig.1). The antagonism of P2Y$_1$ receptors alone, with MRS2500 (1 nmol), was equally effective in reducing the ischemic lesion (2.3 ± 0.3 mm^3; p<0.001 comparing with the MCAO + V group) (Fig. 1).
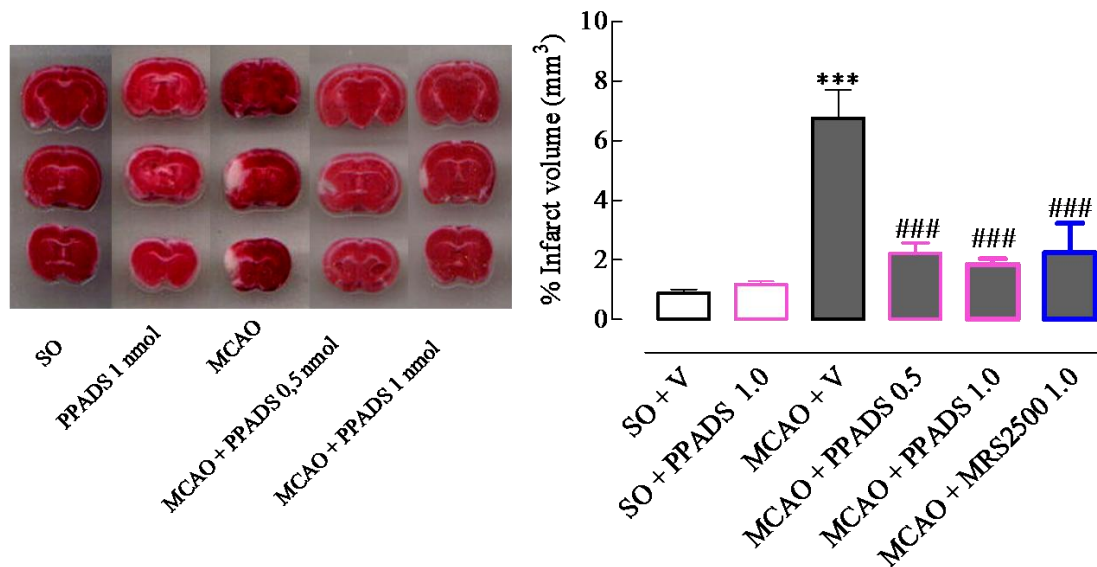
FIGURE 1: Blockade of P2 receptors or of P2Y$_1$ receptors significantly reduces the ischemia-induced infarct area. Swiss male mice (2 months old, weighting between 30-40g) received saline, the non-selective antagonist of P2 receptors PPADS (0.5 nmol or 1 nmol in 1µL) or the selective antagonist of P2Y$_1$ receptors MRS2500 (1 nmol in 1µL), icv, 10 min before middle cerebral artery occlusion (MCAO). The SO (sham-operated) + V (vehicle) is the control group that received saline icv and were operated without the occlusion of the middle cerebral artery (MCA). After 24 hours, animals were sacrificed and their brains were isolated and sliced using a mouse brain slicer matrix (0.5 mm coronal slice intervals). The infarct area was revealed by submersing the brain sections in a 2% in normal saline 2,3,5-Triphenyltetrazolium chloride (TTC) solution at 37ºC during 20 minutes, in the dark. The slices were washed twice in saline solution and fixed in 4% paraformaldehyde for 30 min at room temperature. Slices were again passed twice by saline solution and, afterwards, both sides of sections were scanned on a flatbed colour scanner. The colorless TTC is reduced to the red formazan product by dehydrogenases. The stain intensity correlates positively with the number and functionality of mitochondrias. The infarct volume is presented as a white region in the slice and was calculated integrating the infarct areas of the scanned images corresponding to the whole series of brain slices. Results are presented as a percentage of the total volume of the contralateral hemisphere and are described as mean ± SEM; n= 6 animals *per* group. ***p<0.001 comparing to the SO + V group and ###p<0.001 comparing with the MCAO + V group. Note that both doses of PPADS and the MRS2500 were effective at preventing MCAO-induced ischemic brain lesion.

4.6.2.2 BLOCKADE OF P2 RECEPTORS PREVENTS ISCHAEMIA-INDUCED MOTOR AND SPATIAL MEMORY DEFICITS

A second group of animals was sacrificed three days after MCAO, when the ischemia-induced neurodegeneration is prominent. At this endpoint mice presented significant deficits in motor skills, as evaluated by counting the number of crossings (MCAO + V, 69 ± 7 comparing to 96 ± 8 of the SO + V group; n= 8; $p<0.05$) and rearings (MCAO + V, 10 ± 2 comparing to 20 ± 2 of the SO + V group; n= 8; $p<0.01$) in an open field (Fig. 2). Blockade of P2 receptors with PPADS significantly prevented the motor deficits, in a dose-dependent manner: the number of crossings of animals that received 0.5 nmol of PPADS, icv, before MCAO were 90 ± 4 (n= 8; $p<0.05$ comparing to the MCAO + V group) and the number of crossing of the animals that received 1 nmol of PPADS were of 106 ± 9 (n= 8; $p<0.01$ comparing to the MCAO + V group) (Fig. 2). In terms of rearings, the PPADS 0.5 nmol + MCAO group had a better scoring (27 ± 3) comparing to the MCAO + V group (10 ± 2; n= 8; $p<0.001$) and the same was observed with the PPADS 1 nmol + MCAO group (21 ± 4; n= 8; $p<0.05$). Moreover, the highest dose of PPADS had no effect *per se* in the performance of the animals (SO + PPADS 1.0 ; 90 ± 12 crossings and 21 ± 3 rearings) (Fig 2).

FIGURE 2: Blockade of P2 receptors prevents ischemia-induced locomotor deficits. Swiss male mice (2 months old, weighting between 30-40g) received saline or the non-selective antagonist of P2 receptors, PPADS (0.5 nmol or 1 nmol in 1μL), icv, 10 min before middle cerebral artery occlusion (MCAO). The SO (sham-operated) + V (vehicle) is the control group that received saline icv and were operated, 10 min later, without the occlusion of the middle cerebral artery. After 3 days, locomotor performance was evaluated in an open field and the number of crossings and rearings were counted. The results are presented as mean ± SEM values; n= 8. *p<0.05 and **p<0.01 comparing to the SO + V group and #p<0.05, ##p<0.01 or ###p<0.001 comparing with the MCAO + V group. Note that both doses of PPADS prevented MCAO-induced locomotor deficits.

4.6.2.3 BLOCKADE OF P2 RECEPTORS PREVENTS ISCHAEMIA-INDUCED SPATIAL MEMORY DEFICITS

Since the MCAO-induced lesion affects the cortical areas of the striatum and of the hippocampus, it likely affects spatial working memory (Hodges *et al.*, 1997; Block, 1999). the effects of ischemia and of the PPADS treatment before MCAO in the spatial working memory of mice were evaluated 3 days after MCAO, as the percentage of alternation in a Y-maze. As shown in Figure 3, mice with MCAO (MCAO + V) performed significantly worse ($57 \pm 3\%$ of alternation; $p<0.01$; n= 8) than the sham-operated mice (SO + V; $74 \pm 2\%$ of alternation; $p<0.01$; n= 8). Both doses of PPADS prevented this effect (0.5 nmol PPADS: $77 \pm 2\%$ of alternation; $p<0.01$ comparing with MCAO + V; n= 8; 1 nmol PPADS: $73 \pm 4\%$ of alternation; $p<0.01$ comparing with MCAO + V; n= 8) and had no effect per se in the animal's performance (SO + PPADS 1nmol: $71 \pm 5\%$ of alternation; n= 8) (Fig. 3).
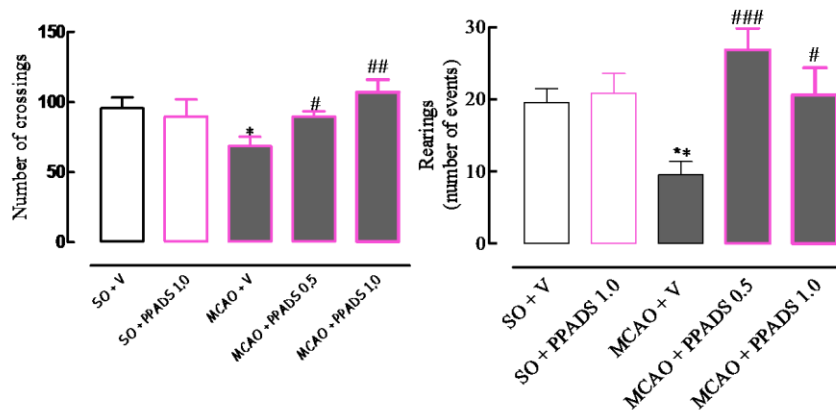

FIGURE 3: Blockade of P2 receptors prevents ischemia-induced spatial working memory deficits. Swiss male mice (2 months old, weighting between 30-40g) received saline or the non-selective antagonist of P2 receptors PPADS (0.5 nmol or 1 nmol in 1μL), icv, 10 min before middle cerebral artery occlusion (MCAO). The SO (sham-operated) + V (vehicle) is the control group that received saline icv and were operated, 10 min later, without middle cerebral artery occlusion. After 3 days, the ability of animals to alternate in a Y-maze was evaluated. Both doses of PPADS prevented MCAO-induced decrease of alternation in the Y-maze. The percentage of alternation was calculated according to the formula: total of alternations / (Total arm entries – 2) X 100 and the results are presented as mean $\pm$ SEM; n= 8. **$p<0.01$ comparing to the SO + V group and ##$p<0.01$ comparing with the MCAO + V group.

4.6.2.3.1 BLOCKADE OF P2 RECEPTORS PREVENTS ISCHEMIA-INDUCED AVERSIVE MEMORY DEFICITS

The MCAO effect on the aversive memory of mice was also tested 3 days after the surgery. As shown in the Figure 4, the group MCAO + V performed poorly in a passive avoidance apparatus, a test commonly used to evaluate aversive memory in rodents. Animals were first habituated to the passive avoidance box, being allowed to explore for 1 minute. Thirty seconds after this exploring period, mice were returned to the box where they received a 0.5 mA shock for 1 s, as soon as they entered the dark compartment. The recent aversive memory of the animals was evaluated by recording the latency time to enter this compartment, fifteen minutes after the shock. The same was done at twenty four hours after the shock to evaluate long-term memory or retention (late aversive memory), allowing a maximum latency time of 300 s. The MCAO + V group had significant worst results in early aversive memory, i.e., 15 min after the electric shock most animals did not remember it and took less time to step-down to the dark compartment of the box than the SO + V group (97.8 ± 26.9 s and 237.5 ± 29.6 s, respectively; $p<0.01$; n= 8) (Fig. 4). The PPADS effect was dose-dependent and significantly improved the performances (MCAO + PPADS 0.5 nmol, 240.8 ± 29.6 s and MCAO + PPADS 1nmol, 299.7 ± 0.29 s; n= 8; $p<0.01$ and $p<0.001$, respectively, comparing to the MCAO + V group) (Fig. 4). The sham-operated animals that received PPADS did not display a different behavior from the sham-operated animals that were administered saline (SO + PPADS 1nmol: 203.2 ± 35.2 s and SO + V: 237.0 ± 29.6 s, respectively) (Fig. 4). In terms of long-term or late aversive memory, tested 24 hours after the electric shock, the MCAO + V group also performed significantly worst than the SO + V group (92.5 ± 24.9 s comparing to 235.0 ± 33.3 s, respectively, $p<0.01$; n= 8) (Fig. 4). In this case, only the highest dose of PPADS prevented the MCAO effect (MCAO + PPADS 1 nmol: 219.6 ± 41.4 s; $p<0.05$; n= 8) even though the 0.5 nmol dose also tended to improve the animal's performance (MCAO + PPADS 0.5 nmol: 175.0 ± 34.6 s; n= 8) (Fig. 4).
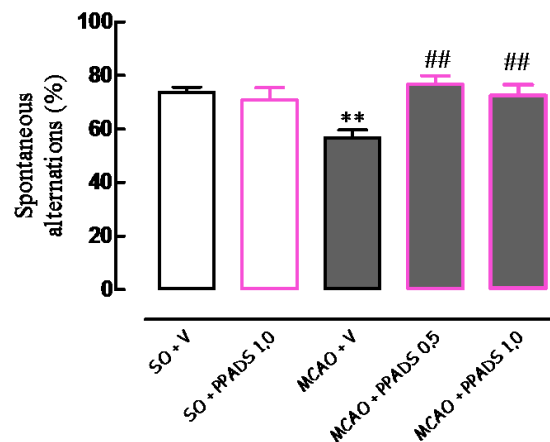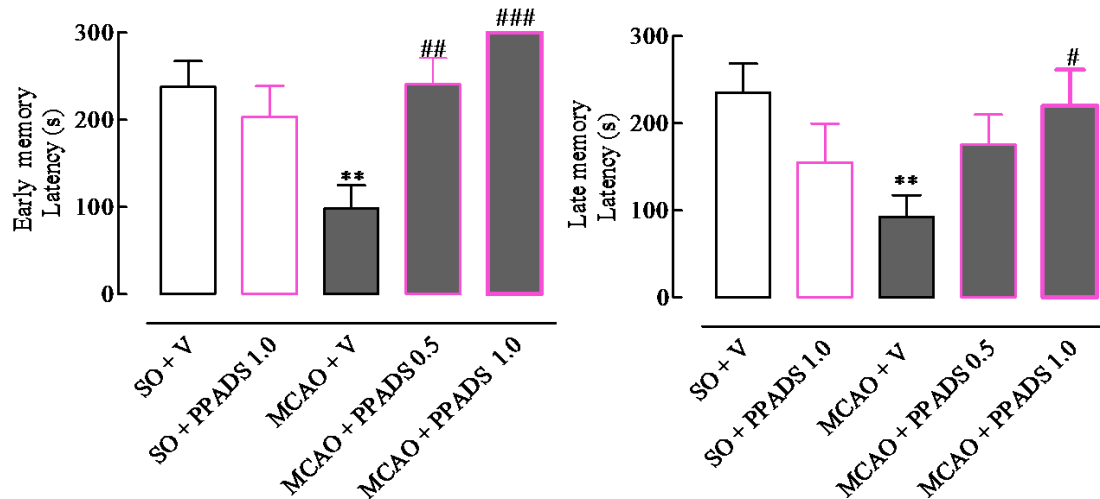
FIGURE 4: Blockade of P2 receptors prevents ischemia-induced early and late aversive memory deficits. Swiss male mice (2 months old, weighting between 30-40g) received saline or the non-selective P2 receptors antagonist PPADS (0.5 nmol or 1 nmol in 1μL, non-selective P2R antagonist), icv, 10 min before the middle cerebral artery occlusion (MCAO). The SO (sham-operated) + V (vehicle) is the control group that received saline icv and were operated, 10 min later, without middle cerebral artery occlusion. After 3 days, the ability of the mice to remember an aversive event (electric shock of 0.5 mA during 1 s) was tested on a passive avoidance box. Fifteen minutes after the shock, the animals were placed on the platform compartment and the time taken for them to step down to the dark compartment where the shock took place (latency) was measured (early memory). The same was done 24 hours after the shock for the evaluation of late memory. The results are expressed as mean ± SEM; n= 8. **$p<0.01$ comparing to the SO + V group and #$p<0.05$, ##$p<0.01$ or ###$p<0.001$ comparing with the MCAO + V group. Note that MCAO resulted in decreased early and late memory performances which were both prevented by PPADS in a dose-dependent manner.

4.6.2.4 BLOCKADE OF P2Y$_1$ RECEPTORS PREVENTS ISCHAEMIA-INDUCED NEUROLOGIC AND MOTOR DEFICITS

The specific blockade of P2Y$_1$ receptors with the selective antagonist MRS2500 (1 nmol in 1μl, icv), 10 min before the MCAO, significantly prevented the ischemia-induced neurological deficits, 24 hours after, evaluated as described in (Hunter *et al.*, 2000). The neurological scoring for the control group, SO + V, was of 18 ± 0.2 scores and for the MCAO + V group was of 15 ± 1.0 scores ($p<0.05$; n= 8). Blockade of P2Y$_1$ receptors effectively prevented this effect of the MCAO (17 ± 0.5 scores, $p<0.01$ comparing to MCAO + V; n= 8). Three days after the MCAO-induced ischemia, the MCAO + V group also presented locomotor deficits comparing with the SO + V group, as evaluated by the number of crossings in an open field (86 ± 12 vs 120 ±7, respectively, $p<0.05$; n= 8) and MRS2500 prevented this effect (130 ± 8; $p<0.05$ comparing to the MCAO + V group; n= 8) (Fig. 5).
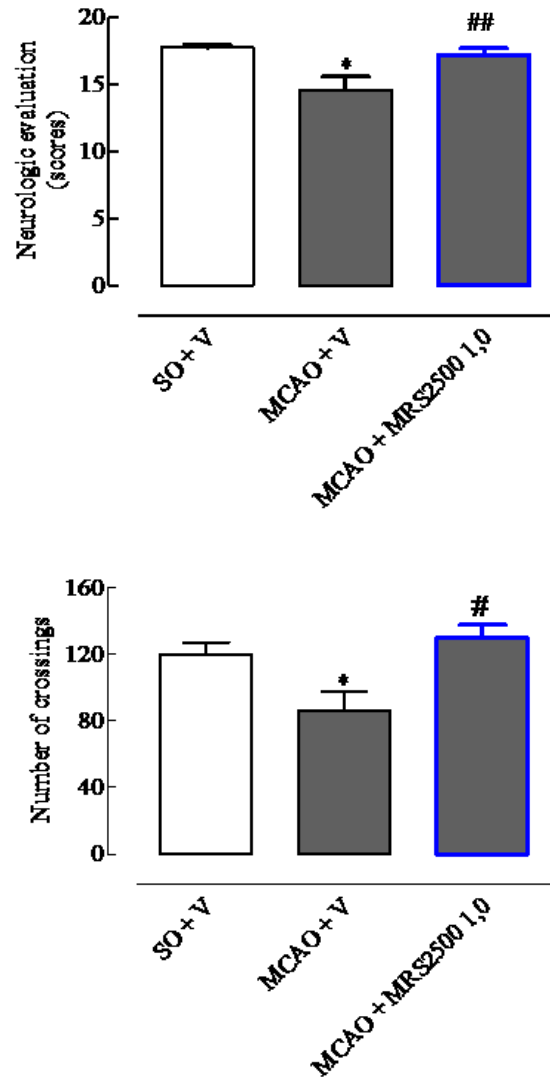
FIGURE 5: Blockade of P2Y$_1$ receptors improves ischemia-induced neurologic scoring and motor deficits. Swiss male mice (2 months old, weighting between 30-40g) received saline or the selective antagonist of P2Y$_1$ receptors MRS2500 (1 nmol in 1μL), icv, 10 min before the middle cerebral artery occlusion (MCAO). The SO (sham-operated) + V (vehicle) is the control group that received saline icv and were operated, 10 min after, without middle cerebral artery occlusion. After 24 hours, neurological scoring was evaluated according to Hunter *et al*., 2000 and the SHIRPA protocol. Three days after ischemia, locomotor performance was evaluated in an open field and the number of crossings was counted. The results are presented as mean ± SEM values; n=8. *p<0.05 comparing with the SO + V group and #p<0.05 comparing with the MCAO + V group. Note that the MRS2500 prevented both the MCAO-induced neurologic and motor deficits.

4.6.2.5 BLOCKADE OF $P2Y_1$ RECEPTORS PREVENTS ISCHEMIA-INDUCED SPATIAL MEMORY DEFICITS

The Morris water maze was used to evaluate the effect of the blockade of $P2Y_1$ receptors on the spatial working memory deficits induced by ischemia. This test was developed by Morris in 1984 (Morris, 1984) and is a useful assay to test spatial memory that depends on the integrity of the hippocampus. The test consists in evaluating the animal's ability to find a platform (placed 1 cm bellow the surface) from visual cues in a room while swimming in a round water pool. The animals were tested in four trials *per* day over three consecutive days and allowed to swim until they reached the escape platform for a maximum of 120 s, after which they were placed on the platform. Spatial memory retention was evaluated 48 hours after the last trial by hiding the platform and allowing the mice to swim for 30 s, recording the time spent on the area where the platform once was. On consecutive days, the MCAO + V group performed significantly worse than the mice of the SO + V group, having spent successively more time to find the platform (Fig. 6). The blockade of $P2Y_1$ receptors (MCAO + MRS2500 group) prevented this effect since the animals performed as well as the non-ischemic mice (Fig. 6, graphic on the left). Moreover, MRS2500 tended to improve memory retention (22.0 ± 2.4 s spent on the platform area) which was affected by the MCAO-induced ischemia (18.9 ± 0.9 s *versus* 25.4 ± 26 s of the SO + V group; n= 8) (Fig. 6, graphic on the right).
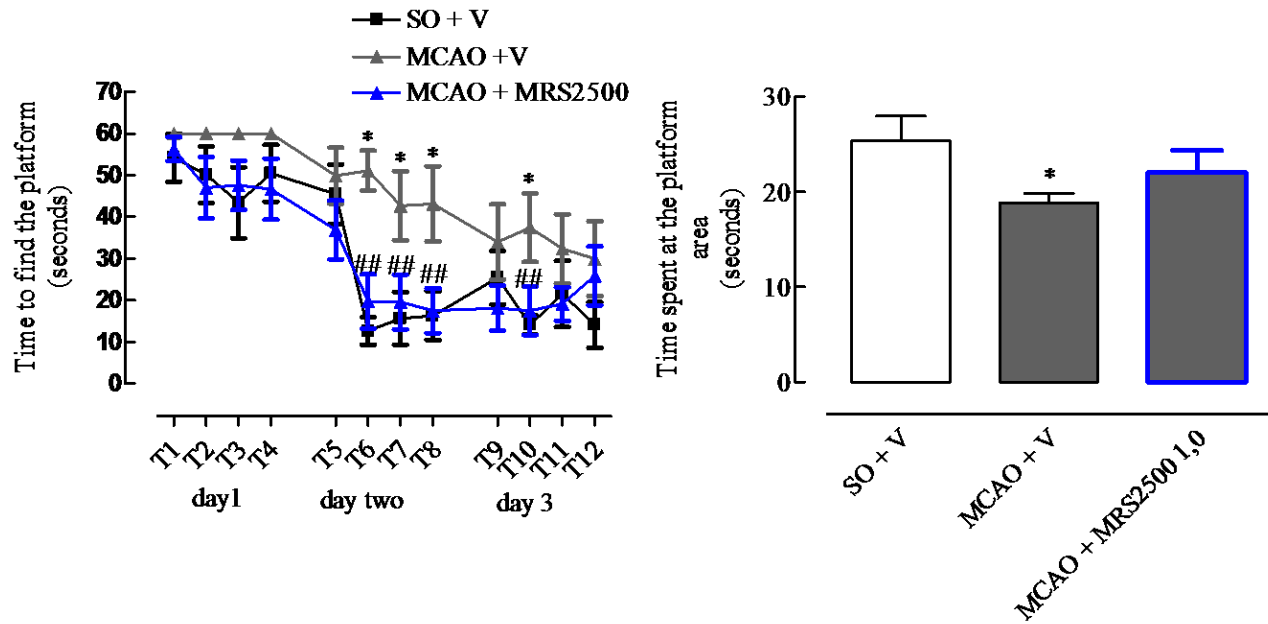
FIGURE 6: Blockade of P2Y$_1$ receptors prevents the ischemia-induced deficits in spatial memory. Swiss male mice (2 months old, weighting between 30-40g) received saline or the selective antagonist MRS2500 (1 nmol in 1μL), icv, 10 min before middle cerebral artery occlusion (MCAO). The SO (sham-operated) + V (vehicle) is the control group that received saline icv and were operated, 10 min after, without middle cerebral artery occlusion. Three days after ischemia, spatial memory performance was evaluated in the Morris water maze by measuring the time to find a platform based on visual cues in a room, during 4 trials on 3 consecutive days (left graph). Memory retention was evaluated 48 hours after the last trial by measuring the time the mice spent on the area of the hidden platform (right graphic). The results are presented as mean ± SEM values; n=8. *p<0.05 comparing to the SO + V group and #p<0.05 comparing with the MCAO + V group. Note that MRS2500 prevented the MCAO-induced spatial memory learning.

4.6.3 DISCUSSION

A role for P2 receptors in the ischemia-induced neurodegeneration and neuroinflammation has been unraveled through *in vitro* studies using superfused brain slices and organotypical cultures, subjected to oxygen and glucose deprivation (Juranyi *et al.*, 1999; Cavaliere *et al.*, 2003; Runden-Pran *et al.*, 2005). However, until recently, the ATP-mediated effects in ischemia models have been mostly attributed to the activation of P2X receptors (Cavaliere *et al.*, 2003; Runden-Pran *et al.*, 2005; Cavaliere *et al.*, 2007; Sperlagh *et al.*, 2007; Milius *et al.*, 2008). Thus, the neuroprotective effects of antagonists of P2 receptors, namely of the non-selective antagonist PPADS, have been argued to occur essentially due to the blockade of these receptors. Even though the involvement of ionotropic P2X receptors, and especially of P2X$_7$ receptors, in mediating excitotoxicity is well described (Apolloni *et al.*, 2009; Skaper *et al.*, 2010), it is also true that P2Y receptors have a prominent role in the patho-physiology of glial cells and in the control of synaptic transmission, as discussed in previous chapters. In particular, the P2Y$_1$ receptors play an important part in many of these mechanisms (Domercq *et al.*,

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2006; Hussl and Boehm, 2006; Di Virgilio *et al.*, 2009). Thus, it was hypothesised that the blockade of these receptors could, therefore, afford a robust neuroprotection against ischemic damage as was found in other disease models. The blockade of P2 receptors, using PPADS, during the *in vivo* MCAO model of ischemia was recently addressed by (Lammer *et al.*, 2006). Here, the authors found that PPADS effectively prevented both morphological and behavioral deficits induced by permanent MCAO. These results also correlate with previous findings using other non-selective antagonist of P2 receptors, suramin (Kharlamov *et al.*, 2002). Moreover, *in vivo* administration of PPADS counteracts the release of glutamate induced by the extracellular ATP (Krugel *et al.*, 2004). To tackle the question of whether the selective blockade of $P2Y_1$ receptors could prevent the ischemic-induced deleterious events in comparison to the general antagonism of P2 receptors, animals were administered either PPADS or MRS2500 (icv) 10 minutes before the MCAO. The choice of PPADS relied on studies showing that among the available P2 receptor antagonists, this one affects a greater number of P2 receptors subtypes ($P2Y_{1,2,4,6}$ receptors; recombinant homomeric $P2X_{1,2,3,5}$ receptors and heteromeric $P2X_{2,3}$ and $P2X_{1/5}$ receptors) (Ralevic and Burnstock, 1998) and, unlike the other antagonists, does not affect glutamate receptors (Tschopl *et al.*, 1992). This late characteristic is particularly important under ischemic conditions since glutamate plays an important part in ischemic-induced excitotoxicity. On the other hand, PPADS also inhibits ecto-nucleotidases resulting in higher extracellular ATP concentrations and less ATP-derived adenosine (Windscheif *et al.*, 1994; Chen *et al.*, 1996), which remains an unsolved problem when dealing with ATP antagonists.

In the present study, both the blockade of P2 receptors in general and the antagonism of $P2Y_1$ receptors alone equally improved the MCAO-induced ischemia outcome in mice, effectively preventing MCAO-induced infarct volume, neurological scores and mnemonic deficits. The results obtained with the blockade of $P2Y_1$ receptors under noxious and generally excitotoxic conditions both *in vitro* and *in vivo*, though promising, seem at odd with the inhibitory role of these receptors under physiological conditions at the synaptic level (Kawamura *et al.*, 2004; Guzman *et al.*, 2005; Rodrigues *et al.*, 2005b) and also with their mediation of cell survival mechanisms in glia (Fujita *et al.*, 2009). However, there is increasing evidence of neuroprotection afforded through the blockade of $P2Y_1$ receptors under ischemic conditions. In this regard, Coppi *et al.* (2007) have recently shown that under normoxia, in hippocampal slices, ATP decreased field excitatory post-synaptic potentials (fEPSP) and population spikes at the CA1 region. This effect was in part mediated by ATP-derived adenosine acting on A_1 receptors and in part by ATP acting on $P2Y_1$ receptors. On the other hand, under ischemic conditions, such as oxygen and glucose deprivation (OGD), the selective blockade of $P2Y_1R$ prevented

OGD-induced appearance of anoxic depression and the consequent irreversible loss of fEPSP. The protective effect of the antagonist of $P2Y_1$ receptors may also pertain to a direct inhibition of intracellular Ca^{2+} in glial cells and consequent reactive gliosis (Troadec *et al.*, 1999; Schipke *et al.*, 2002) and also to the prevention of $P2Y_1$ receptors-mediated release of glutamate from astrocytes (Domercq *et al.*, 2006; Jourdain *et al.*, 2007).

In conclusion, the present work further encourages the view of P2 receptors and specifically of $P2Y_1$ receptors as possible new targets for neuroprotection under excitotoxic and pro-inflammatory conditions.

CONCLUDING REMARKS

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The goal of the work presented in this thesis was to explore the control of neuroinflammation by adenosine A_{2A} receptors and the neuroprotection afforded with the blockade of ATP $P2Y_1$ receptors under deleterious conditions in the hippocampus.

The purinergic system controls most cellular activity and communication in the brain (Burnstock, 2006; Burnstock, 2009b). During noxious conditions, the extracellular concentration of ATP dramatically increases due to a combination of events such as metabolic failure, increased neuronal firing and cell degeneration (Melani *et al.*, 2005; Franke and Illes, 2006). Once in the extracellular space, ATP rapidly originates adenosine (which can also be released *per se* from cells) due to the catabolic activity of ectonucleotidases (Cunha *et al.*, 1998; Zimmermann, 2000; Zimmermann, 2006). Being a more stable molecule, adenosine is a recognizable major neuromodulator (Sebastiao and Ribeiro, 2000; Cunha, 2001), mostly through the activation of A_1 or of A_{2A} receptors, which are the most abundant adenosine receptors in the brain (Cunha, 2005). The excitatory activity mediated by the activation of A_{2A} receptors is particularly relevant upon noxious conditions, since both adenosine and the density of these receptors are increased (Cunha *et al.*, 2006; Duarte *et al.*, 2006; Costenla *et al.*, 2011). Accordingly, the blockade of A_{2A} receptors is neuroprotective in such situations (Cunha, 2005; Gomes *et al.*, 2011) and particularly *in vivo*, in animal models of brain disorders such as Alzheimer's disease (Canas *et al.*, 2009), ischaemia (Latini *et al.*, 1999), epilepsy (Boison, 2010) and Parkinson's disease (Kalda *et al.*, 2006; Yu *et al.*, 2008). The control of neuroinflammation may explain the broad spectrum of pathologies in which this blockade is beneficial. The presented work strongly suggests that A_{2A} receptors can control the recruitment and activation of microglia and thus the genesis of neuroinflammation. In addition, it is also shown that A_{2A} receptors are able to modulate the direct, deleterious impact of neuroinflammation on neurons, such as the priming of neurons to excitotoxicity induced by the pro-inflammatory cytokine interleukin-1β. Overall, these results contribute to further elucidate the beneficial impact of the blockade of A_{2A} receptors in so many different pathologies.

ATP receptors are also involved in the pathophysiology of many brain diseases and their expression and density is frequently and sometimes chronically altered in such situations (Illes and Alexandre Ribeiro, 2004; Franke and Illes, 2006). The instability of extracellular ATP and its rapid conversion to adenosine has complicated the unraveling of its direct effects on cells (Cunha and Ribeiro, 2000). However, the development of new, more potent and selective drugs for the different

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ATP receptors has helped to better understand both their physiology and pathophysiology. Typically, the deleterious effects of ATP have been attributed to the activation of the excitatory P2X receptors and especially to the activation of $P2X_7$ receptors (Aswad and Dennert, 2006; Coutinho-Silva *et al.*, 2007; Bernardino *et al.*, 2008) that form large pores on the cellular membranes linked to the apoptotic pathway (Atkinson *et al.*, 2002). However, the modulatory effects of P2Y receptors are receiving increasing attention.

In the present work, it was found that the antagonism of $P2Y_1$ receptors partially inhibited the signaling induced by the interleukin-1β in hippocampal neurons and attenuated the exacerbation of excitotoxicity by this cytokine. In addition and importantly, the blockade of these receptors completely prevented the noxious effects of glutamate in hippocampal cultured neurons. It was also found that $P2Y_1$ receptors influenced the early effects of glutamate through NMDA receptors and its blockade effectively protected neurons against glutamate-induced neurotoxicity at short-time exposures to the aminoacid. These results seem at odd with the synaptic inhibitory role of $P2Y_1$ receptors since they were found to inhibit glutamate release pre-synaptically (Rodrigues *et al.*, 2005b) and to decrease NMDA currents post-synaptically (Luthardt *et al.*, 2003). However, they also inhibit the M-type k^+ currents in hippocampal pyramidal cells (Filippov *et al.*, 2006) which contributes to increase neuronal excitability. In addition, $P2Y_1$ receptors were implied in the pathophysiology of Alzheimer's disease (Moore *et al.*, 2000b) and in the ischemia-induced damage to hippocampal slices (Traini *et al.*, 2011), which is suggestive of a role in both excitotoxic- and inflammation-induced damage. In fact, these receptors have a widespread distribution in the mammalian brain, in both glial and neuronal structures (Moore *et al.*, 2000a; Moran-Jimenez and Matute, 2000) and are crucial for the formation of the astrocytic calcium waves (Fam *et al.*, 2003; Gallagher and Salter, 2003) and in the control of glutamate transport in these cells (Domercq *et al.*, 2006; Zeng *et al.*, 2009). These findings, added to the ability of $P2Y_1$ receptors to control the glutamate-induced damage in the hippocampus, lead to further exploring their targeting in animal models of diseases where there is an overexcitability of neurons and neuroinflammation-induced damage in the hippocampus, such as in epilepsy. The blockade of $P2Y_1$ receptors was tested in a rat model of temporal lobe epilepsy induced by the systemic administration of kainate. The characterization of the density of P2 receptors in the hippocampal membranes, at different times after the kainate-induced seizures, showed a significant (long-term) altered density of most subtypes of P2 receptors, both in synaptosomes and in total membranes. This altered density evidences the contribution of ATP signaling in the pathophysiology of seizures. Most P2 receptor subtypes were up-regulated in the hippocampus 24 hours after the systemic insult with kainate. Thus, this endpoint

was chosen for the subsequent analysis. In fact, at this time, there was also a clear loss of terminal markers in the hippocampal membranes, an evident microgliosis and neurodegeneration in all hippocampal subregions. In particular, at 24 hours after the kainate administration, the $P2Y_1$ receptors were significantly up-regulated in both synaptic and total membranes, which is indicative of their importance in both synaptic and non-synaptic sites. Moreover, the intracerebroventricular administration of a selective antagonist of $P2Y_1$ receptors (MRS2500) prevented the loss of most terminal markers in hippocampal membranes. This is especially important considering that many neurological disorders may start with a synaptotoxicity before evoluting to neurodegeneration (Coleman *et al.*, 2004; Canas *et al.*, 2009; Cognato *et al.*, 2010) thus, giving a window of opportunity to profilatic treatment in these diseases. In addition, MRS2500 also prevented the strong microgliosis and attenuated the neurodegeneration induced by kainate in the hippocampus.

Finally, the blockade of $P2Y_1$ receptors significantly prevented the ischemia-induced damage in the brain of mice subjected to the permanent occlusion of the medial cerebral artery (MCAO) and abrogated both the neurological and mnemonic deficits induced by MCAO.

Overall, the presented data supports a role for the blockade of A_{2A} receptors in the control of neuroinflammation and the potential targeting of $P2Y_1$ receptors under pro-inflammatory and excitotoxic conditions in the brain.

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