UNIVERSIDADE DE LISBOA

Faculdade de Medicina

Modulation of γ-aminobutyric acid (GABA) uptake by $P2Y_1$ metabotropic purinergic receptor in rat cortical astrocytes

Joaquim Pedro Faria Jacob

Curso de Mestrado em Neurociências (XI Edição)

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A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina da Universidade de Lisboa em reunião de 22 de Novembro de 2011.

The experimental work described in this Master thesis was performed at the Institute of Pharmacology and Neurosciences, Faculty of Medicine and Neurosciences Unit, Institute of Molecular Medicine, University of Lisbon, during the research year of the XI Master degree in Neurosciences and under the supervision of Professor Ana Maria Sebastião.

Resumo

Os astrócitos expressam uma variedade de receptores purinérgicos (P2), envolvidos na comunicação entre astrócitos e indutores de aumentos rápidos na $[Ca^{2+}]_i$. Os receptores metabatrópicos do ATP (P2Y) regulam o Ca^{2+} citoplasmático através da via de sinalização PLC-PKC, a qual está, também, envolvida na regulação da actividade dos transportadores de GABA, nomeadamente GAT-1 e GAT-3. No presente estudo foi analisada, a modulação dos transportadores de GABA nos astrócitos pela breve activação dos receptores P2Y e os seus aumentos de Ca^{2+} concomitantes.

Foram realizadas experiências, de imagiologia de $Ca^{2+,}$ para caracterizar funcionalmente os subtipos de receptores P2Y, expressos em culturas corticais primárias imaturas (11-15 dias de cultura) e maduras (21-25 dias em cultura) enriquecidas em astrócitos. Realizaram-se ensaios, de recaptação de [^{3}H]-GABA, para avaliar se os agonistas moduladores de Ca^{2+} afectam a actividade dos transportadores de GABA em astrócitos.

Neste trabalho demonstrou-se que, tanto astrócitos imaturos como maduros expressam funcionalmente receptores P2Y, sendo o receptor mais preponderante, o $P2Y_1$. Incubação com ATP (100 µM) durante um minuto inibe a actividade dos dois transportadores de GABA analisados. O efeito inibitório foi reproduzido, na presença da adenosina deaminase e pelo agonista específico dos receptores $P2Y_{1,12,13}$ (2-MeSADP). A inibição verificada na presença de 2-MeSADP é perdida, com os antagonistas dos receptores P2 (PPADS) e $P2Y_1$ (MRS2179). O efeito do 2-MeSADP é também perdido, com a incubação com U73122, inibidor da PLC, mas não com GF109203X, inibidor da PKC, indicando que este efeito é mediado por um

mecanismo dependente da PLC. A inibição dos GAT está também associada aos rápidos aumentos do Ca^{2+} intracelular, induzidos por estes receptores.

Concluí-se que, uma activação breve dos receptores $P2Y_1$ em astrócitos maduros induz aumentos intracelulares de Ca^{2+} e inibe os GAT em astrócitos, sugerindo que, estas duas funções astrocitárias podem estar relacionadas no controlo dos níveis extracelulares de GABA.

Palavras-chave: Astrócitos; ATP; Receptores $P2Y_{1;}$ Sinalização por Cálcio; Transportadores de GABA

Abstract

Astrocytes express a wide variety of purinergic (P2) receptors, that are involved in astrocytic communication through fast increases in $[Ca^{2+}]_i$. Of these, the metabotropic ATP receptors (P2Y) regulate the cytoplasmic Ca^{2+} through the PLC-PKC pathway, which is also involved in the regulation of GABA transporters activity, namely GAT-1 and GAT-3. The present study analysed the modulation of GABA transport into astrocytes by the brief activation of P2Y receptors and their concurrent increases in cytoplasmatic Ca^{2+}.

Ca^{2+} imaging experiments were performed to functionally characterize the subtypes of P2Y receptors expressed in immature (11-15 days in culture) and mature (21-25 days in culture) primary cortical astroglial-enriched cultures. [^{3}H]-GABA uptake assays were conducted to ascertain if the agonists that trigger Ca^{2+} increases affect GABA transporters activity in astrocytes.

It is describe that both immature and mature astrocytes express functional P2Y receptors, with the $P2Y_1$ receptor being the preponderant one. One min incubation with ATP (100μM) produced an inhibition on the activity of the two GABA transporters analysed. Inhibition was reproduced in the presence of adenosine deaminase and by a specific agonist for the $P2Y_{1,12,13}$ receptor (2-MeSADP). The effect of 2-MeSADP was completely blocked when the cells were pretreated with general P2 (PPADS) and selective $P2Y_1$ (MRS2179) receptor antagonists. Inhibition by 2-MeSADP was lost by incubation with the PLC inhibitor (U73122) but not by the PKC inhibitor (GF109203X), suggesting an involvement of the PLC pathway by a PKC-independent mechanism. GAT inhibition by $P2Y_1$ receptors is also associated with the rapid increases in intracellular Ca^{2+} produced by the activation of these receptors.

In conclusion, brief activation of $P2Y_1$ receptors in mature astrocytes triggers Ca^{2+} increases and inhibits GABA transport into astrocytes, suggesting that the two main astrocytic functions can be related to control extracellular GABA levels.

Key words: Astrocytes; ATP; Calcium Signalling; GABA Transporters; $P2Y_1$ receptors

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Abbreviations list

2-MeSADP	2-methylthio-adenosine-5'-diphosphate
AA	Arachidonic Acid
AC	Adenylyl Cyclase
ACHC	3-aminocyclohexane-carboxylic acid
ADO	Adenosine
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BAPTA-AM	Acetoxymethyl bis(O-aminophenoxy)ethane-N,N, N',N'-tetraacetate
Ca^{2+}	Calcium
CaM	Calmodulin
CaMKs	Calmodulin-dependent Kinases
cAMP	Cyclic AMP
cGMP	Cyclic GMP
Cl^-	Chloride
CNS	Central Nervous System
CPA	α-Cyclopiazonic Acid
$cPLA_2$	Cytosolic Phospholipase A2
DAG	Diacylglycerol
DIC	Days in Culture
DMEM	Dulbecco's Modified Eagles Medium
EGTA	Ethyleneglycol bis(β-aminoethylester)-N,N,N',N'-tetraacetate
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
Fura-2 AM	Fura-2 Acetoxymethyl Ester
GABA	γ-Amino Butyric Acid
GABA-T	GABA-transaminase
GAD	Glutamate Decarboxylase
GATs	GABA transporters
GC	Guanylyl cyclase
GF109203X	2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) maleimide
GMP	Guanosine Monophosphate
GPCRs	G protein-coupled Metabotropic Receptors
GS	Glutamine Synthetase
GTP	Guanosine-5'-triphosphate
$InsP_3$	Inositol Triphosphate
K^+	Potassium
K_m	Michaelis constant
MAPKs	Mitogen-activated Protein Kinases
MRS2179	N^6-methyl-2´-deoxyadenosine- 3´,5´-bisphosphate
MRS2500	(1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen

	phosphate ester tetraammonium salt
Na^+	Sodium
N-DPB-nipecotic acid	N-4,4-diphenylbut-3-en-1-yl-nipecotic acid
NGS	Normal Goat Serum
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PBS	Phosphate-buffered Saline
PFA	Paraformaldehyde
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-Kinases
PIP2	Phosphatidylinositol Biphosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt
PTX	Pertussis Toxin
SKF89976A	1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride
SNAP-5411	1-[2-[tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3-piperid inecarboxylic acid
TCA cycle	Tricarboxylic Acid Cycle
THAO	4,5,6,7-tetrahydroisoxazolo[4,5-c]azepin-3-ol
THPO	4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol
TMD	Transmembrane Domains
U73122	1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione
UDP	Uridine 5’-Diphosphate
UTP	Uridine 5’-Triphosphate
α-KG	α-ketoglutarate

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1| Introduction

The Central Nervous System (CNS) has a highly refined structure composed by intimate associations of its basic cell types (*e.g.* neurons and glial cells). In the mammalian CNS, glial cells are about 10 times more abundant than neurons, and cover almost 50% of the total cell volume. Oligodendrocytes and microglia have long been recognized as having distinctive and precise functions in the CNS. In contrast, the role of astrocytes (the most predominant glial cell type, in terms of number, surface area and volume) started to be understood much later (Privat et al., 1995), still being a matter of intense research.

1.1. Astrocytes

In 1893, Michael von Lenhossek proposed the term “astrocyte” (astroglial cell) [Kettenmann & Verkhratsky 2008], and Andriezen (1893) described two types of astrocytes: the protoplasmatic (found in grey matter) possess few glial filaments, with irregular contours and extend sheet-like processes; the fibrous (found in white matter) displays many filaments, extended cylindrical branching processes in various directions and shows regular contours (Privat et al., 1995). Afterwards, a third type was identified, the radial cells, which have a preponderant role in neurogenesis, but also in neuronal patterning, migration, specification and/or differentiation during development (Campbell & Götz 2002). Radial glia spans through the entire white matter, perpendicularly to the axis of the ventricles, with unbranched and rectilinear processes arranged in bundles (Privat et al., 1995). After maturation, radial glia disappears in the majority of brain regions, with the exception in the retina (Müller cells), in the cerebellum (Bergmann glia cells) and in the periventricular organs, the

hypophysis and the raphe part of the spinal cord (tanycytes) [Privat et al., 1995]. Besides their difference in morphology and function in the CNS, the different types of astrocytes also differ in their antigenic phenotype and development characteristics (Privat et al., 1995).

Traditionally, astrocytes were only perceived to be the chemical and physical insulators of the neuronal elements, enabling neurons to carry out the multiple functions of the brain. When their role in neuronal survival was acknowledged, they became known as “passive support cells”, and described as having a protective role in the synapse microenvironment, namely through the stabilization of the extracellular potassium concentration (Walz 2000) and the removal of neurotransmitters from the synapse (Henn & Hamberger 1971; Henn et al., 1974).

Several experimental evidences, in the 90s, identified the presence of voltage-gated channels and neurotransmitters receptors on astrocytes, which correspond mostly to G protein-coupled metabotropic receptors (GPCRs) [Verkhratsky et al., 1998]. The identification of these receptors, led to the proposal that astrocytes are also able to participate in other aspects of the nervous system function besides the protection of neurons. Now it is widely accepted that they can be involved in the regulation of synaptic neurotransmission, the control of adult neurogenesis and brain vascular tone. This gave rise to a novel concept, the “tripartite synapse” (Araque et al., 1999)**,** consisting of synaptically associated glia and the traditional pre- and postsynaptic neuronal components.

The seminal works of Cornell-Bell et al. (1990) and Charles et al. (1991) show that astrocytes do not only detect and respond to neuronal activity, but that they are also able to communicate with each other through increases in the intracellular calcium

concentrations ($[Ca^{2+}]_i$). The Ca^{2+} fluctuations emerge as responses to changes in the surrounding milieu, and lead to changes in astrocytic functions. Consequently, the Ca^{2+} increases do not represent passive responses, being the signal through which astrocytes respond, integrate, and transmit information.

Communication between astrocytes occurs in the form of intercellular Ca^{2+} waves via gap junctions (Dermietzel et al., 1989; Finkbeiner 1992). Astrocytes can be seen as a highly dynamic "functional syncytium", contacting with all other cellular elements in the brain, including neurons, oligodendrocytes, $NG2^+$ glial cells, microglia, and vasculature. This "functional syncytium" can exchange molecules for signalling (*e.g*. inositol triphosphate – $InsP_3$) or for metabolic activity (*e.g.* glucose and adenosine 5'-triphosphate – ATP; Orellana et al., 2009). Moreover, Ca^{2+} signalling in astrocytes is also correlated with the release of various substances ("gliotransmitters"), including, among others, glutamate, ATP, D-serine, γ-amino butyric acid (GABA) and prostaglandin (Volterra & Meldolesi, 2005; Perea et al., 2009). These neuroactive substances not only modulate neuronal activity, which demonstrate the existence of neuron-to-astrocyte communication (Parpura et al., 1994; Pasti et al., 1997), but can also act as an autocrine/paracrine signal (Enkvist & McCarthy 1992).

The astrocytic network is distinct from the binary signalling of neuronal networks, offering a chance of analogue information processing in the brain.

1.2. Calcium (Ca^{2+}) Signalling

Cellular signalling via Ca^{2+}, a ubiquitous intracellular second messenger, depends on the encoding and decoding of the Ca^{2+} signals (Berridge et al., 2000). The process of encoding corresponds to the transformation of environmental cues into

intracellular Ca^{2+} variations. Then these variations are interpreted and converted into a broad spectrum of physiological responses (decoding phase). This wide influence is due to the versatility of the Ca^{2+} signalling mechanism (in terms of speed, amplitude and spatiotemporal patterning), which is tightly regulated by a set of different mechanisms. These mechanisms include membrane Ca^{2+} transporters (present in the cell membrane and in the membrane of intracellular organelles) and cytoplasmatic Ca^{2+} buffers, which are involved in the accumulation, storage and release of Ca^{2+} (Berridge et al., 2000). In addition to the fundamental Ca^{2+}-signaling toolkit, the crosstalk between Ca^{2+} and other intercellular pathways is also vital to create a versatile Ca^{2+} signalling network (Berridge et al., 2000).

At rest, glial cells maintain the free $[Ca^{2+}]_i$ at extremely low levels (30-400 nM), and upon stimulation, within milliseconds, the $[Ca^{2+}]_i$ raises up to hundreds of nM or several μM in specific microdomains, while propagating the increase from cell to cell (Verkhratsky et al., 1998).

Two crucial properties of astrocytes allow them to have an important impact on synaptic circuits. First, different stimuli [*e.g.* glutamate (Cornell-Bell et al., 1990); mechanical (Charles et al., 1991); electrical (Nedergaard 1994) and ATP (McCarthy & Salm 1991; van den Pol et al., 1992; Kriegler & Chiu 1993; Salter & Hicks 1994; Bernstein et al., 1996; Centemeri et al., 1997; Newman & Zahs 1997)] leads to an elevation of $[Ca^{2+}]_i$ in culture astrocytes. Second, the increase in $[Ca^{2+}]_i$ propagates, in a wave-like manner that can be restricted to one cell or be transmitted to neighbouring cells (Cornell-Bell et al., 1990; Charles et al., 1991). This spread can be described as a propagating wave, due to the shape of the temporal intercellular Ca^{2+}

change, which is predictable and similar for each point in the path and with a similar velocity between and within cells (Charles et al., 1991).

All astrocytic $[Ca^{2+}]_i$ changes tend to emerge as a wave-like phenomenon, travelling from one region to another as a transient rise in $[Ca^{2+}]_i$. Although, appearing similar, they are composed of separate and distinct phenomena (an initial spike, a sustained elevation, oscillatory intracellular waves, and regenerative intercellular waves; Kim et al., 1994). These distinct components of the astrocytic Ca^{2+} response are due to two different signal transduction pathways that are neither dependent nor entirely independent of each other (Kim et al., 1994). The ionotropic response, leads to a sustained elevation in $[Ca^{2+}]_i$ related to a receptor-mediated sodium (Na^+) and Ca^{2+} influx, depolarization, and voltage-dependent Ca^{2+} influx. In addition, it also evokes regenerative intercellular waves that propagate efficiently and without a decrease from cell to cell, possibly involving the Na^+/Ca^{2+} exchanger (Duffy & Macvicar 1994; Jabs et al., 1994). The metabotropic response, in contrast, evokes a complex biphasic/oscillatory $[Ca^{2+}]_i$ response (Glaum et al., 1990). An initial spatial Ca^{2+} spike that can propagate rapidly from cell to cell and involves the activation of phospholipase (PL) C, and the synthesis of $InsP_3$, which acts on its receptors, and leads to the release of Ca^{2+} from the endoplasmic reticulum (ER). Then, it is followed by a plateau/oscillations phase, of various amplitudes and frequencies that propagates within cells and is sustained only in the presence of external Ca^{2+} (Glaum et al., 1990; Jensen & Chiu 1990).

1.2.1. Extracellular Messenger

Ca^{2+} waves were initially thought to spread as a result of gap-junction metabolic coupling; however, this can only explain a short-term signalling. For long-range Ca^{2+} signalling, the action of the "*de novo*" generation of Ca^{2+} mobilizing second messengers (*e.g.* InsP3) in neighbouring cells induced by a diffusible factor is critical, augmenting the diffusion of InsP3 through gap junctions or acting on its own receptors (Hassinger et al., 1996).

Ca^{2+} waves can still propagate between astrocytes which do not have functional contact with each other, and the properties of the Ca^{2+} signal (extent and direction) are affected by rapid superfusion of the extracellular medium (Hassinger et al., 1996). ATP was later identified as the key extracellular messenger in this system (Guthrie et al., 1999). So, ATP has a pivotal role in the signalling of neuronal-glial networks, allowing homotypic communication (astrocyte-to-astrocyte), as well as heterotypic signalling, involving astrocytes and adjacent CNS cells.

1.2.1.1. Purinergic Transmission

The release of purines and pyrimidines occurs in response to neurotransmitter stimulation and to other physiological states. Furthermore, receptor activation is linked to a host of second messenger systems and other cell-to-cell signalling molecules, such as cytoplasmatic Ca^{2+}, cyclic AMP (cAMP), $InsP_3$, PLC, arachidonic acid (AA) and nitric oxide (Fields & Burnstock 2006). Therefore, ATP mediates a highly specific and diversified range of pathophysiological actions in various organs and systems.

In physiological conditions, the extracellular ATP concentration can be estimated to be between 2 and 100 μM (Ehrlich et al., 1988), although ATP-containing vesicles could reach concentrations up to 150 and 1000 mM. As a result, a transient ATP concentration at synaptic cleft might ascend to 1 mM or higher (Abbracchio et al., 2006).

Separate types of purine receptors P1 for adenosine (ADO) and P2 for ATP and adenosine 5'-diphosphate (ADP) were proposed by Burnstock (1978). The ADO/P1 receptor family comprises the high-affinity receptors (A_1 and $A2_A$) and lower-affinity receptors ($A2_B$ and A_3) [Sebastião & Ribeiro 2009]. The P2 receptors are divided into two main families: ATP-gated non-selective cation channels and G protein-coupled receptors, named P2X and P2Y, respectively (Burnstock & Kennedy 1985; Abbracchio & Burnstock 1994). The distinction into P2X and P2Y receptors is based on the pharmacology (Burnstock & Kennedy 1985), mechanism of action (Dubyak 1991) and molecular cloning (Ralevic & Burnstock 1998) of the nucleotide receptors.

Currently, seven P2X ($P2X_{1–7}$ receptor subtypes) and at least eight P2Y ($P2Y_{1,2,4,6,11,12,13,14}$ receptor subtypes) receptors have been identified, including receptors that are sensitive to purines, pyrimidines and sugar nucleotides (Abbracchio et al., 2006; Burnstock 2007b; Köles et al., 2007). The diversity could be even greater due to heteromeric/oligomeric assembly and alternative splicing of the subunits (Abbrachio et al., 2006).

Astrocytes express a wide repertoire of P2 receptors at different levels. This heterogeneous expression can be related with a differential recruitment and/or insertion into the plasma membrane, depending on the cell functional state and specific pathophysiological conditions (Burnstock 2007a). $P2Y_{1,2,4,6,12,14}$ were already

functionally identified in astrocytes (Fields & Burnstock 2006). The activation of $P2Y_1$ and $P2Y_2$ purinoceptors is both necessary and sufficient to produce a full propagation of Ca^{2+} waves, although exhibiting differences in the properties of this phenomenon (Fam et al., 2000; Fam et al., 2003; Fumagalli et al., 2003; Gallagher & Salter 2003).

1.2.1.1.1. P2Y G Protein-Coupled Nucleotide Receptor

The P2Y G protein-coupled receptors were first cloned in 1993 (Lustig et al., 1993; Webb et al., 1993). In general, the receptors are composed of 308 to 377 amino acids with a mass of 41 to 53 kDa after glycosylation. Although showing a high diversity in the amino acid composition (Shaver 2001), all members of the P2Y purinoceptor family share a characteristic subunit topology of an extracellular amino NH_2 containing several potential glycosylation sites, seven transmembrane domains (TMD), with a high degree of sequence homology between the TM3-, TM6 and TM7-spanning regions, and an intracellular COOH terminus. The third intracellular loops and COOH terminus vary considerably among P2Y subtypes, exhibiting several consensus binding/phosphorylation sites for protein kinases (PKs), thereby influencing the pattern and degree of coupling to the $G_{q/11}$, G_s and G_i proteins (Dubyak 1991; Barnard et al., 1994).

A simplified pharmacological classification differentiates P2Y receptors into four subgroups: 1) purinoceptors such as $P2Y_1$ (most potent agonists is ADP and its analogues), $P2Y_{11}$ (ATP), $P2Y_{12}$ (ADP and its analogues), $P2Y_{13}$ (ADP and its analogues); 2) pyrimidinoceptors responding to either uridine 5'-triphosphate (UTP) [human $P2Y_4$] or uridine 5'-diphosphate (UDP) [$P2Y_6$]; 3) receptors of mixed selectivity responding to both ATP and UTP ($P2Y_2$, rodent $P2Y_4$ and, possibly,

$P2Y_{11}$); and 4) the $P2Y_{14}$ receptor, which exclusively responds to sugar nucleotides. The characteristics of the P2Y receptor subtypes, expressed in rat tissue, are summarized in Table 1.

Table 1 – Characteristics of functionally defined P2Y receptors subtypes expressed in rat tissues.

Receptor Type	Distribution	Principal Agonists	Antagonists	Transduction Mechanism
$P2Y_1$	Brain (*A*), epithelial and endothelial cells, heart, placenta, platelets, prostate and skeletal muscle	2-MeSADP(*ns*)= 2-MeSATP(*ns*)> ADP(*ns*/*E*)	PPADS(*ns*)= suramin(*ns*)>A3P5PS (*ns*)=MR2179(*s*)> MRS2279(*s*)= MRS2500(*s*)	G_q/G_{11}; PLC_β activation
$P2Y_2$	Bone marrow, brain (*A*), epithelial and endothelial cells, heart, kidney tubules, liver, lung, immune cells pancreas, skeletal muscle, spleen, stomach and vascular smooth muscle	UTP(*ns*/*E*)=ATP (*ns*/*E*)	Suramin(*ns*)> Reactive blue 2(*ns*)	G_q/G_{11} and possibly $G_{i/o}$; PLC_β activation
$P2Y_4$	Bone marrow, brain (*A*), endothelial cells, immune cells, intestine, liver, lung, pituitary, placenta and smooth muscle	UTP(*ns*/*E*)=ATP (*ns*/*E*)=ITP(*pa*/*E*)=A p4A(*ns*)	Reactive blue 2 (*ns*)=PPADS(*ns*)> suramin (*ns*)	G_q/G_{11} and possibly $G_{i/o}$; PLC_β activation
$P2Y_6$	Adipose tissue, bone, brain (*A*), epithelial cells, heart, intestine, kidney, lung, placenta, spleen, thymus and vascular smooth muscle	UDP(*ns*/*E*)>UTP (*ns*/*E*)>ADP(*ns*/*E*)> 2-MeSATP(*ns*)	Reactive blue 2(*ns*)> MRS2567(*s*)>PPADS (*ns*)> suramin(*ns*)> MRS2578(*s*)	G_q/G_{11}; PLC_β activation
$P2Y_{11}$	Bone, brain, cartilage, immune cells, intestine, liver, spleen	*not describe for rat*	*not describe for rat*	G_q/G_{11} and G_S; PLC_β activation and AC stimulation
$P2Y_{12}$	Glial cells (*A*), platelets and spinal cord	2-MeSADP(*ns*)> ADP(*ns*/*E*)>ATP (pa/E)	Suramin(*ns*)> Cangrelor(*ns*)> Clopidogrel m.(*ns*)> Prasugrel m.(*ns*)> Reactive Blue 2(*ns*)	$G_{i/o}$; AC inhibition
$P2Y_{13}$	Bone marrow, brain (*A*[1]), heart, liver, lymph nodes, pancreas, peripheral leukocytes and spleen	ADP(*ns*/*E*)> 2-MeSADP(*ns*)>> ATP(*ns*/*E*)	MRS2211(*ns*)> Cangrelor(*ns*)> Prasugrel m.(*ns*)> suramin(*ns*)= PPADS(*ns*)= Reactive Blue 2(*ns*)	$G_{i/o}$ and G_{16}; PLC_β activation and AC inhibition
$P2Y_{14}$	adipose tissue, bone marrow, brain (*A*), heart, intestine, lung, peripheral immune cells, placenta, spleen and stomach	UDP-glucose	*No antagonists tested on rat;* in human UDP (*ca*[2])	Possibly G_q/G_{11} and $G_{i/o}$

The table summarizes cloned rat P2Y-receptors that have been proved to mediate actions of extracellular nucleotides when expressed and studied in functional assay systems in rat.
(*A*), including **A**strocytes by functional evidences, such as Ca^{2+} imaging, protein kinase activation, pharmacological and electrophysiological studies; (*ca*), **C**ompetitive **A**ntagonist; (*E*), **E**ndogenous ligand; (*ns*), **N**on-**s**elective ligand; (*pa*), **P**artial **A**gonist; (*s*), **S**elective ligand; Clopidogrel m., active metabolites of clopidogrel; Prasugrel m., active metabolites of prasugrel; [1]Fumagalli et al., 2004; [2]Fricks et al., 2008.
Table adapted from Abbracchio et al., 2006, Fields and Burnstock 2006 and von Kügelgen 2006.

P2Y receptors can be further subdivided into two groups: the $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ subgroup and the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ subgroup. This new subdivision is based on 1) the phylogenetic and structural (i.e., protein sequence) similarity; 2) the presence of specific amino acid motifs in TM6 and TM7, proposed to be essential for binding to extracellular nucleotides; and 3) the selectivity of G protein coupling (Abbracchio et al., 2006).

The coupling of the various P2Y receptors to certain G proteins was inferred from both indirect evidences, such as the measurement of intracellular levels of inositol phosphates, Ca^{2+}, or cAMP and determination of pertussis toxin (PTX) sensitivity, and direct evidences, like the measurement of the impact of P2Y agonist on guanosine-5'-triphosphate (GTP) hydrolysis. The same P2Y receptor can couple to functionally distinct G proteins and signalling pathways involving different conformations of the receptor (Ralevic & Burnstock 1998). The division of the P2Y receptor subtypes based on G protein subtype preference is: 1) $P2Y_1$ (Filtz et al., 1994; Schachter et al., 1996), $P2Y_2$ (Lustig et al., 1993; Chang et al., 1995) and $P2Y_6$ (Communi et al., 1996) all use as primary coupling G_q/G_{11}, to activate the PLC/$InsP_3$ endoplasmic reticulum Ca^{2+}- release pathway. 2) $P2Y_4$ (Communi et al., 1996) and $P2Y_{11}$ (Communi et al., 1997) are couple to the activation of both PLC and the adenylyl cyclase (AC) pathways; this can result from the induction of more than one conformational state of this receptor, which enables associations with different G_α subunits. 3) $P2Y_{12}$ (Hall & Hourani 1993; Hollopeter et al., 2001), $P2Y_{13}$ (Communi et al., 2001) and $P2Y_{14}$ (Chambers et al., 2000) almost exclusively couple to members of the $G_{i/o}$ family of G proteins, inhibiting AC. Then, the profile of downstream Ca^{2+}-dependent effectors will depend not only on the amplitude and duration of the Ca^{2+}

signals induced through different receptors (Dolmetsch et al., 1997), but also on the receptors expressed in each cell type (Ralevic & Burnstock 1998).

As highlighted above, the primary transduction pathway of P2Y receptors is via interaction with heterotrimeric G proteins composed of α and βγ complex subunits, which then initiates the further downstream events. The traditional downstream effectors, mediating responses in the range of a few seconds, are the G_i activation associated with the inhibition of AC and decreased cAMP production and the coupling to $G_{q/11}$ proteins and subsequent stimulation of the membrane-bound PLC. The PLC activation catalyses the rapid hydrolysis of phosphatidylinositol biphosphate (PIP_2) into two second messengers, InsP3, which mobilizes Ca^{2+}, and diacylglycerol (DAG), which activates PKC – in the presence of Ca^{2+}. The PKC activation is involved in the phosphorylation of intracellular proteins. Ca^{2+} can also establish a complex with the Ca^{2+}-binding protein calmodulin, thus activating the calmodulin-dependent kinases (CaMKs) and phosphatases (Communi et al., 1996; Simon et al., 1995; White et al., 2003; Abbracchio et al., 2006). Additionally, P2Y receptor-induced responses often involve the signalling through second messengers (*e.g.* cAMP, DAG and Ca^{2+}) towards small GTPases and/or the interaction with several signalling pathways, e. g. receptor tyrosine kinases, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3-K), phospholipase A_2 (PLA_2) and phospholipase D (PLD), cell adhesion molecules, like α_v integrins, PDZ domain-containing proteins; P2Y receptors might cross-talk via G_α-dependent signalling with other G protein-coupled receptors. Also, they can cross-talk via $G_{\beta\gamma}$-dependent signalling or indirectly via G_α-dependent signalling with various ion channels, e.g., K^+ channels, voltage-

activated Ca^{2+} channels, Na^{+} channels and Cl^{-} channels (Abbracchio et al., 2006; Köles et al., 2008).

1.2.1.1.2. Complexity of Purinergic Signalling

The biological complexity of purinergic receptors is greatly due to the properties of response elicited by different P2 receptors, being further augmented both by the conversion of ATP into ADO and other metabolites and receptor dimerization.

ATP exerts a biphasic modulatory effect, with the P2Y receptor-mediated responses, being at much slower time scale than those mediated by P2X receptors, because the former are associated with generation of second messenger molecules and can also interact with receptors of other transmitters (Abbracchio et al., 2006). In addition, a significant modulatory effect of the physiological response induced by ATP is its rapid degradation and interconversion in its by-products, namely ADO, by several groups of membrane-associated ectonucleotidases (Zimmermann 2000). A mutual action between ADO and ATP signalling must be considered, as they frequently exert antagonist actions, and could function as a significant mechanism for glia to carry out the fine-tuning of synapses (Fields & Burnstock 2006).

Co-localization of ADO A_1 receptors and $P2Y_1$ receptors occur in numerous regions of CNS (Ochiishi et al., 1999; Moore et al., 2000) and a possible interaction can change their pharmacological properties or generate new functions (Angers et al., 2002). The interaction between these receptors was reported in co-transfected cells (Yoshioka & Nakata 2001; Yoshioka et al., 2002) and also in brain tissue (Yoshioka et al., 2002), including astrocytes (Tonazzini et al., 2007). However, other

types of P2Y receptors can also form hetero-oligomers with A_1 receptors, namely $P2Y_2$ (Dickenson et al., 1998; Suzuki et al., 2006; Namba et al., 2010).

P2Y receptors subtypes are highly diverse in terms of amino acid sequences and respond to several extracellular chemical signals of ubiquitous occurrence, which allow them to have specific physiological roles in cellular signalling. So, this primitive system, due to its diversity of actions, is in a good position to influence, either short-term events that occur in neurotransmission and neuromodulation in the CNS or potent long-term (trophic) roles, including neuronal maturation, neurite outgrowth, expression of transmitter receptors and cytotoxicity (Figure 1; Burnstock 2007a).

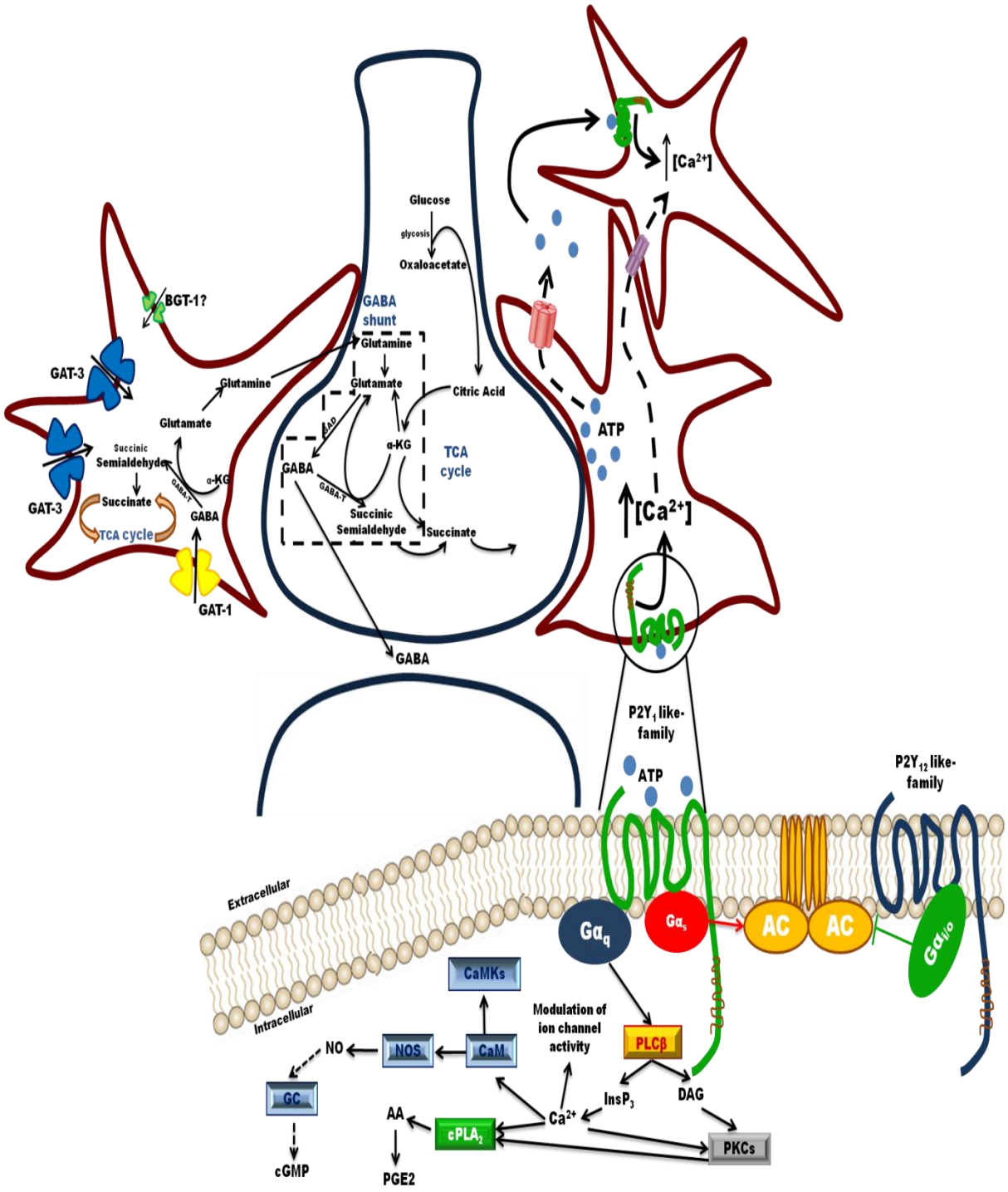

Figure 1 – The tripartite synapse. With special emphasis on the transport of GABA into astrocytes and the intercellular signaling pathways activated by the $P2Y_1$ like-family receptor. This diagram represents a comprehensive account of the metabolism of GABA and the signaling events mediated by the $P2Y_1$ like-family receptor. Arrows with solid lines indicate established responses, whereas, arrows with dashed lines indicate responses not yet clearly elucidated. Full identification of the acronyms in this figure, is reported on the Abbreviations list

1.3. γ – Aminobutyric Acid (GABA)

γ-aminobutyric acid (GABA) was initially described, in mammalian brain tissue, independently by Awapara et al. (1950), Roberts & Frankel (1950) and Udenfriend (1950), and is widely accepted as the main inhibitory neurotransmitter in the adult mammalian CNS (Krnjević & Phillis 1963; Krnjević & Schwartz 1967; Dreifuss et al., 1969), playing a critical role on the modulation of the magnitude and time course of synaptic signalling.

One crucial function of astrocytes is to supply neurons with metabolic intermediates, and although lacking the enzyme responsible for synthesis of GABA from glutamate (Schousboe et al., 1977), astrocytes play a vital role in the *de novo* generation of GABA. Glutamine, the glutamate precursor, is synthesized in astrocytes due to the action of glutamine synthetase (GS), and is the main metabolite transferred from astrocytes to GABAergic neurons. GABA arises from glutamate via decarboxylation by the action of glutamate decarboxylase (GAD) in a pathway named “GABA shunt” (Wingo & Awapara 1950).

In the nerve terminals, GABA can be released to the synaptic cleft via a Ca^{2+} dependent vesicular release (Sihra & Nicholls 1987) or Ca^{2+} independent release via transporter reversal (Belhage et al., 1993). Upon release, GABA interacts with $GABA_A$, $GABA_C$, and $GABA_B$ receptors; the first two are ionotropic and primarily located postsynaptically and the latter are metabotropic and localized both pre- and postsynaptically (Bormann 2000; Owens & Kriegstein 2002). The activation of these synaptic receptors is transient, and the signal is terminated rapidly by the removal of GABA from the extracellular space via a high affinity GABA transport system, after which GABA transaminase and then succinic semialdehyde dehydrogenase

metabolizes GABA into succinate, which enters the tricarboxylic acid cycle (Hertz & Schousboe 1987) and completes the "GABA shunt". This uptake mechanism represents an efficient process for terminating the physiological actions mediated by GABA (Iversen & Neal 1968). The involvement of neurons in this process is well known (Hösli & Hösli 1978). However, GABA is also taken up by surrounding astrocytes (Schousboe et al., 1977; Hertz et al., 1978), through a process that bears the characteristics of a high-affinity transport system closely resembling that in nerve terminals (Hertz & Schousboe 1987).

1.3.1. GABA Transporters (GATs)

Ambient GABA concentration in the synaptic cleft depends on neurotransmitter release, diffusion, and especially transporter function through plasma membrane GABA carriers. The GABA transporters (GATs) display a Michaelis constant (K_m) in the high nanomolar to low micromolar range, which explains the capacity of these transporters to maintain sub-micromolar extracellular transmitter levels (Beckman & Quick 1998).

The GATs belong to the *SLC6* superfamily of Na^+-dependent transporters, showing a net +1 inward charge movement due to the cotransport of two Na^+, one Cl^-, and one GABA molecule per cycle (Mager et al., 1993). Topologically, the members of this superfamily are composed of 12 TMD, with potential glycosylation sites between TM helices III and IV, and with both NH_2- and COOH-termini facing the cytoplasm. These regions contain phosphorylation sites, one for protein kinase A (PKA) and 7 for PKC (Guastella et al., 1990), probably involved in the regulation of the transport process (Kanner 1994).

The importance of the transporters in the termination of neurotransmitter action is determined by the transport rates, the Michaelis constant for the transporter (*as mentioned above*), and the density and localization of these transporters at, or close, to the synapse (Beckman & Quick 1998).

GABA transporters have a slow transport rate of about 10 units/sec (Mager et al., 1993). In synapses in which responses are mediated through GPCRs, transporter-mediated signalling effects could be attributed to the transporter directly (Beckman & Quick 1998). In "fast" synapses (ligand-gated ion-channel synapses) another type of transport action should exist due to the slow turnover rates of these transporters. Evidence suggests that this mechanism could be the sequestration of the transmitter at its binding sites within the transporter (Diamond & Jahr 1997).

Neuronal and astrocytic GATs are pharmacologically heterogeneous (Iversen & Neal 1968; Bowery et al., 1979) and the existence of different populations of GATs was further supported by the cloning of four transporters subtypes in mouse brain, named GAT1-4 (López-Corcuera et al., 1992; Liu et al., 1992; Liu et al., 1993). The molecular cloning studies revealed the expression of 3 subtypes of transporters in rat brain: rGAT-1, rGAT-2 and rGAT-3 (Guastella et al., 1990; Borden et al., 1992). Of these, GAT-1 and GAT-3 are expressed exclusively in the CNS, whereas the GAT-2 is localized in epithelial as well as in glial and neuronal cells (Liu et al., 1993; Borden 1996; Conti et al., 1999). The contribution of a fourth GABA transporter in rat brain, BGT-1, remains to be determined (Borden 1996). GAT-1 exhibits the highest levels of expression in the cerebral cortex of adult rats, and although being mainly considered a neuronal GABA transporter (Swan et al., 1994; Durkin et al., 1995; Ribak et al., 1996a; Ribak et al., 1996b), it is also present, to a smaller extent, in

distal astrocytic processes, both in the brain (Minelli et al., 1995; Ribak & Brecha 1996a; Ribak & Brecha 1996b; DeBiasi et al., 1998) and in the retina (Johnson et al., 1996). The second most expressed GAT in the cortex, GAT-3, is predominantly expressed in distal astrocytic processes in direct contact with GABAergic neurons (Durkin et al., 1995; Minelli et al., 1996). A mature pattern of expression, for the predominantly GATs expressed in the cortex, is reached at postnatal day 30 for GAT-1 and postnatal day 15 for GAT-3 (Conti et al., 2004).

The different pharmacological properties and cellular localization in combination with the relative contribution of astrocytic and neuronal uptake support the idea that they differ functionally. GABA accumulated by astrocytes is either degraded and lost from the GABA pool or returned to neurons in a complex GABA-glutamine-glutamate shuttle (Schousboe 2000). Therefore, the inhibition of astrocytic GABA transport would enhance the pool of synaptic GABA and facilitate reuptake into nerve endings. Thus, in presynaptic neurons, GABA can be reused as a transmitter enhancing GABAergic neurotransmission, leading to an inhibition of excitability and preventing against epileptic seizures and other GABA related dysfunctions (Schousboe 2000). An increase in the extracellular levels of GABA due to the inhibition of GABA transport into astrocytes also leads to an enhanced tonic inhibition mediated by extrasynaptic slow desensitizing $GABA_A$ receptors (Walker & Semyanov 2007). Inhibitors of glial uptake should be of particular importance for the development of anticonvulsant drugs, like tiagabine, which targets GAT-1 (Iversen 2006).

1.3.1.1. Pharmacological Distinction of GABA Transporters

The K_m for GABA transport in neurons and astrocytes is 8 and 32 μM, respectively. Kinetic analysis of the cloned rat GABA transporters, expressed in rat brain, reveal a K_m for GABA of 7, 8 and 12 μM for GAT-1, GAT-2 and GAT-3, respectively (Madsen et al., 2007). Among the classical transporters inhibitors, nipecotic acid and guvacine inhibit GAT-1, 2 and 3 come to the fore, but they have no effect on BGT-1. Diaminobutyric acid and 3-aminocyclohexane-carboxylic acid (ACHC) inhibit GAT-1 more potently than other transporters, whereas β-alanine shows the opposite selectivity (Borden 1996). The structures of another two compounds, 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol (THPO) and 4,5,6,7-tetrahydroisoxazolo[4,5-c]azepin-3-ol (THAO) were a breakthrough in the development of highly potent GABA uptake inhibitors. The addition of a lipophilic diaromatic side chain led to the development of N-4,4-diphenylbut-3-en-1-yl-nipecotic acid (N-DPB-nipecotic acid)/1-(4,4-Diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride (SKF89976A), a selective inhibitor of GAT-1 and 1-[2-[tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3-piperid inecarboxylic acid (SNAP-5114) which preferentially inhibits BGT-1/GAT-2 and GAT-3. Subsequently, a huge number of other compounds based on nipecotic acid and guvacine scaffold with various lipophilic aromatic side chains were synthesized and characterized as GAT inhibitors (Borden 1996; Madsen et al., 2007).

1.3.1.1.1. Pathways involved in the regulation of GABA Transporters

Recent evidence demonstrates that GATs and other neurotransmitter transporters in general are not passive players in the regulation of neuronal signalling. Guastella

et al. (1990) speculated about the possible involvement of a variety of initiating factors and transduction cascades in the modulation of transport function, and since then further evidence has been gathered. This regulation can be receptor mediated (*e.g.* receptor couple to kinases/phosphatases activity and AA production) due to the existence of a signal directly associated with the transport process, or transporter-mediated (*e.g.* pH changes and transmitter chronic treatment) that provides the "trigger" for regulation (Beckman & Quick 1998).

It seems that the most common modulation process of uptake depends on the phosporylation-dephosphorylation states of the transporter for members of the subfamily Na^+/Cl^- dependent carriers. The functional regulation can occur directly on the transporter protein through direct phosphorylation (Casado et al., 1993; Conradt & Stoffel 1997), or by changing the rate of the transmitter flux through the transporter as a result of the associations of the carrier with other synaptic proteins, such as syntaxin 1A (Quick et al., 1997; Beckman et al., 1998; Deken et al., 2000). The regulation of GATs activity by redistributing functional transporters from the plasma membrane to intracellular locations depends mainly on the activity of PKC, tyrosine kinases and phosphatases (Corey et al., 1994; Quick et al., 1997; Law et al., 2000). The stimulation of the AC/cAMP/PKA pathway, through the activation of ADO A_{2A} receptors, is also involved in the facilitation of GAT-1 mediated transport by restraining the tonic PKC-mediated inhibition (Cristóvão-Ferreira et al., 2009). Mechanisms involving calmodulin (CaM) are also known to regulate transporter function (Ramamoorthy et al., 1992; Jayanthi et al., 1995; Gonçalves et al., 1997). Several hypotheses have been advanced to explain the regulation through these mediators, like the involvement of a classical regulatory pathway, alterations in the

rates of exocytosis/endocytosis of the transporter protein, or a sequestration of the transporters in a nonfunctional conformation (Beckman & Quick 1998).

The involvement of pH (Sonders et al., 1997; Cao et al., 1997) and other second messengers, such as AA (Volterra et al., 1992; Trotti et al., 1995; Zhang &. Reith 1996), are also known to modulate the activity of several transporters in neurons as well as in astrocytes, including glutamate, dopamine and GABA.

Slower modulatory effects on transporters are also observed due to changes in mRNA levels or protein synthesis of the transporter after chronic treatment with transport inhibitors, or as a result of the spill over of the transmitter onto their receptors (Rattray et al., 1994; Thomsen & Suzdak 1995).

2| Aims

The majority of synapses in the CNS are tripartite in nature, and signal processing in brain results from the continuous interaction between the elements that compose this structure (the pre- and postsynaptic components and the surrounding astrocytes). Thus, the coordination between the neuronal networks and the glial syncytium is crucial, due to the astrocytic involvement in several supportive functions (*e.g.* the control of neurotransmitters levels) and the gliotransmission phenomenon.

ATP is a ubiquitous signalling molecule, and during its brief time in the extracellular space before being hydrolyzed to its metabolites, is one of the key triggers of calcium signalling responses in astrocytes, inducing the further release of gliotransmitters. Accordingly, it seems that the purinergic cascade plays a major role in both glial-glial networks and glia-neuron communication.

As detailed in the *Introduction*, calcium signalling-coupled to certain subtypes of metabotropic ATP (P2Y) receptors operate through PLC activation (Centemeri et al., 1997), and GABA transport decreases when the PLC transducing system is modulated by specific enzymes (Gomeza et al., 1991; Cristovão-Ferreira et al., 2009). Taking into consideration these findings, the main objective of this work was to clarify if these two astrocytic functions, Ca^{2+} waves and modulation of extracellular GABA levels, are related to each other and to identify the mechanisms involved.

Therefore, to unravel these questions and achieve the proposed aims, primary cultures of rat cortical astrocytes were prepared to:

Task 1 – Characterize, in our culture conditions, which P2Y receptors subtypes mediated the $[Ca^{2+}]_i$ increases in response to a brief application of P2Y receptors agonists. This task was performed in two time points 11-15 days in culture (DIC) and 21-25 DIC;

Task 2 – Investigate the influence of P2Y receptor activation on GABA transport modulation;

Task 2.1. – Study this influence through the application of pharmacological tools, characterized in task 1;

Task 2.1. – Assess if the modulation of GABA transport by P2Y receptor activation is related to calcium signalling. This was approached in two ways: by preventing the P2Y-induced calcium rise and by interfering with the PLC pathway;

Task 3 – Investigate the presence of the most important P2Y receptor functionally expressed in cultured astrocytes, as well as the two main GABA transporters expressed in cerebral cortex in mammals (GAT-1 and GAT-3);

3| Methodology

3.1. Drugs and Antibodies

The following antibodies and drugs were used: goat anti-mouse IgG or anti-rabbit conjugated to Alexa Fluor 488 and 588 from Invitrogen (Barcelona, Spain); rabbit polyclonal antibody anti-GAT-1, anti-GAT-3, mouse polyclonal anti-glial fibrillary acidic protein (anti-GFAP) and mouse polyclonal anti-microtubule-associated protein 2 (anti-MAP2) from Millipore (Bedford, MA, USA); rabbit polyclonal antibody anti-P2Y1R from Alomone Laboratories (Jerusalem, Israel); mouse anti-cluster of differentiation molecule 11b (anti-CD11b) was a gift from R. Franco (University of Barcelona, Spain); goat anti-rabbit IgG conjugated to horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA, USA); rabbit monoclonal anti-GFAP from Sigma-Aldrich (St. Louis, USA); adenosine deaminase [E.C. 3.5.4.4, 200U/mg in 50% glycerol (v/v), 10mM potassium phosphate] was purchased from Roche (Amadora, Portugal); 4-amino-n-[2,3-^{3}H]butyric acid ([^{3}H] GABA, specific activity 89.5 Ci/mmol) from Amersham (Buckinghamshire, UK) and 4-amino-n-butyric acid (GABA) from Sigma (St. Louis, USA); acetoxymethyl bis(O-aminophenoxy)ethane-N,N, N',N'-tetraacetate (BAPTA-AM) was obtained from Molecular Probes (Eugene, OR, USA); 2-methylthio-adenosine-5'-diphosphate (2-MeSADP), α-Cyclopiazonic Acid (CPA), 2-[1-(3-dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X), N^6-methyl-2´-deoxyadenosine- 3´,5´-bisphosphate (MRS2179), (1*R**,2*S**)-4-[2-Iodo-6-(methylamino)-9*H*-purin-9-yl]-2-(phosphonooxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS2500), pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium salt (PPADS), 1-(4,4-Diphenyl-3-butenyl)-

3-piperidinecarboxylic acid hydrochloride (SKF89976A), 1-[2-[tris(4-methoxyphenyl)methoxy]ethyl]-(S)-3-piperid inecarboxylic acid (SNAP 5114), 1-[6-[[(17$_\beta$)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) and uridine-5'-diphosphate disodium salt (UDP) were purchased from Tocris (Avonmouth, UK); adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-triphosphate disodium salt hydrate (ATP), uridine 5′-triphosphate trisodium salt dehydrate and ethyleneglycol bis(β-aminoethylester)-N,N,N',N'-tetraacetate (EGTA) were bought from Sigma (St. Louis, USA). Enhanced chemiluminescence Western blotting system came from Amersham Biosciences (Buckinghamshire, UK). Stock solutions of drugs were prepared with dimethylsulphoxide or distilled water and kept at -20 ºC until used. Solutions of drugs were prepared from stock solutions diluted in culture medium and/or incubation buffer immediately before use.

3.2. Primary Astroglial-enriched Cortical Cultures

Animal handling and experiments were conducted according to the guidelines set in Directive 2010/63/EU of the European Parliament and the Council of the European Union. Primary astroglial-enriched cultures were prepared from offspring (postnatal day 0 to 2) of Wistar rats (Harlan, Barcelona, Spain), as described by Vaz et al. (2011). Briefly, the brains were dissected under sterile conditions. After the brains were dissected, the olfactory bulbs, hippocampal formations, basal ganglia and meninges were carefully removed in cold PBS solution (in mM: NaCL 140, KCl 2.7, KH_2PO_4 1.5 and $NaHPO_4$ 8.1, pH adjusted to 7.4). Cortex tissue were dissected gently by trituration in 4.5g/l glucose Dulbecco's Modified Eagles Medium (DMEM, Gibco, Paisley, UK) and filtered through mesh of 230µm and centrifuged at 200g for

10 minutes (at room temperature). The pellet was resuspended in 4.5g/l glucose DMEM medium and filtered through a cell strainer (70µm; BD Falcon, Erembodegem, Belgium) and centrifuged at 200g for 10 minutes (at room temperature). Astrocytes readily stick to a plastic surface; so, the cells were seeded into 24-well plastic plates for Uptake experiments at a density of 4×10^4 cell/well, into T75 Cell Culture Flasks for Intracellular Calcium (Ca^{2+}) measurements and Immunocytochemistry studies and in Tissue Culture Dish for Western Blot assays.

Cultures of astrocytes were maintained for 11-15 Days in Culture (DIC) for Intracellular Calcium (Ca^{2+}) Measurements in immature astrocytes and 21-25 DIC (mature astrocytes) for Intracellular Calcium (Ca^{2+}) measurements, Uptake experiments, Western Blot assays and Immunocytochemistry studies. The cultures were maintained in appropriate medium [4.5 g/l glucose DMEM medium containing 10% fetal bovine serum (FBS, Gibco, Paisley, UK) with 0.01% antibiotic/antimycotic (Sigma, Steinheim, Germany)] in a humidified atmosphere (5% CO_2) at 37ºC. Culture medium was changed on the third day after seeding and every 7 days thereafter.

The plated cells were then treated with two different procedures (for the Intracellular Calcium (Ca^{2+}) Measurements and for Immunocytochemistry, in order to reduce contamination with microglia). (1) Intracellular Calcium (Ca^{2+}) measurements in immature astrocytes (11-15 DIC): after the cultures reached confluence (7 DIC), the surface cells attached to the confluent astrocyte monolayer were removed by horizontal shaking for 3 hours at 300 rpm (37ºC and 5% CO_2). After this protocol, cells were plated into the dishes four days before the Intracellular Calcium (Ca^{2+}) measurements. (2) Intracellular Calcium (Ca^{2+}) measurements and Immunocytochemistry studies in mature astrocytes (21-25 DIC): after the cultures

reached confluence (7 DIC), the surface cells attached to the confluent astrocyte monolayer were removed by horizontal shaking for 12-15 hours at 300 rpm (37ºC and 5% CO_2) as described by McCarthy & de Vellis (1980). At the 9^{th} DIC, astrocyte monolayers were treated with 8μM of cytosine-β-D-arabinofuranoside (ARA-C) for 2 days to eliminate dividing cells (i.e. microglia). This treatment is not cytotoxic to quiescent contact-inhibited astrocytes (Hamby et al., 2006; Saura 2007). The cell cultures were subsequently shifted back into a drug-free medium and plated into the dishes on DIC 17 and used four days later.

3.3. Intracellular Calcium (Ca^{2+}) Measurements

Four days before the Calcium Imaging Experiments in both immature and mature astrocytes, the cells were plated in γ-irradiated glass bottom microwell dishes (MatTek Corporation, Ashland, MA, USA) with a glass diameter of 14mm and glass thickness of 0.16-0.19mm. These dishes were previously coated with Poly-d-lysine for 1 hour and washed 3 times with sterile water. For Imaging Experiments, astrocytes had to be seeded in a dish with a glass coverslip, and due to its poor adherence to glass surfaces a coating procedure had to be adopted. This procedure was optimized for our culture conditions in our laboratory.

The platting of the cells ($5x10^4$ cells/ml for 11-15 DIC astrocytes and $7x10^4$ cells/ml for 21-25 DIC astrocytes) in microwell dishes, was made by trypsinization (1% trypsin-EDTA) for 2 minutes and this reaction was stopped by the addition of 4.5 g/l glucose DMEM medium containing 10% fetal bovine serum with 0.01% antibiotic/antimycotic. The different platting concentrations in the immature and mature cultures was due to the fact that in the 21-25 DIC assays a higher

concentration of cells were needed to originate regions of cells with a similar number of cells as the ones on the 11-15 DIC assays. Until the day of the experiment, the platted cells were maintained in suitable growing medium in a humidified atmosphere (5% CO_2) at 37ºC.

At the day of Ca^{2+} Imaging Experiments, astrocytes were loaded with the Ca^{2+}-sensitive fluorescent dye fura-2 acetoxymethyl ester (fura-2 AM; 5μM) at 22ºC for 45 minutes. After loading, the cells were washed three times in external physiological solution (composition in mM: NaCl 125, KCl 3, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, D(+)-glucose 10 and HEPES 10; pH 7.4 adjusted with NaOH; Rose et al., 2003). The dishes with the plated cells were then placed on an inverted microscope with epifluorescent optics (Axiovert 135TV, Zeiss) equipped with a xenon lamp and band-pass filters of 340 and 380 nm wavelengths. Throughout the experiments, the cells were continuously superfused at 1.5 ml/min by means of a roller pump with physiological solution. The tested P2Y receptor ligands were applied directly to single astroglial cells by pressure using a FemtoJet microinjector (Eppendorf, Hamburg, Germany) with an injection pressure of 15 psi for 0.3 seconds. Low Ca^{2+} solution (0.5 mM $CaCl_2$ + 1mM EGTA), CPA, or various blocking drugs (e.g. PPADS, MRS2179) were superfused 15-30 minutes before and during the third pressure application of an agonist (Fischer et al., 2009).

Image pairs obtained every 250 ms by exciting the preparations at 340 and 380 nm were used to obtain ratio images. Ratiometric dyes, such as fura-2 AM, correct for unequal dye loading, bleaching and focal-plane shift. Excitation wavelengths were changed using a high speed wavelength switcher, Lambda DG-4 (Sutter Instrument, Novato, CA, USA), and the emission wavelength was set to 510 nm. Image data

were recorded by a cooled CCD camera (Photometrics CoolSNAP fx) and processed and analysed using the software MetaFluor (Universal Imaging, West Chester, PA, USA). Regions of interest were obtained by defining the profile of the cells and averaging the fluorescence intensity within the delimited area. Intensity values were converted to $[Ca^{2+}]$. For calibration purposes, the external Ca^{2+} of fura-2 AM-loaded cells were washed out for 10 min (0 mM Ca^{2+}/10 mM EGTA) before the ionophore ionomycin free acid (10 µM; Ascent, Bristol, UK) was added to permeabilize the cell membrane. After equilibration between intracellular and external Ca^{2+} (20 min), a 2 mM Ca^{2+} solution was added. After another 10 min, a Ca^{2+}-free + 2 mM Mn^{2+}-containing solution was washed in. Mn^{2+} enters the cells and quenches the Fura-2 fluorescence revealing the Fura-2-independent background fluorescence, which has to be subtracted from all measured fluorescence values for background correction (Lohr & Deitmer 2010). R is the ratio of fluorescence values measured with excitation wavelengths of 340 nm versus 380 nm throughout the experiment. R_{min} (ratio value under Ca^{2+}-free conditions), R_{max} (ratio value under Ca^{2+}-saturated conditions), S_{f2} (fluorescence value measured at an excitation of 380 nm under Ca^{2+}-free conditions) and S_{b2} (fluorescence value measured at an excitation of 380 nm under Ca^{2+}-saturated conditions) were read from the calibration curves, with the background-corrected. It was assumed a dissociation constant (K_D) of fura-2 and Ca^{2+} of 145 nM (Simpson 2005) and the free cytoplasmatic Ca^{2+} concentration in nM ($[Ca^{2+}]_i$) was calculated according to the formula given by Grynkiewicz et al. (1985):

$$[Ca^{2+}] = K_D \times \frac{(R - R_{\min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}.$$

The determination of $[Ca^{2+}]_i$ values were performed using MetaFluor software.

For Ca^{2+} imaging experiments in astrocyte cultures, *n* represents the number of individual responsive cells in each experiment. A field with at least 40 cells was selected in which experiment, and the response of these cells to P2 agonists in different conditions was averaged.

The wave amplitude was calculated by subtracting the maximum peak wave (12 cycles of data acquisition around the maximum point) to the baseline level (25 cycles of data acquisition) before the stimulation. The percentage of alteration by manipulations with other agonists or incubation with antagonists (S_3) was calculated with respect to the second response to the tested agonist (S_2). In all assays, a first stimulation (S_1) was used to establish the good functional viability of the tested cells and after the incubation with a drug, a new stimulation without the drug (S_4/washout) was performed. The stimulations were separated by 30 min intervals. Within each experiment the average response (of all responsive cells in the field) in S_1 was not appreciably different from S_2 and neither from S_4.

Statistical analyses of Ca^{2+} imaging data were performed using GraphPad Prism 5 (San Diego, CA, USA) software. Data are expressed as the mean ± S.E.M., and two-sample comparisons were made using *t* tests and multiple comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni correction post-test.

3.4. GABA Uptake Assays

Determination of GABA uptake was obtained as described by Vaz et al. (2011). Briefly, astrocytes were preincubated for 3 hours at 37ºC (5% CO_2) in serum-free 1g/l glucose DMEM (Gibco, Paisley, UK). Following preincubation, cells were rinsed once

in serum-free 1g/l glucose DMEM and allowed to equilibrate for 10 minutes in this medium. This medium was then exchanged with control serum-free 1g/l glucose DMEM or drug containing serum-free 1g/l glucose DMEM. In the experiments with ATP and 2-MeSADP, they were added 1 or 5 min before starting the GABA transport assays, to simulate, as much as possible, the fast and transient actions of P2Y agonists in astrocytes. The transport was initiated by addition of 30 µM [^{3}H]GABA in a transport buffer composed of (in mM): NaCl 137, KCl 5.4, $CaCl_2.2H_2O$ 1.8, $MgSO_4$ 1.2 and HEPES 10, pH adjusted with Tris-Base to 7.40. Transport was stopped 1 minute after [^{3}H]GABA addition by rapidly washing the cells twice with ice-cold stop buffer composed of (in mM): NaCl 137 and HEPES 10, pH adjusted with Tris-Base to 7.40) followed by solubilisation with 250µl of lyses buffer (NaOH 100mM and 0.1% SDS) at 37ºC for 1 hour. The amount of [^{3}H]GABA taken up by astrocytes was quantified by liquid scintillation counting. GAT-1 and GAT-3 mediated GABA uptake was taken as the difference between the [^{3}H]GABA uptake in the absence and in the presence of GAT-1 blocker, SKF 89976A (20µM) and the GAT-3 blocker, SNAP 5114 (40µM), respectively. GAT-1 and GAT-3 blockers were added to astrocytes at the same time as the other drugs (ADA, BAPTA-AM, CPA, GF109203X, P2 antagonists and U73122). Neither the incubation with SKF 89976A nor the incubation with SNAP 5114 alters the Ca^{2+} responses induced by 2-MeSADP 100 µM (Appendix I).

Statistical analyses of the uptake data were performed using GraphPad Prism 5 (San Diego, CA, USA) software. Data are expressed as the mean ± S.E.M., *n* represents the number of independent cultures. Two-sample comparisons were conducted using *t* tests; multiple comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni correction post-test.

3.5. Western Blot Analysis

Astroglial-enriched cultures (at the 21 DIC stage), were resuspended by homogenization in a 0.32M sucrose solution containing 10 mM Hepes, 1 mM EDTA and BSA 1 mg/ml with protease inhibitors (1 mM PMSF). The homogenates were incubated with rotation for 20 minutes at 4ºC and then centrifuged at 16,000 *g* for 15 minutes at 4ºC. The supernatants were collected and the protein determined by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Equal amounts of protein were denaturated [GAT-1 and GAT-3 (150 µg): at 37ºC for 30 minutes; P2Y1 receptor (80 µg): at 70ºC for 10 minutes] in 1x sample buffer (10 mM Tris-HCl at pH 6.8, 7% sodium dodecyl sulphate (SDS), 2% glycerol, 20 mM dithiothereitol and 0.02% bromphenol blue) and subjected to SDS-PAGE (10% SDS-polyacrylamide gel). Proteins were electrotransferred onto PVDF membranes, previously soaked in methanol, for 90 minutes at 400 mA in a transfer buffer. Membranes were incubated for 1 hour in a blocking solution with 5% of non-fat dry milk in phosphate buffer solution with 0.05% Tween 20 (10 x) at room temperature and probed with rotation, for 2 hours at room temperature, with appropriately diluted primary antibodies in 3% BSA in PBS-Tween 20 and 0.02% sodium azide: rabbit polyclonal anti-GAT-1 (1:100), rabbit polyclonal anti-P2Y1 (1:200) and rabbit polyclonal anti-GAT-3 (1:100) antibodies followed by secondary antibody goat anti-rabbit IgG conjugated to horseradish peroxidise (1:10.000). Immunocomplexes were detected by enhanced chemiluminescence (ECL system). The protein amount was normalized by rabbit polyclonal antibody anti-β-actin (1:10.000), and the images were analysed using Image J software (NIH).

3.6. Immunocytochemistry

At DIC 18, the cells were plated ($4x10^4$ cells/ml) into 24-well plates containing a glass coverslip coated with Poly-d-lysine as described in *Intracellular Calcium (Ca^{2+}) Measurements* section of the Methods.

The astroglial-enriched cultures, 5 days after platting, were washed twice for 5 minutes in phosphate-buffered saline (PBS pH 7.40) and fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. After washing twice with PBS, the cells were preincubated for 30 minutes with PBS containing normal goat serum (NGS; 10%) and 0.03% Triton X-100. For double-labelling astrocytes and microglia and astrocytes and neurons, cultures were incubated (at room temperature for 1 hour) with the primary antibodies rabbit anti-GFAP (1:200) and mouse anti-CD11b (1:500), and rabbit anti-GFAP (1:200) and mouse anti-MAP2 (1:100), respectively. For $P2Y_1$ receptor localization, cultures were incubated (at room temperature for 3 hours) with the primary antibodies mouse anti-GFAP (1:200) and rabbit anti-$P2Y_1$ (1:400). For GATs localization, cultures were incubated (at room temperature for 3 hours) with the primary antibodies rabbit anti-GFAP (1:200), mouse anti-GAT-1 (1:100) or mouse anti-GAT-3 (1:100). Next, the cells were rinsed in PBS-Tween 20 0.05% for 3 times, 5 minutes each. Visualization of GFAP, CD11b, MAP-2, $P2Y_1$ receptor, GAT-1 and GAT-3 positive cells was accomplished upon 1 hour incubation, at room temperature, with the secondary antibodies (in 10% NGS-PBS-Triton X-100 0,03%) anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500) for GFAP and anti-mouse IgG conjugated to Alexa Fluor 588 (1:500) for GAT-1; anti-rabbit IgG conjugated to Alexa Fluor 588 (1:500) for GFAP and anti-mouse IgG conjugated to Alexa Fluor 488 (1:500) for GAT-3; anti-mouse IgG conjugated to Alexa Fluor 488

(1:500) for GFAP and anti-rabbit IgG conjugated to Alexa Fluor 588 (1:500) for $P2Y_1$; anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500) and anti-mouse IgG conjugated to Alexa Fluor 588 (1:500) for CD11b and MAP-2.

In negative controls, the primary antibody was omitted. Cells were washed with PBS-Tween 20, 3x5 minutes, before and after incubation with DAPI (1:15.000) for 5 minutes. The cells were one last time washed in PBS, carefully dried and mounted on a small drop of Mowiol on a slide and dried for 24 hours at room temperature. Then the slides were kept at 4ºC. About 600 cells were counted in each culture to evaluate the percentage of microglia and neurons. The number of C11b- and MAP-2-positive cells was expressed as a percentage of the total number of cells counted. Images were acquired using a Zeiss (Thornwood, NY, USA) Axiovert 200 and analysed with the help of the Image J software.

4| Results

4.1. Pharmacological characterization of the P2Y receptors functionally expressed on astrocytes

The purpose of these first series of Ca^{2+}-imaging experiments was to functionally characterize the purinoceptors which mediates the Ca^{2+} signalling in rat primary astroglial-enriched cortical cultures with 10 to 15 DIC. This astroglial-enriched culture was obtained by a routinely laboratory procedure from our group, which was reported to have ≥ 95% GFAP positive cells.

The mean resting $[Ca^{2+}]_i$ was 191.33 nM ± 3.29 nM (*n* = 659 cells). Challenge of cultured rat cortical astrocytes with several ATP concentrations (from 10^{-9} to 10^{-3} M) for a brief period of time (0.3 s) elicited a concentration dependent increase in the $[Ca^{2+}]_i$ (Figure 2a). The dynamic of these responses was characteristic of a response mediated by the activation of P2 metabotropic purinoceptors (P2Y), where a transient $[Ca^{2+}]_i$ rise followed by a fast decline to the resting level (Figure 2b) was observed. A stimulation time of 0.3 seconds was used, since in previous work from our laboratory this duration of stimulation was used to functionally characterize the P2Y receptors in the glial-derived C8-D1A cell line (Vaz et al., 2008), and also due to the fast time course of nucleotide degradation by ectonucleotidases (Cunha et al., 1994; Dunwiddie et al., 1997; Cunha et al., 1998). The amplitude of the initial $[Ca^{2+}]_i$ transient and the number of responding cells is highly dependent on ATP concentration, the threshold for the Ca^{2+} elevation above basal levels being approximately between 1-10 μM ATP (Figure 2a; *n* = 100 responsive cells from two independent cultures, $P < 0.01$). An activation near the maximal was reached in the

presence of 100 μM ATP (Figure 2a; *n* = 100 responsive cells from two independent cultures, *P* < 0.01).

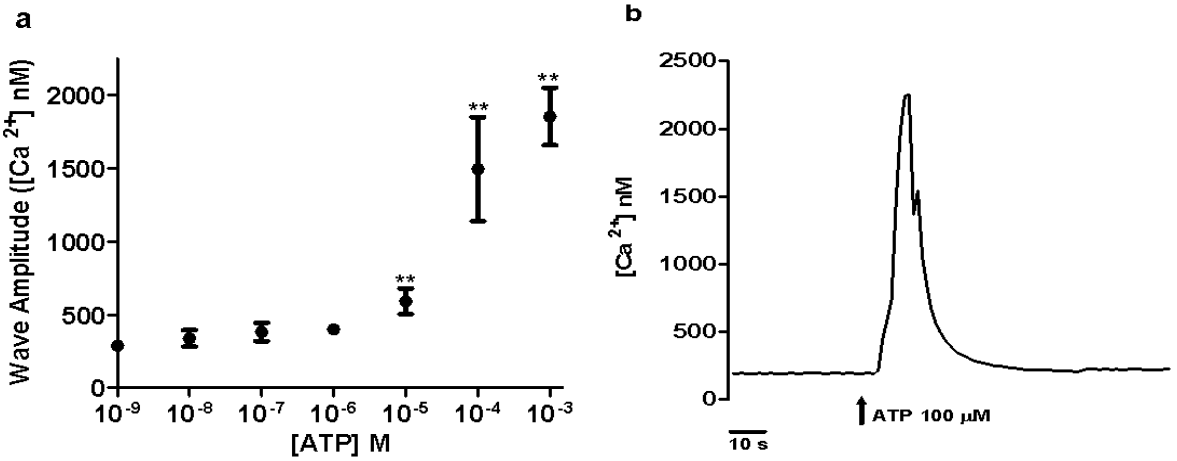

Figure 2 - Effect of ATP on $[Ca^{2+}]_i$ responses. (a) Dose-dependent changes in $[Ca^{2+}]_i$ elicited by various ATP concentrations (10^{-9} -10^{-3} M). ATP was pressure applied at the same cells, for 0.3 s at 20 min intervals between concentrations. Mean ± S.E.M. of 100 responsive cells from 2 independent cultures; **, *P* < 0.01 vs basal levels $[Ca^{2+}]_i$, assessed by one-way ANOVA followed by Bonferroni correction. The results represent only responsive cells. (b) Representative change in $[Ca^{2+}]_i$ signal illustrating the transient response upon a brief application (0.3 s) of ATP (100 μM) at time point 30 s (arrow) consisting of an initial $[Ca^{2+}]_i$ peak followed by a fast decline.

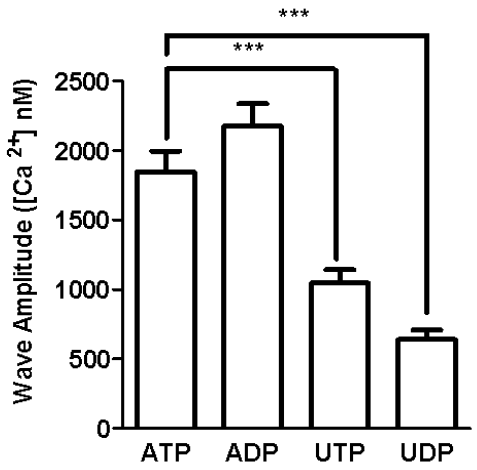

Figure 3 – P2Y receptor agonist-induced $[Ca^{2+}]_i$ responses on cortical astrocytes (11-15 DIC). All tested agonists (100 μM) were pressure applied at the same cells, for 0.3 s at 20 min intervals between different agonists. Agonist effects are shown as the response (wave amplitude) during S_3 - agonist-induced response signal. Mean ± S.E.M. of 49-101 responsive cells from 1-2 independent cultures; ***, *P* < 0.001 significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

Application of ATP and ADP (100 μM each for 0.3 s) caused a marked and reproducible $[Ca^{2+}]_i$ increase in the majority of the cells (83%± 2.4%; total analysed cells = 114 from two independent cultures) with a similar Ca^{2+} wave amplitude, 1846 nM ± 148.8 nM and 2176 nM ± 160.9 nM, respectively (Figure 3). Although UTP evokes $[Ca^{2+}]_i$ increases in 77 % of studied cells (total analysed cells = 131 of one culture), the amplitude of the evoked Ca^{2+} response is significantly smaller (1045 nM ±

96.71 nM; *n* = 101 responsive cells from one culture, *P* < 0.001) from the one mediated by ATP (Figure 3). The stimulation with UDP only evokes a response in 44% ± 2.5% of the analysed cells (111 cells), with an increase in intracellular Ca^{2+} of 639.7 nM ± 67.60 nM (*n* = 49 responsive cells from two independent cultures, *P* < 0.001; Figure 3).

The agonist profile is in agreement with the presence of $P2Y_{1,2,4,6,12,13}$ receptors, so which P2Y receptor-subtypes are functionally coupled to $[Ca^{2+}]_i$ increases induced by ATP and ADP (100 μM) using antagonists for P2 and P2Y receptors was analysed (Figure 4).

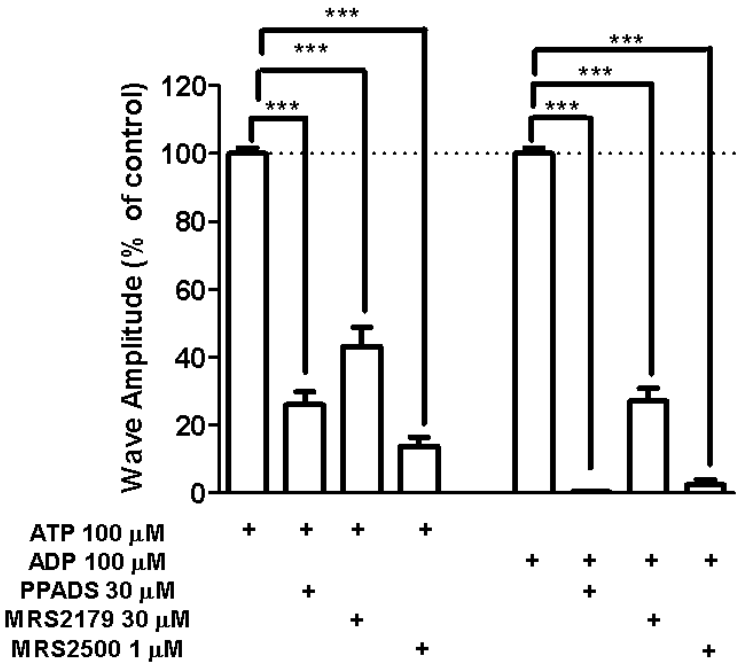

Figure 4 – Effects of inhibitors of P2/P2Y receptor antagonists on the $[Ca^{2+}]_i$ response to ATP and ADP. Summary plot of the influence of PPADS (30 μM), MRS2179 (30 μM) and MRS2500 (1 μM) on the $[Ca^{2+}]_i$ response to ATP (100 μM) and ADP (100 μM). ATP and ADP were pressure-applied four times for 0.3 s each at intervals of 20 min. After establishing two stables responses to the agonists, ATP or ADP (S_1 and S_2), an antagonist-containing solution was superfused 15 min before and during the third agonist application (S_3), followed by washout for another 30 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon drug incubation vs. S_2 - the control signal (ATP or ADP stimulation in a drug-free medium). Mean ± S.E.M. of 12-27 responsive cells from 2-4 independent cultures; ***, *P* < 0.001; significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

The involvement of P2 receptors on this effect, as seen in figure 4, was confirmed by significantly reduced response to ATP (in terms of wave amplitude) in the

presence of nonselective P2X/P2Y receptor antagonists, PPADS (% of inhibition = 74% ± 3.8%; *n* = 25 responsive cells from 4 independent cultures, *P* < 0.001). The bath application (for 15 minutes) of the $P2Y_1$ competitive antagonist, MRS2179 (30 μM), and the more selective $P2Y_1$ antagonist, MRS2500 (1 μM), depressed the ATP-induced $[Ca^{2+}]_i$ response (Figure 4). Bath application of MRS2179 (30 μM) reduce the Ca^{2+}-response induced by ATP to 43% ± 5.6% of its original amplitude (*n* = 26 responsive cells from 2 independent cultures, *P* < 0.001) and bath application of MRS2500 (1 μM) reduce the Ca^{2+}-response induced by ATP to 14% ± 2.7% of its original amplitude (*n* = 26 responsive cells from 2 independent cultures, *P* < 0.001). Exposure of astrocytes to ADP (physiological agonist of $P2Y_{1,12,13}$) resulted in a similar response of the ATP induced Ca^{2+}-increases (Figure 3). The ADP-induced response was fully abolished by PPADS and MRS2500 (% of inhibition = 99% ± 0.03% in PPADS- treated cultures, *n* = 39 responsive cells from 2 independent cultures, *P* < 0.001; % of inhibition = 97% ± 1.5% in MRS2500-treated cultures, *n* = 12 responsive cells from 2 independent cultures, *P* < 0.001). Similarly to the ATP-induced response, MRS2179 caused a pronounced inhibition of ADP-induced $[Ca^{2+}]_i$ response, reducing the response to 27% ± 3.9% of its original amplitude (*n* = 27 responsive cells from 2 independent cultures, *P* < 0.001; Figure 4).

When ATP or ADP were applied in a Ca^{2+}-low bath solution (0.5 mM $CaCl_2$ + 1 mM EGTA for 15 min; free Ca^{2+} = 123 nM and bound Ca^{2+} = 0.49964 mM. Data obtained using the “Chelator” program by Schoenmakers et al. 1992) to reduce the contribution of extracellular Ca^{2+}, the Ca^{2+} response still exhibited the characteristic transient $[Ca^{2+}]_i$ raise, although to a lesser extent of its original amplitude, 67% ± 3.2% (*n* = 88 responsive cells from 4 independent cultures, *P* < 0.001) and 65% ±

2.3% (*n* = 56 responsive cells from 2 independent cultures, *P* < 0.001), respectively (Figure 5, left panel). The incubation of CPA (10 μM for 15 min), the Ca^{2+}-ATPase pump inhibitor of the endoplasmic reticulum, was used to deplete the intracellular Ca^{2+} pools (Figure 5, right panel). The CPA incubation produced a rise in the basal $[Ca^{2+}]_i$ level (mean resting $[Ca^{2+}]_i$ of 469 nM ± 77 nM on the ATP assay and 761 nM ± 194 nM on the ADP assay) presumably due to "capacitative Ca^{2+} entry" or store-operated Ca^{2+} entry (Koizumi et al., 2002; Bouron et al., 2005; Rubini et al., 2006). The ATP and ADP effects on the $[Ca^{2+}]_i$ response were inhibited in the presence of CPA (ATP-induced response reduced to 67% ± 1.8% of its original amplitude, *n* = 111 responsive cells from 3 independent cultures, *P* < 0.001; ADP-induced response reduced to 56% ± 3.2% of its original amplitude, *n* = 56 responsive cells from 3 independent cultures, *P* < 0.001; Figure 5, right panel).

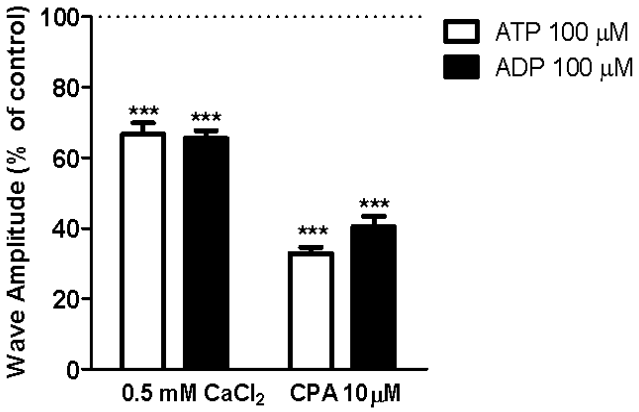

Figure 5 – Influence of Ca^{2+} low medium or depletion of intracellular Ca^{2+} stores on the $[Ca^{2+}]_i$ response to ATP and ADP. Summary plot of the influence of Ca^{2+} low medium (0.5 mM $CaCl_2$ + 1 mM EGTA) or CPA (10 μM) incubation for 15 min, on the $[Ca^{2+}]_i$ response to ATP (100 μM; white bars) and ADP (100 μM; black bars). After establishing two stables responses to the agonists, ATP or ADP (S_1 and S_2), a Ca^{2+}-free solution or CPA were superfused 15 min before and during the third agonist application (S_3), followed by washout for 30 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon specific medium (Ca^{2+}-low or CPA) incubation vs. S_2 - the control signal (ATP or ADP stimulation in a normal medium). Mean ± S.E.M. of 56-111 responsive cells from 2-4 independent cultures, ***, *P* < 0.001; significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

In summary, the results indicated that, as already described by others (Fumagalli et al., 2003; Fischer et al., 2009), immature cortical astrocytes express functional P2Y receptors, namely $P2Y_1$, and other subtypes are likely to be present, such as the

UTP sensitive-receptors ($P2Y_{2,4}$), being difficult to discriminate between $P2Y_2$ and $P2Y_4$ due to the lack of agonists and antagonists.

Functional maturity of GABA transporters in astrocytes is reached approximately at the third week in culture (Conti et al., 2004). During astrocytic development, the expression levels of P2Y receptors also change. Zhu & Kimelberg (2001) reported an up-regulation of $P2Y_2$ receptors during hippocampal astrocytes development (from 5% to 38% of GFAP positive cells), whereas the expression of $P2Y_1$ receptors was similar at all ages (around 31% of GFAP positive cells). The receptor mRNAs were always translated into functional $P2Y_1$ receptors, but the up-regulation of mRNA for $P2Y_2$ receptors was not accompanied by more $P2Y_2$ functional receptors (Zhu & Kimelberg 2001).

Thus, due to the objective of this work, the next series of experiments were designed to functionally characterize the subtypes of P2Y receptors in cultured astrocytes with 21 to 25 DIC. Besides leading to increases in intracellular Ca^{2+} on astrocytes, ATP can also act on various purinoceptors in neurons and microglia. After reaching confluence (7-10 DIC), astrocytes stop proliferating due to contact inhibition, and microglia starts to propagate rapidly (Saura 2007); then, the culture conditions were set to minimize microglial proliferation, and estimation of the proportion of microglia was evaluated.

Astrocytes used after 21-25 DIC in culture were previously subjected to an additional treatment of 12-15 hours of orbital shaking (at 7 DIC) and then at 9^{th} DIC, ARA-C (8 μM) was added to the DMEM medium for 2 days. For the characterization of the culture, astrocytes were immunostained for the classic glial marker, glial fibriallary acidic protein (GFAP), the neuronal marker, microtubule-associated protein

2 (MAP2) and the microglial marker, cluster of differentiation molecule 11b (CD11b). Both the astrocytes in culture subjected to the normal medium change as the astrocytes subjected to the shaking and the ARA-C treatment can be described as monolayers of astrocytes exhibiting a flattened and polygonal morphology. Cultures grown without any treatment besides the normal medium change contained a substantial number of contaminating microglia (11% ± 1.3%, *n* = 5 cultures; Figure 6a; Appendix II). The number of CD11b-positive cells was substantially reduced (Figure 6b; Appendix II), but not eradicated, by the shaking and ARA-C treatment (3.6% ± 1.2%, *n* = 6 cultures; Appendix II) and 93% ± 0.93% (*n* = 6 cultures; Appendix II) of cultured rat astrocytes stained positively for GFAP. Neuronal contamination in both conditions was null (*n* = 6 cultures; Appendix II).

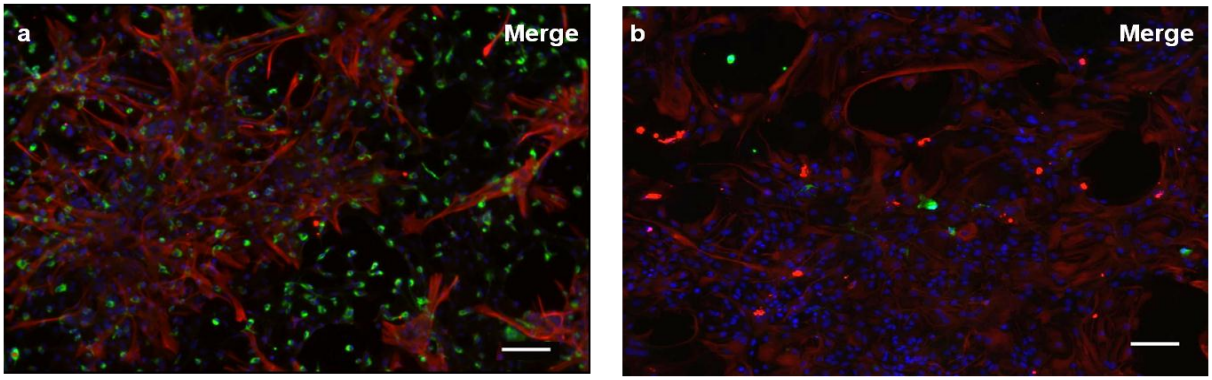

Figure 6 - Characterization of primary enriched-astroglial cultures containing different percentage of microglia. Astrocytes are labelled in red, microglia in green and nuclei in blue. Representative immunofluorescent micrographs of the two types of cultures with 21-25 DIC: (a) without treatment (*n* = 5 cultures) and (b) shake (15 h) + ARA-C (*n* = 6 cultures) for GFAP and CD11b. Scale bar: 25 μM

In the three-week cultures (21-25 DIC), spontaneous $[Ca^{2+}]_i$ oscillations occurred more frequently by comparison with cultures with 10-15 DIC, although the mean baseline level $[Ca^{2+}]_i$ was slightly lower (150 nM ± 30 nM, n = 406 responsive cells). After 21-25 DIC, the tested agonists evoked $[Ca^{2+}]_i$ increases in astrocytes (Figure 7a), with a similar dynamics (an initial $[Ca^{2+}]_i$ peak followed by a fast decline) as observed after 11-15 DIC. Pressure-application of ATP (100 μM) induced a

substantial increase on the $[Ca^{2+}]_i$, with the threshold for the Ca^{2+} elevation above basal levels between 1-10 μM of ATP (Figure 7b). The concentration-response relationships (Figure 7b) for ATP and 2-MeSADP (activates $P2Y_{1,12,13}$ receptors) show that the minimal concentration at which 2-MeSADP evoked a Ca^{2+}-response above the baseline level was lower than that required for ATP, with 2-MeSADP being about 1000 times more potent than ATP (*n* = 84 responsive cells of 2 independent cultures).

Application of 2-MeSADP induced a maximal response at 100 μM. At this

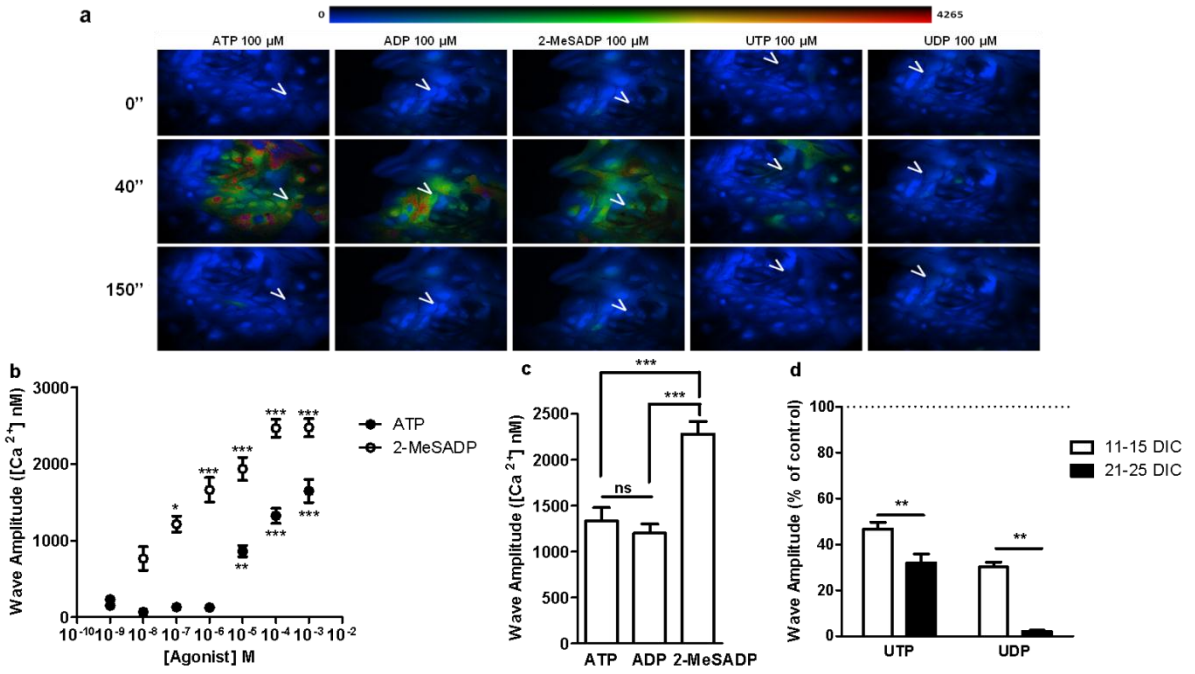

Figure 7 - P2Y receptor agonist-induced $[Ca^{2+}]_i$ responses on cortical astrocytes (21-25 DIC). (a) $[Ca^{2+}]_i$ maps showing three time points (0, 41 and 150 seconds) with all tested agonists pressure-applied for 0.3 s at time point 30 seconds. (b) Dose-dependent changes in $[Ca^{2+}]_i$ elicited by various ATP (⊸) and 2-MeSADP (⊸) concentrations (10^{-9}-10^{-3} M). ATP and 2-MeSADP were pressure applied at the same cells, for 0.3 s at 20 min intervals between concentrations Data are expressed as Mean ± S.E.M. of 84 responsive cells from 2 independent cultures; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs basal levels $[Ca^{2+}]_i$, assessed by one-way ANOVA followed by Bonferroni correction. (c) Summary plot of the P2 and $P2Y_1$ agonist effects of on the $[Ca^{2+}]_i$ in astrocytes with 21 to 25 DIC. (d) Summary plot of the effects of UTP and UDP on the $[Ca^{2+}]_i$ in astrocytes, in two different maturation time points (11-15 DIC – white bars; 21-25 DIC – black bars). Agonist effects (in c and d) are shown as the response (%) during S_3 - agonist-induced response signal vs. S_2 - the control signal (ATP stimulation). Mean ± S.E.M. 37-76 responsive cells from 2 independent cultures; **, $P < 0.01$; ***, $P < 0.001$; significant differences between S_3 vs. S_2; ns, not significant, assessed by the Student's *t*-test. The results represent only responsive cells.

concentration 2-MeSADP causes a Ca^{2+}-response 1.71 times more potent than the one induced by 100 µM ATP (*n* = 84 responsive cells from 2 independent cultures, *P* < 0.001; Figure 7c) and a Ca^{2+}-response 1.90 times more potent than the one induced by 100 µM ADP (*n* = 75 responsive cells from 2 independent cultures, *P* < 0.001; Figure 7c). At 100 µM the responses induced by ATP and ADP are similar (Figure 7c).

In Figure 7d, it can be observed that the detected responses to the pyrimidine nucleotide UTP ($P2Y_{2,4}$ agonist) and UDP ($P2Y_6$ agonist) on the functional mature astrocytic cultures were lower than the response evoked on 10-15 DIC cultures (% of UTP induced response (% of control) = 47% ± 3% at 10-15 DIC and 32% ± 4% at 21-25 DIC, *n* = 40 responsive cells from 2 independent cultures, *P* < 0.01; % of UDP induced response (% of control) = 30% ± 2% at 10-15 DIC and 2% ± 0.52% at 21-25 DIC, *n* = 37 responsive cells from 2 independent cultures, *P* < 0.01).

Consistent with the observations in immature astrocytes, PPADS completely abolished the ATP-induced transient $[Ca^{2+}]_i$ elevations (Figure 8), with an inhibition of 98% ± 0.91% of the ATP-induced response (*n* = 39 responsive cells from 2 independent cultures, *P* < 0.001; Figure 8) and additionally the 2-MeSADP-induce $[Ca^{2+}]_i$ elevation is also completely abolished (inhibition of 99% ± 0.23%; *n* = 31 responsive cells from 3 independent cultures, *P* < 0.001; Figure 8). The same effects were observed when the cells were pretreated with MRS2179 (ATP-induced response: inhibition of 76% ± 4%, *n* = 60 responsive cells from 3 independent cultures, *P* < 0.001; 2-MeSADP-induced response: inhibition of 97% ± 1%, *n* = 68 responsive cells from 3 independent cultures, *P* < 0.001; Figure 8). The residual effects of ATP on Ca^{2+}-wave, when $P2Y_1$ receptor was blocked, could be explained

by the presence of other P2Y receptor, probably of the $P2Y_{2,4}$ subtype. Indeed, although the responses to UTP are only detected in a few cells (10 out of 50 cells), in the presence of the $P2Y_{1,12,13}$ antagonists, MRS2179, UTP evoked a mean response of 47% ± 17% of its original amplitude in 8 of the 10 responsive cells (1 culture).

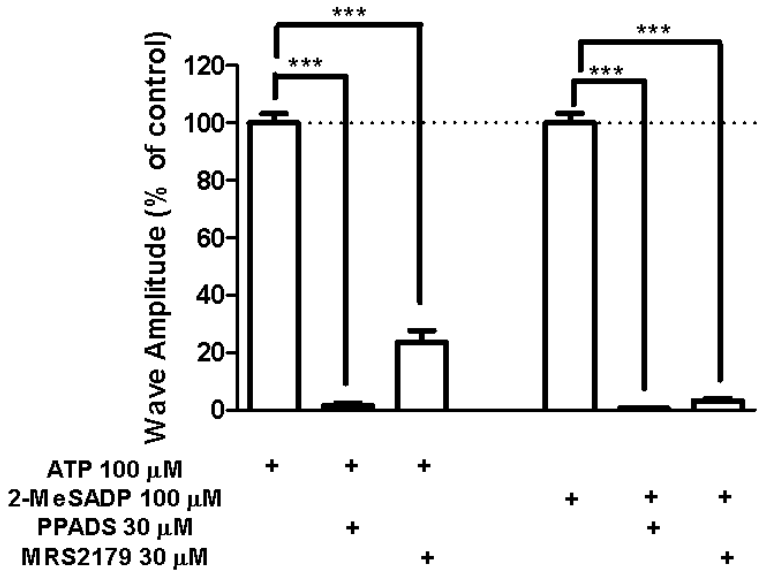

Figure 8 – Effects of inhibitors of P2/P2Y receptor antagonists on the $[Ca^{2+}]_i$ response to ATP and 2-MeSADP. Summary plot of the influence of PPADS (30 µM) and MRS2179 (30 µM) on the $[Ca^{2+}]_i$ response to ATP (100 µM) and 2-MeSADP (100 µM). ATP and 2-MeSADP were pressure-applied four times for 0.3 s each at intervals of 20 min. After establishing two stables responses to the agonists, ATP or 2-MeSADP (S_1 and S_2), an antagonist-containing solution was superfused 15 min before and during the third agonist application (S_3), followed by washout for another 20 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon drug incubation vs. S_2 - the control signal (ATP or 2-MeSADP stimulation in a drug-free medium). Mean ± S.E.M. of 31-68 responsive cells from 2-3 independent cultures; ***, $P < 0.001$; significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

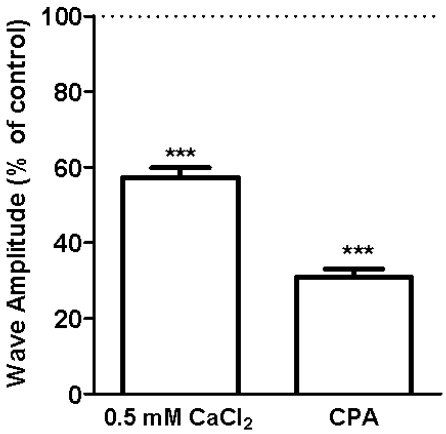

Figure 9 – Influence of Ca^{2+} low medium or depletion of intracellular Ca^{2+} stores on the $[Ca^{2+}]_i$ response to 2-MeSADP. Summary plot of influence of Ca^{2+} low medium (0.5 mM $CaCl_2$ + 1 mM EGTA) or CPA (10 µM) incubation for 15 min, on the $[Ca^{2+}]_i$ response to 2-MeSADP (100 µM). After establishing two stables responses to the agonist, 2-MeSADP (S_1 and S_2), a Ca^{2+}-free solution or CPA were superfused 15 min before and during the third agonist application (S_3), followed by washout for another 20 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon specific medium (Ca^{2+}-low or CPA) incubation vs. S_2 - the control signal (2-MeSADP stimulation in a normal medium). Mean ± S.E.M. of 94-168 responsive cells from 3 independent cultures, ***, $P < 0.001$; significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

The brief application of 2-MeSADP, in these culture conditions, is also mainly dependent on the Ca^{2+} release from intracellular stores located within the ER, as

seen in figure 9. Stimulated Ca^{2+} increases induced by 2-MeSADP are attenuated in a low-Ca^{2+} concentration medium (% of inhibition of 42.7% ± 2.6%, *n* = 168 responsive cells from 3 independent cultures, *P* < 0.001), and when intracellular Ca^{2+} stores were depleted by incubation with CPA, they were significantly reduced (% of inhibition of 69% ± 2.2%, *n* = 94 responsive cells from 3 independent cultures, *P* < 0.001).

Taken together, the results of the present study suggest that the main P2Y receptor subtype functionally expressed on astrocytes is a $P2Y_1$ receptor, coupled to release of intracellular Ca^{2+}, and shows that these purinoceptors participate in the propagation of Ca^{2+} elevations between cortical astrocytes. The results also show that the pharmacological characteristics of the predominant P2Y receptor, coupled to Ca^{2+} signalling in astrocytes, is similar in both immature and mature astrocytes.

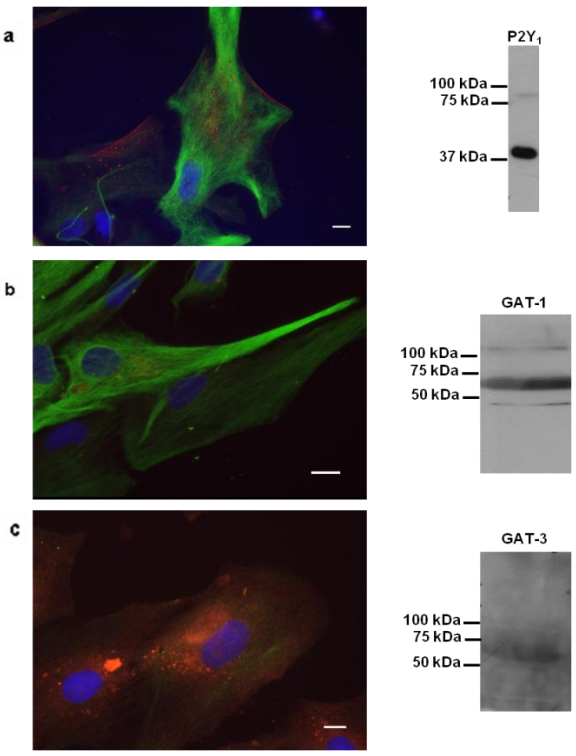

Figure 10 – Presence of $P2Y_1$ receptor, GAT-1 and GAT-3 on astrocytes (21-25 DIC). (a) Representative immunocytochemistry, astrocytes labeled green and $P2Y_1$ receptor labeled red (left) and Western blot showing the expression of $P2Y_1$ receptor (37 kDa). (b) Representative immunocytochemistry, astrocytes labeled green and GAT-1 labeled red (left) and Western blot showing the expression of GAT-1 (67 kDa). (c) Representative immunocytochemistry, astrocytes labeled red and GAT-3 labeled green (left) and Western blot showing the expression of GAT-3 (70 kDa).

4.2. Presence of $P2Y_1$ receptor, GAT-1 and GAT-3 on mature astrocytes

The presence of $P2Y_1$, GAT-1 and GAT-3 on 21-25 DIC enriched-astrocytic cultures was addressed and confirmed by Western blot analysis (Figure 10, right panel). Two immunoreactive bands of apparent

molecular weights of 37 and approximately 90 kDa were recognised by the anti-$P2Y_1$ antibody. The antibodies against GAT-1 and GAT-3 revealed a single band at the apparent molecular mass of 67 and 70 kDa, respectively. In addition, immunocytochemical studies clearly showed that both GATs and $P2Y_1$ purinoceptor are localised on mature astrocytes (Figure 10, left panel).

4.3. $P2Y_1$ receptor modulation of GABA Transporter activity on astrocytes

The uptake of GABA depends on two main transporters, GAT-1 and GAT-3, which under our culture conditions accounted for 41% ± 2.4% (*n* = 20 cultures) and 52% ± 2.5% (*n* = 22 cultures) of the total transport, respectively.

Before evaluating the influence of any drug on GABA uptake, experiments were designed to define the incubation time (one or five min) and concentration (1-100 μM) of the endogenous ligand, ATP (Figure 11a). Effects of 1 min and 5 min were compared to know if 1 min incubation was a too short time period to induce a significant modulation on GABA transport into astrocytes. Due to technical constrains, periods of incubation shorter than 1 min cannot be used. Incubation times longer than five minutes were considered to be too long and that they did not mimic the temporal patterns of a physiological model of Ca^{2+} signalling in astrocytes. Incubations with 1 min of ATP reveal a different dynamics of the Ca^{2+}-response comparing to the 0.3 s stimulation, in Ca^{2+}-imaging experiments (Appendix III). The different dynamics is probably associated to a response depending on both a metabotropic and ionotropic receptors (Kim et al., 1994). At 1 min incubation, ATP (100 μM) caused an inhibition of 22% ± 6.3% (*n* = 6, *P* < 0.05; Figure 11a, left panel) on GAT-1 mediated transport. GAT-3 mediated transport was also reduced (% of

inhibition = 28% ± 5.3%, *n* = 5, *P* < 0.05; Figure 11a, right panel) under similar experimental conditions (100 μM of ATP for 1 minute). The effect of ATP after 5 min incubation was not significantly different from that observed after 1 min incubation time (Figure 11a), thus, in the remaining experiments, a 1 min incubation time period with 100 μM was used. To ascertain whether extracellular ADO endogenously released by astrocytes could influence the [^{3}H]GABA uptake modulation by ATP, astrocytes were incubated for 15 min with adenosine deaminase (1 U/mL), an enzyme that catabolises adenosine into inosine. [^{3}H]GABA was then added after the ATP incubation, and its uptake was compared with the uptake by astrocytes

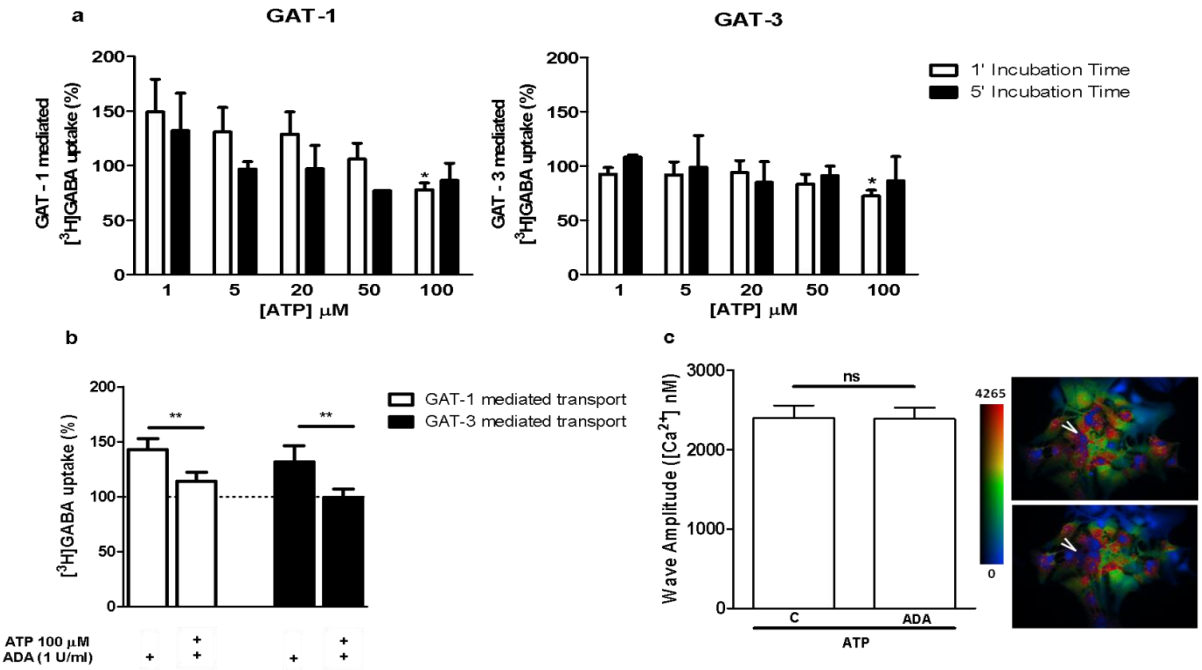

Figure 11 – Influence of ATP on GAT-1 and GAT-3 mediated transport on cortical astrocytes. (a) Concentration-dependent of ATP at two different incubation time: 1 min (white bars) and 5 min (black bars) on [^{3}H]GABA uptake mediated by GAT-1 (left panel) and GAT-3 (right panel). The ordinates represent [^{3}H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. Data are expressed as Mean ± S.E.M.; *, *P* < 0.05, assessed by one-way ANOVA followed by Bonferroni correction. (b) Exogenous application of ATP (100μM) for 1 minute inhibited GABA Transporter 1 (white bars) and GABA Transporter 3 (black bars), even in the presence of adenosine deaminase, ADA (1U/ml), which was added 30 minutes before the addition of ATP. Data are expressed as in (a), **, *P* < 0.01, assessed by the Student's *t*-test (c) Summary plot of the influence of incubation with ADA, which inhibits the accumulation of extracellular adenosine, on the $[Ca^{2+}]_i$ response to ATP in astrocytes (on the $[Ca^{2+}]_i$ maps, stimulation point is represented by an arrowhead). After establishing two stables responses to the agonists, ATP (S_1 and S_2), an ADA-containing solution was superfused 30 min before and during the third agonist application (S_3), followed by washout for another 30 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon drug incubation vs. S_2 - the control signal (ATP stimulation in a drug-free medium - **c**). Mean ± S.E.M. of 67 responsive cells from 2 independent cultures; ***, *P* < 0.001; ns, not significant, assessed by the Student's *t*-test.. The results represent only responsive cells.

incubated in the absence of ATP and in the presence of ADA. The incubation with ADA had no effect on ATP-induced modulation of GAT-1 and GAT-3 (n = 5, P < 0.01; Figure 11b) nor the Ca^{2+}-response induced by ATP (n = 67 responsive cells from 2 independent cultures, P < 0.001; Figure 11c). This data indicates that the effect on GABA transport is due to ATP itself, rather than the action of ADO, since ADO mediated effects were abolished by incubation with ADA.

According to the data in Intracellular Ca^{2+} measurements, the $P2Y_1$ receptor is probably the major contributor for the fast increases in Ca^{2+} in mature astrocytes. Therefore, the $P2Y_1$ receptor involvement on this modulation of [^{3}H]GABA uptake in astrocyte-enriched primary cortical culture was examined through the application of its agonist (2-MeSADP) and P2 (PPADS) and $P2Y_1$ (MRS2179) antagonists. First, three different 2-MeSADP concentrations (1-100 µM) were tested (Figure 12a). GAT-1 mediated transport was significantly decreased when astrocytes were incubated with 10 µM 2-MeSADP (% of inhibition = 21% ± 8.9%, n = 5, P < 0.05). A similar result was obtained with a 100 µM concentration (% of inhibition = 23% ± 10%, n = 5, P < 0.01). 2-MeSADP only induced a significant inhibition of GAT-3 mediated transport at the highest concentration tested (100 µM), inhibiting the transport by 33% ± 7% (n = 5, P < 0.05). Incubations with 1 min of 2-MeSADP reveal a similar dynamics of the Ca^{2+}-response comparing to the 0.3 s stimulation, in Ca^{2+}-imaging experiments (Appendix III).

The inhibitory effects of 2-MeSADP (100 µM) on GAT-1 and GAT-3 were lost when astrocytes were previously incubated with P2 and $P2Y_1$ antagonists (Figure 12b and 12c), PPADS (30 µM; GAT-1: n = 5, P < 0.001; GAT-3: n = 6, P < 0.01) and MRS2179 (30 µM; GAT-1: n = 5, P < 0.05; GAT-3: n = 6, P < 0.01). The results

indicate that in these conditions, GABA transport is inhibited by the activation of $P2Y_1$ receptor.

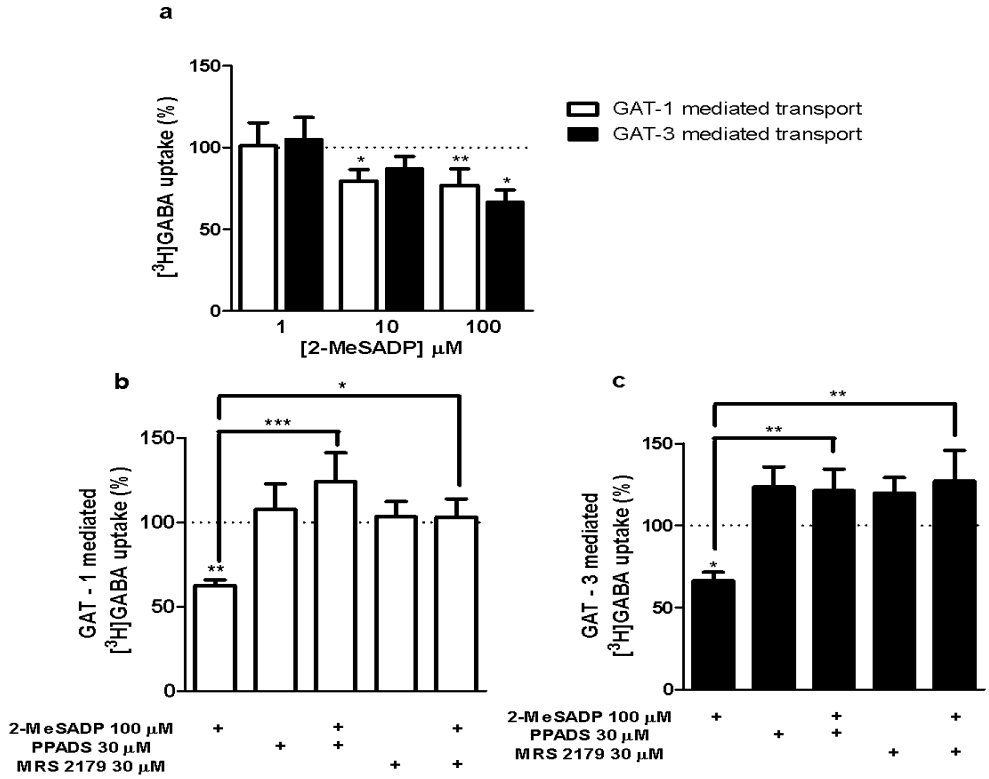

Figure 12 – Influence of $P2Y_1$ on GAT-1 and GAT-3 mediated transport on cortical astrocytes. (a) Concentration-dependent effect of 2-MeSADP (1-100 μM) on [^{3}H]GABA uptake mediated by GAT-1 (white bars) and GAT-3 (dark bars), after 1 min incubation. Application for 1 minute of 2-MeSADP (100μM), causes an inhibition of GABA Transporter 1 (b) and GABA Transporter 3 (c), and this effect is inhibited when astrocytes where preincuabated with P2 non selective (PPADS) and $P2Y_1$ (MRS2179) antagonists for 15 min. The ordinates represent [^{3}H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. Data are based on 1-min uptake and are expressed as Mean ± S.E.M.; *, $P < 0.05$; **, $P < 0.01$; assessed by one-way ANOVA followed by Bonferroni correction.

$P2Y_1$ receptor modulates GATs activity, so it was hypothesized that this action could be related to the main physiological actions evoked by this receptor, the $[Ca^{2+}]_i$ response. To test this hypothesis, astrocytes were preloaded with BAPTA-AM (20 μM, for 30 min) to chelate intracellular calcium, as expected 2-MeSADP did not lead to significant increase in $[Ca^{2+}]_i$ (n = 174 responsive cells from 3 independent cultures, $P < 0.001$; Figure 13a). As shown in figure 13b, the preincubation of

BAPTA-AM greatly attenuated the inhibitory action of 2-MeSADP on GAT-1 (% GABA uptake = 95% ± 7.9%; *n* = 4, *P* < 0.05; Figure 13b, left panel - white bars) and on GAT-3 (% GABA uptake = 94% ± 9.6%; *n* = 4, *P* < 0.05; Figure 13b, left panel – black bars). After depleting the Ca^{2+} stores through incubation with CPA (10 μM, for 15 min), the effect of 2-MeSADP on [^{3}H]GABA uptake, in both GAT-1 and GAT-3, was also lost (*n* = 5, *P* < 0.05; Figure 13b, right panel).

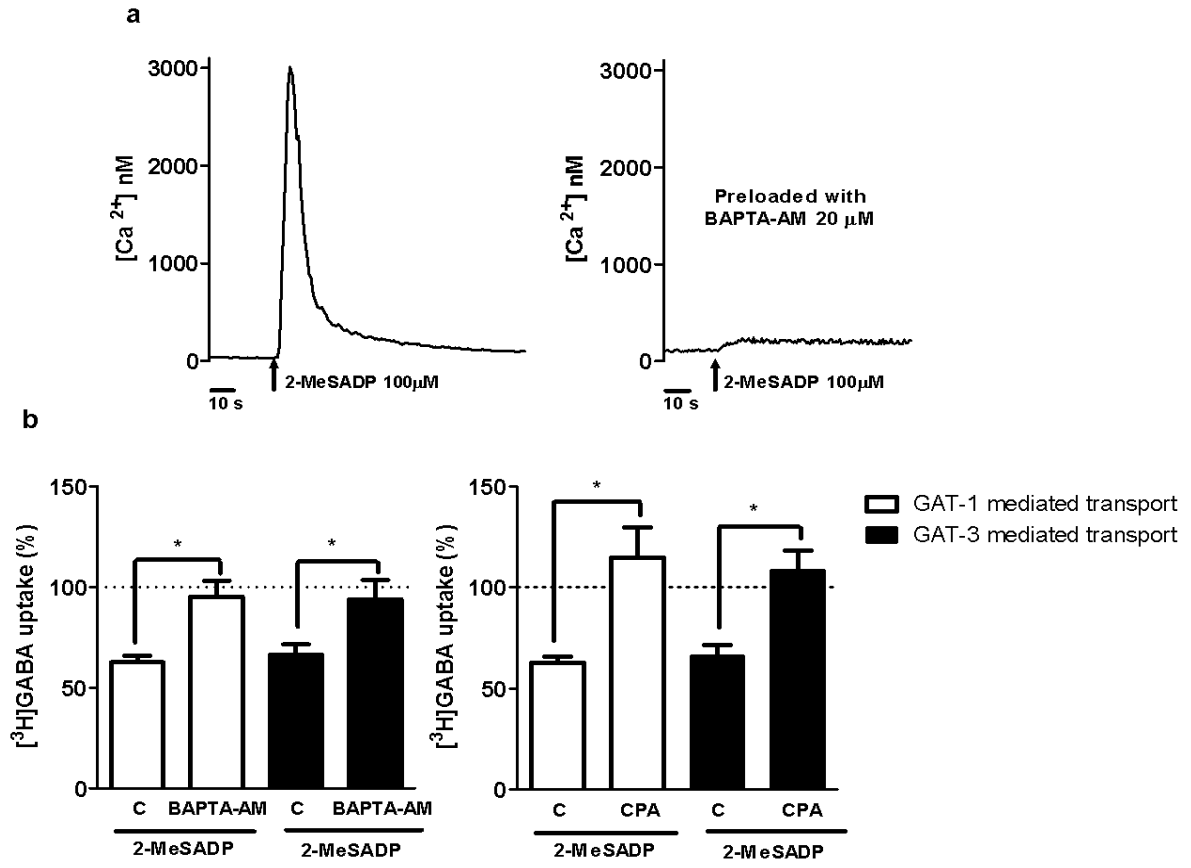

Figure 13 – Influence of Ca^{2+} on GAT-1 and GAT-3 mediated transport modulation induced by 2-MeSADP on cortical astrocytes. (a) Time course changes in the $[Ca^{2+}]_i$ in response to the 0.3 s stimulation of 100 μM 2-MeSADP in astrocytes control cells (*left*) and in cells preloaded for 30 min with 20 μM BAPTA-AM (*right*). Extracellular solution contained 2 mM $CaCl_2$. This one experiment is representative of the 3 experiments done. (b) Exogenous application for 1 minute of 2-MeSADP (100μM), does not inhibited the GAT-1 (white bars) and GAT-3 (black bars) transport activity in BAPTA-AM-loaded cells (20 μM for 30 min; *left*) and after depletion of intracellular Ca^{2+} stores with CPA (10 μM for 15 min; *right*), ***c*** represents the effect of 2-MeSADP in the absence of BAPTA-AM or CPA. The ordinates represent [^{3}H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. Data are expressed as Mean ± S.E.M.; *, *P* < 0.05; assessed by the Student's *t*-test.

Activation of P2Y receptors induces activation of different transduction signal cascades, namely PLC/IP_3/DAG/Ca^{2+}-release/PKC pathway (Abbracchio et al., 2006). Therefore, the requirement of PLCγ and PKC on the inhibition of GAT-1/3 mediated transport induced by 2-MeSADP was evaluated. As shown in figure 14a, the stimulation of $[Ca^{2+}]_i$ increases induced by 2-MeSADP was almost completely prevented by pretreatment of 20 min with a supramaximal concentration (3 μM) of U73122, the membrane-permeable inhibitor of PLC (inhibition of 92% ± 1.6%; *n* = 116 responsive cells from 3 independent cultures, $P < 0.001$; Figure 14a).

By itself, U73122 slightly inhibited [^{3}H]GABA uptake, although not significantly different from that observed in the control condition (% of GABA uptake upon U73122 incubation for GAT-1 = 83% ± 14%, *n* = 3; % of GABA uptake upon U73122 incubation for GAT-3 = 82% ± 11%, *n* = 4; Figure 14b). The inhibitory action of 2-MeSADP on GAT-1- and GAT-3-mediated transport was fully prevented by U73211 (Figure 14b). The GAT-1 and GAT-3 transport after 1 min exposure to 100 μM 2-MeSADP was respectively 61% ± 3% (*n* = 11) and 66% ± 5.2% (*n* = 14) of the value in the control condition (without 2-MeSADP or U73122). In the presence of U73122, and after adding 2-MeSADP, GAT-1 and GAT-3 transport was respectively 88% ± 8% (*n* = 3, $P < 0.01$) and 103% ± 7.7% (*n* =4, $P < 0.01$) of the value in the control condition (without 2-MeSADP or U73122). The GAT-1 and GAT-3 mediated transport when preincubated with U73122 alone did not differ significantly from [^{3}H]GABA uptake induced by 2-MeSADP after preincubation with U73122 (GAT-1: *n* =3; GAT-3: *n* = 4; Figure 14b).

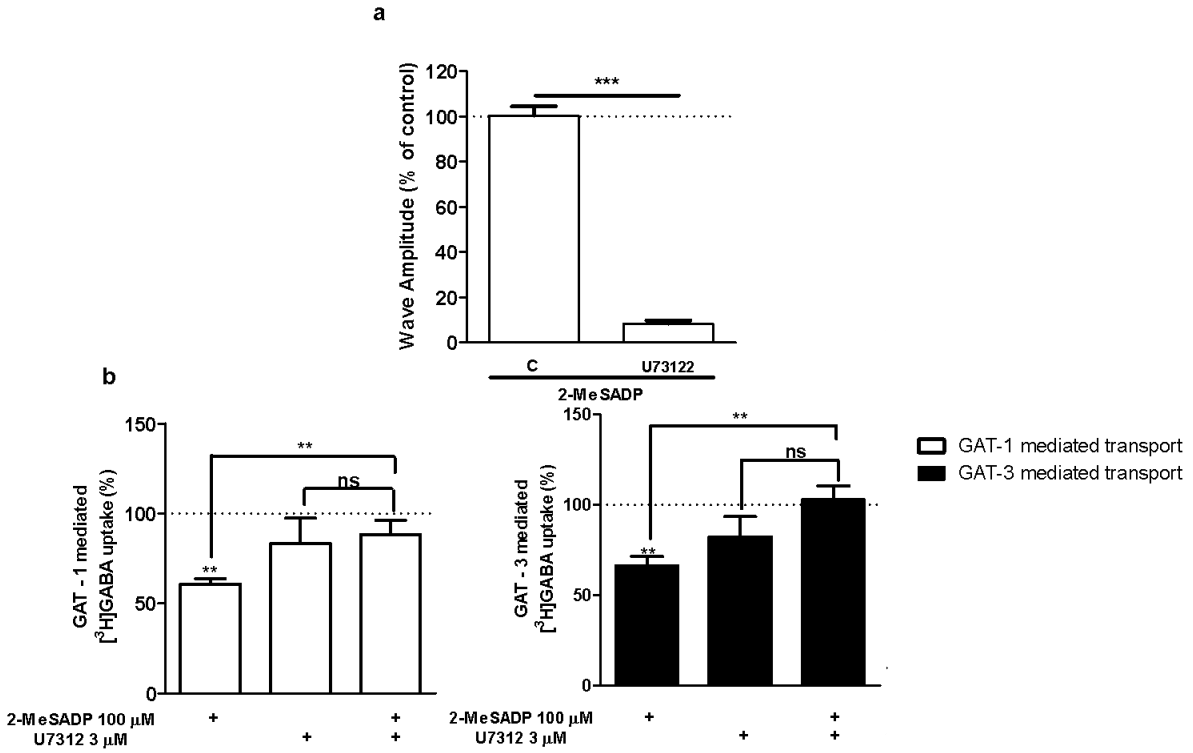

Figure 14 – $P2Y_1$ modulates GABA uptake in cortical astrocytes, through a PLC-dependent mechanism. (a) Summary plot of the influence of U73122 (3 µM) incubation on the $[Ca^{2+}]_i$ response to 2-MeSADP (100 µM). After establishing two stables responses to the agonist, 2-MeSADP (S_1 and S_2), a U73122 solution was superfused 20 min before and during the third agonist application (S_3), followed by washout for another 20 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon U73122 incubation vs. S_2 - the control signal (2-MeSADP stimulation - **c**). Mean ± S.E.M. of 116 responsive cells from 3 independent cultures, ***, $P < 0.001$; significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells. (b) Application for 1 minute of 2-MeSADP (100µM), does not inhibited the GAT-1 (white bars) and GAT-3 (black bars) transport activity in U73122-loaded cells. The ordinates represent $[^3H]$GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. Data are expressed as Mean ± S.E.M.; **, $P < 0.01$; assessed by the Student's *t*-test.

The influence of PKC upon GABA uptake (Figure 15) was investigated by using its general inhibitor, GF109203X (1 µM for 20 min). The results in figure 15b show that in the presence of GF109203X alone, GAT-1 mediated transport increased up to 127% ± 10.8% ($n = 5$, $P < 0.05$) and GAT-3 mediated transport increased up to 157% ± 18%, $n = 6$, $P < 0.05$) in comparison with the control condition (without GF109203X); however, the PKC inhibition did not prevent the 2-MeSADP-induced decrease in GAT-1 ($n = 5$; $P < 0.05$; Figure 15a, white bars) and GAT-3 ($n = 6$, $P < 0.05$; Figure 15a, black bars).

In addition, it was observed that pretreatment with GF109203X enhanced the transient Ca^{2+}-signal induced by 2-MeSADP, with a 1.2 fold increase of the response to 2-MeSADP after preincubation with the PKC inhibitor (*n* = 150 responsive cells from 4 independent cultures, $P < 0.01$ Figure 15b).

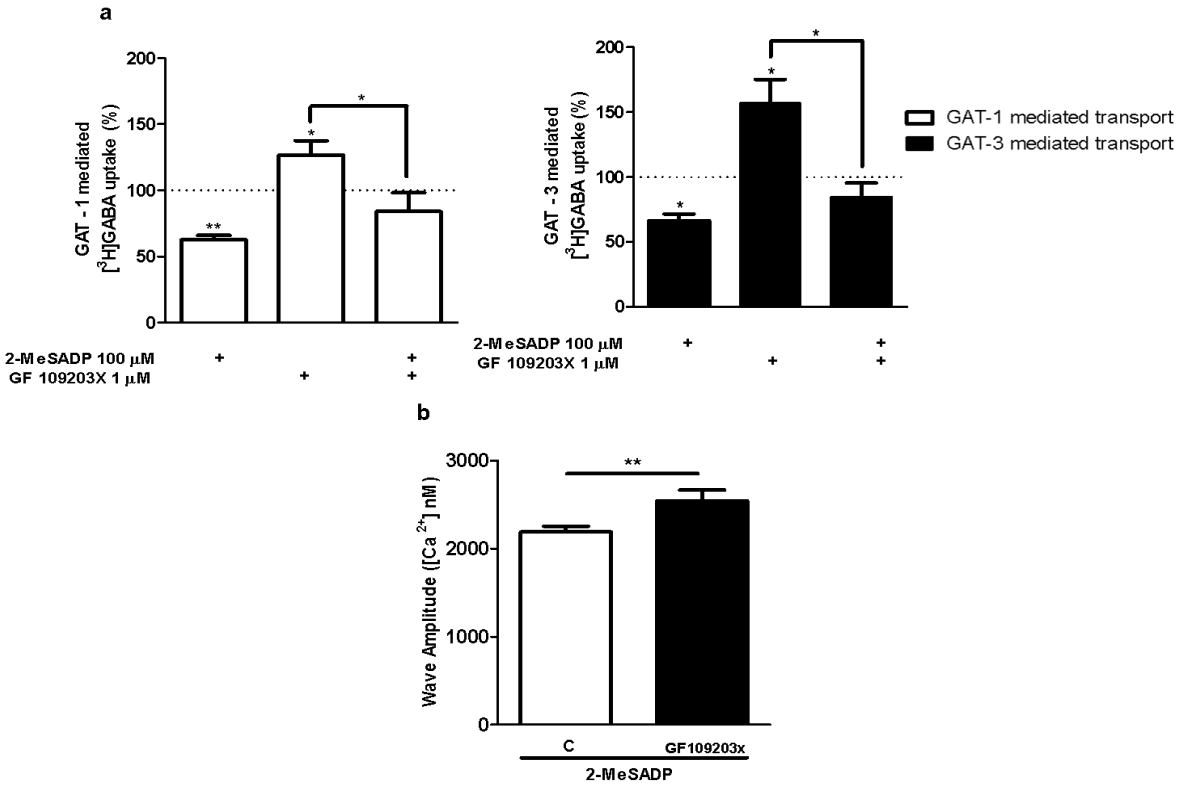

Figure 15 – $P2Y_1$ modulates GABA uptake in cortical astrocytes, through a PKC-independent mechanism. (a) Consequence of manipulating PKC activity upon GAT-1 (white bars) and GAT-3 (black bars) mediated GABA transport. Inhibiting PKC activity, by GF109203X (1 µM), enhanced the baseline uptake. Application for 1 minute of 2-MeSADP (100 µM), still inhibited the GAT-1 (white bars) and GAT-3 (black bars) transport activity in GF109203X-loaded cells. The ordinates represent [^{3}H]GABA uptake as percentage of the control value in the same experiments, which was taken as 100%. Data are expressed as Mean ± S.E.M., *, $P < 0.05$, **, $P < 0.01$, ns, not significant, assessed by the Student's *t*-test. (b) Summary plot of the influence of GF109203X (1 µM) incubation on the $[Ca^{2+}]_i$ response to 2-MeSADP (100 µM). After establishing two stables responses to the agonist, 2-MeSADP (S_1 and S_2), a GF109203X solution was superfused 20 min before and during the third agonist application (S_3), followed by washout for 30 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon GF109203X incubation vs. S_2 - the control signal (2-MeSADP stimulation - **c**). Mean ± S.E.M. of 150 responsive cells from 4 independent cultures, ***, $P < 0.001$; significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

5| Discussion

The present study revealed that, as described in immature astrocytes (Fumagalli et al., 2003; Fischer et al., 2009), a brief activation of $P2Y_1$ receptors on mature astrocytes also generates transient Ca^{2+} elevations. Furthermore, it is shown, for the first time that the activation of $P2Y_1$ receptors leads, to an inhibition of GAT-1 and GAT-3 activity in mature astrocytes. The pharmacological profile of the receptors resembles the ADP-preferring receptor $P2Y_1$ phenotype (Abbracchio et al., 2006; von Kügelgen 2006), since a subtype-specific antagonist and a general P2 receptor antagonist prevented the transporter modulations. The expression studies data reported here also demonstrates the presence of the $P2Y_1$ receptor as well as the GATs (GAT-1 and GAT-3), in agreement with previous studies (Minelli et al., 1995; Minelli et al., 1996; Burnstock 2007a). The $P2Y_1$ receptor exerts its biological effects through the activation of downstream signalling pathways, such as the PLC/DAG/$InsP_3$/Ca^{2+} mobilization/PKC, and it seems that the PLCβ signalling is involved in the inhibition of the GABA transport mediated by $P2Y_1$ both on GAT-1 and GAT-3. However, the modulation of both GATs induced by $P2Y_1$ activation does not depend on PKC activity.

The conclusion that $P2Y_1$ receptor is involved in Ca^{2+} increases in mature astrocytes (21-25 DIC) derives from 5 pieces of evidence:

(i) A brief application of the P2 agonists (*e.g.* ATP and 2-MeSADP) induces a spatial Ca^{2+} spike that rapidly returns to baseline levels. The Ca^{2+} response induced by ATP is mainly dependent on the intracellular Ca^{2+} stores, and only partially on the extracellular Ca^{2+}, and the same dependence is observed in the response mediated by 2-MeSADP. When the extracellular [Ca^{2+}] was reduced (from 2 mM to 0.5 mM),

the response dynamics seen is the same as when P2 agonists were applied in normal medium. These properties of the Ca^{2+} increases are characteristic of a metabotropic response mediated by P2Y receptors (Kim et al., 1994);

(ii) ATP, ADP and 2-MeSADP are $P2Y_1$ receptor agonists (Table 1). 2-MeSADP has a higher affinity than ADP (Waldo & Harden 2004) and ATP is a partial agonist with a reduced intrinsic activity when compared with that of ADP, and its actions in native $P2Y_1$ receptors in tissues are likely to be due to ADP formation from ATP by the action of ectoenzymes (Zimmermann 2000). 2-MeSADP is not selective for rat $P2Y_1$ receptors and can bind to other P2Y receptors (Table 1), in particular $P2Y_{12}$ (Hollopeter et al., 2001; Simon et al., 2002) but also to the $P2Y_{13}$ receptor (Fumagalli et al., 2004). The $P2Y_{13}$ receptor in rat, in contrast with $P2Y_1$ and $P2Y_{12}$, displays a higher relative potency for ADP than for 2-MeSADP (Fumagalli et al., 2004). Consequently, the possibility of other P2Y receptors contributing to the Ca^{2+}-increaes cannot be entirely ruled out;

(iii) The selective $P2Y_1$ antagonist MRS 2179 (Boyer et al., 1998) antagonized the response to ATP and to a larger extent than the one induced by 2-MeSADP. MRS2179 does not inhibit P2Y subtypes other than the $P2Y_1$ receptor at the concentration used (von Kügelgen 2006), although it can have some antagonistic activity at the $P2X_1$ receptor, but is 11-fold more selective for $P2Y_1$ receptors vs. $P2X_1$ (Brown et al., 2000);

(iv) PPADS (30 μM) antagonizes the response of both ATP and 2-MeSADP. This drug antagonizes both P2X and P2Y receptors, being more efficient at the $P2Y_1$ receptor subtype than at the $P2Y_{12/13}$ receptor (Nicholas 2001; Marteau et al., 2003);

(v) $P2Y_1$ is expressed in these cells. In Western blot studies, a multiline pattern (37 and approximately 90 kDa) was detected by an antibody against $P2Y_1$ receptor; which agrees with the data obtained by Quintas et al. (2011) in 30 DIC astrocytes using the same primary antibody. The $P2Y_1$ receptor has a predicted molecular mass (expected from datasheet) of 66 kDa, so the 37 kDa band obtained here could represent deglycosylated form of the receptor (Yoshioka 2001). The band with a higher molecular weight may result from $P2Y_1$ receptor oligomerisation (Tonazzini et al., 2008) or be due to a high temperature protein denaturation (Waldo & Harden 2004).

After establishing the nature of the P2Y receptors responsible for Ca^{2+}-signalling in mature astrocytes, I focused on the major objective of the work and found that a brief (1 min) application of P2 receptors agonists (ATP and 2-MeSADP) in enriched mature-astroglial primary cultures led to the inhibition of activity in GABA transporter, the effect being shown either into GAT-1 and GAT-3 activity. ATP released has a short (< 200 ms) *in situ* half-life in the hippocampus (Dunwiddie et al., 1997; Cunha et al., 1998) due to its rapid enzymatic degradation to ADP and ADO, although in the cerebral cortex its action is more prolonged (Cunha et al., 1994). ADO modulates both pre- and postsynaptic components and also has nonsynaptic actions in the nervous system, mainly through activation of the A_1 and A_{2A} high affinity receptors (Sebastião & Ribeiro 2009). The extracellular accumulation of this important neuromodulator occurs as a breakdown product of ATP metabolism and also by its release from neurons and glial cells (Sebastião & Ribeiro 2000). Previous studies from our group reported that ADO, at a low concentrations, reduces [^{3}H]GABA uptake by acting on A_1 receptors (Cristóvão-Ferreira et al., 2011). Very low ADO

levels are detected over a long period of incubation with ATP in cortical astrocytes (Lai & Wong 1991; Wink et al., 2003), which could be explained *in situ* by the presence of a feed forward inhibition mechanism of ecto-5'-nucleotidase (Zimmermann 1996). In the assays reported here, it seems that the inhibitory action of extracellular ATP in the GATs activity, during a 1 min treatment, does not depend on its hydrolysis into adenosine, since: (i) removal of extracellular ADO (i.e. in the presence of ADA) does not affect the inhibition of [^{3}H]GABA uptake induced by one minute incubation with 100 μM ATP, (ii) the ADP analogue, 2-MeSADP, caused a slightly higher inhibition of the GABA transport than the one induced by ATP, which also suggests that their effects are not mediated by extracellular catabolism into ADO, and (iii) the inhibitory action of 100 μM ATP is slightly lower after an incubation for 5 min in comparison with an incubation of only 1 min.

An alternative approach to assess the possible involvement of adenosine in the inhibitory effect of [^{3}H] GABA uptake mediated by ATP would be the inhibition of the activity of the ecto-enzyme NTPDase 2. This ecto-enzyme is widely expressed in rat cortical astrocytes in culture (Wink et al., 2006). However, the sole commercial available inhibitor of ecto-ATPases, 6-N,N-Diethyl-D-b-g-dibromomethylene adenosine triphosphate (ARL 67156), only inhibits effectively the action of the human NTPDase2 (Iqbal et al., 2005; Lévesque et al., 2007). On the other hand, the influences of ATP on GAT activity, even in the presence of ADA, do not definitively exclude the involvement of A_1 receptors on the modulation of GABA uptake, because no assay was performed when the A_1 receptor was blocked with DPCPX. This procedure was not adopted due to the possible formation of A_1-$P2Y_1$ receptor heteromers. Due to the formation of these heteromers, A_1 receptor signalling

pathway could be impaired upon incubation with 2-MeSADP, the $P2Y_1$ receptor agonist (Tonazzini et al., 2008).

The pharmacological characterization of the effects observed in GAT activity strongly suggests that the observed inhibition on GABA uptake into astrocytes is mediated by the ADP-preferring receptors $P2Y_1$. $P2Y_1$ receptors are involved in this effect since a subtype-specific $P2Y_1$ receptor antagonists and a general P2 receptor antagonists completely inhibit the modulation of the transporter induced by 2-MeSADP. The influence of nucleotide metabolism in the effect of 2-MeSADP also has to be considered due to the metabolic instability of the 2-MeSADP (Alvarado-Castillo et al., 2005). In tested conditions (1 min incubation of 100 μM 2-MeSADP) the formation of metabolites are probably negligible, taking into account the work of Quintas et al. (2011) in mature astrocytes. Thus, after 1 hour of incubation of mature astrocytes with 100 μM 2-MeSADP, there is an accumulation of approximately 30 μM 2-MeSAMP, and in the case of 2-MeSADO (which activates ADO A_3 receptor) the accumulation of this metabolite can even be neglected even after 3 hours of 2-MeSADP incubation (Quintas et al., 2011). An increase in the [3H]GABA uptake was observed upon pretreatment of astrocytes with PPADS (P2 receptor antagonist) and MRS2179 ($P2Y_1$ receptor antagonist), suggesting that GAT activity could be under tonic modulation by endogenous adenine nucleotides, such as it has been observed in glycine transporters (Jiménez et al., 2011).

In the CNS, GABA release is under the modulation of P2Y (Illes & Ribeiro, 2004), either enhancing it via the $P2Y_1$ receptor (Saitow et al., 2005) or inhibiting the release through the $P2Y_4$ receptor activation (Donato et al., 2008). In glial cells, the activation of P2X receptors (*e.g.* $P2X_1$ and $P2X_2$/$P2X_3$ heteromer) modulates the extracellular

GABA levels, probably through a reduction in the efficacy of the uptake process (Neal et al., 1998). Wang et al. (2002) also reported an enhancement of GABA release mediated by the activation of $P2X_7$ in astrocytes, although this process occurs through a mechanism independent from GABA transporter activity. Outside the CNS, the renal betaine/GABA transporter (BGT1) activity is inhibited by both P2 receptors (via uracil nucleotide-preferring receptors, $P2Y_{2,4}$) or by ADO, through A_1 receptors (Kempson et al., 2008). This redundant function of P2 receptors may be associated to the existence of a dual role for each P2 subtype, depending on the conditions of ATP exposure (James & Butt 2002). A transient exposure to micromolar levels of ATP activates purinoceptors of high-affinity (P2Y and the rapidly desensitising P2X receptors, such as $P2X_1$), causing an increase in glial $[Ca^{2+}]_i$ that is linked to the release of gliotransmitters (*e.g.* ATP and glutamate; Volterra & Meldolesi, 2005). Consequently, ATP will accumulate in the extracellular space, reaching higher concentrations (in the millimolar range), which are sufficient to activate P2Y receptors and also low-affinity $P2X_7$ receptors (James & Butt 2002). The neurochemical studies reported here are broadly consistent with the findings of a GATs modulation due to an activation of P2 receptors, namely the $P2Y_1$ receptor subtype.

In astrocytes, increases in extracellular GABA may be caused either by a functional inhibition of the transporter by a wide variety of signalling cascades (Gadea & López-Colomé 2001), a Ca^{2+}-independent reversal of the carrier-mediated transport system at the level of the plasma membrane (Levi & Gallo 1995) or by a Ca^{2+}-independent reversal of GATs triggered by glutamate uptake (Héja et al., 2009). As reported here, the stimulation of $P2Y_1$ purinoceptors in rat astrocytes causes a

propagation of Ca^{2+} increases, mainly dependent on the Ca^{2+} ions liberated from internal stores. Therefore, experiments were designed to emphasize the importance of intracellular Ca^{2+} and its signalling system via the PLC/$InsP_3$ pathway on the regulation of GABA transporter activity by the 2-MeSADP. When Ca^{2+} mobilization was blocked using BAPTA-AM or CPA during the acute incubation with 2-MeSADP, the $P2Y_{1,12,13}$ agonist no longer induces an inhibition on [^{3}H]GABA uptake. Although incubation of BAPTA-AM and CPA modulate the $[Ca^{2+}]_i$, these compounds by itself did not affect [^{3}H]GABA uptake, suggesting that the 2-MeSADP-induced inhibition of uptake is not a direct effect of $[Ca^{2+}]_i$ increase and actually a modulation of uptake by down-stream Ca^{2+}-dependent pathways. More direct evidence for the existence of a functional link between 2-MeSADP-induced raise in $[Ca^{2+}]_i$ and inhibition of [^{3}H]GABA uptake is provided by the results showing that the inhibitory actions of 2-MeSADP on GAT-1 and GAT-3 transport are blocked by U73122 (PLC inhibitor), suggesting a role for PLC and, accordingly, for $InsP_3$ on this process. A complementary approach could be made using U73343, an inactive analogue of U73122 (Bleasdale et al., 1990). It would be expected that the incubation with this compound does not affect the inhibitory modulation of GATs activity induced by 2-MeSADP. Therefore, this release of intracellular Ca^{2+} can provide a plausible explanation for the findings that show that the activation of the $P2Y_1$ receptor reduces GABA transporter activity. A regulatory Ca^{2+}-dependent mechanism had already been reported to act on neurotransmitter transporters (Parpura et al., 1995; Nishio et al., 1995), and to couple to some pathways (Figure 16), like PLA_2 (Chéramy et al., 1996; Duarte et al., 1996; Cunha & Ribeiro 1999) or CaM (Llinás et al., 1985; Sitges et al., 1995; Gonçalves et al., 1997; Gadea et al., 2002). These pathways are also induced upon P2Y receptor activation

in astrocytes (Bronstein et al., 1988; Bruner & Murphy 1990; Erb et al., 2006; Figure 1).

The PLA_2 reaction is the primary pathway through which AA is cleaved from the *sn-2* position of membrane phospholipids (Balsinde et al., 1999). AA formation will stimulate the activation of some specific PKC isoforms (Murakami & Routtenberg 1985; Shinomura et al., 1991; Hardy et al., 1994), and also leads to an increase in extracellular GABA levels in a PKC-dependent way (Chéramy et al., 1996) but also independently of the activation of this intercellular modulator (Breukel et al., 1997). However, in the former case, the observed effects are largely due to a marked facilitation of the release process rather than to uptake inhibition (Chéramy et al., 1996).

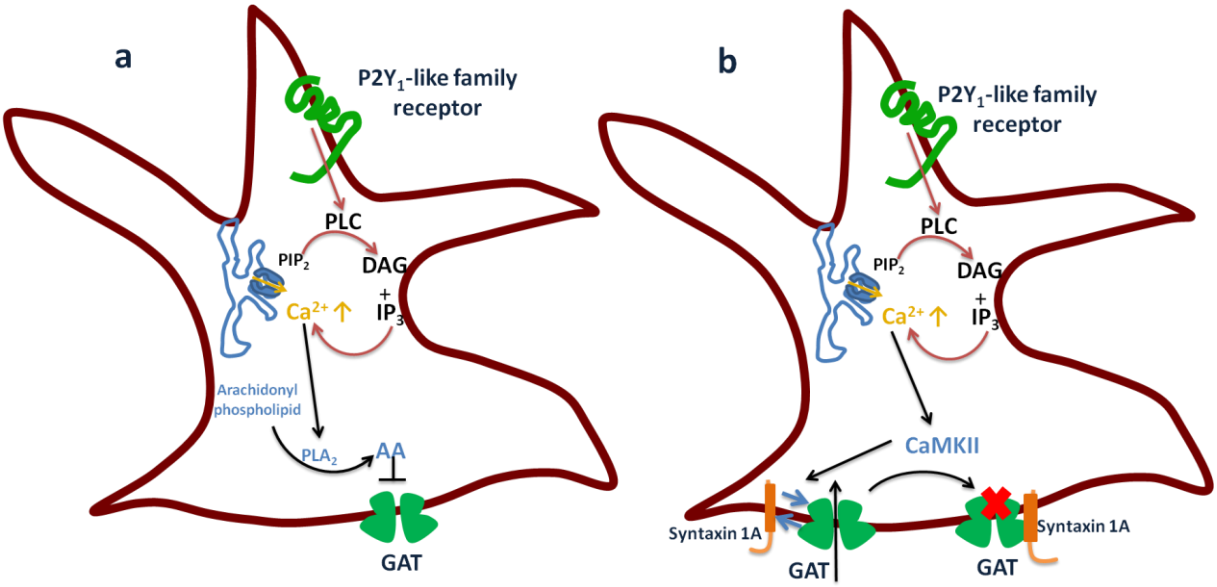

Figure 16 – Possible models of GABA transport modulation on astrocytes. (a) When the membrane phospholipids are acted upon phospholipase A2 (PLA_2), arachidonic acid (AA) is formed. AA can modulate GABA transporter (GAT) activity through a direct interaction with the transporter or through changes in membrane fluidity. (b) In the absence of phosphorylation, syntaxin 1A can bind to many partners, including the GAT. Phosphorylation by Ca^{2+}/calmodulin kinase II (CaMKII) strengthens the GAT/syntaxin 1A interaction, resulting, inhibiting GAT function. For more details see text.

The possibility that PKC could mediate the [^{3}H]GABA uptake inhibition is very strong, since the PLC-PKC pathway is one of the main pathways elicited after $P2Y_1$

activation (Abbracchio & Burnstock 1994), and this pathway is also involved in the transporter activity modulation in astrocytes (Gomeza et al., 1991). Considering the results reported here, it seems that PKC activation is not essential to the inhibition of the high-affinity transport system for GABA exerted by 2-MeSADP in glial cells, because the 2-MeSADP effect was insensitive to the PKC inhibitor, GF109203X. This result can be explained by the role of PKC in the astrocytic Ca^{2+} increases, as PKC is essential in the negative regulation of $P2Y_1$ receptors, fundamental after high frequency stimulation (Fam et al., 2003; Hardy et al., 2005; Weng et al., 2008). The negative regulation was confirmed, in the conditions studied, as a pretreatment with GF109203X greatly increased the 2-MeSADP-induced Ca^{2+} mobilization and induced a higher relative percentage of inhibition of [^{3}H]GABA uptake in comparison to when 2-MeSADP was incubated alone. GF109203X increases [^{3}H]GABA uptake, because endogenous PKC activity can, by itself, tonically inhibit GATs activity (Cristóvão-Ferreira et al., 2009). To complete these observations, it would be important to perform assays where astrocytes were incubated with a known PKC activator, in order to evaluate if the effect of 2-MeSADP on transporter activity was lost in the presence of the PKC activator. An activation of PKC is associated with a decrease in surface expression of GATs (Corey et al., 1994) and the inhibition of the $[Ca^{2+}]_i$ increases in astrocytes (Weng et al., 2008).

As reported herein, Ca^{2+} seems to play a pivotal role in modulating [^{3}H]GABA uptake. The modulation of the GABA transport independently of PKC that was identified in this work, suggests that GABA transporter modulation can be due to an alteration on the binding affinity of the substrate or other associate proteins, rather than, for example, being regulated by the transporter trafficking as an acute response

to PKC activation (Chen et al., 2004). Hence, a mechanism that might explain a PKC-independent modulation of GAT is through an action of AA (Breukel et al., 1997) directly interfering with the transporter molecule or be due to secondary changes in the membrane fluidity (Chan et al., 1983; Barbour et al., 1989; Volterra et al., 1992; Trotti et al., 1995).

Another Ca^{2+}-linked mechanism for regulating GABA transporter activity is due to the Ca^{2+}/calmodulin complex. In synaptossomes, GABA uptake was already shown to be greatly reduced by Ca^{2+} in a concentration-dependent manner, in which, at least in the micromolar range of concentration of Ca^{2+} (1-10 μM), it appears to involve the Ca^{2+}/calmodulin-stimulated phosphatase 2B, calcineurin (Gonçalves et al., 1997; Gonçalves et al., 1999). In the present work, it was observed that Ca^{2+} elevations induced by 2-MeSADP, on average, evoke an increase of 1 to 2 μM in $[Ca^{2+}]_i$, at which concentrations GABA uptake is still significantly inhibited in synaptossomes (Gonçalves et al., 1997) and in which the calcineurin can be activated (Ingebritsen et al., 1983; Wera & Hemmings 1995). CaM-dependent protein kinase II (CaMKII) was also reported to participate in the modulation of glycine transport in Müller glia cells, and the effect of CaMKII was mimicked by a transient Ca^{2+} increase (Gadea et al., 2002). In agreement with the results of Gonçalves et al. (1997), the former study also reported that trifluoperazine (a calmodulin inhibitor) does not alter the normal GABA uptake process (Gadea et al., 2002). However, this drug is also known to inhibit PKC activity (Le Peuch et al., 1983), thus an effect of CamKII on GAT activity independently of PKC could not be excluded in the work of Gonçalves et al. (1997), so a modulation of the transporter activity through an action of CaMKII appears to be a better candidate.

Ca^{2+}/calmodulin complex modulates the activity of several target molecules, such as CaMK, protein phosphatases, adenylyl cyclases, phosphodiestereases (Van Eldik et al., 1982), and nitric oxide synthase (Stuehr 1999), and it can also interact with transporter proteins (Jayanthi et al., 1994; Jayanthi et al., 2000; Fog et al., 2006) or syntaxins (Ohyama et al., 2002), through CaMKII. Among these molecules, syntaxin 1A has a very important action on endogenous regulation of transport function, and it has been reported to be expressed in astrocytes (Parpura et al., 1995; Jeftinija et al., 1997). This molecule is a nervous system-specific plasma membrane protein which is better known as a regulator of vesicle fusion and trafficking and vesicle docking of synaptic vesicles (Rothman 1996). Syntaxin 1A is also capable of regulating GABA transporters, both functionally and physically (Quick et al., 1997; Beckman et al., 1998) and acts as a positive regulator of GAT-1 surface expression (Deken et al., 2000; Horton & Quick 2001). Mechanisms of the effects of syntaxin 1A include not only changes in transporter trafficking but also alterations in the rate of transport. The NH_2 tail of GAT-1 can bind to the H3 domain of syntaxin 1A, and this interaction limits the tail participation in substrate translocation, causing a decrease in substrate transport (Deken et al., 2000). Thus, a good hypothesis to explain a PKC independent regulatory mechanism would be through a regulation of the interaction between GABA transporters and syntaxin 1A due to CaMKII activation. A similar modulation was already observed in other transporters, such as in the dopamine (Fog et al., 2006) and serotonin transporter (Ciccone et al., 2008), both of them belong to the sodium- and chloride-dependent neurotransmitter transporter family SLC6, like GATs. The effects of CaMKII can be related to a change in the binding

between the transporter and syntaxin 1A (Ciccone et al., 2008), or even directly in the syntaxin 1A protein (Ohyama et al., 2002; Ciccone et al., 2008).

The inhibition of the GABA transport reported in this work could be in agreement with the fact that ATP released during high neuronal activity can act through P2 receptors exerting a neuroprotective mechanism, namely through increased extracellular GABA levels. From all P2Y receptors described, only the ones that are known to be couple to G_q/G_{11} proteins ($P2Y_{1-6}$) have been implicated in CNS disorders (Burnstock 2008), pointing towards the importance of the calcium signalling in astrocytes. $P2Y_1$ receptors are implicated in epileptogenesis in a Ca^{2+}-dependent manner (Kumaria et al., 2008), and their inhibition and, consequently, the inhibition of Ca^{2+} increases evoked by the activation of this receptors, was suggested as an important therapeutic strategy for epilepsy (Burnstock 2008). Interestingly, however, an acute administration of the $P2Y_1$ receptor agonist (ADP*β*S) induces an anxiolytic-like behavioural profile in rats, and this effect is antagonized PPADS and MRS2179 (Kittner et al., 2003), suggesting that the anxiolytic-like effect of ADP*β*S is mediated through the PLC pathway and nitric oxide formation (Kittner et al., 2003), which is known to reduce GABA uptake in hippocampal synaptossomes (Cupello et al., 1997). Although, 2-MeSADP is not so metabolically stable as ADP*β*S, the latter agonist is only effective in the endogenously expressed $P2Y_{1,12}$ receptor of humans and $P2Y_{13}$ in both humans and mouse (von Kügelgen 2006). Then, data reported in this work could provide a direct evidence for this action, in which a purinergic effect dependent on PLC activity modulates the activity of GABA transporters. Meanwhile, the importance of nitric oxide on the modulation of GABA transporters was revealed to be related with its effect on the interaction between syntaxin 1A and GAT-1 (Fan et

al., 2006). In addition, an acute treatment (30 min) with the GAT inhibitor tiagabine (Borden 2006) also induces anxiolytic-like effects in rats (Schmitt et al., 2002), and the results were confirmed by in *GAT1* knockout mice (Liu et al., 2007).

Therefore, the present observed interaction between $P2Y_1$ receptor-mediated signalling pathway and GAT activity might be of particular interest for further studies, including the design of new therapeutic strategies in the treatment of anxiety.

6| Conclusions

This aptitude of Ca^{2+} increases, mediated by the $P2Y_1$ purinoceptor to regulate the GABA transport activity raises the question as to the physiological significance of this action. This regulation acquires even more relevance both due to the diverse functions of purines in the control of several behavioural pathways and to their role in the coordination of the long-distance glial signalling pathway (Ca^{2+} waves), since in addition to providing physiological modulatory actions, astrocytic function could be related to neurological disorders and psychiatric conditions.

The data in this report fits the hypothesis advanced by Ciccone et al. (2008), according to which calcium-mediated signals act as a trigger for controlling neurotransmitter transporters. One of the calcium triggers involved in this action can be evoked by the $P2Y_1$ receptors, as described in this report. The influence of Ca^{2+} on GABA transport activity can occur through a modulation of the interaction between syntaxin 1A and the transporter protein (Deken et al., 2000), thus keeping the transporter functionally silent, awaiting a cell signal “informing” that it is important to remove the neurotransmitter from the synaptic cleft.

In conclusion, a brief activation of $P2Y_1$ receptor in astrocytes triggers Ca^{2+} waves and inhibits GABA transport into astrocytes, suggesting that the two main astrocytic functions can be related; therefore, this could represent a feedback regulation mechanism through astrocytes, controlling the extracellular GABA levels.

7| References

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8| Appendix I

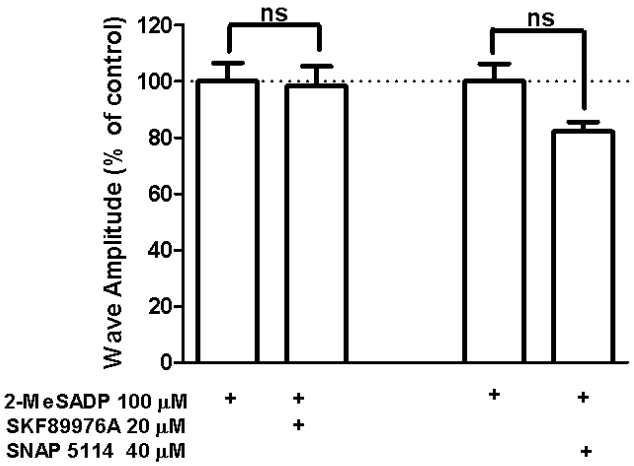

Figure A-I – Effects of GABA Transporters inhibitors on the $[Ca^{2+}]_i$ response to 2-MeSADP. Summary plot of the influence of SKF89976A (20 µM) and SNAP5114 (40 µM) on the $[Ca^{2+}]_i$ response to 2-MeSADP (100 µM). 2-MeSADP was pressure-applied four times for 0.3 s each at intervals of 30 min. After establishing two stables responses to the agonists, 2-MeSADP (S_1 and S_2), an GAT inhibitor-containing solution (either SKF89976A or SNAP5114) was superfused 20 min before and during the third agonist application (S_3), followed by washout for another 30 min (S_4) Effects are shown as the response (%) during S_3 - agonist-induced response signal upon drug incubation vs. S_2 - the control signal (2-MeSADP stimulation in a drug-free medium). Mean ± S.E.M. of 57-67 responsive cells from 1 culture; ns, not significant differences between S_3 vs. S_2, assessed by the Student's *t*-test. The results represent only responsive cells.

9| Appendix II

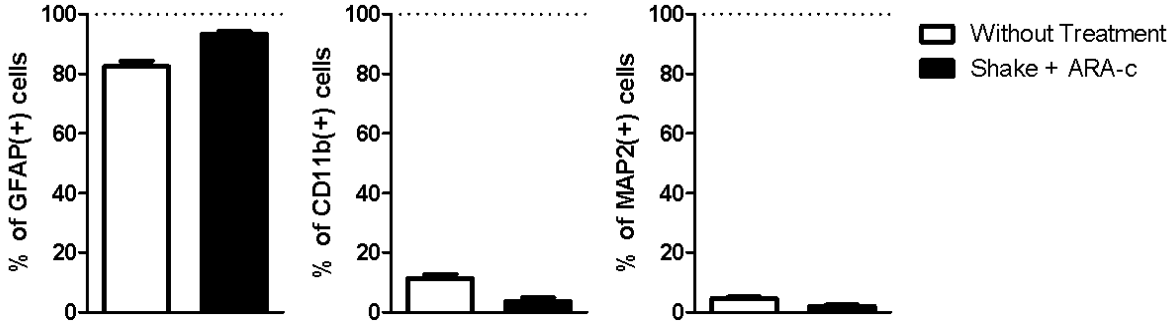

Figure A-II – Quantification of GFAP-, CD11b and MAP2-positive cells in two different astrocytic culture conditions. Summary of percentage of cells showing GFAP-, CD11b and MAP2-positive cells, from cultures (25 DIC) without any treatment (besides normal medium change; white bars) and cultures (25 DIC) subjected to shake + ARA-C procedure (15 h orbital shaking + 8 µM of ARA-C for 2 days; black bars).

10| Appendix III

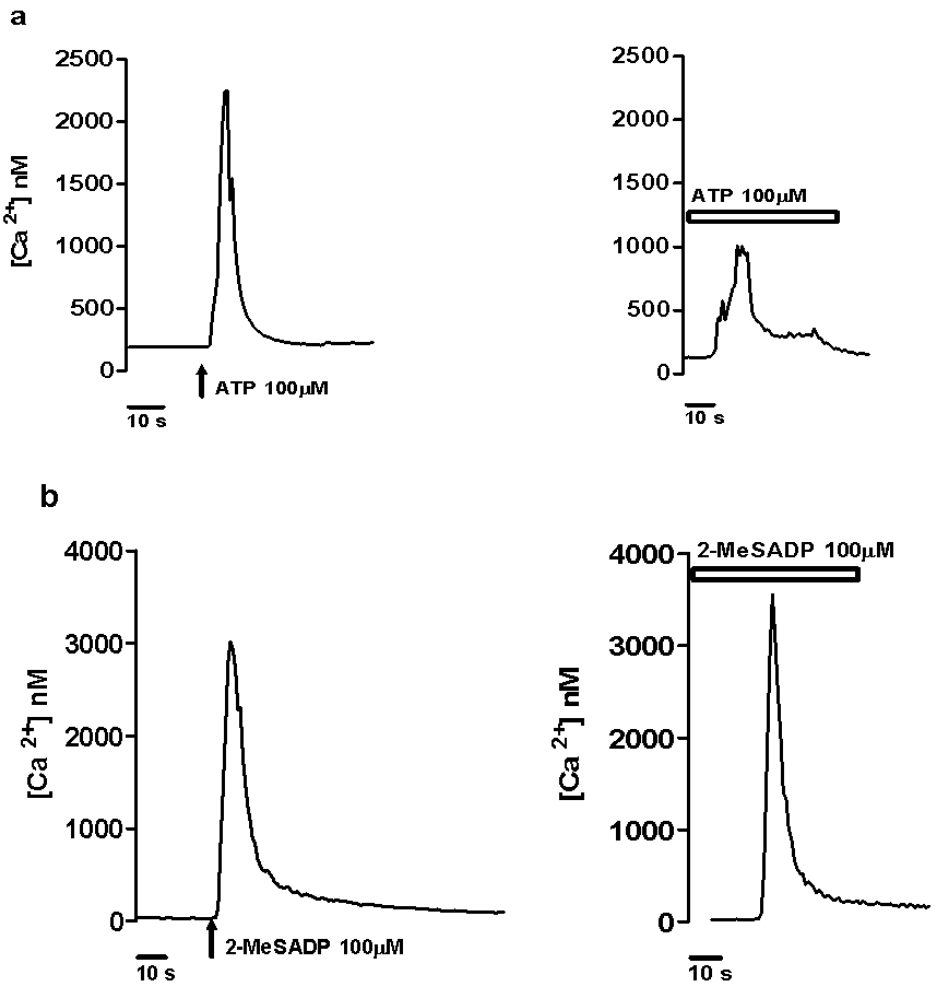

Figure A-III – Effect of ATP and 2-MeSADP with two stimulations times (0.3 s and 60 s). (a) Left, representative change in $[Ca^{2+}]_i$ signal illustrating the transient response upon a brief application (0.3 s) of ATP (100 µM) at time point 30 s (arrow) consisting of an initial $[Ca^{2+}]_i$ peak followed by a fast decline. *Right*, representative change in $[Ca^{2+}]_i$ signal illustrating the biphasic response upon prolonged application (60 s, horizontal bar) of ATP (100 µM) consisting of an initial $[Ca^{2+}]_i$ peak followed by a lower but sustained $[Ca^{2+}]_i$ plateau. (b) Left, representative change in $[Ca^{2+}]_i$ signal illustrating the transient response upon a brief application (0.3 s) of 2-MeSADP (100 µM) at time point 30 s (arrow) consisting of an initial $[Ca^{2+}]_i$ peak followed by a fast decline. *Right*, representative change in $[Ca^{2+}]_i$ signal upon prolonged application (60 s, horizontal bar) of 2-MeSADP (100 µM), showing a response with a similar dynamics as the response upon 0.3 s stimulation. Mean ± S.E.M. of 45 responsive cells from 1 culture. The results represent only responsive cells.