UNIVERSITÀ DI PISA

Scuola di Dottorato di Ricerca in
"Scienza del Farmaco e delle Sostanze Bioattive"

Dottorato di Ricerca in
"Scienza del Farmaco e delle Sostanze Bioattive"

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" Purinergic system: regulations and interactions "

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“ All truths are easy to understand once they are discovered;
the point is to discover them ”

Galileo Galilei

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Abbreviations

A_1R, A_1 adenosine receptor;

A_{2B}R, A_{2B} adenosine receptor;

ADA, adenosine deaminase;

ADF, human astrocytoma cells;

ADP, adenosine 5'-diphosphate;

ARs, adenosine receptors;

ATP, adenosine 5'-triphosphate;

CA1 & **CA3**, cornu ammonis 1 & 3 (hippocampal subfields);

cAMP, cyclic adenosine 5'-monophosphate;

CGS 21680, 2-(carboxyethylphenylethylamino)adenosine-5'-carboxamide;

CHA, N^6-cyclohexyladenosine;

CNS, Central Nervous System;

DPCPX, 8-cyclopentyl-1,3 dipropyl xanthine;

FSK, forskolin;

GDP, guanosine 5'-diphosphate;

GPCR, G protein-coupled receptor;

GTP, guanosine 5'-triphosphate;

GTPγS, guanosine-5'-(γ-thio)triphosphate;

HC, hippocampal membranes;

MeSADP, 2-methylthio-adenosine 5'-diphosphate;

MRS 1220, 9-chloro-2-(2-furyl)-5-phenylacetamino[1,2,4]triazolo[1,5-c]quinazoline;

MRS 1706, N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin8-phenoxyl]acetamide;

MRS2179, 2'-deoxy-N^6-methyl adenosine 3',5'-diphosphate;

NECA, 5'-N-ethylcarboxamidoadenosine;

$P2Y_1$R, purinergic $P2Y_1$ receptor;

PSD, postsynaptic density;

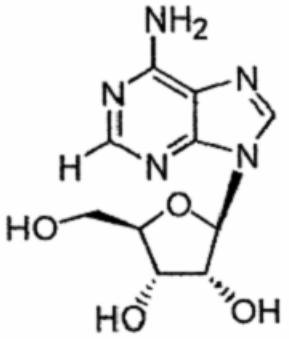
PTX, Pertussis-toxin;

R-PIA, (-)-N^6-(2-Phenylisopropyl)-adenosine;

SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine;

TNF-α or **TNF-alpha,** tumor necrosis factor-alpha.

Purine & Purinergic ligands



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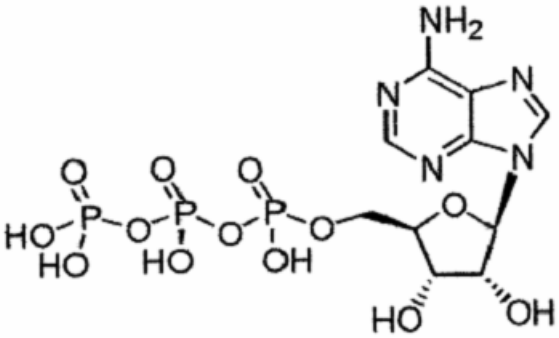

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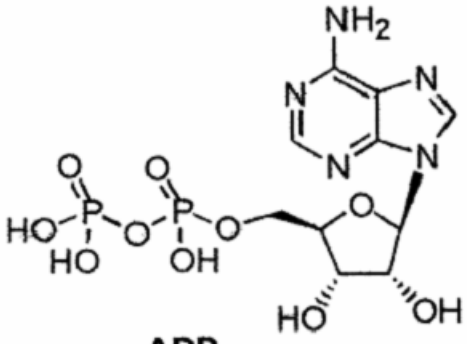

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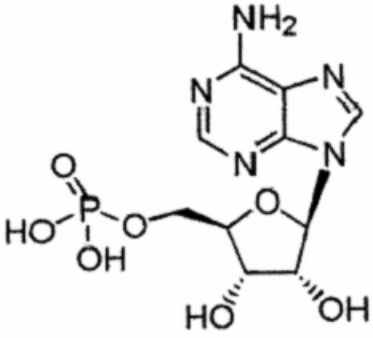

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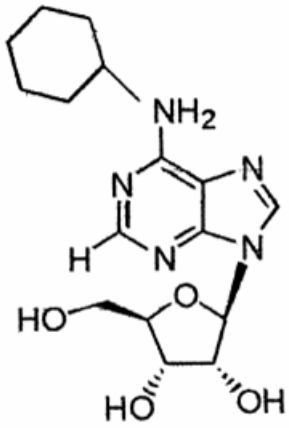

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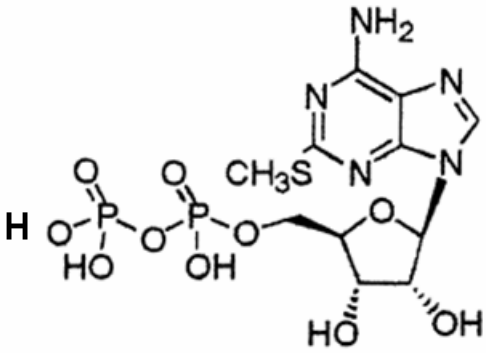

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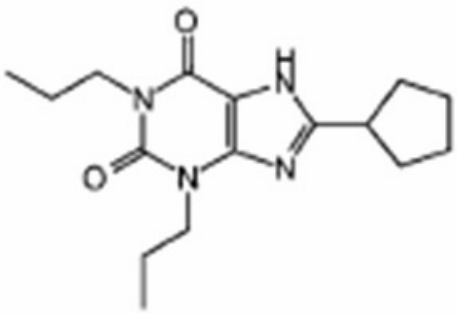
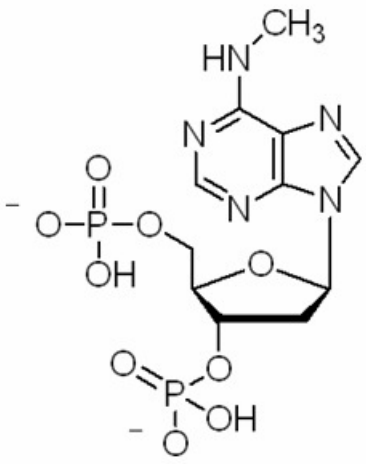
DPCPX





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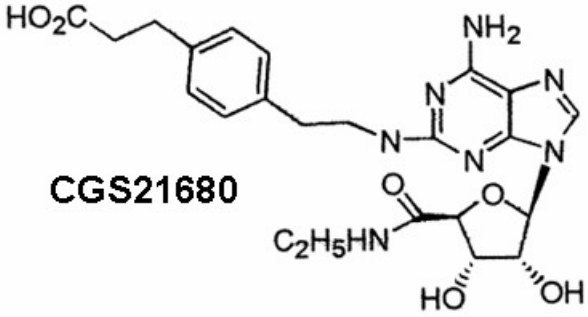

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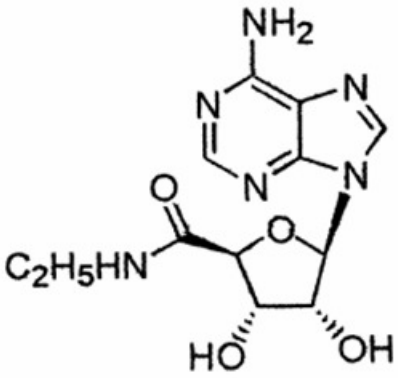

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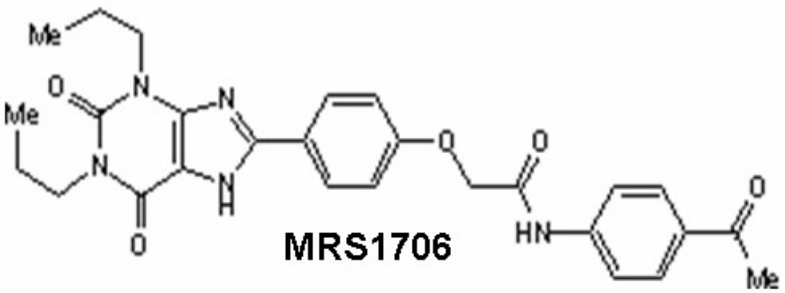

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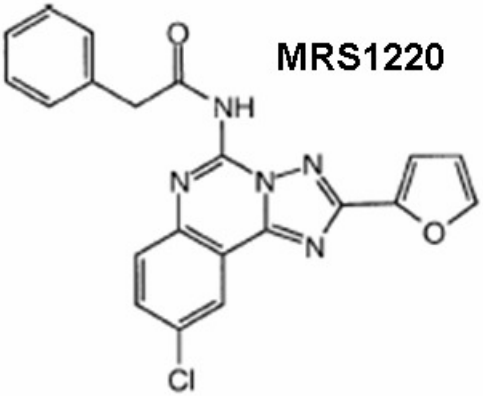

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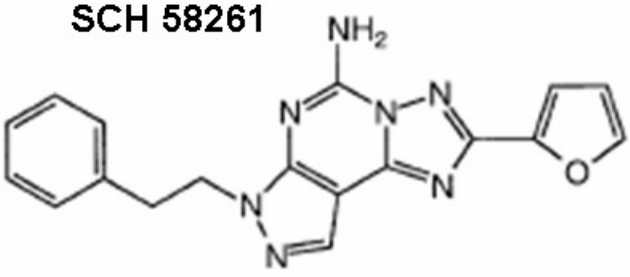

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Chapter 1:
Introduction

1.1 Purinergic system

Purines have major roles in the activities of non-neuronal cells as well as neurons. This includes fast signalling roles in exocrine and endocrine secretion, platelet aggregation, vascular endothelial cell-mediated vasodilation and nociceptive mechano-sensory transduction, as well as acting as a co-transmitter and neuromodulator in most, if not all, nerve types in the peripheral and central nervous systems. More recently, slow (trophic) purinergic signalling has been implicated in cell proliferation, migration, differentiation and death in embryological development, wound healing, restenosis, atherosclerosis, ischemia, cell turnover of epithelial cells in skin and visceral organs, inflammation, neuroprotection and cancer (Burnostock, 2006).

In the late 1960s the search for the transmitter responsible for the non-adrenergic and non-cholinergic nerve responses led to the surprising proposal that ATP or a related nucleotide might be the transmitter involved in both the gut and the bladder (Burnstock *et al*., 1972). This "*purinergic hypothesis*" met considerable resistance for the next 20 years, partly perhaps because ATP was recognized at that time as an intracellular molecule contained in all cells and of particular importance as an energy source, and it was considered that such a ubiquitous molecule was unlikely to act as a neurotransmitter, even though the presence of powerful ecto-enzymes for the breakdown of ATP was already known.

Later co-transmission turned out to be the rule, rather than the exception, and in fact the significance of purinergic transmission was much extended by this concept. Evidence for ATP as a co-transmitter, even if with variable levels of contribution, has been found for all peripheral or central nerves so far investigated (Burnstock, 2004).

Purinergic receptors were first defined in the late 1970s and a basis for distinguishing two types of **purinoceptors**, named **P1** and **P2** for adenosine and ATP/ADP, respectively, was proposed (Burnstock, 1978).

In the early 1990s, receptors for purines were cloned: four P1 receptor subtypes and seven P2X ionotropic and eight P2Y metabotropic receptor subtypes are currently recognized and characterized.

P1 and P2Y purinergic receptors belong to the family of ***G protein coupled receptors*** (**GPCRs**), the largest family of cell-surface molecules involved in signal transmission, activated by a wide variety of ligands, including peptide and non-peptide neurotransmitters, hormones, growth factors, odorant molecules and light. The large number of GPCRs and the importance of their physiological roles have made the search for novel therapeutic drugs an important and constantly expanding activity in the pharmaceutical industry: indeed these receptors are the target of more than 50% of the current therapeutic agents on the market (Marinissen and Gutkind, 2001).

These receptors share a seven trans-membrane helixes common structure, connected by three extracellular and three intracellular loops and constituted by an extracellular amino terminus and an intracellular carboxyl tail. Activated receptors interact with heterotrimeric G proteins that, through their α and $\beta\gamma$ subunits, modulate the activity of membrane effectors such as enzymes (adenylyl and guanylyl cyclases, phosphodiesterases, phospholipases) or ionic channels. These membrane effectors generate second messengers (such as cAMP, diacylglycerol or inositol trisphosphate) and/or alter the intracellular concentration of critical ions (such as Ca^{2+}, Na^{+}, K^{+}), which trigger phosphorylation-dephosphorylation cascades that propagate the signal intracellularly to produce the final actions (**Fig. 1**). It has been recently shown that most biological responses mediated by GPCRs are not dependent on a single biochemical route, but result from the integration of the functional activity of an intricate network of intracellular signalling pathways. Furthermore G protein-coupled receptors are dynamically regulated, frequently through phosphorylation-dephosphorylation cycles (Vazquez-Prado *et al.*, 2003).

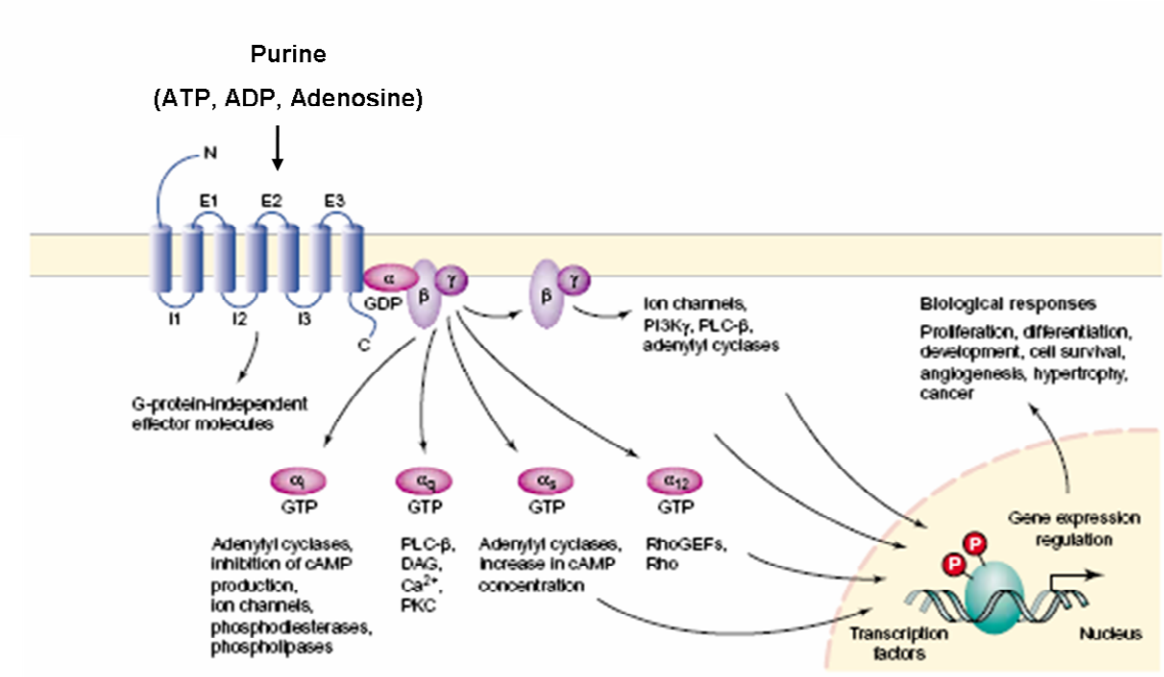


Fig. 1: Diversity of G-protein-coupled receptors (GPCRs). A wide variety of ligands, including purine, use GPCRs to stimulate cytoplasmic and nuclear targets through heterotrimeric G-protein-dependent and -independent pathways. Such signaling pathways regulate key biological functions such as cell proliferation, cell survival and angiogenesis. Abbreviations: DAG, diacylglycerol; GEF, guanine nucleotide exchange factor; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLC, phospholipase C. Modified from Marinissen and Gutkind (2001).

1.2 Adenosine and adenosine receptors

The purine nucleoside adenosine plays a central role in the energy metabolism of any cell and its formation, by breakdown of ATP in the extracellular space, is closely related to the energy consumption of the cell: in fact its concentration, although strictly regulated by deamination or phosphorylation metabolism, can increase dramatically when there is an imbalance between energy use and energy supply (Fredholm, 1997). It is recognized as a very important substance in the homeostasis of CNS cells; due to the complex regulation of energy metabolism and adenosine levels, the extracellular adenosine concentration in all body fluids is rather constant under basal conditions (30-300 nM), but oxygen depletion induces a rise in extracellular adenosine to 10 μM or even higher (Schulte and Fredholm, 2003).

Under basal conditions and in particular in emergency situations, adenosine plays a major role in the central nervous system, in the cardiovascular system, as an endogenous pain modulator, the immune system, mast cell degranulation, asthma, cell growth, proliferation and apoptosis (for review Fredholm *et al.*, 2001 and 2005).

The purine nucleoside adenosine acts on a family of GPCRs, collectively called ***adenosine receptors*** (AR) or ***P1*** purinoceptors: four distinct adenosine receptor subtypes, A_1, A_{2A}, A_{2B} and A_3, have been cloned and biochemically-pharmacologically characterized (Fredholm, 1995; Fredholm *et al.*, 2001).

They are coupled to an intricate network of signalling pathways involving classical second messenger pathways, such as modulation of cAMP production or the phospholipase C (PLC) pathway, and in addition interact with mitogen-activated protein kinases (MAPK), which could give them a role in cell growth, survival, death and differentiation.

The original classification of adenosine receptors was based on their effects on cAMP levels in different tissues: A_1 and A_3 adenosine receptors mediate a decrease in cAMP via $G_{i/o}$, whereas the two A_2 receptors, the high-affinity A_{2A} and low-affinity A_{2B} subtypes, mediate an increase in cAMP via G_s (Schulte and Fredholm, 2003).

In particular:

- The A_1 adenosine receptor (**A_1R**), highly affine for adenosine, acts through inhibition of adenylyl cyclase, activation of several type of K^+ channels, inactivation of N, P and Q-type Ca^{+2} channels, activation of phospholipase C and acti-

vation of ERK ½. A_1Rs mediate inhibitory actions both presynaptically, by reducing transmitter release, and postsynaptically, by hyperpolarizing neurons (but also astrocytes): A_1Rs protect in this manner CNS cells from the excitotoxicity present during pathophysiological states (Haas and Selbach, 2000). In fact, lines of evidence for an A_1R neuroprotective role have been extensively supplied by both in vitro and in vivo studies (reviewed by Schubert *et al.*, 1997; Ribeiro *et al.*, 2003).

- The A_{2B} adenosine receptor (**A_{2B}R**), named "low affinity" receptor (Beukers *et al.*, 2000), generally couples to G_s, but several studies implicated signalling via $G_{q/11}$. Little is known about the cell-specific expression of the A_{2B}R in vivo or about their functional significance, also because there is a lack of specific agonists for this receptor. Despite this limitation, the A_{2B}R has been implicated in several important biological events, such as mediating vasodilation, inhibiting growth of rat aortic smooth muscle cells and controlling the production of cytokines and inflammation (reviewed by Feoktistov *et al.*, 1998; Yang *et al.*, 2006).

Moreover in the CNS, adenosine is a "fine-tuner neuromodulator" (Ribeiro *et al.*, 2002) and, as a central substance in energy metabolism, can effectively regulate neuronal firing and communication: in general, it has an inhibitory activity, mainly mediated by A_1Rs, correlated with low energy reserve. Increasing demand and decreasing availability of energy as they occur during excessive neuronal activity, hypoxia or hypoglycemia are associated with high adenosine levels that provide a protective feedback in such pathological situations (Haas and Selbach, 2000).
Although adenosine is not a neurotransmitter on its own, it shares many properties, via A_1R activation, with inhibitory neurotransmitters (i.e. GABA), such as the control of glutamatergic transmission in CNS; nevertheless adenosine might indirectly control GABAergic functioning (Ribeiro *et al.*, 2003). Excitatory actions, on the other hand, are mediated by ATP and high levels of intracellular ATP provide for high neuronal excitability when ATP-sensitive K^+ channels are closed (Haas and Selbach, 2000).

1.3 ATP and purinergic receptors

Extracellular adenosine 5'-triphosphate (ATP) has been recognized as a ubiquitous signalling molecule, acting as a fast neurotransmitter and modulator of transmitter release and neuronal excitability and participating in cell differentiation, proliferation and survival, as well as a toxic agent that mediates cellular degeneration and death (Burnstock, 2006). There is a large gradient for ATP transport or secretion out of cells; this happens via ATP transporters, channels or via Ca^{2+}-mediated exocytosis from synaptic vesicles, together with classical neurotrasmitters. Intracellular ATP concentrations are in the millimolar range, whereas extracellular ATP concentrations rely on the balance between release and degradation (the concentration estimates range from nanomolar to micromolar) (reviewed by Franke and Illes, 2006). A dramatic release of ATP and purines occurs after cellular death, via the damaged cell membranes.

Ecto-nucleotidase family enzymes limit the extracellular actions of ATP by enhancing its removal as well as by producing adenosine, which can functionally antagonize some effects of ATP. In neuronal tissue, AMP, ADP, and ATP were shown to be rapidly converted to adenosine with a $t_{1/2}$ of 200 msec (Zimmermann, 2000).

Potential sources of extracellular purines in the CNS include neurons, glia, endothelium and blood. Purinergic mechanisms may be involved in the ethiopathology of many neurodegenerative conditions, especially because massive extracellular release of ATP, adenosine and other neurotransmitters occurs. During different kinds of "acute" (i.e. ischemia, hypoxia, mechanical stress) and "chronic" pathological conditions (i.e. pain, epilepsy, drug exposure, retinal diseases, Alzheimer's disease and possibly Parkinson's disease), purinergic receptor activation could either be a cause or a consequence of neuronal cell death/glial activation and may be related to detrimental and/or beneficial effects (reviewed by Franke and Illes, 2006).

In 1972, Burnstock proposed the concept of ATP as a neurotransmitter (Burnstock, 1972); subsequently the existence of special cellular sites of action for nucleotides as ***P2*** purino-receptors was published in 1978 (Burnstock, 1978).

There are two principal families of P2 receptors (Abbracchio and Burnstock, 1994), widely expressed in the CNS:

- P2X receptors (P2XR), which are ligand-gated ion channels;
- P2Y receptors (**P2YR**), which belong to the group of GPCRs.

P2Rs are expressed on the surface of almost all cells. Up to now 7 mammalian P2XR subtypes ($P2X_{1\text{-}7}$) and 8 mammalian P2YR subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) have been cloned and functionally defined as P2Rs (Ralevic and Burnstock, 1998; Abbracchio *et al.*, 2003) (**Fig. 2**). As recommended by the IUPHAR nomenclature committee, the term "P2Y" will be used for cloned GPCRs that have been shown to mediate effects of extracellular nucleotides (Fredholm *et al.*, 1997).

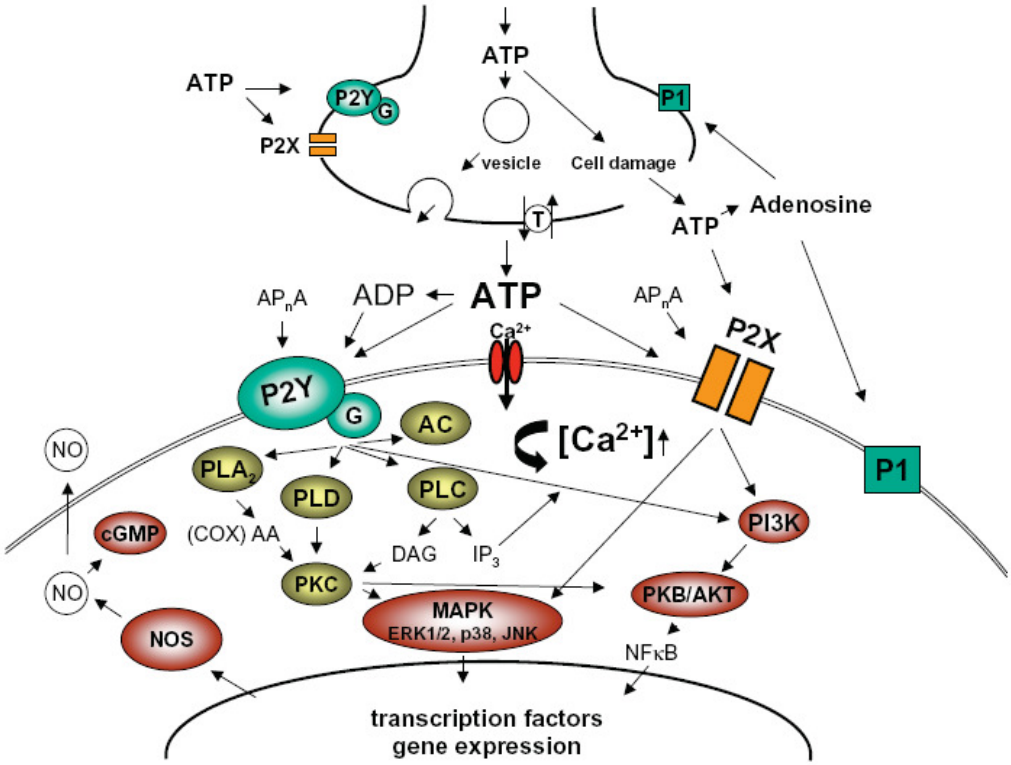


Fig. 2: Schematic diagram illustrating ATP actions at synapses: phospholipase A2 (PLA2), phospholipase D (PLD), protein kinase C (PKC), adenylate cyclase (AC), phospholipase C (PLC), diacylglycerol (DAG), inositol-(1,4,5)-trisphosphate (IP3), nitric oxide synthase (NOS), phosphoinositide 3-kinase (PI3K), serine-threonine kinase (AKT), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNKs), cyclic guanosine monophosphate (cGMP), arachidonic acid (AA), cyclooxygenase (COX), nitric oxide (NO) and nuclear factor kappa B (NFnB).

All known P2YRs possess at their extracellular domains 4 cysteine residues, which are likely to form two disulfide bridges (Hoffmann *et al.*, 1999). The receptors of a first group ($P2Y_{1\text{-}2\text{-}4\text{-}6\text{-}11}$ R), all couple via G_q proteins to stimulation of phospholipase C followed by increases in inositol phosphates and mobilization of Ca^{2+} from intracellular stores. The receptors of a second group ($P2Y_{12\text{-}13\text{-}14}$ R) all couple via G_i proteins to inhibition of adenylate cyclase, followed by a decrease in intracellular cAMP levels (Von Kugelgen, 2006).

Members of this P2 receptor family are widely expressed in the CNS and are involved in glia-glia and glia-neuron communications, whereby they play important physiological and patho-physiological roles in a variety of biological processes.

In particular, the **$P2Y_1R$**, first cloned from late-embryonic chick brain (Webb *et al.*, 1993) and then detected in other mammalian species, is selective for adenine nucleotides, principally activated by ADP nucleotides. The agonist compound MeSADP has a 10 times higher affinity at the human $P2Y_1R$ than ADP; however, ATP itself is a partial agonist with a reduced intrinsic activity when compared with that of ADP (Waldo and Harden, 2004). There are $P2Y_1R$ subtype-selective antagonists available, such as the MRS2179 (Camaioni *et al.*, 1998): the affinity constant of MRS2179 at the human $P2Y_1R$ amounts to about 100 nM (Waldo *et al.*, 2002) and it doesn't change responses mediated by other P2YRs (Von Kugelgen, 2006).

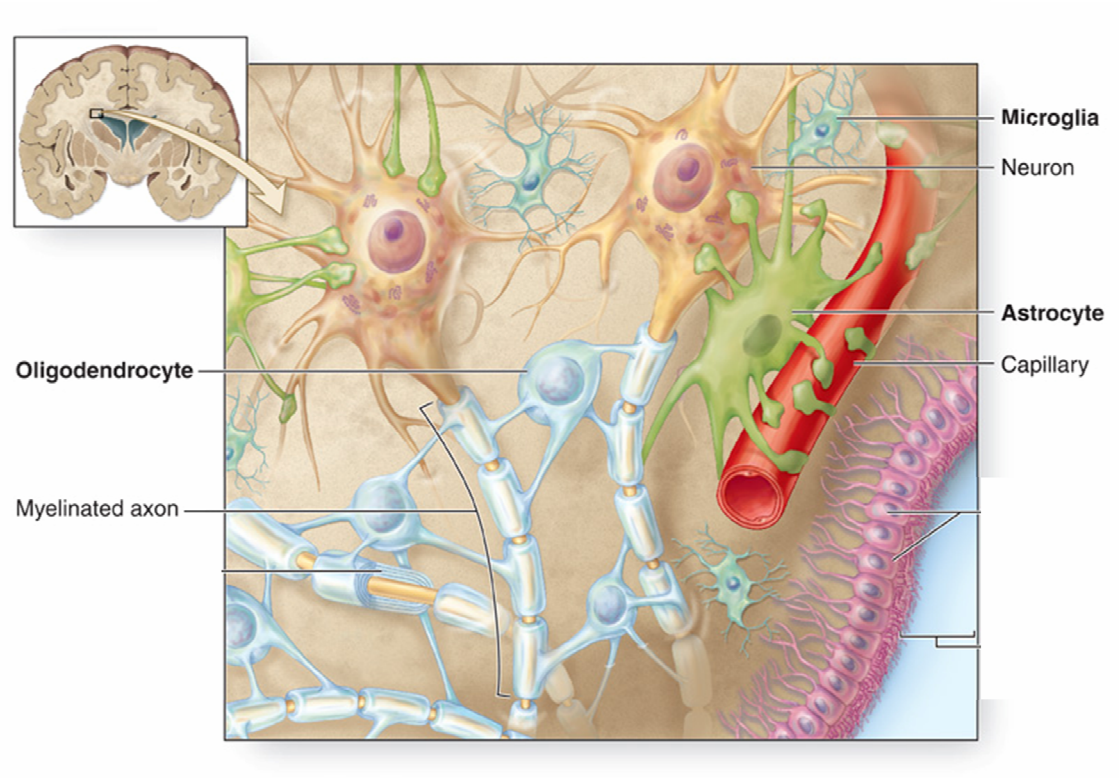
$P2Y_1Rs$: mediate muscle relaxation and the release of endothelium-derived relaxing factors or prostaglandins, thus contributing to the ADP-induced platelet aggregation (Fabre *et al.*, 1999); result localized at characteristic Alzheimer's disease structures in the hippocampus and cortex (Moore *et al.*, 2000); are involved in apoptotic events (Sellers *et al.*, 2001; Mamedova *et al.*, 2006) as well in the gliotic response under physiological and pathological conditions (Franke *et al.*, 2001; 2004).

Type	Species	Access. no.	Chr. l.	Tissue distribution	Principal agonists
$P2Y_1$	Human	U42029, U42030, S81950, AJ006945	3q25.2	Wide including platelets, heart, skeletal muscle, neuronal tissues,	(*N*)-mc-2-MeSADP>2-MeSADP> ADP=ADPβS≫ATP
	Bovine	X87628		digestive tract	2-MeSATP>ADP>ATP
	Rat	U22830	2q31		2-MeSADP=2-MeSATP>ADP
	Mouse	U22829	3E1		2-MeSATP>2Cl-ATP>ATP
$P2Y_2$	Human	NM_002564	11q13.5	Wide including lung, heart, skeletal muscle,	UTP=ATP>INS37217>Ap4A>ATPγS
	Canine	XM_542321	21	spleen, kidney	UTP≥ATP>ADP>2-MeSATP
	Porcine	AY620400			UTP>ITP>ATP>UDP
	Rat	L46865, U09402	1q32		UTP=ATP>CTP>GTP
	Mouse	L14751	7F1		UTP=ATP>Ap4A
$P2Y_4$	Human	U40223, X91852, X96597	Xq13	Placenta, lung, vascular smooth muscle, brain,	UTP>UTPγS
	Rat	Y11433, Y14705	Xq31	liver	UTP=ATP=ITP=Ap4A
	Mouse	AJ277752	XC2		UTP=ATP
$P2Y_6$	Human	U52464, AF007891	11q13.5	Wide including lung, heart, aorta,	UDP=5Br-UDP>>UTP>2-MeSADP
	Rat	D63665	1q32	spleen, placenta, thymus, intestine,	UDP>UTP>ADP>2-MeSATP
	Mouse	NM_183168	7E1	brain	UDP>UTP>ADP>2-MeSATP
$P2Y_{11}$	Human	NM_002566	19p13.2	Spleen, intestine, immunocytes	ARC67085≥ATPγS=BzATP> ATP,(UTP)[a]>2-MeSATP
	Canine				ADPβS=2-MeSADP≥2-MeSATP>ATP
$P2Y_{12}$	Human	AF313449, AF321815, AB052684	3q25.1	Platelets, neural tissues	2-MeSADP >ADP>>(*N*)-mc-2-MeSADP
	Bovine	AJ623293			2-MeSADP ≫ADP,ATP
	Rat	AF313450	2q31		2-MeSADP >ADP>ATP
	Mouse	AJ312130, NM_027571	3D		2-MeSADP >ADP>ADPβS
$P2Y_{13}$	Human	NM_023914, NM_176894	3q24	Spleen, leuco-cytes, bone marrow,	2-MeSADP>(=)ADP>ADPβS
	Rat	AY639875	2q31	liver, brain	ADP>2-MeSADP ≫ATP
	Mouse	NM_028808	3D		ADP=2-MeSADP=ADPβS>ATP
$P2Y_{14}$	Human	NM_014879	3q21-25	Placenta, adipose	UDP-glucose>UDP-galactose
	Rat	U76206	2q31	tissue, intestine,	UDP-glucose
	Mouse	NM_133200	3D	brain, spleen	UDP-glucose

Table 1: The table summarizes cloned mammalian P2YRs that have been proved to mediate actions of extracellular nucleotides when expressed and studied in functional assay systems (Access No: GenBank accession No; Chr. l: chromosomal location). Table modified from Von Kugelgen (2006).

1.4 Astroglia

Astroglial cells are the most abundant cells in the CNS and it appears that the ratio of astrocytes to neurons increases with the increasing complexity of the CNS (Pekny and Pekna, 2004). In fact in the CNS astroglia is involved in multiple brain functions under physiological conditions, including neuronal development, synaptic activity and homeostatic control of the extracellular environment (Ciccarelli *et al.* 2001; Anderson *et al.*, 2003). In particular, astrocytes (**Fig. 3**) are involved in the most integrated functions of the CNS: on one hand they may contribute to damage by propagating spreading depression or by sending pro-apoptotic signals to healthy tissue via gap junction channels or by inhibiting regeneration by formation of the glial scar; on the other hand, astrocytes are important in neuronal antioxidant defence, secrete growth factors, promote neurogenesis and regeneration in the chronic phase after injury (Anderson *et al.*, 2003).



Astrocytes actively participate in the processes triggered by brain injury, which are initially aimed at limiting and repairing brain damage but may eventually contribute to neuronal cell death. Under pathological conditions, such as hypoxia or ischemia, they in fact turn into an activated form called “reactive astrogliosis” (Brambilla and Abbracchio

2001; Brambilla *et al.* 2003). Although it is accepted that activated astrocytes contribute to isolate damaged brain areas from surrounding healthy cells and participate in neuronal recovery synthesizing neurotrophins and pleiotrophins, these cells also release several potentially toxic compounds (i.e. nitric oxide and cytokines; Neary *et al.*, 1996) and an excessive and prolonged astrogliosis, such as that found in acute and chronic neurodegenerative diseases, may contribute to brain damage. In addition, astrocytes exert an important role in the modulation of extracellular glutamate, which is the main excitatory neurotransmitter within the CNS, in pathological conditions characterized by neurotoxic glutamate accumulation, such as cerebral ischemia and traumatic brain injury.

Nevertheless glia has been found to be more vulnerable to apoptosis than neurons in focal ischemic infarction (Petito *et al.*, 1998).

Astrocytes, which play a critical role in brain homeostasis in that they control the local environment in normal as well as in pathological conditions, have recently been identified as a source for a wide variety of glio-trasmitters that modulate synaptic activity (Martin *et al*., 2007).

Growing evidence indicates that purines are widely involved in the molecular mechanisms underlying the multiple functions of astrocytes, either by exerting their influence on key intracellular activities (energy metabolism, nucleic acid synthesis) or activating their membrane receptors (Neary *et al.,* 1996; Abbracchio and Burnstock 1998).

1.5 Aim of the work

1.5.1 A_1-$P2Y_1$ receptors

In support of the assumption that cerebral ischemia or mechanical damages aggravate brain injury via the efflux of ATP, evidence suggests that the interference with the purinergic system (in particular the ATP excitatory system) could provide neuroprotection, thus improving functional recovery and diminishing cell death in the peritraumatic zone (Franke and Illes, 2006). Anyhow detailed investigations on the systemic application of these compounds, either to animals or human volunteers/patients, are still missing.

Whereas the neuroprotective role of adenosine analogues active at the A_1R subtype has been known for several years, the function of $P2Y_1$ rceptor has not been fully characterized, enough if its broad distribution in a variety of cell-types throughout the brain of different species (Zhu *et al*., 2001; Moran-Jimenez and Matute, 2000; Webb *et al*., 1998) indicates important physiological and patho-physiological functions; indeed, changes in the expression of $P2Y_1Rs$ are reported in different cell types and tissues, as consequence of a wide range of pathological conditions (Moore et al, 2000; Neary *et al*., 2003; Lammer *et al.*, 2004; Franke *et al*., 2004; Luttikhuizen *et al.*, 2004).

The synthesis of $P2Y_1R$ antagonistic compounds, permeable through the blood-brain barrier, has been proposed with a therapeutic potential, for example, by decreasing the excessive glutamate release in the CNS or by reducing the size of the glial scar following ischemic attacks (Franke and Illes, 2006). Anyway in the same time ATP, but no adenosine, showed to protect astrocytes against the cell death induced by oxidative stress damage, via $P2Y_1R$ activation (Shinozaki *et al.*, 2005).

It is now well ascertained that GPCRs can be directly associated as either mono- or hetero-oligomers, thus altering their functions (Maggio *et al*., 2005) during physiopathological conditions; the activation of one particular signalling pathway of a GPCR can either amplify or inhibit the intracellular pathway of another.

A_1 receptors have shown to act synergistically with P2Y receptors and, in particular, with $P2Y_1Rs$ (Masino *et al*., 2002; Fredholm *et al.*, 2003). Recently it has been demonstrated that A_1Rs can form a heteromeric complex with $P2Y_1Rs$ (Yoshioka *et al.*, 2001; 2002): heterodimerization between A_1 and $P2Y_1$ receptors thus generates an adenosine receptor which has a P2Y-like receptor agonistic pharmacology and acts also through adenylyl cyclase signalling pathway.

So, considering the relevance of the A_1R-, $P2Y_1R$- and astroglia-mediated actions in the CNS, the *aim of this work* has been:

- As a first step, to investigate the subcellular/cellular localization/co-localization of A_1Rs and $P2Y_1Rs$ in hippocampus, a particularly damage-sensitive area, focusing in particular on the glutamatergic synapses and surrounding areas, and their functional cross-talk at membrane levels, in crude hippocampal membranes;

- As a second step, to investigate the A_1R-P2Y_1R cross-talk in human astroglial cells, in particular the functional implications of P2Y_1R activation on A_1R functioning.

1.5.2 A_{2B} receptors

Adenosine has been identified as a significant paracrine inhibitor of inflammation (Linden, 2005), but it has not been certain which of its receptors mediate this effect. There is growing interest in elucidating the mechanisms by which adenosine inhibits the inflammation, since the adenosine receptors are promising targets for new anti inflammatory therapies.

Recently A_{2B}R has been identified as a critical regulator of inflammation. After a number of previous reports indicating that A_{2B}R activation can be pro-inflammatory (Linden, 2006), a recent work (Yang *et al.*, 2006) reports a pro-inflammatory phenotype resulting from deletion of the gene encoding the A_{2B}R in mice and suggests that activation of A_{2B}Rs can also have anti-inflammatory effects, as already reported in macrophages (Xaus *et al.*, 1999; Kreckler *et al.*, 2006). In line with this, Rosi and co-workers (2003) found that LPS-mediated chronic brain inflammation was associated with microglia activation and neuronal A_{2B}R down-regulation. Nevertheless, an up-regulation of A_{2B}Rs, in hippocampal astrocytes, mediated an important role in the protective effects of cerebral ischemic preconditioning in rats (Zhou *et al*., 2004). At the present the role of the A_{2B}R remains enigmatic since its activation can either stimulate or inhibit the release of proinflammatory cytokines, in different cells and tissues. Nevertheless the effects of adenosine on astrocyte functions and the role of various AR subtypes, especially A_{2B} receptor, are still not well defined in astroglial cells.

In response to injury or infection, resident CNS cells generate pro-inflammatory cytokines which, through the recruitment of immune cells and the autocrine activation of glial cells, may contribute to acute and chronic brain disease pathogenesis as well to psychiatric disorders (for review see Lucas *et al*., 2006). However, cytokines may have a dualistic role, with detrimental acute effects but also beneficial effects in long-term repair and recovery (Lucas *et al*., 2006).

Recently, a functional cross-talk between cytokines and ARs has been demonstrated in different cell lines, including glial cells (Fredholm and Altiok 1994; Xaus *et al.* 1999;

Khoa *et al.* 2001; Trincavelli *et al.* 2002); A_{2B}Rs, activated only under hypoxic or ischemic conditions, has been implicated in the control of cytokine release (Fiebich *et al.,* 1996; Feoktistov *et al*., 2002; Zhong *et al*., 2004; Zhang *et al*., 2005; Kreckler *et al*., 2006; Yang *et al*., 2006), that in turn can exacerbate or reduce inflammation.

In particular, studies from our laboratory demonstrated that A_{2B}Rs are regulated by chronic treatment with the proinflammatory cytokine TNF-alpha (TNF-α) in astroglial cells: the exposure to TNF-α for 24 hours, although did not affect either the A_{2B} mRNA and protein expression level, induced an up-regulation of the receptor responsiveness, thus impairing the agonist-mediated receptor phosphorylation and inducing, in turn, a delay in A_{2B}R desensitization processes. The up-regulation of A_{2B}R responsiveness plays an important role also in mediating chronic astrogliosis, thus suggesting a role for this receptor subtype in the long-term control of astrocytic function (Trincavelli *et al.*, 2004).

While the effects elicited by A_2Rs on proinflammatory cytokines have been extensively studied, even if with contrasting results, the cytokine (i.e. TNF-α) influence on adenosine receptors has not been yet extensively investigated.

Moreover, considering that the release of endogenous mediators during brain injuries mainly occurred in a few (1-4) hours, in order to better clarify the role of A_{2B}R in the acute phase of brain damage, the *aim of the present work* has been:

- To investigate the effects of the short-term TNF-α exposure on A_{2B}R expression and functioning in human astrocytoma ADF cells and the intracellular pathways involved.

1.6 References

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Chapter 2:

Co-localization and functional cross-talk between A_1 and $P2Y_1$ purine receptors in rat hippocampus

2.1 Abstract

Adenosine and ATP, via their specific P1 and P2 receptors, modulate a wide variety of cellular and tissue functions, playing a protective or degenerative role in damage conditions. Although in the brain, in general, adenosine inhibits excitability and ATP functions as an excitatory transmitter in the central nervous system, recent data suggest the existence of a heterodimerization and a functional interaction between P1 and P2 receptors in the brain, in particular the adenosine A_1 (A_1R) and the purine $P2Y_1$ receptors ($P2Y_1R$), playing a potentially important role in the purinergic signalling cascade. In the present work, we investigated A_1R and $P2Y_1R$ sub-cellular localization/co-localization and their functional cross-talk at the membrane level in rat hippocampus. This is a particularly vulnerable brain area, which is sensitive to adenosine- and ATP-mediated control of glutamatergic transmission. The postembedding immuno-gold electron microscopy technique showed that A_1R and $P2Y_1R$ are co-localized at the synaptic membranes and surrounding astroglial membranes of glutamatergic synapses.

To investigate the functional cross-talk between the two types of purinergic receptors, we evaluated the reciprocal effects of their activation on their G protein coupling. $P2Y_1R$ stimulation impaired the ability of A_1R coupling to G protein, whereas the stimulation of A_1R increased the functional responsiveness of $P2Y_1R$.

The results demonstrated an A_1R-$P2Y_1R$ co-localization at glutamatergic synapses and surrounding astrocytes and a functional interaction between these receptors in hippocampus, suggesting ATP and adenosine can interact in purine-mediated signalling. This interaction may be particularly important during pathological conditions, when large amount of these mediators are released.

2.2 Introduction

ATP and adenosine, via their specific P2 and P1 purinergic receptors (Fredholm *et al*., 1994), mediate a wide variety of physiological processes including neuromodulation and neurotrasmission. Moreover, the purinergic system has been involved in many pathological and neurodegenerative conditions, in which massive release of ATP and, in turn, ADP and adenosine production, occur from damaged or dying cells, *i.e.* following ischemia, necrosis or injuries (Braun *et al*., 1998; Rathbone *et al*., 1999; Burnstock, 2004). Several reports have described a dualistic neuroprotective-neuromodulatory role of ATP interacting with the specific ionotropic receptors (P2XR) and G-protein coupled receptors (GPCRs) (P2YR) (Fredholm *et al*., 1994). Among P2YRs, $P2Y_1$ receptors ($P2Y_1R$) appear to be of particular interest in patho-physiological mechanisms both with detrimental or beneficial effects (Franke and Illes, 2006). On the other hand, through the activation of the inhibitory A_1 adenosine receptors (A_1R) coupled to G proteins (Dunwiddie and Masino, 2001), adenosine inhibits the release of excitatory neurotransmitters and decreases neuronal excitability, exerting a neuroprotective role (Wardas, 2002).

Recent data have provided evidences for the existence of an association between A_1 and $P2Y_1$ receptors. In a co-transfected cell line model, Yoshioka and Nakata (2004) have demonstrated that these receptors directly interact to generate a hetero-oligomer, which has novel pharmacological and functional characteristics indicating a potential role in the purinergic-signalling cascade. Moreover, in specific rat brain regions (*i.e*. hippocampus) a high degree of co-localization of both these receptors has been demonstrated by immunofluorescence and immunoprecipitations experiments (Yoshioka *et al*., 2002). In CNS, although $P2Y_1$ and A_1 receptors have been involved in modulation of brain damage and contribute, alone or in combination, to neuro-degenerative/regenerative processes (Neary *et al*., 2003; Franke and Illes, 2006; Von Kugelgen, 2006), no data are at the present available on the precise localization/co-localization of A_1 and $P2Y_1$ receptors at cellular and sub-cellular levels and on their reciprocal modulation/functional interaction in native tissues following specific agonist activation.

The hippocampus, a central component of the limbic system, has been identified as a brain area with a specific vulnerability to injuries, in particular to ischemia (Harry and Lefebvre d'Hellencourt, 2003). In the hippocampus, A_1 and $P2Y_1$ receptors are particularly abundant (Gottlieb and Matute, 1997; Zhu *et al*., 2001; Ochiishi *et al*., 1999; Jimenez et al., 2000; Moran-Jimenez and Matute, 2000) and involved in the modulation

of glutamate release (Rudolphi *et al*., 1992; Mendoza-Fernandez *et al*., 2000; Masino *et al*., 2002; Koizumi *et al*., 2003; Kawamura *et al*., 2004; Rodrigues *et al.,* 2005; Jourdain *et al*., 2007), hence contributing to neurotransmission and neuro-degenerative and -regenerative processes.

In the present work, we investigated the localization/co-localization of A_1R and $P2Y_1R$ in rat hippocampus, focusing in particular on the glutamatergic synapses and surrounding areas, using electron microscopic (EM) quantification of postembedding immunogold labelling; this technique allows the precise localization of targets to be identified at sub-cellular resolution, on different parts of astrocytes and neurons.

As a first step to investigate the functional A_1 and $P2Y_1$ receptors cross-talk, we studied the A_1R activation following $P2Y_1R$ stimulation and vice versa in rat crude hippocampal membranes. For this purpose the [^{35}S]guanosine-5'-(γ-thio)-triphosphate ([^{35}S]GTPγS) binding assay (Lorenzen *et al*., 1993 and 1996) was used: the GTP binding represents the initial step of any GPCR activation and of the intracellular signalling cascade mediated by GPCRs (Lorenzen *et al*., 1996).

2.3 Materials and methods

2.3.1 Materials

The A_1R antibody (rabbit) was supplied by Alpha Diagnostic (San Antonio, TX, USA), while the $P2Y_1R$ antibody (rabbit) from Alomone Labs (Jerusalem, Israel). The A_1R antibody was raised against a 14 amino acid peptide corresponding to amino acid residues 163-176 of the rat or human receptor protein, on the extracellular N-terminal domain. The $P2Y_1R$ antibody was raised against a 17 amino acid peptide (C)RALIYKDLDNSPLRRKS, corresponding to residues 242-258 of rat or human $P2Y_1R$; the epitope location is in 3rd intracellular loop (i3) between the TM5 and TM6 domains. Goat anti-rabbit immunoglobulins coupled to 10 nm or 15 nm gold particles were obtained from Aurion (Wageningen, The Netherlands). Secondary antibody goat anti-rabbit IgG-HRP conjugate was from Calbiochem (EMD Biosciences, affiliate of Merck KgaA, Darmstadt, Germany). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA); full range Rainbow Molecular Weight Markers (range 10–250 kDa) was obtained from Amersham Biosciences (Freiburg, Germany).

[^{35}S]GTPγS (specific activity 1000-1250 Ci/mmol) was purchased from Amersham Biosciences Europe GmbH (Freiburg, Germany); adenosine deaminase (ADA) was from Roche Diagnostics GmbH (Mannheim, Germany). N^6-cyclohexyl adenosine (CHA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), GDP, guanosine-5'-(γ-thio)triphosphate (GTP□S), 2-methylthio-adenosine 5'-diphosphate (MeSADP), 2'-deoxy-N^6-methyl adenosine 3',5'-diphosphate (MRS2179), and protease inhibitors were obtained from Sigma Chemical Co (St. Louis, MO, USA).

The protein concentration of the samples was established using the Protein Assay based on Bradford method from Bio-Rad (Hercules, CA, USA), using bovine serum albumin as a standard. All chemicals were of analytical grade and all solutions were prepared in demineralised and purified water obtained with the Milli-Q Millipore water purifying system (Millipore, Billerica, MA, USA).

2.3.2 Postembedding Immunogold Cytochemistry

Immunogold electron microscopy quantification was used to study A_1 and $P2Y_1$ receptors in rat hippocampus, focusing on glutamatergic synapses. Receptor immunocytochemistry was performed as previously described in Bergersen *et al*. (2005), with some modifications.

Adult male Wistar rats (300 g, n=3) were anesthetized by pentobarbital i.p. and subjected to transcardiac perfusion (50 ml/min for 20 min) with a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer (NaPi) pH 7.4 at 4ºC. The brains were left *in situ* overnight (4ºC). Then isolated hippocampal specimens were cryoprotected by immersion in graded concentrations of glycerol (10, 20 and 30%) in 0.1 M NaPi for 30 min. at each step and then overnight in 30% glycerol in 0.1 M NaPi at 4ºC. Samples were then plunged into liquid propane cooled at -190ºC with liquid nitrogen in a Universal Cryofixation System KF80 (Reichert-Jung, Wien, Austria). For freeze-substitution (Muller *et al*., 1980), tissue samples were immersed in a solution of anhydrous methanol and 0.5% uranyl acetate overnight at -90ºC. The temperature was raised stepwise in 4ºC increments per hour from -90º to -45ºC, where it was kept for the subsequent steps. Tissue samples were washed several times with anhydrous methanol to remove residual water and uranyl acetate. The infiltration in Lowicryl HM20 went stepwise from Lowicryl/methanol 1:2, 1:1 and 2:1 (1 h each) to pure Lowicryl (overnight). For polymerization, the tissue was placed in a pre-cooled embedding mall. The polymerization was catalyzed by UV light at a wavelength of 360 nm for

2 days at -45ºC followed by 1 day at room temperature. Ultrathin sections (80 nm) were cut with a diamond knife on a Reichert-Jung ultramicrotome and mounted on nickel grids (300 mesh square, Electron Microscopy Sciences, USA) using an adhesive pen (Coat-Quick "G", Electron Microscopy Sciences, USA).

The sections were processed at room temperature in solutions of 0.05 M Tris HCl buffer, pH 7.4 containing 0.3% (for $P2Y_1R$ antibody) or 0.1% (for A_1R antibody) NaCl and 0.1% Triton X-100 (TBST) and completed as stated below.

After "etching" in sodium ethanolate to remove plastic from tissues, sections were left in TBST containing 2% human serum albumin (HSA) for 10 minutes and then incubated overnight (20 hours) with primary antibodies against A_1R (dilution 1:500: 2 µg/ml) as well as for the $P2Y_1R$ (dilution 1:200: 4 µg/ml), diluted in TBST containing 2% HSA. Sections were then incubated with goat anti-rabbit immunoglobulins coupled to 10 nm gold particles, diluted 1:20 in TBST with 2% HSA and, for A_1R experiments, with 2 mg/ml polyethylene glycol.

The ultrathin sections were processed both with single labelled and double labelled procedures. In double labelling experiments (Ottersen *et al*., 1992), sections were treated with the antibody against $P2Y_1R$ (dilution as above) in the first step (followed by 10 nm gold-labelled secondary antibody) and with the A_1R antibody (dilution as above) in the next step (revealed by 15 nm gold-labelled secondary antibody): exposure to formaldehyde vapours (80ºC, 1 hour) was used between the two immunolabeling steps (Wang and Larsson, 1985), to destroy by formaldehyde the remaining free anti-IgG binding sites on the first primary and secondary antibodies. Potential cross-reactivity arising from the subsequent use of another secondary antibody that would be directed against the same species is prevented in this manner, allowing the simultaneous detection of two different antigens when using two primary antibodies from the same species. A_1 and $P2Y_1$ receptors were distinguished by means of different gold particle sizes (10 nm for $P2Y_1R$, 15 nm for A_1R). The double labelling approaches gave similar patterns as the single labelling protocol.

Negative control experiments also were performed, replacing the incubation with the primary antibodies with 2% HSA in TBST: staining was absent on sections that had been incubated in such a solution. Ultrathin sections were contrasted in uranyl acetate and lead citrate and observed in a Philips CM100 electron microscope.

2.3.4 Immunocytochemistry Quantitative Analysis

Electron micrographs were randomly taken in the hippocampus (magnification 34000x). Gold particles signalling both receptors ($P2Y_1R$ or A_1R) were quantified as number of gold particles/μm^2 at glutamatergic synapses (i.e., asymmetric synapses on dendritic spines) in the stratum radiatum of area CA1 and CA3, as well as in the juxtagranular part of the dentate molecular layer. The former are formed by terminals of ipsilateral (Schaffer collaterals) and commissural axon collaterals from CA3 pyramids, the latter by terminals of mossy cells in the dentate hilus. Only synapses with clearly visible post-synaptic membrane and postsynaptic density were selected for analysis; essentially all synapses with this morphology are glutamatergic in these areas (for identification criteria, see Gylterud Owe *et al*., 2005). Quantitative analyses were carried out on sections single labelled for either $P2Y_1R$ or A_1R. Specific membrane compartments were defined and used for quantifications: they correspond to the presynaptic vesicles membranes, the membrane overlying the postsynaptic density (PSD), presynaptic membrane (opposite to the PSD), pre- and post-perisynaptic membranes (corresponding to membrane lateral to the presynaptic active zone and the PSD, extending laterally by half the length of the PSD), extrasynaptic membranes (belonging to either presynaptic terminals or postsynaptic spines/dendrites but excluding the synaptic and perisynaptic membranes), postsynaptic intracellular membranes, astroglia membranes and mitochondrial outer membranes (**Fig. 1**) (cf. Bergersen *et al*., 2005).

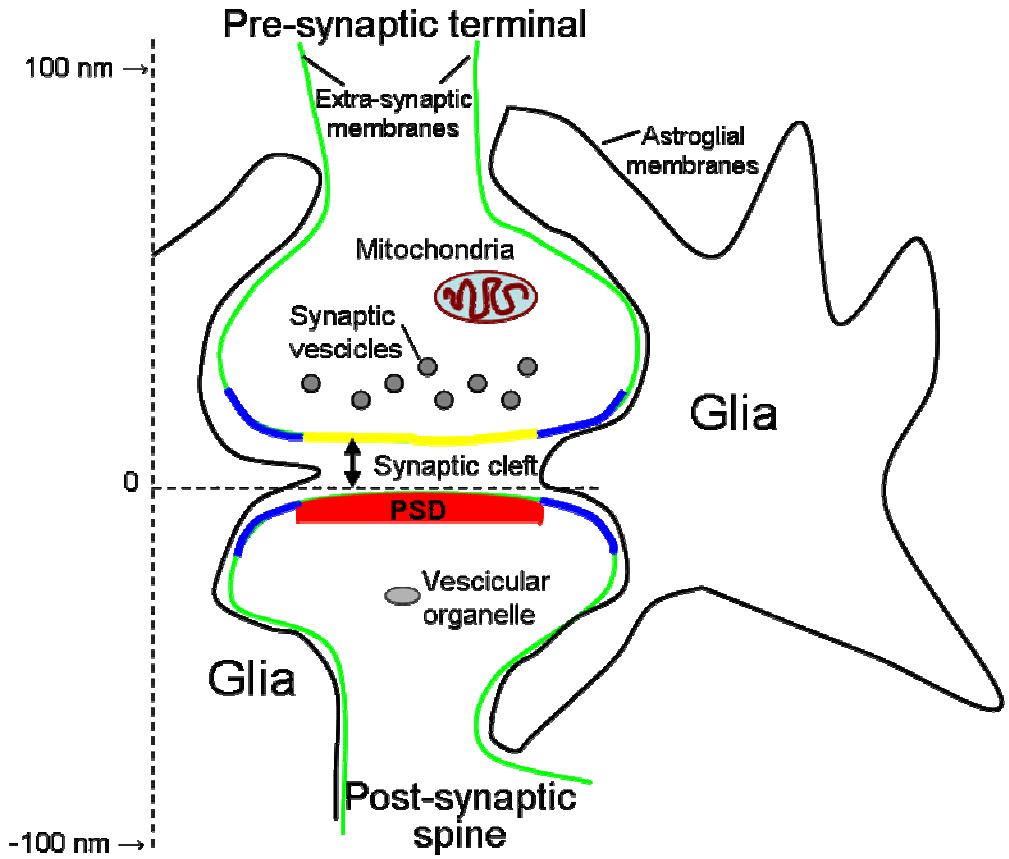

Fig. 1: Schematic illustration of a synapse between a presynaptic terminal and a postsynaptic spine. Analysis of gold particle density (number of gold particles/μm^2) was performed in specific membrane compartments that were defined as: presynaptic vesicles membranes, the postsynaptic membrane overlying the postsynaptic density (PSD; *red*), presynaptic membrane 'active zone' (i.e. opposite to the PSD; *yellow*), pre- and post-perisynaptic membranes on each side of the active zone (*blue*), extrasynaptic membranes (belonging to either presynaptic terminals or postsynaptic spines/dendrites but excluding the synaptic and perisynaptic membranes; *green*), postsynaptic intracellular membranes, astroglial plasma membranes (*black*) and mitochondrial outer membranes (*brown*). Note that the length of perisynaptic membrane considered corresponds to half the total length of the PSD, on each side (the same is valid for the presynaptic perisynaptic membrane). Figure modified from Bergersen *et al*. (2005).

In addition, gross distribution of gold particles was recorded over the following location categories: presynaptic terminal, postsynaptic spine, synaptic cleft, astrocytes, mitochondria, extracellular and undefined.

Particles located within 40 nm (perpendicular distance between the center of gold particle and the membrane) from different membranes were recorded: this distance was cho-

sen because 40 nm is about equal to the lateral resolution of the immunogold method, i.e., the max distance from the centre of the gold particle and the epitope, determined experimentally (Chaudhry *et al.,* 1995; Nagelhus *et al*., 1998). The areas sampled were determined by grid point analysis (Gundersen H.J. *et al.*, 1988; Gundersen V. *et al.*, 1998), and the densities expressed as gold particles / μm^2. The distance between the centres of gold particles, representing receptors, and the external face of the postsynaptic density was determined along a perpendicular axis. All gold particles located within the postsynaptic spine and the presynaptic terminal were recorded; the width of the synaptic cleft was also measured. The distribution of gold particles was compared with that of grid points placed randomly over the sampled area (Gundersen V. *et al*., 1998; Bergersen *et al*., 2003) in order to relate the distributions of A_1R and $P2Y_1R$ to a random distribution.

2.3.5 Rat brain membrane Lysates and Western blotting

Western blot analysis on rat hippocampus and full brain membranes has been used to confirm the specificity of the antibodies and the presence of the receptors, as further control. The whole brain or the hippocampus were removed from Wistar rats (200–300 g; n=3) and immediately processed, keeping on ice. The tissues were suspended in 20 volumes of ice-cold 50 mM Tris HCl, 2 mM $MgCl_2$ buffer, pH 7.4, containing EDTA 1 mM and protease inhibitors (Benzamidine 0.16 mg/ml, Trypsin inhibitor 0.03 mg/ml and Bacitracin 0.2 mg/ml) (buffer A). The tissues were then homogenized with a Polytron homogenizer and after centrifugation (48000 *g* for 10 minutes at 4°C) the membrane pellet were resuspended and re-homogenized in buffer A with ADA 2 U/ml to get a concentration of 50 mg/ml (from original tissue weight). After incubation for 30 minutes at 37°C, the samples were centrifuged at 4°C and each pellet was resuspended to use concentration, boiled in Laemmli solution for 5 minutes, centrifuged at room temperature for 5 minutes and the supernatant used for electhrophoresis (or kept at –20°C until use). Tissue membrane homogenates (50 µg) were so processed by immunoblot following the method previously described (Trincavelli *et al.,* 2004) with minor modifications. Briefly, samples were resolved by SDS-PAGE (10%), transferred to nitrocellulose membranes and incubated with primary antibodies against A_1 (1:500) or $P2Y_1$ receptor (1:200) overnight at 4°C. Blots were developed using the Millipore Immobilon TM Western chemiluminescent HRP Substrate reagents (Millipore, MA, USA).

2.3.6 Rat hippocampal membrane preparation for [^{35}S]GTPγS binding assay

Hippocampus, from male and female Wistar rats (200–300 g; n=3) were rapidly removed and dissected on ice. Tissues were then homogenized in 20 volumes of ice-cold 50 mM Tris–HCl, 2 mM $MgCl_2$ buffer, pH 7.4, containing 0.1 mM dithiothreitol (DTT), protease inhibitors (phenylmethylsulfonyl fluoride 1 mM, benzamidine160 μg/ml, bacitracin 200 μg/ml, trypsin inhibitor 20 μg/ml) (buffer B) and 0.32 M sucrose, using a polytron homogenizer. The homogenate was centrifuged at 1000 *g* for 10 minutes at 4°C: the supernatant was collected and centrifuged at 48000 *g* for 20 minutes at 4°C. The resulting pellet was resuspended in 10 volumes of buffer B and centrifuged again at 48000 *g* for 20 min. at 4°C. The pellet was then resuspended in 5 volumes of the same buffer containing ADA 3 U/ml and incubated at 37°C for 30 min. to remove endogenous adenosine. The membrane homogenate was centrifuged at 48000 *g* for 20 min. at 4°C and the final pellet was stored as aliquots at −80°C until needed. The protein concentration of the sample was established.

2.3.7 [^{35}S]GTPγS binding assay on rat hippocampal membranes

A_1R coupling to G proteins was evaluated assessing the ability of the selective A_1R-agonist CHA to stimulate [^{35}S]GTPγS binding in rat hippocampal membranes (HC) pre-treated for 10 minutes with buffer alone (Control HC) or 100 nM MeSADP (MeSADP-treated HC). In parallel, aliquots of control membranes were also pre-incubated with the A_1R selective antagonist DPCPX (50 nM) for 10 minutes before the CHA-stimulation.

In the same way, $P2Y_1R$/G protein coupling was evaluated assessing the ability of the agonist MeSADP to stimulate [^{35}S]GTPγS binding in membranes pre-treated for 10 minutes with buffer alone (Control HC) or 100 nM CHA (CHA-treated HC). In parallel, control membranes were also treated with the $P2Y_1R$ selective antagonist MRS2179 (10 μM), for 10 minutes before MeSADP-stimulation (MRS2179-treated HC).

Rat hippocampal membranes were resuspended in 25 mM Hepes NaOH, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl (buffer C) containing 1 mM DTT. Aliquots of HC (10 μg) were incubated in 0.1 ml of buffer C containing ADA (0.2 U/ml), 10 μM GDP and 0.3 nM [^{35}S]GTPγS in the presence (stimulated) and absence (basal) of a range of concentrations of CHA or MeSADP (0.1 nM-10 μM). ADA was added to assay to eliminate the interference of endogenous adenosine in the basal [^{35}S]GTPγS binding. Incubation was carried out at 25°C for 2 hours. Unspecific binding was defined in the

presence of 100 μM GTPγS and it resulted less than 10% of total binding. Binding reactions were terminated by rapid filtration under vacuum through Whatman GF/C glass fiber filters (Millipore Corporation): the filters were washed three times with 3 ml of 50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.4 and then counted in a scintillation cocktail. The concentration-dependent increase in specific [^{35}S]GTPγS bindings by agonists was expressed as a percent increase above the basal unstimulated binding (fixed as 100%). All the experiments were performed in duplicate.

2.3.8 Data analysis

Data from immunogold cytochemistry localization were statistically analysed with One-Way ANOVA and/or with Student's *t*-test (two-tails, unpaired) by program GraphPad PRISM Version 4.00 (GraphPad Software, San Diego, CA, USA) and reported as mean±SEM; statistical significance refers to results where $P < 0.05$ was obtained.

Agonist dose-response curves were analyzed by the non-linear regression curve-fitting computer program GraphPad PRISM Version 4.00 and the EC_{50} values were derived. Data are reported as mean±SEM of four different experiments (performed in duplicate). Statistical analysis (Student's *t*-test, two tails, unpaired) was performed using the GraphPad Prism program; significance refers to results where $P < 0.05$ was obtained.

2.4 Results

2.4.1 Immunolocalization

Postembedding immunogold electron microscopy was used to study A_1 (**Fig. 2**) and $P2Y_1$ (**Fig. 3**) receptors in rat hippocampus, focusing on glutamatergic synapses (*i.e.*, small terminals with asymmetric synapses on dendritic spines) and surroundings glia. For quantitative analysis single labelled sections, were randomly selected from CA1 and CA3 stratum radiatum, and juxtagranular part of the dentate molecular layer, regions that are particularly high in nerve terminal glutamate and glutamatergic markers (*e.g.*, Cotman *et al.*, 1987). As no overt differences were noticed between the areas, they were analysed together. Both A_1R and $P2Y_1R$ were detected on synaptic and glial membranes (**Fig. 2-3**).

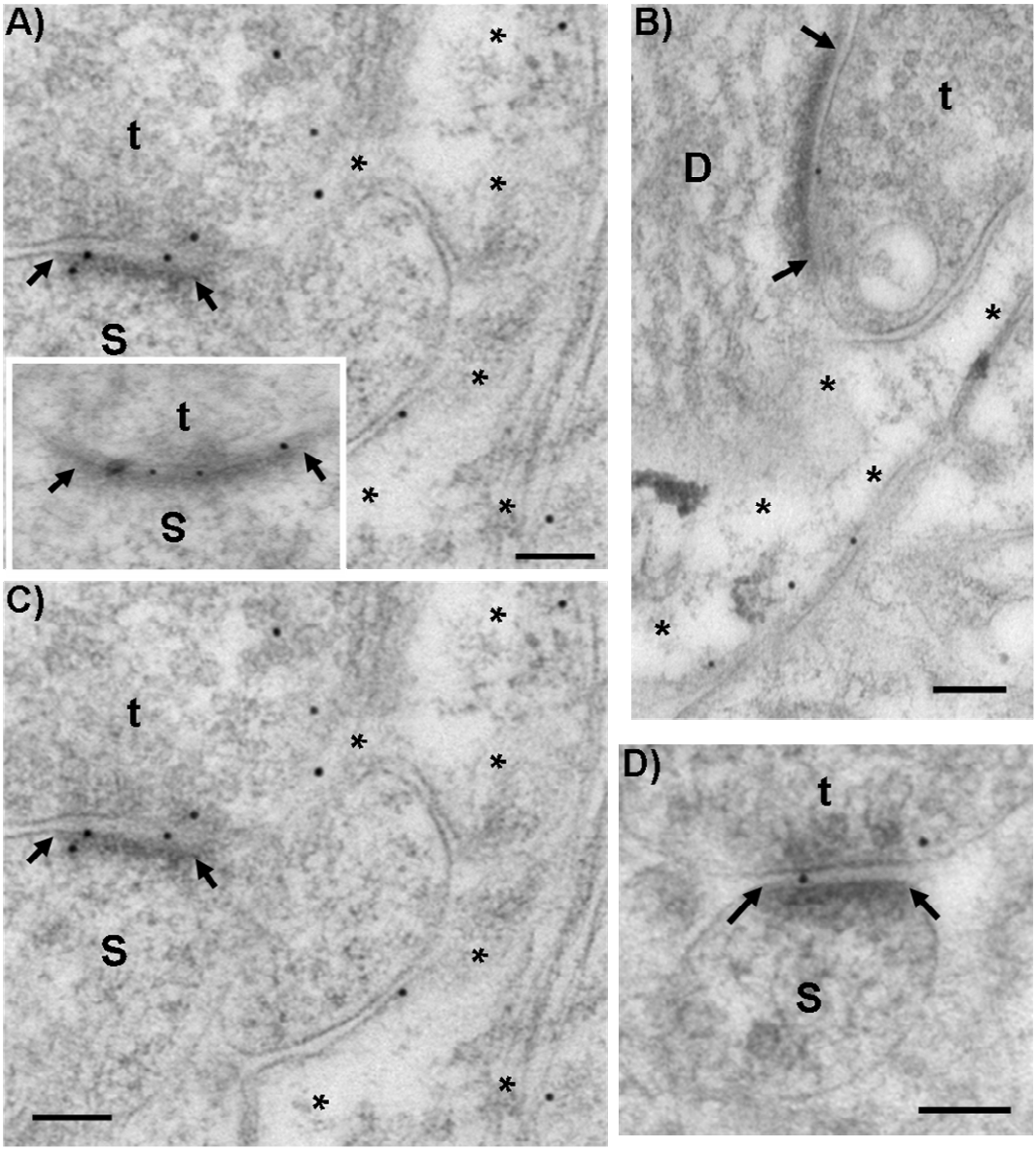

FIG. 2: Localization of purinergic receptors in glutamatergic synapses in rat hippocampus by immunogold labelling. Electron micrographs of sections single labelled for A_1R are illustrated by examples from CA1 stratum radiatum (A main picture, B), CA3 stratum radiatum (A inset, C) and hilus (D). Note that A_1Rs were detected both in the postsynaptic density (black arrows) and in the presynaptic (active zone) membranes at glutamatergic synapses and in glia (*).
Scale bar: 100 nm. m= mitochondria; S= postsynaptic dendritic spine; t= presynaptic axon terminal; * = astroglia.

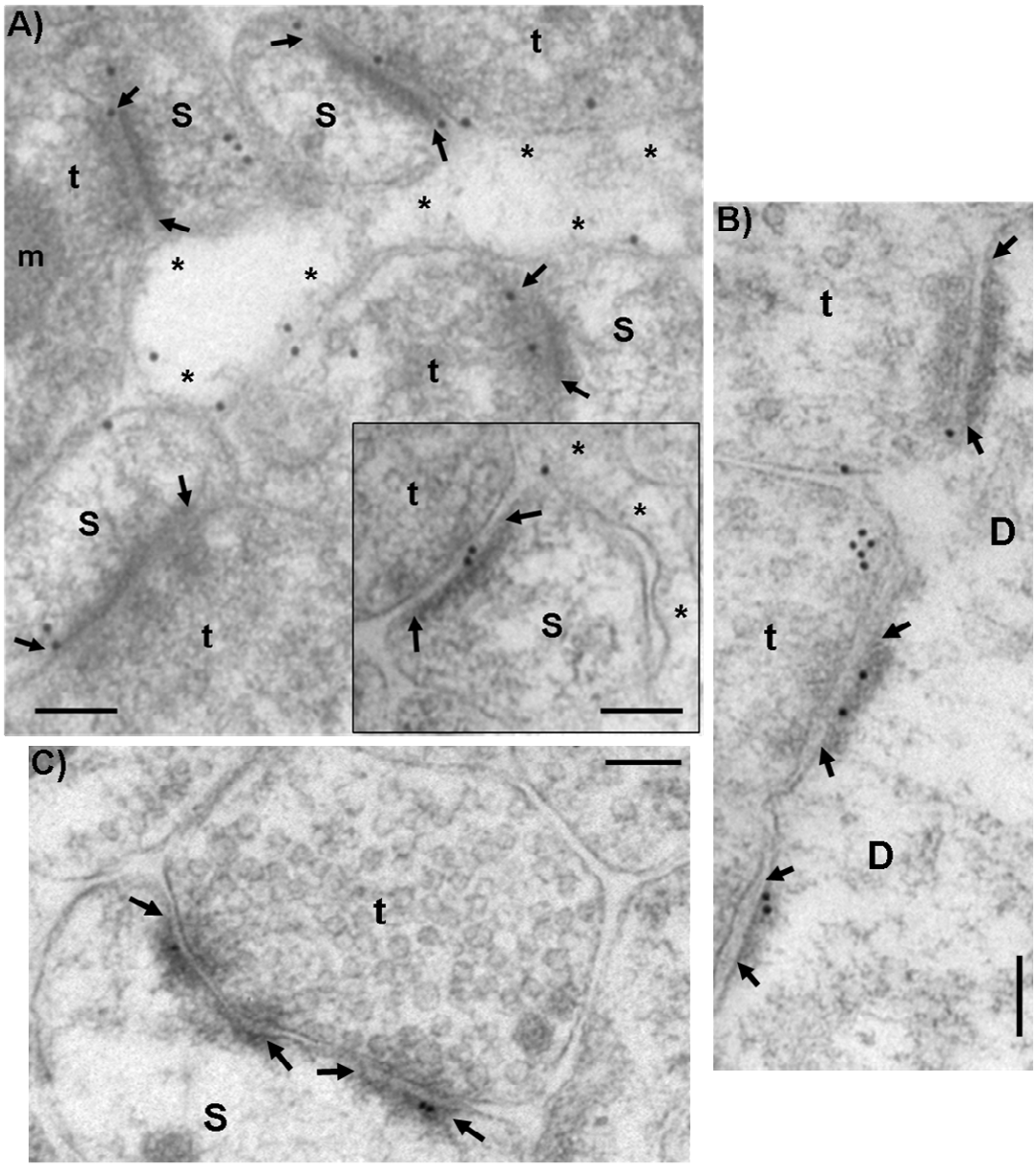

Fig. 3: Localization of purinergic receptors in glutamatergic synapses in rat hippocampus by immunogold labelling. Electron micrographs of sections single labeled for $P2Y_1R$ are illustrated by examples from area dentata (A main picture), CA1 stratum radiatum (A inset), hilus (B) and neuropil layer (close to granular cell layer, C). Note that $P2Y_1Rs$ were detected on synaptic and glial membranes: both in the postsynaptic density (black arrows) and in the presynaptic (active zone) membranes at glutamatergic synapses and in glia (*). Scale bar: 100 nm. m= mitochondria; S= postsynaptic dendritic spine; t= presynaptic axon terminal; * = astroglia.

Immunolabeling negative control experiments have been also performed; omitting the primary antibodies abolished labelling (**Fig. 4**), indicating a low unspecific signal due to the detection system.

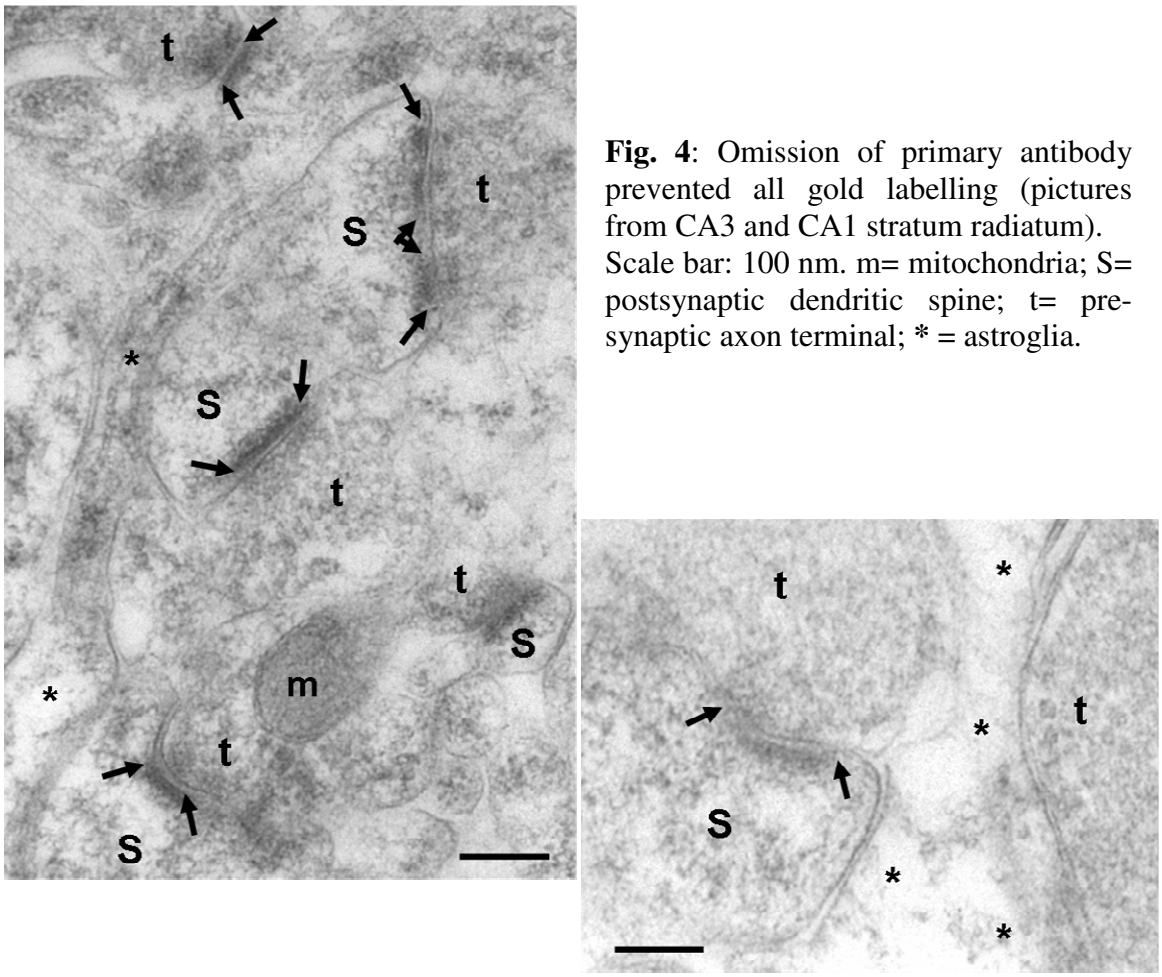

Fig. 4: Omission of primary antibody prevented all gold labelling (pictures from CA3 and CA1 stratum radiatum). Scale bar: 100 nm. m= mitochondria; S= postsynaptic dendritic spine; t= pre-synaptic axon terminal; * = astroglia.

A_1R and $P2Y_1R$ antibody specificity was further addressed by western blot analysis, using rat whole brain and hippocampus membrane fractions (**Fig. 5**): rat tissues were tested with the same antibodies used for immunocytochemistry. Results confirmed the presence of the two receptors and their antibody specificity. A_1R immunoreactive bands, around 80 kDa, correspond to the dimer form of A_1Rs (79 kDa) while the $P2Y_1R$ multi-line pattern shows immunoreactive bands at 76, 40 and 35 kDa, corresponding to dimeric and monomeric variants, in agreement with literature data (Hoffmann *et al.*, 1999; Blum et al., 2002; Moore *et al.*, 2002; Waldo and Harden, 2004). The main band is that around 40-42 kDa, while the lower band is likely a partially degraded product of $P2Y_1R$ and the higher a dimer. In particular, Waldo and Harden (2004) reported that the

incubation at higher temperature increased the occurrence of $P2Y_1R$ multimeric immunoreactive species, with higher molecular mass. A multimeric pattern, due to the sample extraction protocol, is also in agreement with reports of supplier company (Alomone Lab), which indicates that the antibody can produce one band in rat brain extracts under certain conditions, indicating that the different bands shown in Fig. 5B are not due to false immunoreactivity directed against other proteins than $P2Y_1Rs$.

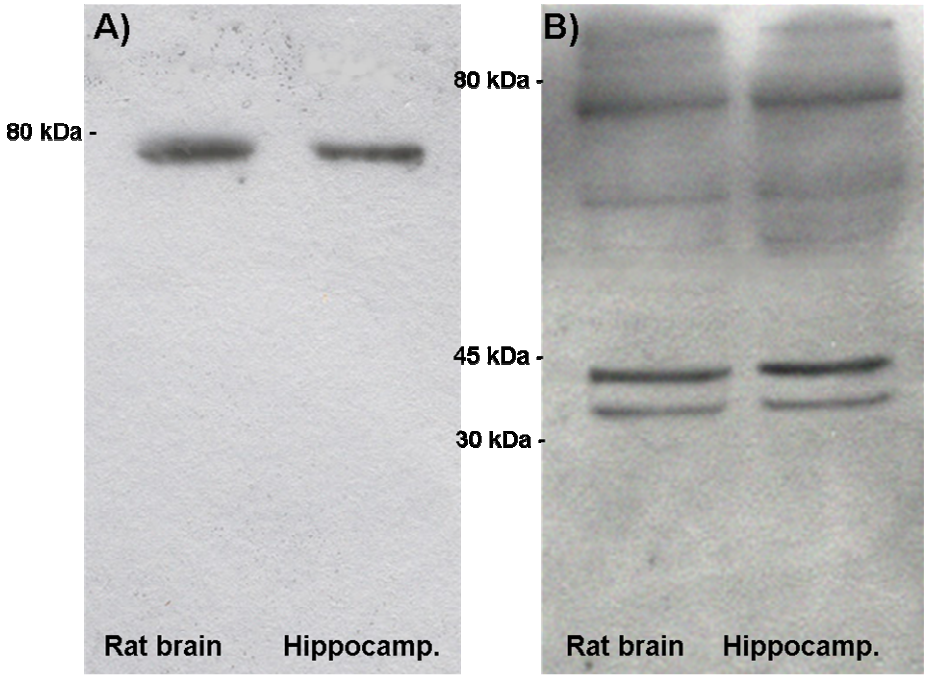

Fig. 5: Western blot analysis of A_1R (**A**) and $P2Y_1R$ (**B**), in rat whole brain and hippocampus membrane homogenates. Rat tissue membranes were lysed, separated (50 µg) by 10% polyacrylamide SDS-PAGE and tested with the same antibodies used for immunocytochemistry.

The receptor distribution and localization was quantified in different subcellular structures (**Fig. 6 A-B**). The localization of both receptors was expressed as the areal density, the number of gold particles/μm^2, in different structures in the vicinity of hippocampal glutamatergic synapses, determining the area by point analysis (Gundersen, H.J. *et al*., 1988). Assuming the mitochondria do not contain the receptors, the density of gold particles over mitochondria may be taken to represent unspecific background binding of antibodies. Both A_1R and $P2Y_1R$ were highly concentrated in the synaptic cleft area and in adjacent astrocytes, which were the only compartments significantly higher than mitochondria (**Fig. 6 A-B**).

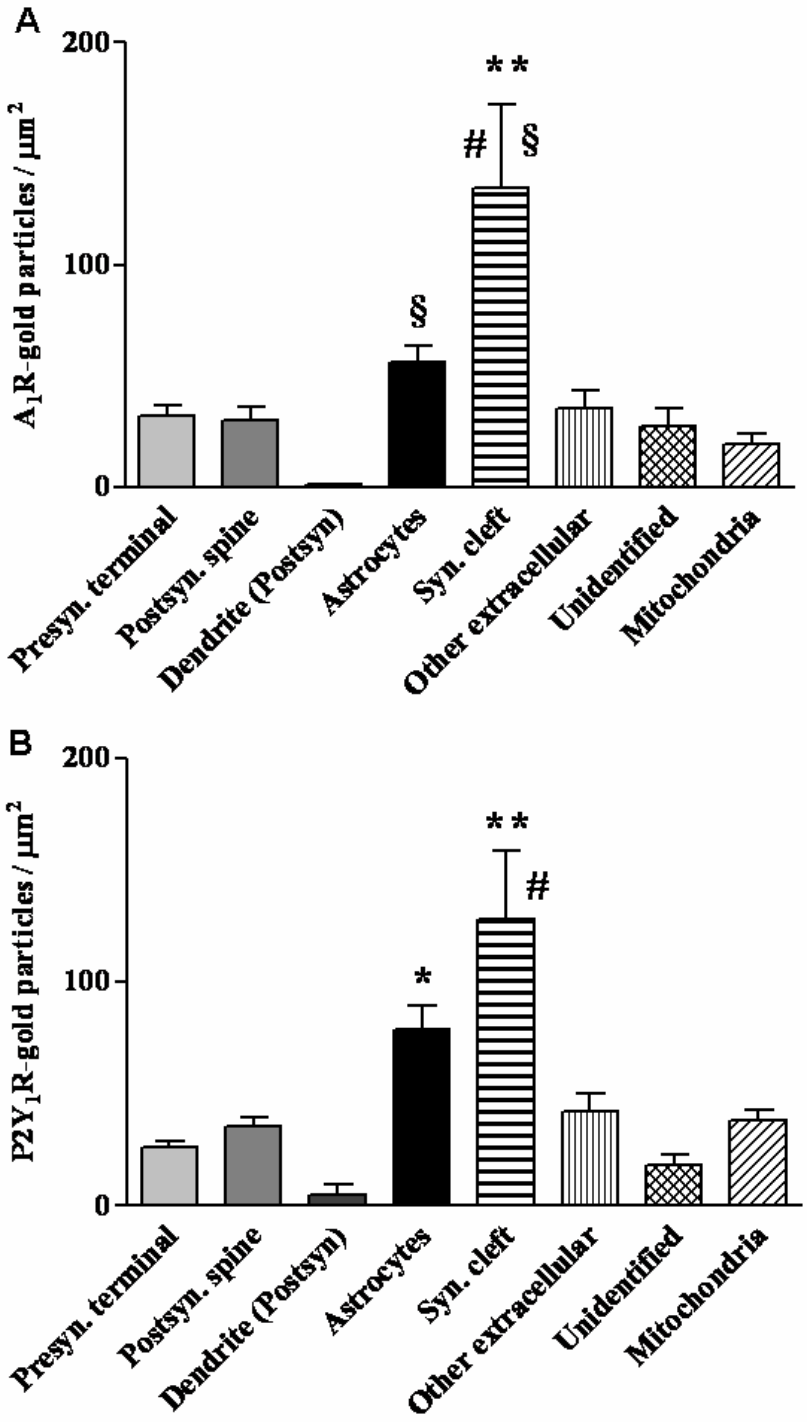

FIG. 6: Distribution of immunogold particles indicating A_1R (**A**) or $P2Y_1R$ (**B**) in different intra- and extracellular compartments. Note that both receptors are enriched at synapses and astroglia, compared to all other compartments. Data, from single labelling experiments, report the localization of receptors as the density (gold particles/µm²; mean±SEM) in the different compartments in the vicinity of hippocampal glutamatergic synapses (randomly selected, from n = 3 animals; 5 grids analyzed for A_1R and 6 grids for $P2Y_1R$); the areas of the compartments were measured by point analysis (see Methods). The numbers of gold parti-

cles on each structure are, in order: 323, 230, 1, 52, 24, 31, 15 for A_1R; 330, 311, 5, 112, 35, 31, 32 for $P2Y_1R$. Areas analysed for each compartments are, in order: 10.8; 7.7; 0.1; 1.1; 0.2; 0.6; 1.3; 1.0 $\square m^2$ for A_1R; 12.1; 8.8; 0.2, 2.1; 0.2; 0.9; 1.7; 0.8 μm^2 for $P2Y_1R$. **A)** A_1R. ** $P<0.01$ all compartments compared to mitochondria (One-way ANOVA, Dunnett's multiple comparison test). Synaptic cleft is significantly different compared to all other domains (# $P<0.01$, One-way ANOVA, Tukey's multiple comparison test). Astrocytes and synaptic cleft columns showed higher receptor density compared to mitochondria (§ $P<0.05$, Student's *t*-test, 2-tails, unpaired). **B)** $P2Y_1R$. * $P<0.05$, ** $P<0.01$ all domains compared to mitochondria (One-way ANOVA, Dunnett's multiple comparison test). Synaptic cleft is significantly different compared to all other domains: # $P<0.001$ vs. all except Astrocytes and $P<0.01$ vs. astrocytes (One-way ANOVA, Tukey's multiple comparison test).

Because the spatial resolution of our immunolabeling method is of the same order of magnitude as the distance between different compartments / membranes in the tissue, an individual gold-particle cannot be directly ascribed to any single structure. Therefore, the association of gold particles with membranes was analysed in several different ways.

First, we investigated the distribution of gold particles associated with each kind of cellular membrane, in the vicinity of hippocampal synapses, recording only particles within a distance ≤10 nm from each kind of membrane to minimize "cross firing" effects from immunoreactivity in nearby structures. Immunogold single-labelling of hippocampal sections showed that A_1R (**Fig. 7 A**) as well as $P2Y_1R$ (**Fig. 7 B**) are mainly associated with presynaptic membranes, postsynaptic membranes overlying the PSD and astroglial membranes. The high A_1R presence on presynaptic-perisynaptic membranes (* $P<0.05$, One-way ANOVA, value vs. mitochondria) could be influenced from receptors that are located in the active zone or as "reserve" (Rebola *et al*., 2003); anyway the values on perisynaptic and extrasynaptic membranes, both pre- and post-, can be influenced from receptors that are instead on astroglia membranes, for the tight contact between these and neurons. No other columns differed from the background level, as indicated by the density of gold particles over mitochondrial outer membranes ($P>0.05$, One-way ANOVA). Further, values at the presynaptic membrane and at the membrane covering the PSD were significantly different compared to all other locations, confirming that A_1Rs are associated with these membranes.

For $P2Y_1R$, the density was significantly higher in PSD than in other membrane compartments, except for the presynaptic and astroglial membranes (# $P<0.01$, One-way ANOVA), meaning these compartment are particularly enriched in the $P2Y_1Rs$.

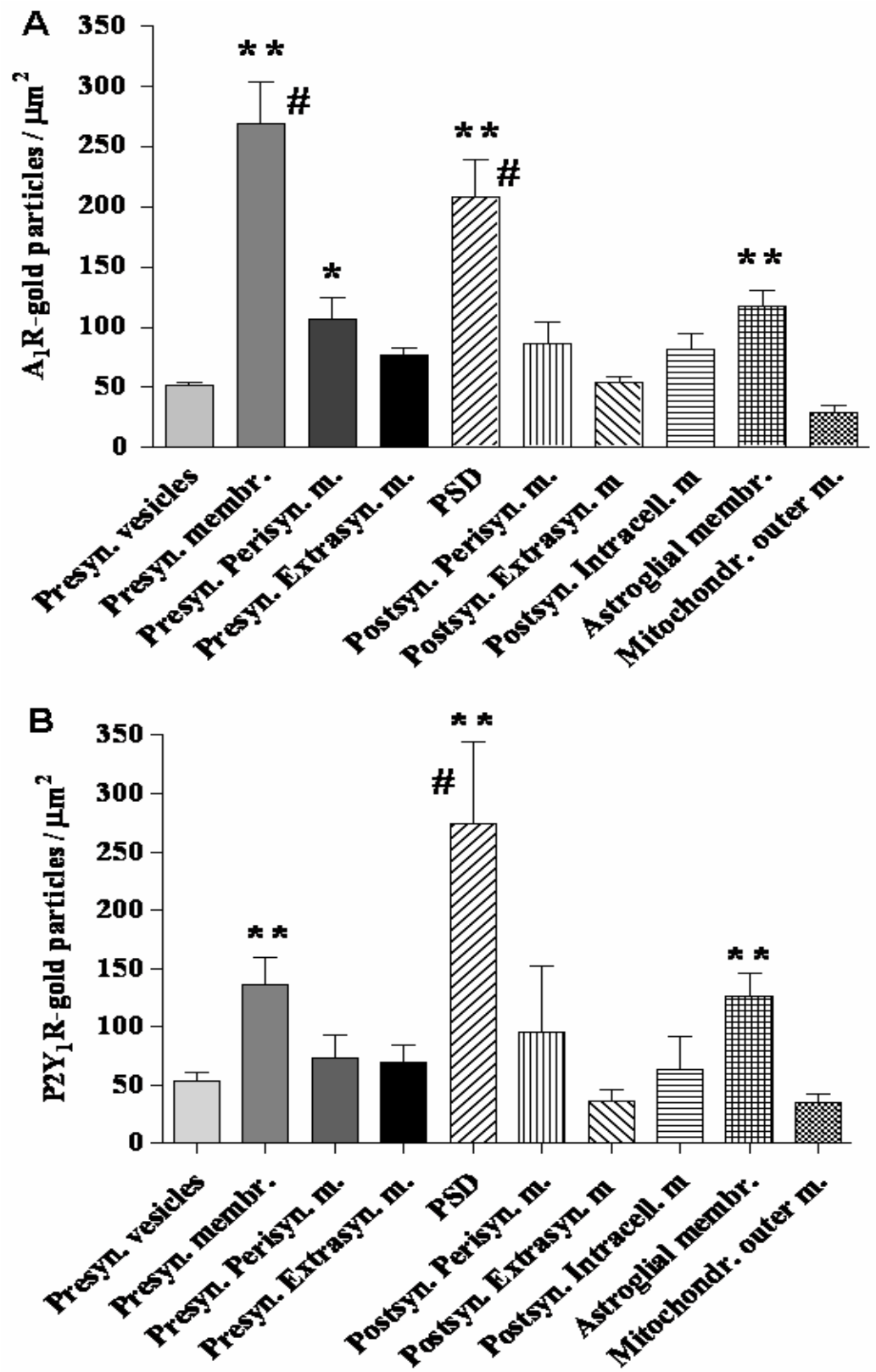

FIG. 7: Distribution of immunogold particles indicating A_1R (**A**) or $P2Y_1R$ (**B**) over different categories of cellular membranes in the vicinity of hippocampal synapses. Both receptor types are enriched over presynaptic as well as postsynaptic membranes and over astroglial membranes. Only particles located within a distance ≤10 nm from each kind of membrane were recorded, to minimize "cross firing" effects from immunoreactivity in nearby structures. The data are presented in the same way, and are from the environments of the same synapses, as are the data in FIG. 3. The sizes of the areas sampled for gold particles (±10 nm on each side of the membrane) were determined by point analysis. Areas analysed for each category are, in order: 3.8; 0.5; 0.3; 1.4; 0.7; 0.3; 1.1; 0.3; 0.6; 0.6 μm^2 for A_1R; 5.1; 0.8; 0.3; 1.6; 0.8; 0.4; 2.0; 0.6; 1.2; 0.7 μm^2 for $P2Y_1R$. The numbers of gold particles within 10 nm from each membrane column are, in order: 196, 126, 22, 98, 141, 19, 54, 32, 57, 12 for A_1R; 260, 81, 15, 101, 124, 26, 74, 36, 116, 27 for $P2Y_1R$. The density of gold particles over the outer membrane of mitochondria gives an estimate of the background level. **A)** * $P<0.05$, ** $P<0.01$, all columns compared to mitochondria background level (One-way ANOVA, Dunnett's multiple comparison test). Presynaptic membrane and PSD are significantly different compared to all other membrane categories (# $P<0.01$, One-way ANOVA, Tukey's multiple comparison test). **B)** ** $P<0.01$ all columns compared to mitochondria background level (One-way ANOVA, Dunnett's multiple comparison test). PSD is significantly different compared to all other columns except for presynaptic membranes and astroglial membranes (# $P<0.01$, One-way ANOVA, Tukey's multiple comparison test).

We then compared the distribution of gold particles representing A_1R and $P2Y_1R$ with the distribution of points spread randomly over the pictures (**Fig. 8**). The gold particles/random points ratio was determined at different distances, from different membrane domains, sorted into bins (10-20-30-40 nm). For the shortest distance (10 nm) bins, the highest ratio values (about 2) were obtained, for both A_1R and $P2Y_1R$, at presynaptic active zone membranes, postsynaptic membranes overlying the PSD and astroglial membranes (**Fig. 8 A-B**), i.e., the same membrane domains that show the highest labelling according to the analysis of Fig. 7. Also several other neuronal membranes, but not mitochondrial outer membranes, had ratios higher than one, consistent with a moderate enrichment of receptors in them. As previously noted, the densities in perisynaptic and extrasynaptic membranes could be influenced from gold particles in active zone or in astroglial membranes.

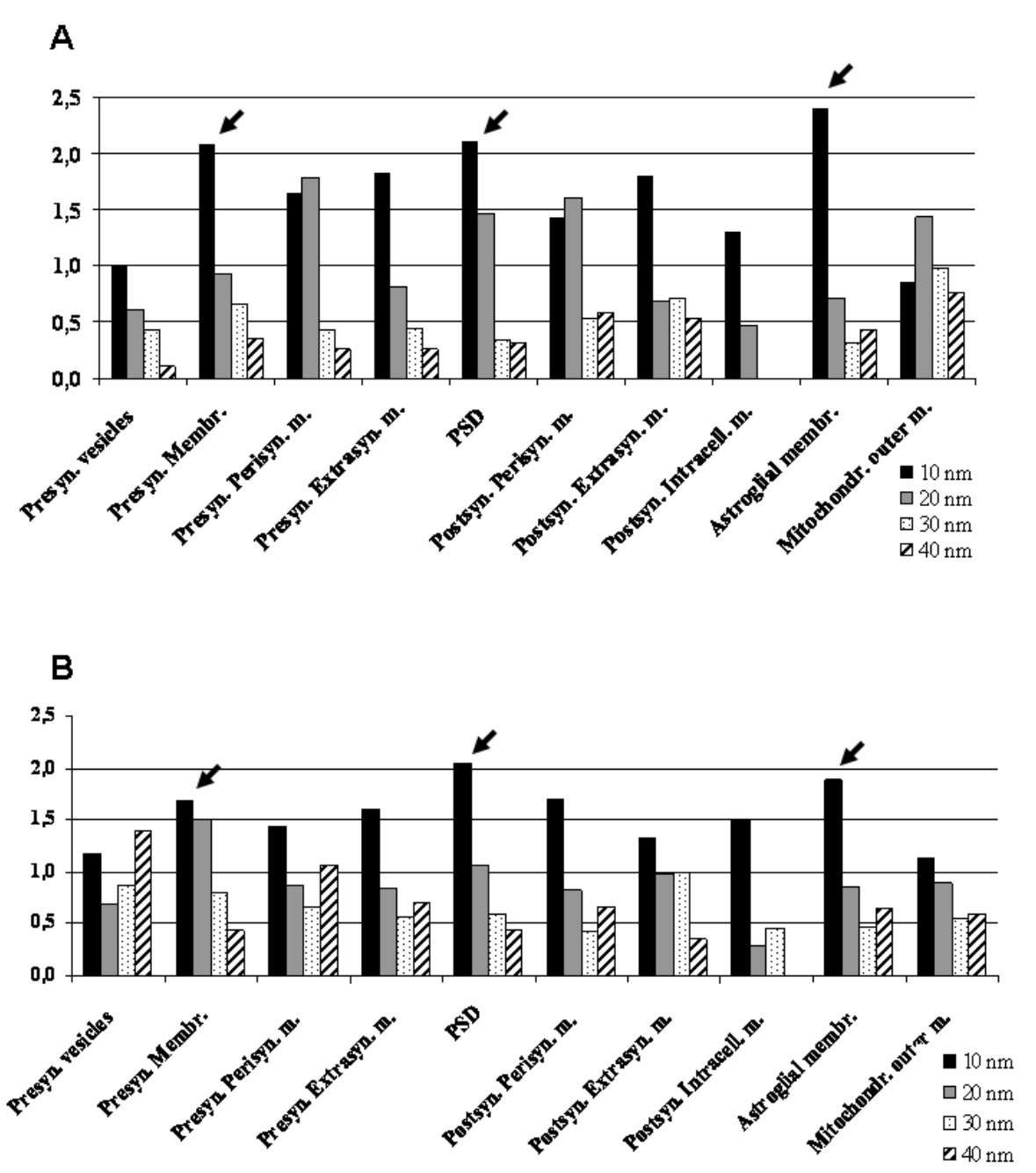

FIG. 8: Histogram of gold particles/random points ratio: gold particles were grouped in bins depending the distance (10-20-30-40 nm) from each kind of membrane, at hippocampal glutamatergic synapses and surrounding glia. The % of the total number of gold particles that occurred in each bin was divided by the % of the total random points (within 40 nm distance) that occurred in the same bin: the ratio gives the distribution of receptors relative to a random distribution. A ratio >1 for the shorter distance bins (10 and 20 nm), indicates a close association with the membrane observed. Note the higher ratios (arrows) for short distances at presynaptic active zone membranes, postsynaptic membranes overlying the PSD and astroglial membranes, for both A_1R (**A**) and $P2Y_1R$ (**B**).

The average distance of immunogold particles from membranes was then measured and compared with a random distribution (**Fig. 9**). Note that for both A_1R (**A**) and $P2Y_1R$ (**B**) the mean distance of gold particles is shorter than that of random points in all membranes, except in mitochondria outer membranes (which represent an estimate of background labelling).

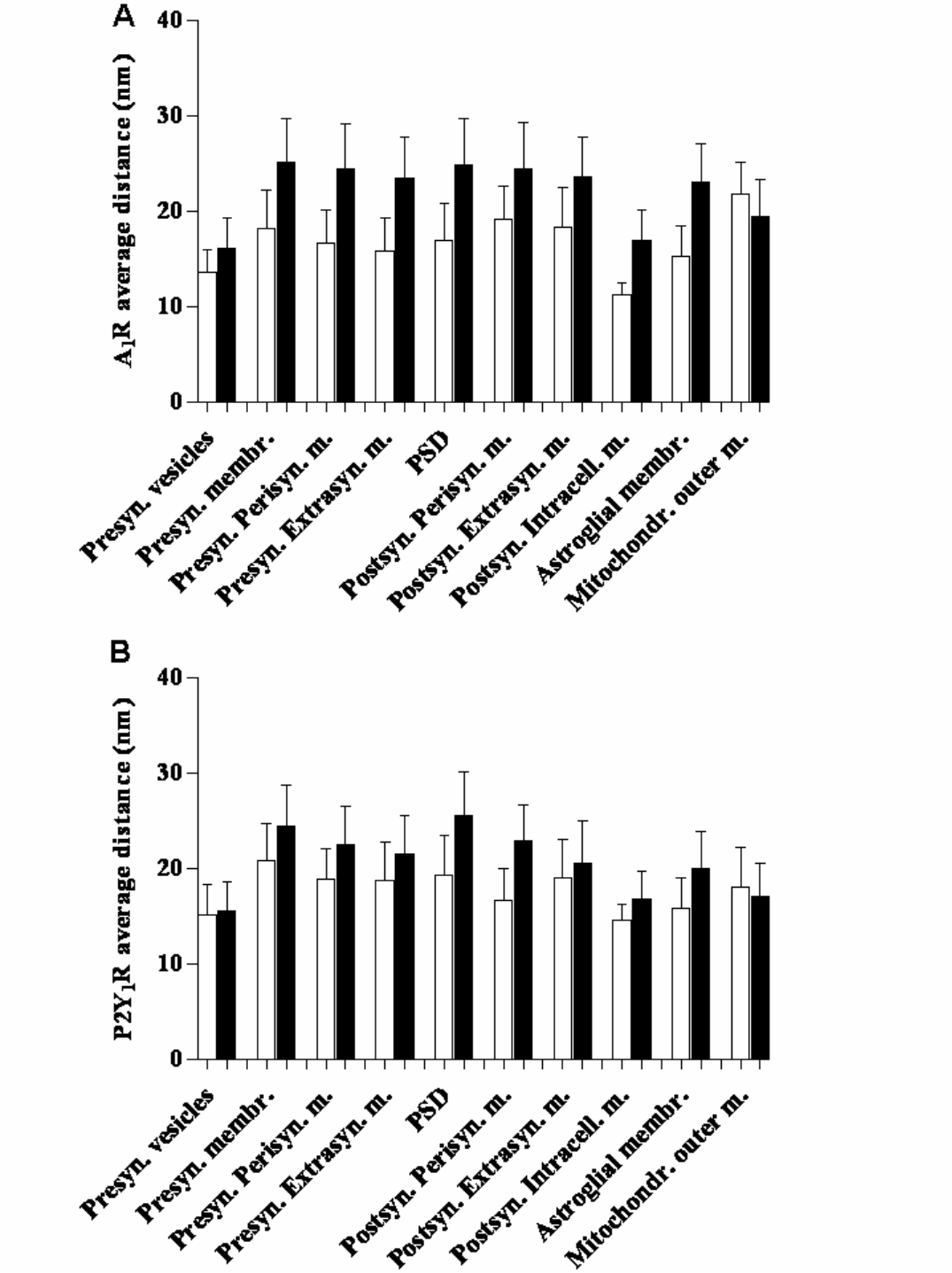

FIG. 9: Average distances of immunogold particles from membranes. The values are the mean±SEM of the distances of gold particles (white bars) or random points (black bars) located within a distance ≤40 nm from different membrane categories, in the vicinity of glutamatergic synapses in rat hippocampus. Note that for both A_1R (**A**) and $P2Y_1R$ (**B**) the mean distance of gold particles is shorter than that of random points in all membranes, except in mitochondria outer membranes (which represent background labelling). The total number of particles counted is: for A_1R gold particles: 286; 241; 34; 159; 230; 38; 101; 37; 91; 241; for P2Y1R gold particles: 392; 238; 41; 183; 247; 47; 145; 44; 207; 44. For A1 random points: 457; 212; 46; 313; 218; 48; 187; 39; 149; 177; for P2Y1 random points: 457; 127; 47; 270; 132; 55; 269; 60; 162; 63.

To further dissect the synaptic localization of A_1R and $P2Y_1R$, all the hippocampal glutamatergic synapses previously analyzed were processed to record the % distribution of both receptors as a function of the perpendicular distance from the postsynaptic membrane overlying the PSD (**Fig. 10**). The % distribution of A_1Rs in the synaptic area (**Fig. 10 A**, *left*) showed that A_1Rs are located over both the PSD and the presynaptic membrane, although the density is highest over the latter. The influence of the extracellular location of the epitope recognized by the A_1R-antibody may have contributed to the high level of gold particles located in the synaptic cleft. A_1R-gold particles situated over the synaptic cleft were preferentially associated with the presynaptic membrane (**Fig. 10 A**, *right*), indicating a shared distribution of the receptor between the pre- and postsynaptic sides. $P2Y_1Rs$ were relatively more distributed towards the PSD membrane (**Fig. 10 B**, *left*), compared to A_1Rs, and gold-particles in the cleft showed less enrichment on the presynaptic side (**Fig. 10 B**, *right*). These results are consistent with a high degree of co-localization of the two types of receptor, but with relatively less of $P2Y_1R$ than of A_1R on the presynaptic side.

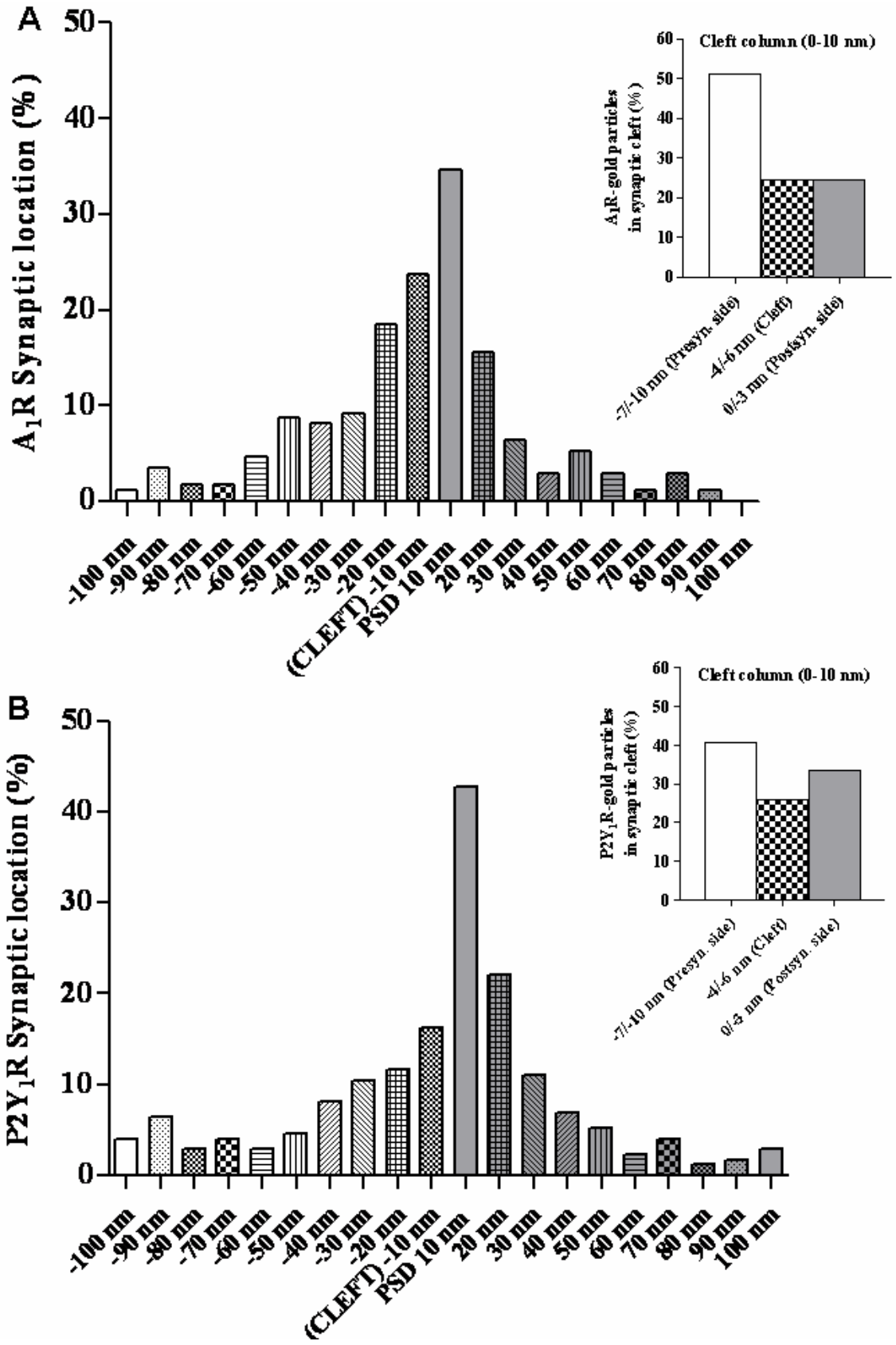

FIG. 10: Distribution of immunogold particles across the synaptic cleft. The *histograms on the left* show the % of gold particles representing A_1R (**A**) and $P2Y_1R$ (**B**) in the synaptic area, as a function of the dis-

tance up to 100 nm from the postsynaptic membrane at the PSD. The distance between the centre of gold particles and the external face of the PSD was determined along a perpendicular axis (cf. Fig. 1). Positive columns (10 to100 nm) represent the postsynaptic spine; negative columns (-20 to -100 nm) the presynaptic terminal; the -10 nm column represents the synaptic cleft (i.e. position 0 to -10 nm). The actual width of the synaptic cleft was 11.5±0.4 nm (mean±SEM, n= 79). Numbers of gold particles considered in the analysis are: n= 266 for A_1R; n= 296 for $P2Y_1R$. All synapses previously analyzed have been processed in this analysis. Note that the highest particle counts for A_1R as well as $P2Y_1R$ were in the PSD bin. The *histograms on the right* show the distribution of immunogold particles within the synaptic cleft. The % of the total number of gold particles located in the synaptic cleft (n= 41 for A_1R; n= 28 for $P2Y_1R$), in function of the distance from the outer face of the PSD; pre-synaptic side = position between 0 and -3 nm; post-synaptic side = between -6 and -10 nm; middle of cleft = between -3 and -6 nm. The distributions within the synaptic cleft indicate that both A_1R (**A**) and $P2Y_1R$ (**B**) are located pre- as well as post-synaptically.

In conclusion, A_1R and $P2Y_1R$ are co-localized in synaptic and astroglial membranes in glutamatergic synapses and surrounding glial membranes in the hippocampus. The quantitative analyses of single labelling data were confirmed by the qualitative analysis of double labelling experiments (**Fig. 11**). The double labelling approach gave similar patterns as the single labelling protocol: the receptors were detected together in the same structures and both were associated with synaptic and astroglial membranes.

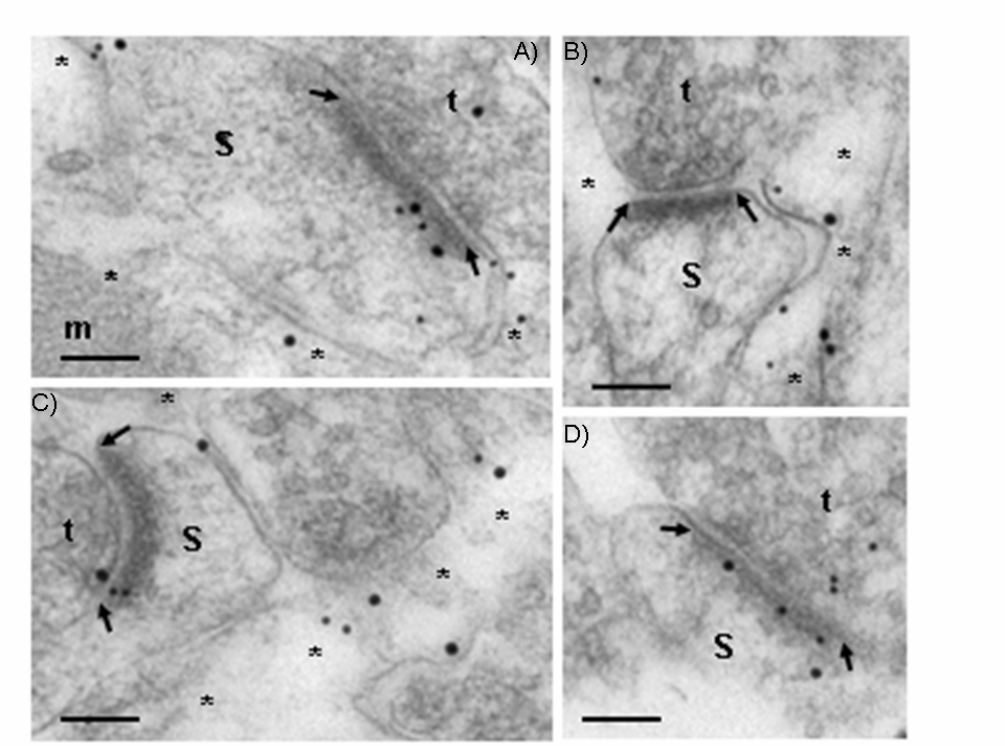

Fig. 11: Qualitative co-localization of $P2Y_1R$ and A_1R in glutamatergic synapses (randomly selected) and surrounding glia, in rat hippocampus CA3 (A, B) and CA1 (C, D) stratum radiatum. Electron micrograph of a section *double labelled* for A_1R (large gold particles, 15 nm) and $P2Y_1R$ (small gold particles, 10 nm). The double labelling approach gave similar patterns as the single labelling protocol. Note that the receptors were detected together in the same structures. A_1R and $P2Y_1R$ appeared co-localized (qualitative data) on the PSD (black arrows) and on astroglia (*). Scale bar: 100 nm. m= mitochondria; S= post synaptic spine; t= pre-synaptic terminal; * = astroglia.

2.4.2 Functional assays

In HC the effect of $P2Y_1R$ activation on A_1R/G protein coupling was measured by evaluating the ability of the A_1R agonist CHA to stimulate G protein activation following 100 nM MeSADP pre-incubation (for 10 minutes). In Control HC, CHA stimulated G protein activation with an EC_{50} of 30.60±3.99 nM (**Fig. 12**). The CHA-mediated effect was abolished in the presence of the selective A_1R antagonist DPCPX (50 nM), confirming A_1R specific activation (*data not shown*).

Membrane pre-incubation with 100 nM MeSADP induced a right-shift of the CHA dose/response curve (EC_{50}=106.26±16.39 nM), suggesting a significant impairment in A_1R-G protein coupling when $P2Y_1$ receptor is activated (*t*-test, two-tails, unpaired: ** $P<0.01$ for EC_{50} of MeSADP-treated HC vs. Control HC) (**Fig. 12**).

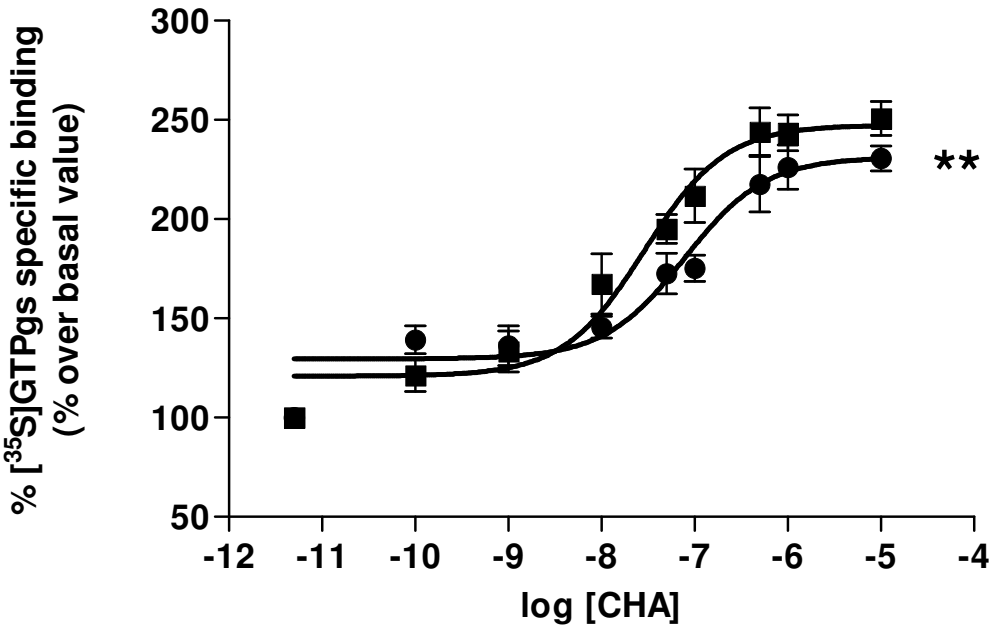

FIG. 12: Effect of $P2Y_1R$ activation on A_1R agonist-mediated G protein coupling. Dose-response curves of CHA-stimulated [^{35}S]GTPγS binding were obtained incubating Control HC (■) and MeSADP-treated (100 nM) HC (●) with different agonist concentration (0.1nM-10μM). Data are expressed as % of [^{35}S]GTPγS specific binding over basal value, set to 100%, and are reported as mean±SEM (n=4), all performed in duplicate. Student's *t*-test, two-tails, unpaired: ** P<0.01 EC_{50} of Control HC vs. MeSADP-treated HC.

On the other hand, the A_1R activation effect on $P2Y_1R$/G protein coupling was measured by evaluating the ability of the $P2Y_1R$ agonist MeSADP to stimulate G protein activation in the absence or the presence of 100 nM CHA (preincubated for 10 minutes). In Control HC, MeSADP stimulated [^{35}S]GTPγS binding with an EC_{50} of 0.851±0.147 nM (**Fig. 13**).

To test the selectivity of MeSADP for $P2Y_1$ receptor subtype in our model, we also stimulated HC in presence of the selective $P2Y_1R$ antagonist 10 μM MRS2179 (preincubated for 10 minutes). MRS2179 blocked MeSADP-mediated G protein coupling in HC (**Fig. 13**), showing this G protein activation is selectively driven by $P2Y_1R$ and other P2Y receptors sensitive to MeSADP ($P2Y_{12\text{-}13}$) have no significantly signal component in rat hippocampus. Membrane CHA pre-incubation induced an significant increase in MeSADP potency to activate $P2Y_1R$-G protein coupling, with an EC_{50} of

0.393±0.058 nM (*t*-test; two-tails, unpaired: * P<0.05), without affecting significantly the G protein coupling efficacy level of the agonist dose/response curve (**Fig. 13**).

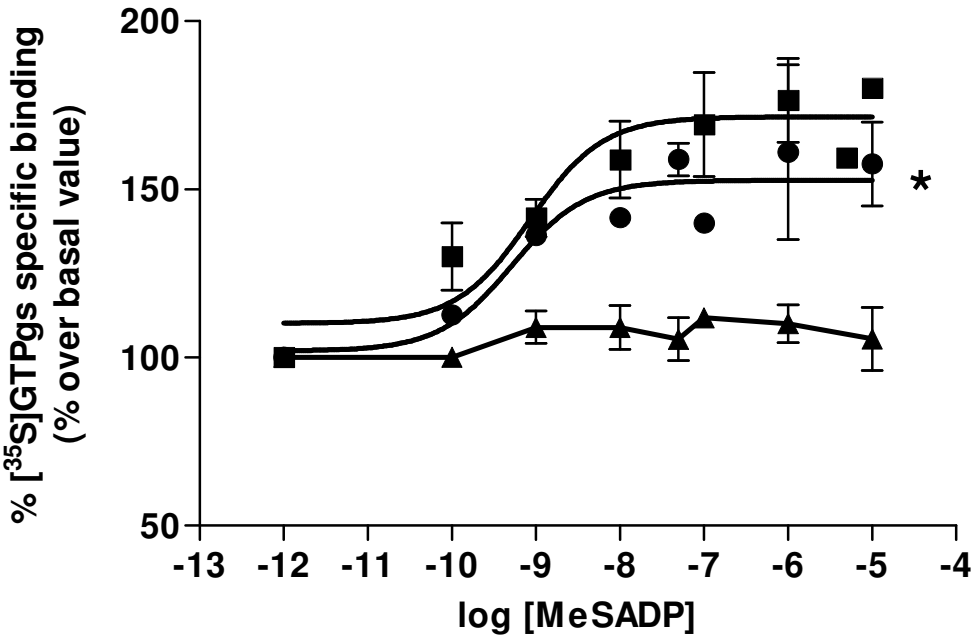

FIG. 13: **A**) Effect of A_1R activation on $P2Y_1R$ agonist-mediated G protein coupling. Dose-response curves of MeSADP-stimulated [^{35}S]GTPγS binding were obtained incubating Control HC (■) and CHA-treated (100 nM) HC (●) with different agonist concentration (0.1nM-10□M). Data are expressed as % of [^{35}S]GTPγS specific binding over basal value set to 100% and are reported as mean±SEM (n=4), all performed in duplicate. Student's *t*-test, two-tails, unpaired: * P<0.05 EC_{50} of Control HC vs. CHA-treated HC. **B**) Aliquots of HC were also pre-exposed to the selective $P2Y_1R$ antagonist MRS2179 (10μM) (MRS2179-treated HC) (▲) and then stimulated by MeSADP (0.1nM-10μM). Data are expressed as % of [^{35}S]GTPγS specific binding over basal value set to 100% and are reported as mean±SEM (n=4), all performed in duplicate.

2.5 Discussion

2.5.1 Immunolocalization and co-localization

This is the first study on A_1R and $P2Y_1R$ localization and co-localization in hippocampus by postembedding immunogold electron microscopy analysis: this high resolution technique is able to show the precise subcellular localization of receptors in different subcellular compartments and cell populations (*e.g.*, Bergersen *et al.*, 2003). The purinergic receptors proved to be mainly associated with membrane domains. Single labelling immunolocalization data showed a significant enrichment of both A_1R and $P2Y_1R$ mainly in postsynaptic membranes at the PSD, in presynaptic active zones, and in astroglial membranes at glutamatergic synapses and surrounding glia in the rat hippocampus. The same conclusion was arrived at by analysing the data in different ways, in order to partly overcome the limits posed by "cross firing" effects antigen located in closely spaced neighbouring membranes. Because of these, the exact labelling densities of the individual membranes cannot be determined. While the three membrane categories mentioned contain higher levels of both of the purinergic receptors studied, low or moderate labelling may exist in other membrane categories. The data suggest that there may be relatively higher densities of A_1R than of $P2Y_1R$ at the presynaptic compared to the postsynaptic membrane. Part of this observed difference might be attributable to the fact that the antibodies were to extracellular and intracellular epitopes, respectively. However, the main conclusion is that the two receptor types are similarly distributed, compatible with a high degree of co-localization. This was born out by double labelling experiments which showed A_1R and $P2Y_1R$ to be closely spaced along synaptic and glial membranes.

Our high resolution data serve to extend and reconcile previous reports obtained with lower resolution methods. Thus A_1R immunoreactivity has been reported in hippocampus both at pre- and post-synaptic terminals but not at glial cells (Ochiishi *et al.*, 1999); an immunohistochemical study in the rat hippocampus has concluded that A_1Rs were mostly located in axons rather than in nerve terminals (Swanson *et al.*, 1995), whereas work on synaptosomal fractions (Rebola *et al.*, 2003) has suggested that A_1Rs are enriched in nerve terminals and are mainly located in synapses, both in the presynaptic active zone and in the PSD membranes. $P2Y_1R$ immunoreactivity has been found in astroglia and in different kinds of neurons in hippocampus (Moran-Jimenez & Matute,

2000), especially in ischemic sensitive areas while at the same time another study reported a striking neuronal localization for $P2Y_1R$ (human brain, Moore *et al*., 2000). $P2Y_1Rs$ have been reported to be present and active on astrocytes all around the brain (Franke *et al*., 2001; Volontè *et al*., 2006). A high degree of co-localization of A_1R and $P2Y_1R$ has been found in rat hippocampus by immunofluorescence experiments but without cellular and sub-cellular identification (Yoshioka *et al*., 2002).

We studied A_1Rs and $P2Y_1Rs$ in the hippocampal region considering the fact that the hippocampus has been identified as a major target site for numerous disease processes (Bachevalier & Meunier, 1996; Harry & Lefebvre d'Hellencourt, 2003), and considering the generally assumed involvement of purinergic receptors in patho-physiological mechanisms and in the modulation of brain damage (Fredholm, 1997; Franke *et al*., 2006b). Ischemia, to which hippocampus is particularly vulnerable, produces a marked increase in glutamate within the brain extracellular space (Benvensiste *et al*., 1984; Hagberg *et al*., 1985), thereby triggering excitotoxic injuries (Choi & Rothman, 1990). Because of the importance of glutamate in pathological conditions and because its release, in neurons and in astrocytes, is modulated both through A_1R (Rudolphi *et al*., 1992; Masino *et al*., 2002) and $P2Y_1R$ (Rodrigues *et al*., 2005; Franke *et al*., 2006a; Jourdain *et al*., 2007), the present study was focused on A_1R-$P2Y_1R$ localization and co-localization within and in the vicinity of glutamatergic synapses.

Our results provide direct morphological support for the previous suggestions that both of these receptors contribute to and interact in the modulation of glutamate release (Rudolphi *et al*., 1992; Mendoza-Fernandez *et al*., 2000; Masino *et al*., 2002; Kawamura *et al*., 2004; Rodrigues *et al.,* 2005).

2.5.2 Functional interaction

The functional interaction of A_1Rs and $P2Y_1Rs$ suggested by the morphological observations was subsequently confirmed through measurement of G protein activation initiated by the A_1R-agonist CHA or the $P2Y_1R$-agonist MeSADP, respectively, and modification of the response through preincubation with the other agonist. Because the receptors on study are coupled to different G protein subtypes (Munshi *et al*., 1991; Yoshioda and Nakata, 2004) and to different intracellular signalling pathways, the [^{35}S]GTPγS binding method was chosen to investigate the A_1R-$P2Y_1R$ interaction and their reciprocal modulation at the membrane level, allowing any change in their func-

tioning to be determined independently of the second messenger systems activated (Lorenzen *et al*., 1996).

According to literature data (Gao *et al*., 2003; Dixon *et al*., 2004; Niebauer *et al*., 2005), the selected agonist preincubation times and concentrations pre-stimulating A_1R and $P2Y_1R$ (100 nM CHA and 100 nM MeSADP, respectively) allow a selective and maximal receptor activation. The EC_{50} for CHA in stimulating A_1R-G protein coupling ranged around 30 nM: CHA-mediated G protein activation was abolished in the presence of the highly selective A_1R antagonist DPCPX (Klotz, 2000), confirming further the selective A_1R activation. Although CHA has been reported to block [^{3}H]DPCPX binding at A_1R with a K_i around 5 nM in rat hippocampus (Maemoto *et al*., 1997), higher EC_{50} values for different A_1R agonists in the [^{35}S]GTP□S binding assay have been found (Lorenzen *et al.*, 1996), correlating with the low affinity state values (K_L) of the receptor. In line with these findings, even an EC_{50} around 100 nM has been reported for CHA in a [^{35}S]GTPγS assay in CHO cells transfected with the human A_1 receptor (Cordeaux *et al.*, 2004).

On the other hand, in our results, MeSADP showed subnanomolar potency in stimulating $P2Y_1R$. Because of the absolute potency of nucleoside tri- and di-phosphates for P2Y receptors is dependent on the levels of receptor protein expression, typical EC_{50} values are not easily defined for specific agonists at particular P2Y receptor subtypes in different tissue preparations and cell lines (Volontè *et al*., 2006). In accordance with our results, low nanomolar EC_{50} values have been reported for human $P2Y_1R$ expressed in astrocytoma 1321N1 cells (Palmer *et al*., 1998; Niebauer *et al*., 2005) and rat $P2Y_1R$, expressed in HEK 293 cells, was reported to be functionally activated by MeSADP at subnanomolar concentrations (Vohringer *et al*., 2000).

MeSADP is the principal agonist not only at $P2Y_1R$ but also at $P2Y_{12\text{-}13}$ receptors, that are coupled to G_i proteins and are expressed (mRNA) in the brain (Hollopeter *et al*., 2001; Zhang *et al*., 2002; Sasaki *et al*., 2003), even if not at high level in the hippocampus (Fumagalli *et al*., 2004; Amadio *et al*., 2006). To confirm that in our model the MeSADP-mediated G protein activation was mainly driven by $P2Y_1R$, we also stimulated hippocampal membranes in the presence of the selective $P2Y_1R$ antagonist MRS2179: MRS2179 was able to block the MeSADP-mediated response, confirming the $P2Y_1R$ involvement since no antagonistic effects have been demonstrated on $P2Y_{12\text{-}13}$ receptors at the MRS2179 concentrations used (Moro *et al.,* 1998; Von Kugelgen, 2006).

The results obtained on A_1R-P2Y_1R cross-talk in hippocampus showed that, stimulating one receptor, the functioning of the other was changed: in particular, P2Y_1R pre-activation caused an impairment in A_1R-G protein coupling with a reduction in A_1R agonist potency; on the other hand, A_1R pre-activation induced an increase in P2Y_1R functional coupling to G proteins. Our results are in agreement with the previously reported reduction in the A_1R ligand affinities in cells co-expressing both A_1R and P2Y_1R (Yoshioka *et al*., 2001). Various studies have reported that ATP, massively released after brain damage, acts to modulate not only its own P2Y_1R but also A_1R (Hourani *et al*., 1991; Piper and Hollinsworth, 1996; Masino *et al.*, 2002; Fredholm *et al*., 2003; Yoshioda and Nakata, 2004).

The functional consequence of this A_1-P2Y_1 receptor cross-talk is complicated by the availability time and the balance of their endogenous ligands. Extracellular ATP, rapidly available due to direct release into the extracellular space, and adenosine, available after ATP breakdown, are tightly regulated by rapid metabolism and re-uptake (Zimmermann, 2000) and can be differently regulated in physiological or pathological conditions: in fact the ecto-nucleotidase chain has proved to be up-regulated in ischemically damaged tissues (Braun *et al*., 1998).

Data, at present, have shown the A_1R -P2Y_1R interaction mechanism may be used to fine-tuning the purinergic signalling, including the inhibition of neurotransmission (Nakata *et al*., 2004). Considering the new information available and the A_1R and P2Y_1R involvement in glutamatergic transmission modulation (Mendoza-Fernandez *et al*., 2000; Masino *et al.*, 2002; Kawamura *et al*., 2004; Rodrigues *et al.,* 2005), we can speculate that there is an A_1R-P2Y_1R cross-talk in rat hippocampal glutamatergic synapses and surroundings glia, where these receptors are co-localized. This might therefore be one of the mechanisms for the adenine nucleotide-mediated inhibition of glutamatergic neurotransmitter release. Therefore, as suggested for adenosine A_1 and A_{2A} receptors in striatal (Ciruela *et al*., 2006) and hippocampal (Rebola *et al*., 2005) glutamatergic nerve terminals, a cross-talk/heteromerization of A_1R-P2Y_1R could exert a fine-tuning modulation of glutamatergic neurotransmission, providing a switch mechanism by which low and high concentrations of adenosine or purines could regulate glutamate release.

Because of the high level of complexity of purinergic receptor signalling (Volontè *et al*., 2006) and the regulation of glia–neuron and glia–glia communications by extracellular

purines (Franke *et al*., 2006b; Jourdain *et al*., 2007), the present work opens the way to further investigation of the A1R-P2Y1R system interaction: on astrocyte cell populations, which communicate bidirectionally with neurons (Newman, 2003; Bezzi *et al*., 2004; Jourdain *et al*., 2007) and contribute to damage or to regeneration after CNS injury (Franke *et al*., 2001; Anderson *et al*., 2003).

2.6 References

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Chapter 3:

Functional cross-talk between A_1 and $P2Y_1$ purine receptors in human astroglial cells

3.1 Introduction

The purinergic system is involved in many physiological and pathological events, especially because ATP massive release occurs from damaged or dying cells following brain ischemia, necrosis or reduced perfusion of brain tumors (Ravelic and Burnstock, 1998; Rathbone *et al*., 1999; Burnstock, 2004). Extracellular ATP levels are strictly regulated by the balance between release and degradation, through a family of ectoenzymes that rapidly (Dunwiddie *et al*., 1997) idrolyzes or interconverts extracellular nucleotides, thereby terminating their signalling action or producing new active metabolites (ADP, AMP or adenosine), which can continue or functionally antagonize some effects of ATP (Zimmermann, 2000). Purinergic nucleotides participate in cell differentiation, proliferation and survival as well as in degeneration and death (Franke and Illes, 2006), thus acting through their specific P2 receptors, subclassified into P2X (ligand-gate cation-selective channels receptors) and P2Y (G-protein coupled receptors, GPCRs) subtypes (Fredholm *et al*., 1994). In particular, $P2Y_1$ receptors ($P2Y_1Rs$), which are selectively activated by ADP nucleotides and act via $G_{q/11}$ protein/ phospholipase C/ Ca^{+2} pathway, are involved, both with detrimental and/or beneficial effects (Franke and Illes, 2006), in platelet aggregation (Fabre *et al.*, 1999), in apoptotic events (Sellers *et al.*, 2001; Mamedova *et al.*, 2006), in microglial inflammatory functions (Von Kugelgen, 2006), in brain ischemic damages (Lammer *et al*., 2004) and trauma (Neary *et al*., 2003; Franke *et al*., 2004), in Alzheimer's disease structures in hippocampus (Moore *et al*., 2000), in the induction of proliferation and reactive astrogliosis (Franke *et al*., 2001; 2004).

Adenosine is principally a regulator of cellular homeostasis, thus acting through its specific P1 adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) (Ribeiro *et al*., 2003). It decreases neuronal excitability, especially through the activation of inhibitory A_1 receptors (A_1R), which are the most abundant among the adenosine receptors in the brain (Dunwiddie and Masino, 2001; Ochiishi *et al*., 1999). The A_1R, which is functionally coupled to a

pertussis toxin-sensitive $G_{i/o}$ protein, acts via adenylyl ciclase inhibition, activation of K^+ channels and inhibition of Ca^{+2} influx (Fredholm *et al*., 2001).

Whereas the neuroprotective role of adenosine analogues active at the A_1 receptor subtype have been known for several years (Wardas, 2002), the role of $P2Y_1$ receptor have not been fully characterized enough if there are indications about important pathological functions (reviewed by Franke and Illes, 2006), according to the fact that purine-mediated induction and maintenance of astrogliosis can be considered as a first response to limit the loss of neuronal tissue after insults (Franke *et al*., 2004).

$P2Y_1$ and A_1R receptors, both widely expressed around the brain (Lenz *et al*., 2000; Jimenez *et al.*, 2000; Moran-Jimenez and Matute, 2000; Ochiishi *et al*., 1999) seems localized in the same region, thus suggesting a potential interaction between these receptors in precise areas. Literature reports have showed A_1 and $P2Y_1$ receptors interact both synergically and functionally (Hourani *et al.,* 1991; Mendoza-Fernandez *et al*., 2002; Fredholm *et al*., 2003; Kawamura *et al*., 2004). Moreover data supporting the existence of a high co-localization and of a heteromeric complex with novel pharmacological and functional properties between A_1 and $P2Y_1$ receptors have been reported in co-trasfected cells and in rat brain tissues (Yoshioda *et al*., 2002; Yoshioka and Nakata, 2004).

In particular, previous results from our group showed a high co-localization between A_1 and $P2Y_1$ receptors on glutamatergic synaptic and astroglia membranes in rat hippocampus and a functional interaction at G protein coupling level between these receptors in hippocampal crude preparations (*see Chapter 2*).

The purinergic system has an high level of complexity and this is further augmented, considering that receptors can cross-talk and also form homomers/heteromers (Nakata *et al*., 2004) and that the composition of the oligomers profoundly affects the biological response of these receptors (Volontè *et al*., 2006). Heterodimerization of GPCRs may results: in changes in the affinity of the receptors for ligands; in mechanisms where one ligand modulates the efficacy and/or potency of another ligand specific for the neighbouring receptor; in the localization of downstream signalling components to specific areas on the cell; in aggregation of downstream signalling or switching off/down one of receptors (Rios *et al*., 2001).

The functional implications of the $P2Y_1R$ heterodimerization with the A_1R and in particular of the $P2Y_1R$ activation influence on the neuroprotective A_1R role still await further characterizations.

In the present work we investigated the A_1R-$P2Y_1R$ cross-talk in human astroglial cells, in particular we evaluated the effects of $P2Y_1R$ activation on A_1R-mediated G protein coupling and intracellular functioning. Although classically thought to mediate a supportive role, there is now evidence to demonstrate that astrocytes actively participate in informing processes by affecting intrinsic properties of synapses (Newman, 2003) and are involved in the most integrated functions of the central nervous system. Based on these data and considering astroglia contributes to damage but also to regeneration and neurogenesis after injuries in CNS (Anderson *et al*., 2003), we envisage that the characterization of A_1R-$P2Y_1R$ cross-talk in astrocytes may have important functional implications.

3.2 Materials and methods

3.2.1 Materials

[^{35}S]GTPγS (specific activity 1000-1250 Ci/mmol) was purchased from Amersham Biosciences Europe GmbH (Freiburg, Germany) and [^{3}H]DPCPX (specific activity 116 Ci/mmol) from Perkin Elmer Life Sciences (Boston, MA, USA). GDP, GTPγS, CHA, MeSADP, DPCPX, MRS2179, R-PIA, Pertussis-toxin (PTX), protease inhibitors and rabbit IgG were obtained from Sigma Chemical Co (St. Louis, MO, USA). ADA was from Roche Diagnostics GmbH (Mannheim, Germany). Cell culture media RPMI1640 and Dulbecco's modified Eagle's medium (DMEM F-12), fetal bovine serum, L-glutamine, penicillin and streptomycin were purchased from Cambrex Bio-Science (Verviers, Belgium). Dimethyl sulfoxide of microbiological quality was purchased from Fluka (Buchs, Switzerland).

Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA). A_1R antibody (rabbit) was supplied by Alpha Diagnostic (San Antonio, TX, USA), while the $P2Y_1R$ antibody (rabbit) from Alomone Labs (Jerusalem, Israel). Secondary antibody goat anti-rabbit IgG-HRP conjugate was from Calbiochem (EMD Biosciences, affiliate of Merck KgaA, Darmstadt, Germany). Protein A Sepharose^{TM} was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). The protein concentration of the samples was established by the Protein Assay based on Bradford method from Bio-Rad (Hercules, CA, USA), using bovine serum albumin as a standard. All other reagent grade

chemicals were supplied from standard commercial sources and all solutions were prepared in demineralised and purified water obtained with the Milli-Q Millipore water purifying system (Millipore, Billerica, MA, USA).

3.2.2 Cell culture

Human astrocytoma cells (ADF) were kindly supplied by Prof. Maria Pia Abbracchio (Department of Pharmacological Sciences, University of Milan). ADF cells (Malorni *et al*., 1994) were grown adherently and maintained in culture in standard conditions (37°C in humidified atmosphere, 95% humidity, 5% CO_2) in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 1% non-essential amino acids (complete medium) as previously described (Trincavelli *et al.,* 2002). When cells were grown at confluence, cells were treated with Trypsin 0,25% for few minutes to detach cells from surface and then complete medium was added to neutralize trypsin; cells were then collected, centrifuged at 180 g for 5 minutes, resuspended in new complete medium and expanded. All operations were carried out under laminar flux hut, with an UV sterilization system.

3.2.3 Cell treatments

When the cells were grown to sub-confluence, the complete medium was replaced with fresh RPMI1640 or DMEM F-12 (only for cAMP assay) medium containing treatment-compounds.

Cells were treated without (Control) or with different MeSADP concentrations (1nM-10□M) for different times (5-60 minutes) (MeSADP) and then harvested for immunoblotting analysis, [^{3}H]DPCPX binding assay, [^{35}S]GTPγS assay and cAMP assay.

Further experiments were carried out after the pre-incubation of cells with the selective $P2Y_1R$ antagonist MRS2179 (10 µM) for 15 minutes before the MeSADP exposure (MRS2179+MeSADP). In selected experiments, the inhibition of Pertussis-toxin (PTX)-sensitive G_i proteins was obtained by incubating cells with 100 ng/ml PTX for 18 hours before membrane preparation (Fumagalli *et al*., 2004).

3.2.4 ***Lysates, immunoprecipitates and Western blotting***

The presence of A_1R and $P2Y_1R$ in control and MeSADP-treated ADF cells was assessed by Western blot analysis of separated proteins from cell lysates. Control and MeSADP-treated ADF cells were processed by immunoblot following the method pre-

viously described (Trincavelli *et al.,* 2004), with some modifications. Separation on SDS-PAGE was performed according to the Laemmli procedure.

Treated and untreated ADF cells were scraped and lysed in PBS (NaH_2PO_4 9.1 mM, Na_2HPO_4 1.7 mM, NaCl 150 mM, pH 7.4) containing 0.5% Deoxycholic acid sodium salt, 0.1% SDS, 1% Nonidet P-40 and proteases inhibitors (phenylmethylsulfonyl fluoride 1 mM, sodium orthovanadate 1 mM, aprotinin 0.04 mg/ml) (RIPA buffer); cell lysates were so centrifuged to get rid of large cellular debris (15000 g for 45 minutes at 4°C).

Cell lysates (50 µg), after being resuspended in Laemmli solution and boiled for 5 minutes, were resolved by SDS-PAGE (10%), transferred to nitrocellulose membranes and probed overnight at 4°C with anti-A_1R (2 µg/ml) or anti-$P2Y_1$R (4 µg/ml) antibody. Membranes were then incubated with the corresponding peroxidase-linked secondary antibody (goat anti-rabbit), washed and developed using the Millipore Immobilon TM Western chemiluminescent HRP Substrate reagents (Millipore, MA, USA). Density of immuno-reactive bands was quantified by densitometric scanning and by a gel documentation system (ImageJ Program, National Institutes of Health, USA).

Immunoprecipitation/co-immunoprecipitation experiments were also carried out to evaluate the dimerization between A_1 and $P2Y_1$ receptors. For co-immunoprecipitation experiments, cell lysates (1 mg) were pre-cleared by incubating directly with Protein A Sepharose (1h at 4°C). Samples were then centrifuged for 10 minutes at 4°C (14000 g): the supernatants were incubated with anti-A_1 or anti-$P2Y_1$ receptor antibody (3 µg/sample) overnight at 4°C under constant rotation and then immunoprecipitated with Protein A Sepharose (2 hours at 4°C). Immuno-complexes, after being washed, resuspended in Laemmli solution and boiled for 5 minutes, were resolved by SDS-PAGE (10%), transferred to nitrocellulose membranes and probed overnight at 4°C with primary antibody anti-A_1R (2 µg/ml). Membranes, after incubation with the secondary antibody, were developed using the Millipore chemiluminescent reagents (see above). Unspecific immunoprecipitation was assessed using non-immune rabbit IgG (3 µg, same amount of antibody).

For molecular mass determination, polyacrylamide gels were calibrated by Full range Rainbow Molecular Weight Markers within the range of 10-250 kDa (Amersham Biosciences, Freiburg, Germany).

3.2.5 ADF membrane preparation for [^{35}S]GTPγS and [^{3}H]DPCPX binding assays

After different treatments, ADF cells were scraped, lysed with 5 mM Tris HCl, 2 mM EDTA, pH 7.4 (Lysis buffer) and homogenized in a polytron homogenizer, keeping on ice. Cell homogenates were then centrifuged at 48000 g at 4°C: membrane pellets were then re-suspended in assay buffer A (50 mM Tris HCl, 10 mM $MgCl_2$, pH 7.4) or B (25 mM Hepes, 100 mM NaCl, 5 mM $MgCl_2$, EDTA 1 mM, pH 7.4), re-homogenized and the protein concentration was determinated. Aliquots of cell membranes were then used for assays or stored at -80°C, after being quickly frozen in N_2.

3.2.6 [^{3}H]DPCPX binding assay

The influence of $P2Y_1R$ activation on A_1R equilibrium binding parameters was evaluated by [^{3}H]DPCPX saturation binding studies: the ligand affinity (K_d) and the maximal density of A_1R binding site (B_{max}) were determinated. For saturation binding studies, Control and MeSADP-treated ADF cell membranes were resuspended in binding buffer A (50 mM Tris HCl, 10 mM $MgCl_2$, pH 7.4) containing ADA (2 U/ml). Then, ADF membrane fractions (20 μg) were incubated for 3 hours at 25°C in binding buffer A containing ADA 0.2 U/ml and different [^{3}H]DPCPX concentrations (0.5-15 nM).

Moreover competition curves of MeSADP were performed by binding assays in the presence of 2.5 nM [^{3}H]DPCPX: ADF cell membranes (40 μg) were incubated for 3 hours at 25°C in binding buffer A containing ADA 0.2 U/ml, [^{3}H]DPCPX 2.5 nM and different MeSADP (0.1nM-10μM) concentrations.

Non-specific binding was determined in presence of 100 μM R-PIA and it resulted less than 10% of total binding. Binding reactions were terminated by rapid filtration under vacuum through Whatman GF/C glass fiber filters (Millipore Corporation): the filters were washed three times with 3 ml of binding buffer A and then counted in a scintillation cocktail. For test-compounds dissolved in DMSO, the final solvent concentration never exceed 2%.

3.2.7 [^{35}S]GTPγS binding assay on ADF cell membranes

A_1R-G protein coupling was evaluated assessing the ability of the specific A_1R agonist CHA to stimulate [^{35}S]GTPγS binding. Cell membranes were suspended in binding buffer B (25 mM Hepes, 100 mM NaCl, 5 mM $MgCl_2$, EDTA 1 mM, pH 7,4) containing DTT 1 mM, BSA 0.1% and ADA (2 U/ml). Aliquots of membrane homogenate (20

μg) were incubated for 30 minutes at 30°C in 0.1 ml of binding buffer B containing 10 μM GDP and 0.2-0.3 nM [^{35}S]GTPγS and stimulated with CHA (0.1-100 nM) or MeSADP (1 μM). ADA was added to assay to eliminate the interference of endogenous adenosine in the basal [^{35}S]GTPγS binding. Non-specific binding was determined in presence of 20 μM GTPγS and it resulted less than 10% of total binding. Binding reactions were terminated by rapid filtration under vacuum through Whatman GF/C glass fiber filters (Millipore Corporation): the filters were washed three times with 3 ml of binding buffer B and then counted in a scintillation cocktail. The results were expressed as a percent increase above the basal unstimulated binding (fixed as 100%). All the experiments were performed in duplicate.

3.2.8 cAMP assay on ADF cells

Two days before the experiment, cells (50000 cells/well) were seeded in 24-well plate, to have sub-confluence conditions in 48 hours. On the day of the cAMP assay, ADF cells were treated with or without MeSADP (1 μM) for different time points (0-60 minutes) and then placed in fresh DMEM F-12 medium with 2 U/ml ADA and 10 μM phosphodiesterases inhibitor 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 201724) for 15 minutes at 37°C. Cells were then stimulated with 10 μM forskolin for 5 min and with CHA (0.5 nM-1 μM) or MeSADP (0.01 nM-1 μM) for 10 minutes.

After incubation, cells were lysed with HCl 0,1 M for 15 minutes at 37°C and assayed for cellular cAMP accumulation using the competitive cAMP enzyme immunoassay system kit (Sigma–Aldrich, St. Louis, MO, USA), following the manufacture's instruction. Briefly cell lysates were centrifuged at 1000 g for 10 minutes and supernatant were used for assay. Luminescence was read at 405 nm with a Wallac Victor$^{2}_{TM}$ 1420 Multilabel plate reader (Perkin Elmer Life Sciences). For test-compounds dissolved in DMSO, the final solvent concentration never exceed 2%.

3.2.9 Data analysis

A non-linear multipurpose curve-fitting computer program (Kell RadLig, Elsevier-Biosoft, USA) was used for analysis of saturation data. Data from agonist dose–response were analyzed by the non-linear regression curve-fitting computer program GraphPad Prism Version 4.00 (GraphPad Software, San Diego, CA, USA) and the EC_{50}

values were derived. The K_i values for competition binding assays were calculated from the EC_{50} values by the Cheng and Prusoff equation (Cheng and Prusoff, 1973).

Data are reported as mean±SEM of three different experiments (performed in duplicate). Statistical analysis was performed with One-Way ANOVA (Tuckey's multiple comparison test) or Student's *t*-test (two-tails, unpaired), using GraphPad Prism program: significance refers to results where $P < 0.05$ was obtained.

3.3 Results

3.3.1 Immunoprecipitation and Western-blotting

The presence of A_1AR and $P2Y_1R$ on ADF cells was addressed by Western blot analysis. Lysates from Control and 1 µM MeSADP (30 min)-treated cells were resolved by SDS-Page and probed with the specific A_1R and $P2Y_1R$ primary antibodies. The results demonstrated both A_1AR and $P2Y_1R$ were expressed in ADF cells (**Fig. 1**). The A_1R immunoreactive band was detected at the apparent molecular mass of 39 kDa (**Fig. 1A**): furthermore, the A_1R expression level was not affected by MeSADP exposure. The principal $P2Y_1R$ immunoreactive bands were instead around 54 and 48 kDa (**Fig. 1B**), with an additional band around 95 kDa.

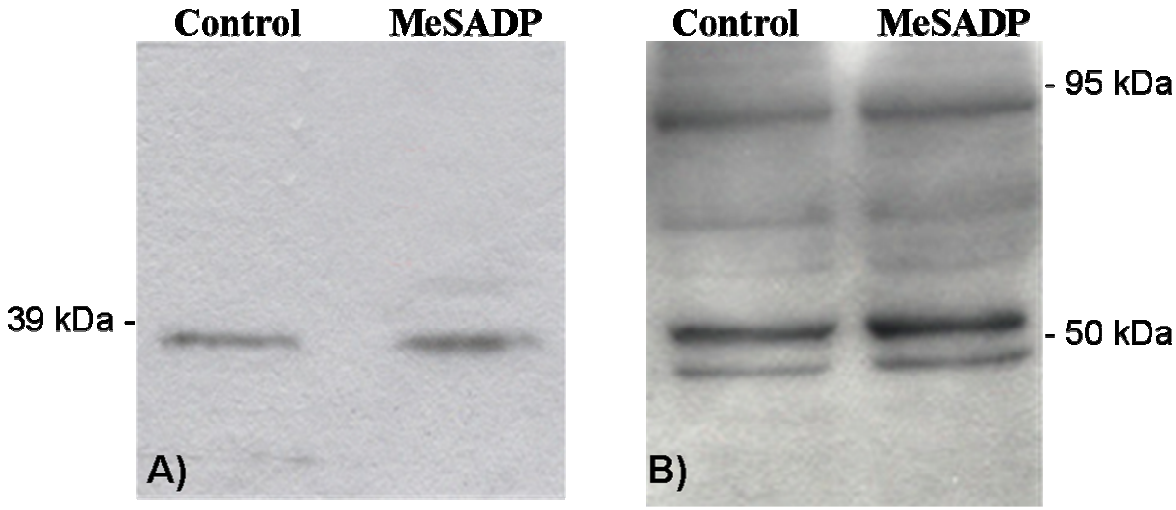

Fig. 1: Western Blot analysis of A_1R (**A**) and $P2Y_1R$ (**B**), in lysates of Control and MeSADP (1µM for 30')-treated ADF cells. Receptors were lysed, resolved by 10% polyacrilamide SDS-PAGE and tested with specific antibodies.

The existence of A_1R-$P2Y_1R$ heteromeric complexes, in basal conditions, was thus investigated by co-immunoprecipitation experiments (**Fig. 2**). Untreated ADF cells were lysed and then A_1R or $P2Y_1R$ were immunoprecipitated by primary antibody and Protein A, resolved by SDS-PAGE and probed with the anti-A_1R antibody. The results showed A_1Rs and $P2Y_1Rs$ are able to interact to form a heteromeric complex in basal condition, in ADF cells. In fact it has been possible to detect the A_1R immunoreactive band in $P2Y_1R$-immunoprecipitate sample, thus suggesting the anti-$P2Y_1R$ antibody was able to immunoprecipitate A_1R protein and so the two receptors are covalently bound on cell membranes.

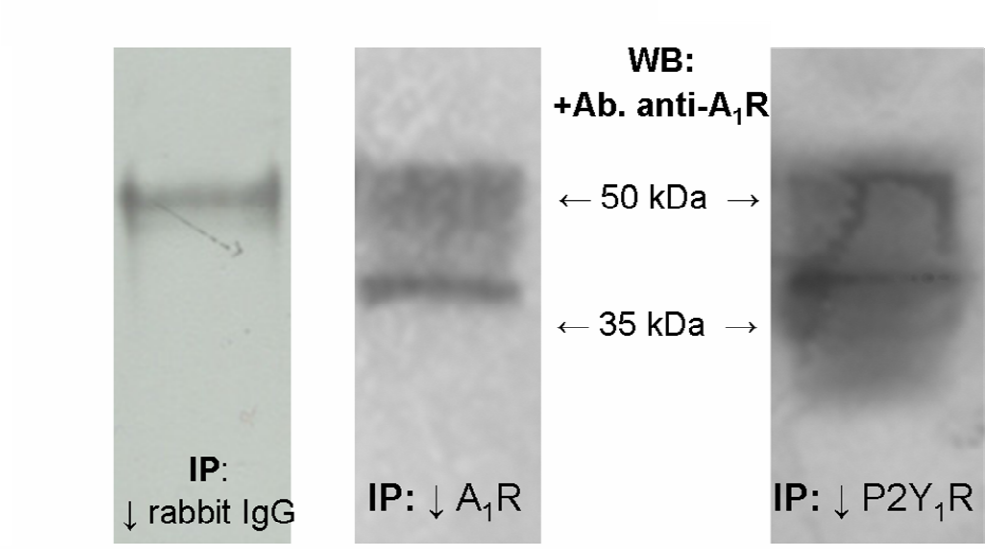

Fig. 2: Immunoprecipitation of A_1R and $P2Y_1R$. A_1R or $P2Y_1R$ were immunoprecipitated by incubating cell lysates with anti-A_1R or –$P2Y_1R$ antibody and Protein A, processed by SDS-PAGE and probed with the anti-A_1R antibody. *Left panel*: Unspecific immunoprecipitation of rabbit IgG solution (3 µg/lane).

3.3.2 [^{3}H]DPCPX Binding Assay

The influence of $P2Y_1R$ activation on A_1R equilibrium binding parameters was evaluated by [^{3}H]DPCPX saturation binding experiments.

As illustrated in **Fig. 3**, Scatchard analysis of [^{3}H]DPCPX saturation binding data demonstrated the presence of a homogenous population of A_1AR binding sites in ADF cells with a K_d value of 8.8±0.7 nM and a B_{max} value of 1340±110 fmol/mg proteins. Cell pre-incubation with 1 µM MeSADP for 30 minutes induced a significant decrease in the A_1R radioligand affinity constant value (K_d= 4.1±0.2 nM; * $P<0.05$ vs. Control, Stu-

dent's *t*-test) without affecting significantly the receptor density value (B_{max}= 1140±95 fmol/mg proteins; P>0.05 vs. Control, Student's *t*-test). Results suggested the A_1R affinity for its antagonist ligand increases when $P2Y_1R$ is also activated, thus confirming further that the A_1R expression level was not affected by MeSADP exposure.

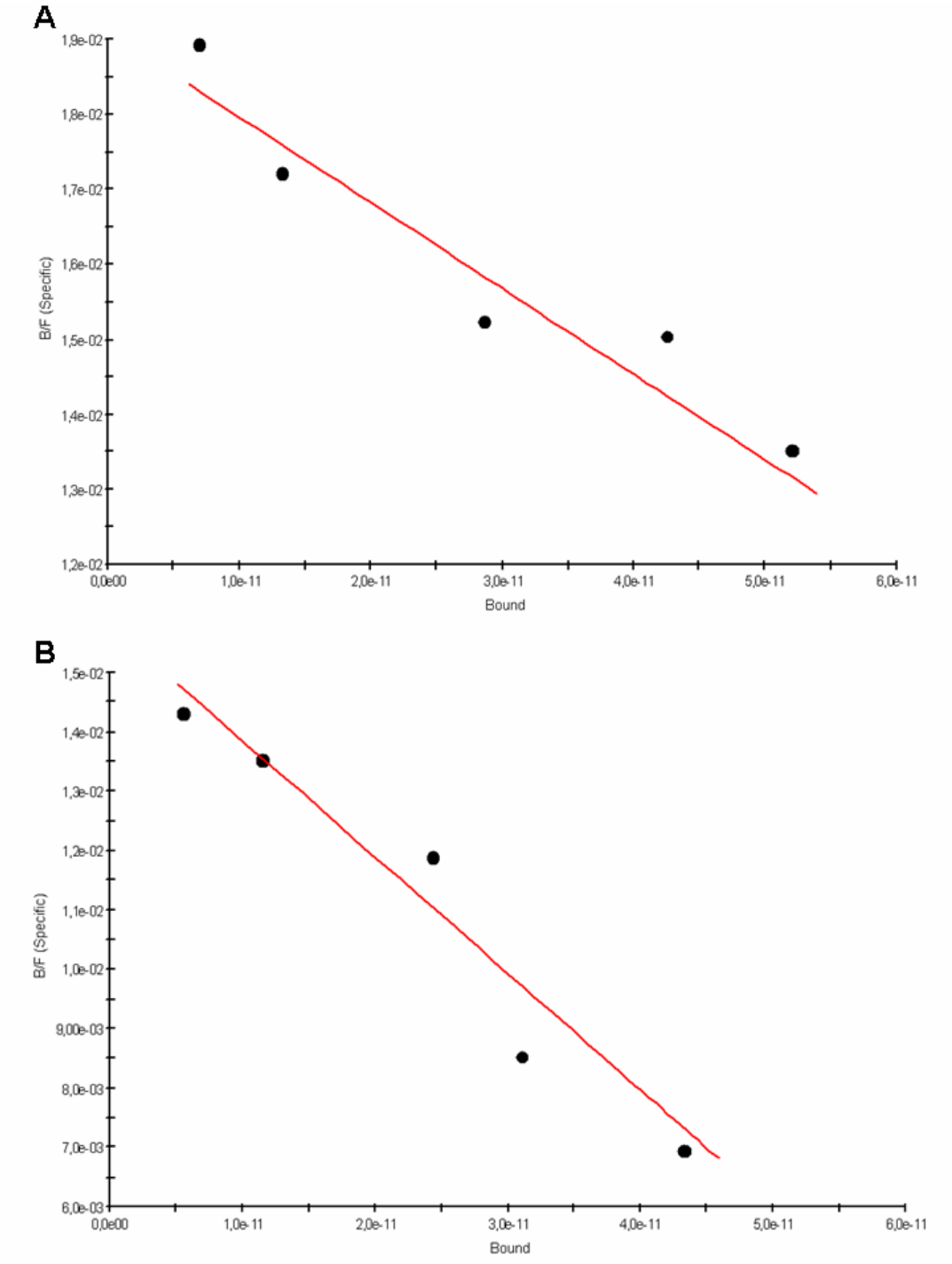

Fig. 3: [^{3}H]DPCPX Scatchard analysis assay. Cell membranes, obtained from Control (**A**) and MeSADP (1 µM for 30')-treated (**B**) cells, were incubated for 3 hours at 25°C with different [^{3}H]DPCPX concentrations

(0.5-15 nM). The figure depicted a representative Scatchard analysis of [^{3}H]DPCPX saturation binding data; results are reported as mean±SEM (n=3). * P<0.05 Control vs. MeSADP, Student's *t*-test.

To evaluate whether MeSADP effects were caused by a direct interaction of $P2Y_1R$ agonist on A_1R binding site, competition experiments were performed using different MeSADP concentrations (0.1nM-10μM). As showed in **Fig. 4**, MeSADP resulted not able to compete with [^{3}H]DPCPX for binding to A_1R site, thus showing no direct interaction with A_1R binding sites.

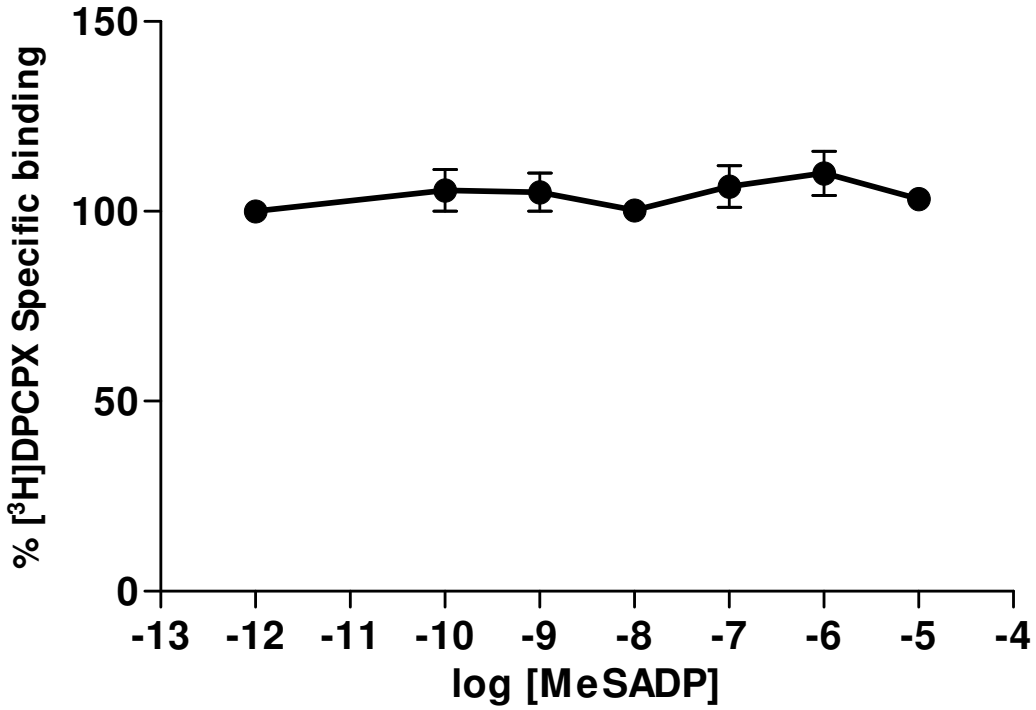

Fig. 4: Membranes, obtained from Control ADF cells (■), were incubated for 3 hours at 25°C with increasing MeSADP concentrations (1 nM-10 μM) and 2.5 nM [^{3}H]-DPCPX. Data are expressed as mean±SEM of % of specific binding (n=3).

3.3.3 [^{35}S]GTPγS binding assay

As first step, we investigated the basal G protein activation level in Control and 1 μM MeSADP (30') pre-treated cells. Cell treatment with MeSADP didn't induce any significant alterations in the basal [^{35}S]GTPγS binding value, thus suggesting it has no ef-

fect on G protein functional state (**Fig. 5**): Control= 15.26±3.1 fmol/mg protein, MeSADP= 16.48±4.8 fmol/mg protein (P>0.05 Control vs. MeSADP, Student's *t*-test).

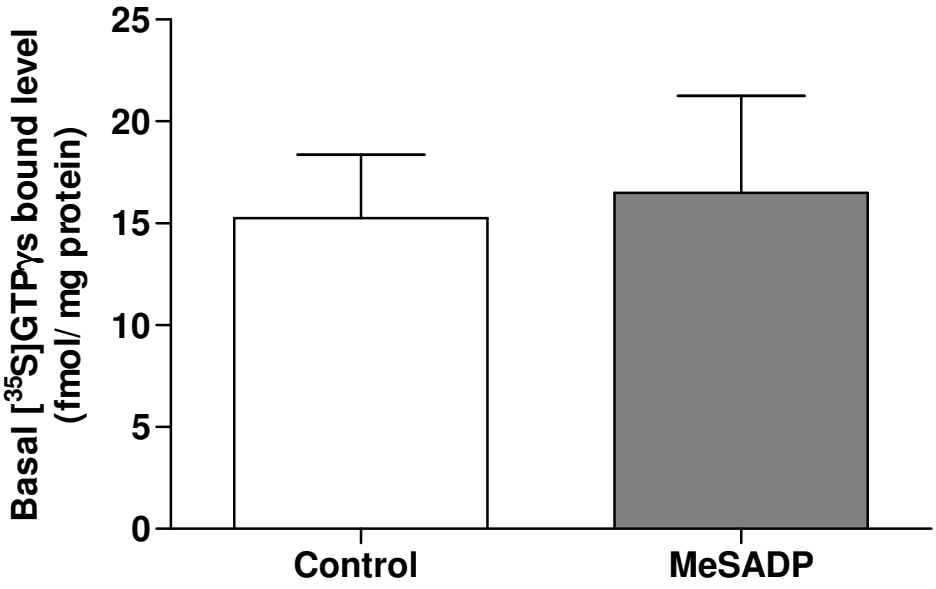

Fig. 5: Basal G protein activation levels in membranes from Control (*white*) and 1μM MeSADP-treated (*grey*) ADF cells. Data are expressed as fmol/mg of proteins and represent the mean±SEM of three experiments. P>0.05 Control vs. MeSADP, Student's *t*-test.

The effect of $P2Y_1R$ pre-activation on A_1R/G protein coupling was then assessed by evaluating the ability of the selective A_1R agonist CHA to stimulate [^{35}S]GTPγS binding in Control and 1 μM MeSADP-treated cell membranes.

In control ADF cells, CHA stimulated G protein activation with an EC_{50} value of 2.47±0.43 nM. CHA-mediated effects were abolished in the presence of the selective A_1R antagonist DPCPX (50 nM), thus confirming the agonist response is mediated by A_1R activation (*data not shown*).

Cell pre-incubation with 1 μM MeSADP for 30 min induced a right-shift of the agonist dose/response curve, with an EC_{50} value for CHA of 10.72±3.22 nM (** P<0.01 Control vs. MeSADP; One-way ANOVA) (**Fig. 6**). Data suggested a significant impairment in the A_1R/G protein coupling when $P2Y_1$ receptor is activated.

To discern if the functional interaction between these receptors at level of receptor/G protein coupling occurred at membrane sites was mediated by intracellular signalling pathways, aliquots of Control cell membranes were incubated directly in the assay with MeSADP 1 μM, 10 minutes before CHA stimulation: EC_{50} for CHA resulted 9.68±1.38 nM (** P<0.01 Control+MeSADP on assay vs. Control; P>0.05 Control+MeSADP on assay vs. MeSADP; One-way ANOVA) (**Fig. 6**). The effects of $P2Y_1R$ on A_1 receptor-G protein activation occurred at membrane level and were not mediated by intracellular signalling pathways.

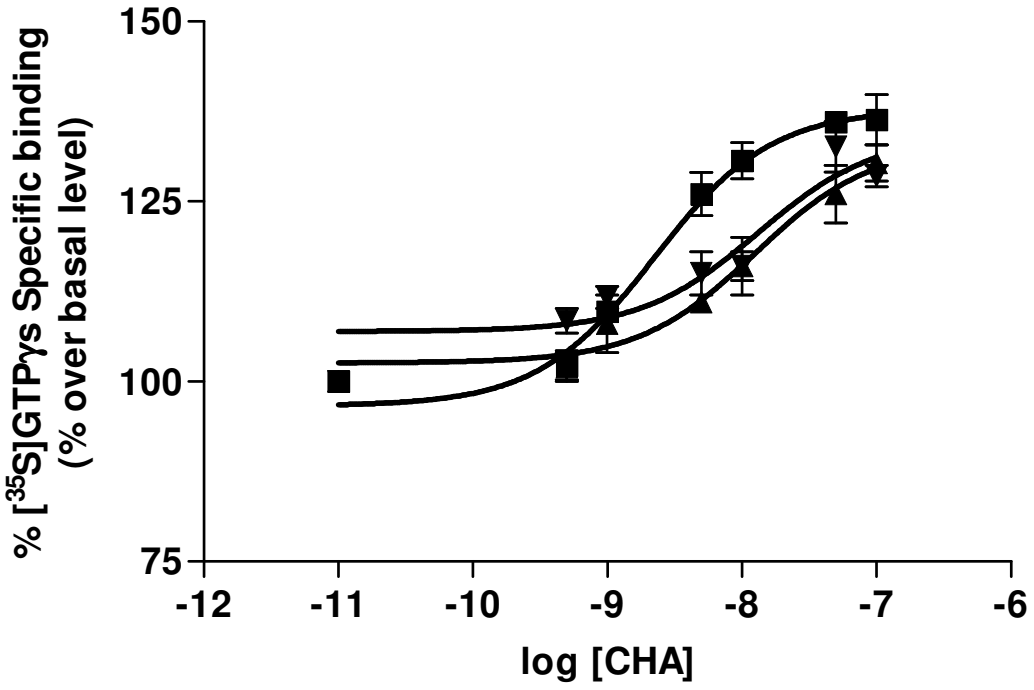

Fig.6: Effects of MeSADP-mediated $P2Y_1R$ activation on A_1R/G-protein coupling. Cell membranes, from Control (■) and MeSADP (1μM for 30')-treated (▲) ADF cells, were prepared and the ability of the A_1R agonist CHA to stimulate GTPγs binding was assessed. Similar results were obtained incubating directly the membranes with MeSADP 1μM on the assay (▼). Data are expressed as % of basal [^{35}S]GTPγS binding (set to 100%) and represent the mean±SEM (n=3).
*** P<0.001 Control vs. MeSADP; ** P<0.01 Control vs. Control+MeSADP on assay; P>0.05 MeSADP vs. Control+MeSADP on assay (Student's *t*-test).

Moreover, to ascertain the MeSADP effects were really mediated by $P2Y_1$ receptor subtype, we also stimulated ADF cells in presence of the selective $P2Y_1R$ antagonist MRS2179 (10 μM). MRS2179 was able to block the MeSADP-mediated impairing effect on A_1R-G protein coupling in ADF cells (* $P<0.05$ MRS2179+MeSADP vs. MeSADP; $P>0.05$ MRS2179+MeSADP vs. Control; One-way ANOVA) (**Fig. 7**) and to restore the A_1R-G protein coupling level as in Control cells. Data showed MeSADP effects are mediated by $P2Y_1R$ and didn't involve other P2Y receptors present on astrocytes and sensitive to the agonist (i.e. $P2Y_{12\text{-}13}$).

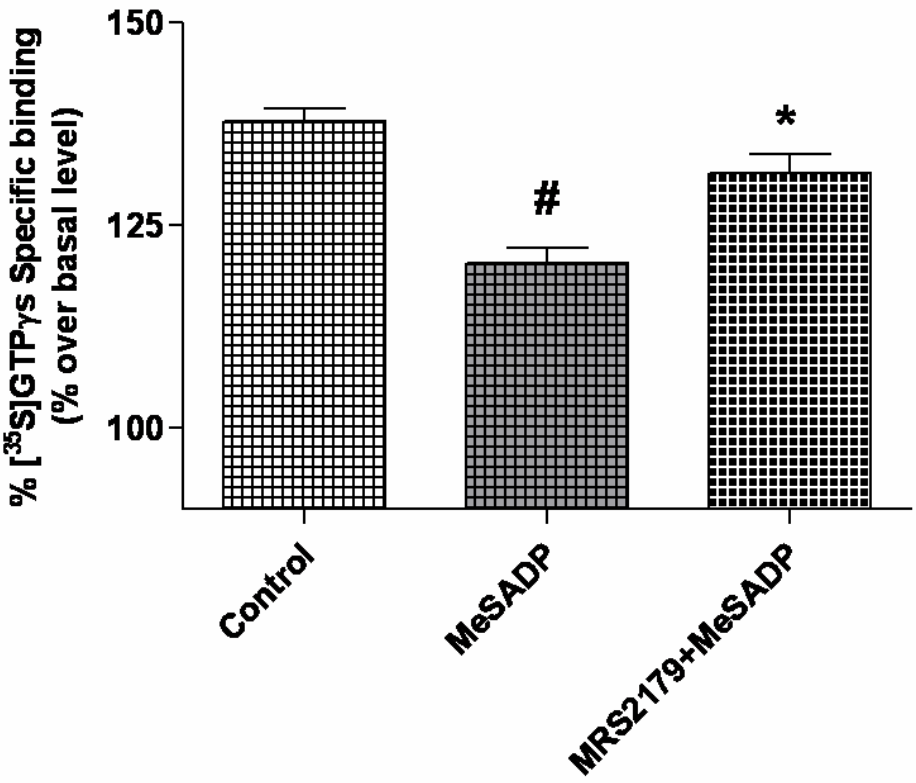

Fig. 7: $P2Y_1R$ selectivity of MeSADP-mediated effects on A_1R/G-protein coupling impairment. Cell membranes, from Control (*white squares*), MeSADP (1μM for 30')-treated (*grey squares*) and 10 μM MRS2179 plus MeSADP-treated (*black squares*) ADF cells, were stimulated with 100 nM CHA to asses GTPγs binding. Data are expressed as percentage of basal [^{35}S]GTPγS binding (set to 100%) and represent the mean±SEM (n=3).
* $P<0.05$ MRS2179+MeSADP vs. MeSADP; $P>0.05$ MRS2179+MeSADP vs. Control; # $P<0.01$ MeSADP vs. Control (One-way ANOVA).

Moreover, we investigated the MeSADP-stimulated coupling to G_i protein, to assess the possible interference of other P2YRs (i.e. $_{12\text{-}13}$), that are achievable by MeSADP and coupled to G_i proteins, in ADF cells. The 1 μM MeSADP-stimulated G protein coupling was assayed in control and 18 hour PTX-treated cells (**Fig. 7**), in which PTX treatment inhibited G_i protein functioning. The G protein activation levels didn't differ significantly between treated and untreated cells (P>0.05 Control vs. PTX, Student's *t*-test). The results confirmed further the MeSADP activity in ADF cells is $P2Y_1R$-selective and not mediated by other P2YRs; nevertheless results also suggested the MeSADP-mediated $P2Y_1R$ coupling to G_i protein component is of not relevance and the A_1R-G protein reduced coupling was not caused by modulation of G_i activation state by MeSADP.

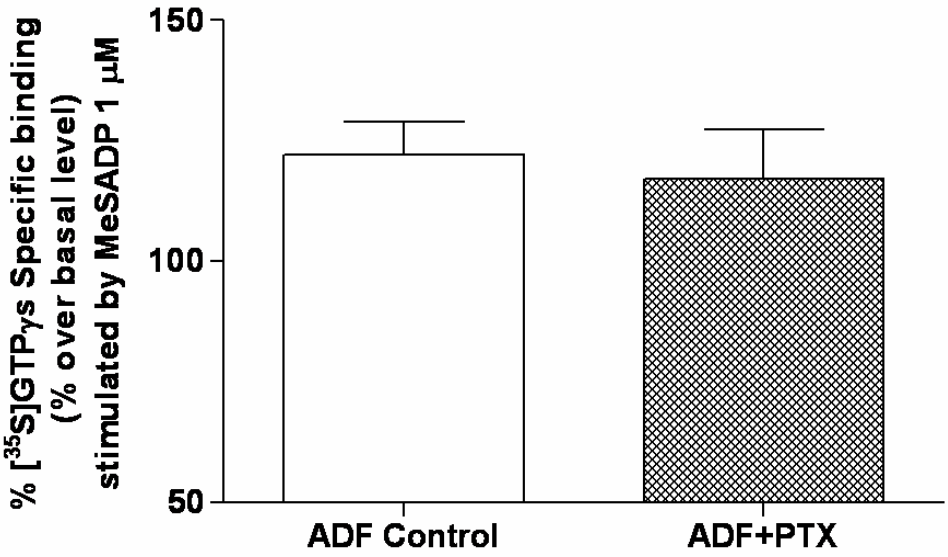

Fig. 8 $P2Y_1R$ coupling to G_i protein. The 1 μM MeSADP-stimulated G protein coupling was assayed in Control (*white*) and 18 hours PTX (100 ng/ml)-treated (*grill pattern*) ADF cells. Following incubation, membrane fractions were prepared and the ability of 1 μM MeSADP to stimulate GTPγs binding was assessed. Data are expressed as % of basal [^{35}S]GTPγS binding (set to 100%) and represent the mean±SEM (n=3). P>0.05 Control vs. PTX, Student's *t*-test.

3.3.4 cAMP Assay

The effect of $P2Y_1R$ pre-activation on A_1R functional responsiveness was then assessed by evaluating the ability of the selective A_1R agonist CHA to inhibit cAMP production in Control and MeSADP-treated cell membranes.

As a first step, we investigated the effects of MeSADP treatment (1 μM for 30') on basal and 10 μM FSK-stimulated cAMP levels. As showed in **Fig. 9**, MeSADP pre-treatment *per se* didn't influence cAMP levels in ADF cells, both in basal and FSK-stimulated conditions (P>0.05 Control vs. MeSADP and Control+FSK vs. MeSADP+FSK; Student's *t*-test).

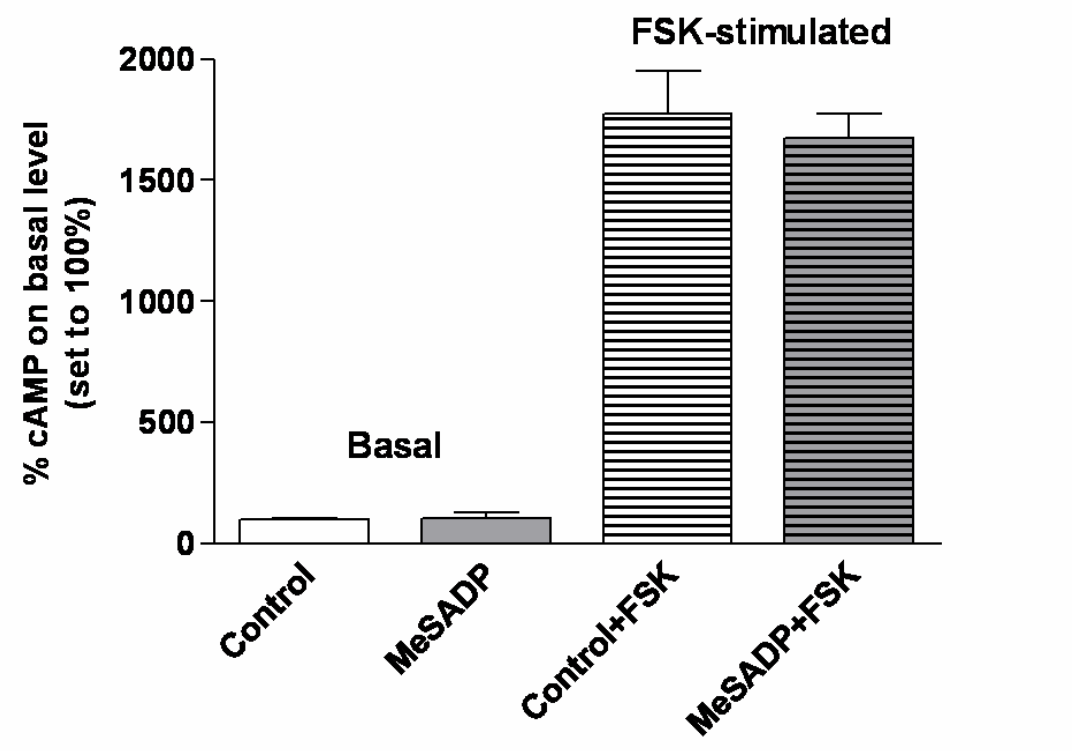

Fig. 9: Basal (*full*) and 10 μM FSK-stimulated (*stripes*) cAMP level in Control (*white*) and MeSADP treated (*grey*) cells. Results are expressed as % of FSK-stimulated cAMP on basal levels (set to 100%) and as mean±SEM (n= 3). P>0.05 Control vs. MeSADP, Student's *t*-test.

Then the A_1R functional responsiveness was evaluated by measuring the ability of CHA to inhibit cAMP production stimulated by 10 μM FSK.

In Control cells CHA inhibited the production of cAMP in a concentration dependent manner with an EC_{50} value of 1.085±0.17 nM (**Fig. 11**).

Cell exposure to MeSADP (1 μM) induced a significant decrease in A_1R functional responsiveness in a time dependent manner with a maximal effect following 30 minutes incubation (**Fig. 10**). These results suggested 1 μM MeSADP was able to induce a short-term $P2Y_1R$-mediated heterologous A_1AR desensitization.

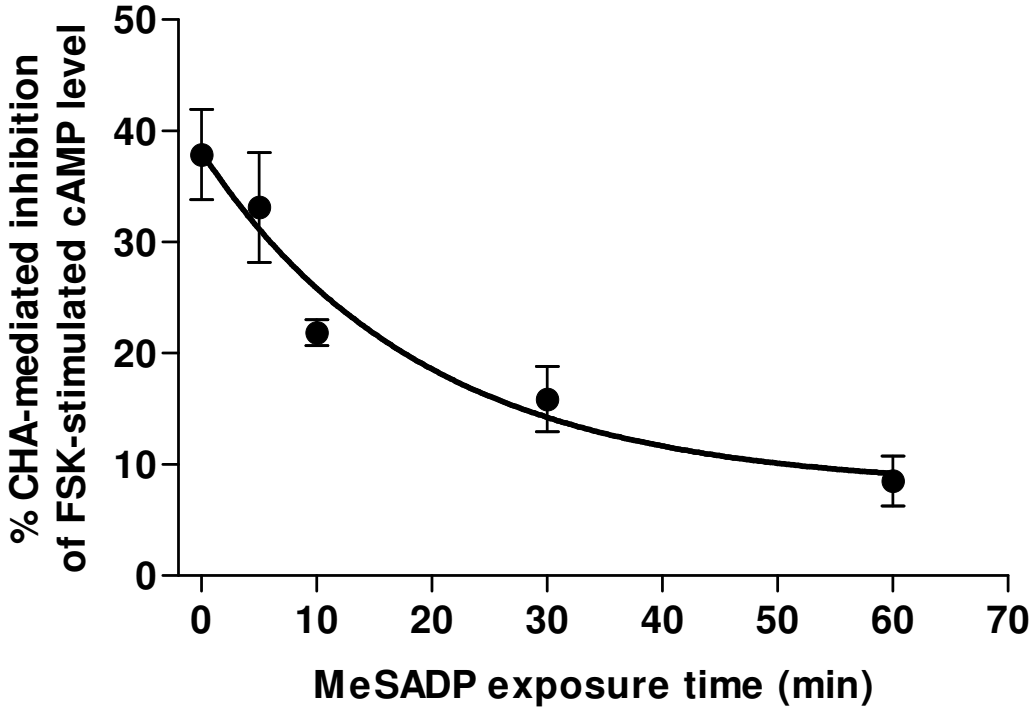

Fig. 10: MeSADP exposure time-dependence effects on A_1R functional responsiveness. Cells were treated with or without 1 μM MeSADP for different times (5-60 min); then the ability of 10 nM CHA to inhibit 10 μM FSK-stimulated cAMP accumulation was investigated. Data are expressed as percentage of cAMP on 10 μM FSK-stimulated level (set to 100%) and represent the mean±SEM (n=3).

When cells were pre-treated with MeSADP (1 μM for 30 min) a significant reduction in the CHA potency was detected (EC_{50}= 4.306±0.51 nM; *** P<0.001 Control vs. MeSADP-treated cells, Student's *t*-test; **Fig. 11**). Data suggested a significant impairment of A_1R functional coupling to adenylyl cyclase pathway when $P2Y_1$ receptor is activated.

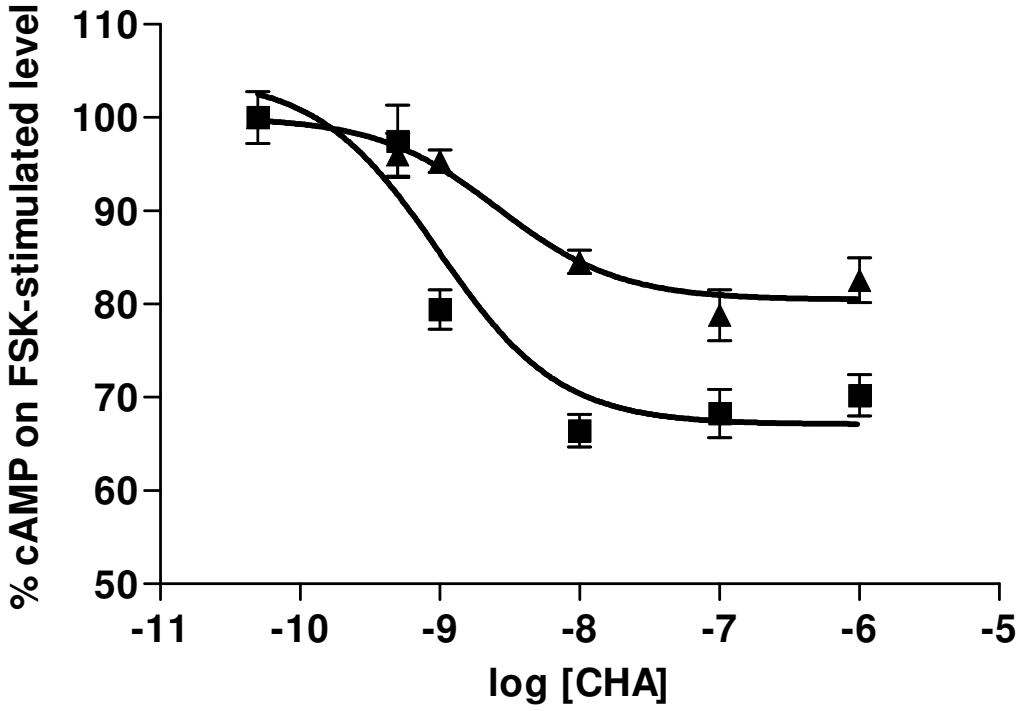

Fig. 11: CHA concentration-dependence effects on A_1R functional responsiveness. The ability of the A_1R agonist CHA to inhibit the 10 μM FSK-mediated cAMP production was assessed in cell membranes, from Control (■) and MeSADP(1μM for 30')-treated (▲) ADF cells. Data are expressed as percentage of cAMP on 10 μM FSK-stimulated level (set to 100%) and represent the mean±SEM (n=3). Control EC_{50}= 1.085±0.17 nM; MeSADP EC_{50}= 4.306±0.51 nM. *** P<0.001 Control vs. MeSADP, Student's *t*-test.

To confirm the selectivity of MeSADP for $P2Y_1Rs$, we also stimulated ADF cells in presence of the selective antagonist MRS2179 (10 μM). MRS2179 blocked the MeSADP-mediated A_1R functional impairment (** P<0.01 MeSADP vs. MRS2179+MeSADP and vs. Control; P>0.05 MRS2179+MeSADP vs. Control, One-way ANOVA), thus restoring the A_1R functional responsiveness up to control levels (P>0.05 MRS2179+MeSADP vs. Control) (**Fig. 12**). Data showed the MeSADP-mediated A_1R functional impairment is driven by $P2Y_1R$ and doesn't involve any other P2Y receptor sensitive to MeSADP and expressed in astrocytes (i.e. $P2Y_{12\text{-}13}$).

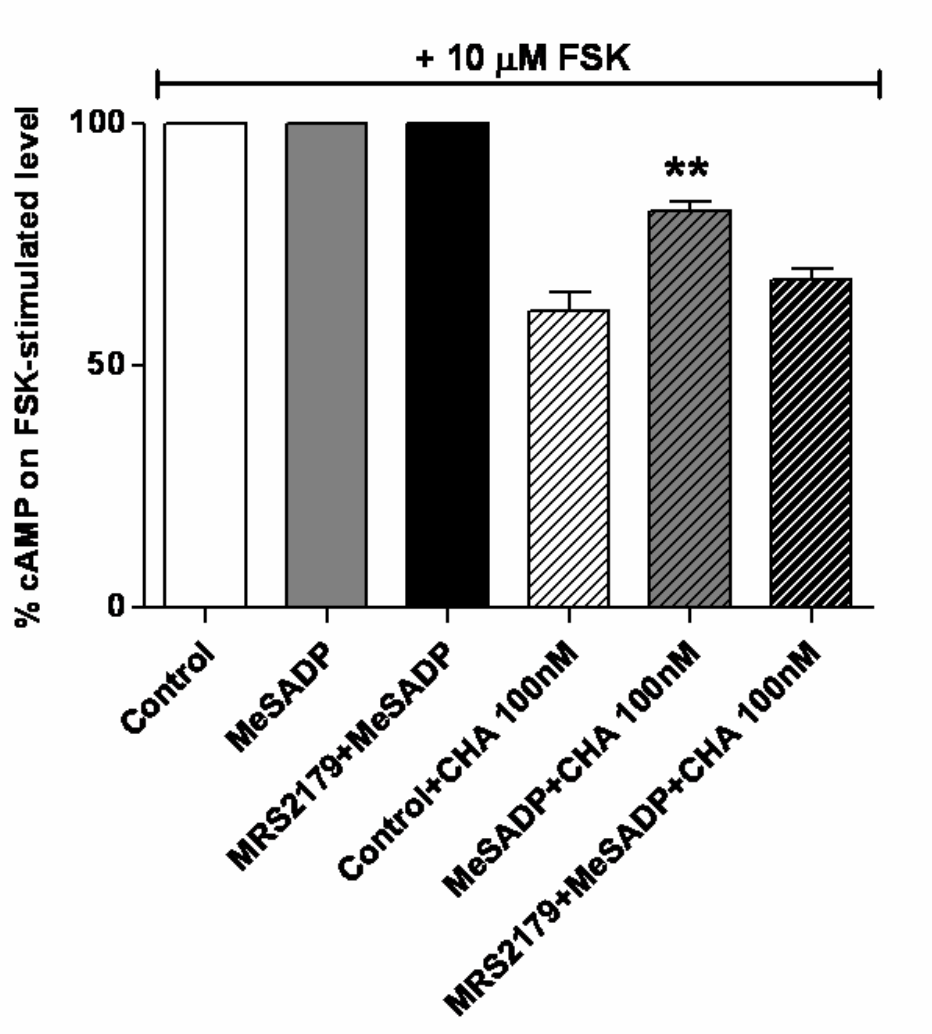

Fig.12: $P2Y_1R$ selectivity of MeSADP-mediated effects on A_1R responsiveness impairment. Cell membranes, from Control (*white*), MeSADP (1μM for 30')-treated (*grey*) and 10 μM MRS2179 plus MeSADP-treated (*black*) ADF cells, were stimulated without (*full colour*) or with 100 nM CHA (*stripes*) to asses the 10 μM FSK-mediated cAMP inhibition. Data are expressed as percentage of cAMP on 10 μM FSK-stimulated level (set to 100%) and represent the mean±SEM (n=3).
** P<0.01 MRS2179+MeSADP and Control vs. MeSADP; P>0.05 MRS2179+MeSADP vs. Control (One-way ANOVA).

In the end, to investigate if $P2Y_1R$ was able to act also through the A_1R-adenyly cyclase pathway, as reported by Yoshioka and co-workers (2001), in ADF cells we evaluated the ability of MeSADP to inhibit 10 μM FSK-mediated intracellular cAMP production in ADF cells. Increasing concentrations of MeSADP resulted not able to reduce cAMP

levels: these results suggest $P2Y_1R$ is not able to act through adenyly cyclase signalling pathway in ADF cells (**Fig. 13**).

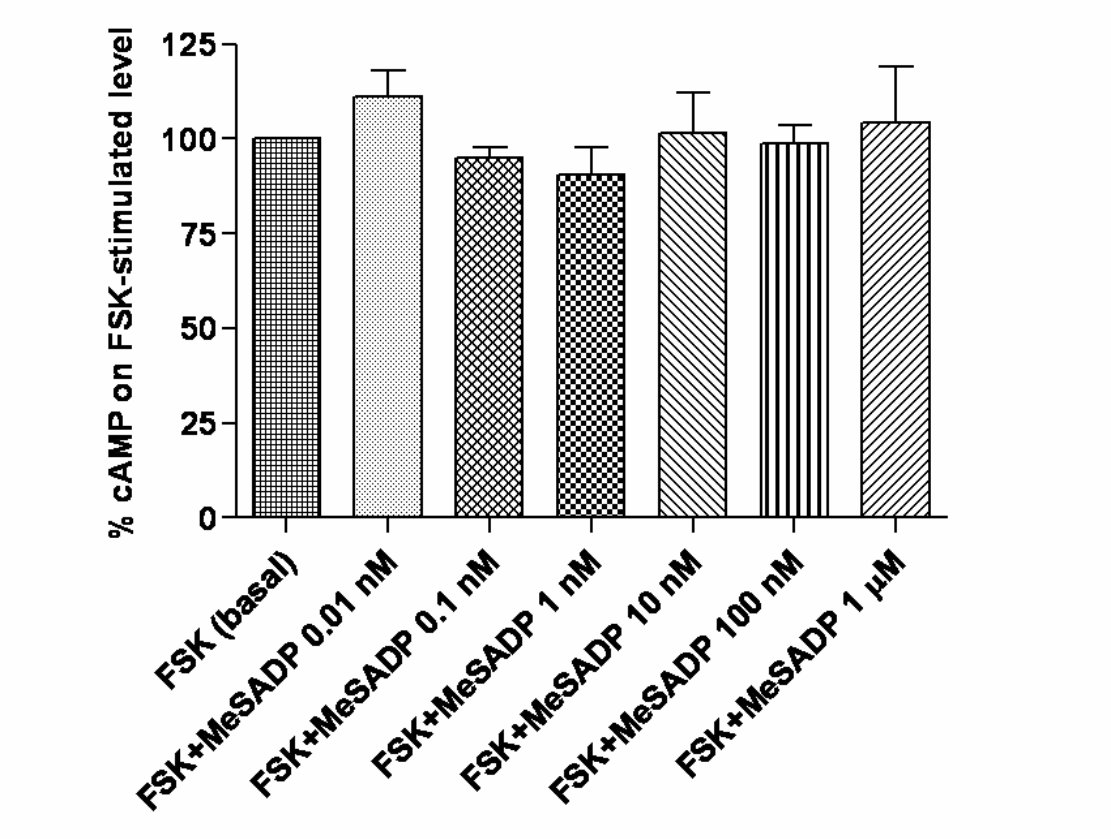

Fig. 13: MeSADP concentration-dependence effects on 10 µM FSK-stimulated cAMP intracellular levels in ADF cells. Membranes, obtained from Control ADF cells, were stimulated without or with increasing Me-SADP concentrations (0.01 nM-1 µM), in the presence of 10 µM FSK. Data are expressed as percentage of cAMP on 10 µM FSK-stimulated level (set to 100%) and represent the mean±SEM of three different experiments. P>0.05 all columns vs. FSK basal (One-way ANOVA).

3.4 Discussion

The present study investigated the A_1R-$P2Y_1R$ cross-talk in human astroglial ADF cells: in particular alterations in the A_1R binding and functioning parameters following the $P2Y_1R$ activation by its selective agonist MeSADP were evaluated.

As a first step, it has been demonstrated that ADF cells express both purinergic A_1 and $P2Y_1$ receptors. Immunoblotting results, from cell lysates, showed the A_1R (39 kDa) and the $P2Y_1R$ multi-line pattern (48-54-95 kDa), in agreement with literature data (Hoffmann *et al*., 1999; Sellers *et al*., 2001; Moore *et al*., 2002; Waldo and Harden, 2004). The main band is that around 54 kDa, while the lower band (48 kDa) is likely a partially degraded product of $P2Y_1R$ and the higher a multimeric form. Waldo and Harden (2004) reported that the incubation at higher temperature increased the occurrence of $P2Y_1R$ multimeric immunoreactive species, with higher molecular mass; moreover, the 95 kDa band is in agreement with the antibody data sheet. The multimeric pattern, due probably to sample preparation protocols and to detergent use, is also in agreement with a report from the antibody supplier company (Alomone Labs).

The co-immunoprecipitation results indicated that A_1R is able to interact with $P2Y_1R$ to form a heteromeric complex in ADF cell membranes, just in basal condition, as previously reported in A_1-$P2Y_1$ receptor co-transfected cell model and in rat brain (Yoshioka *et al.*, 2002).

The functional interaction between A_1 and $P2Y_1$ receptors was then investigated among the effects of ADF cell treatment with the $P2Y_1R$ agonist MeSADP on A_1R functional responses. According to preliminary experiments and to literature data concerning P2YR agonist potency in different systems (reviewed by Volontè *et al*., 2006), the 1 □M MeSADP cell exposure for 30 minutes was chosen to stimulate a maximal $P2Y_1R$ activation. MeSADP is the principal agonist not only at $P2Y_1R$, with a 10 times higher affinity reported at this receptor (human) than ADP (Waldo and Harden, 2004), but also at $P2Y_{12\text{-}13}$, that are both expressed in astrocytes and coupled to G_i proteins (Von Kugelgen, 2006). In our study, the MeSADP actions have been demonstrated to be $P2Y_1R$-mediated using the selective $P2Y_1R$ antagonist MRS2179 (10 □M); in fact, MRS2179 doesn't affect responses mediated by other P2Y receptors (Moro *et al.,* 1998), helping to identify $P2Y_1R$-mediated responses (Von Kugelgen, 2006).

In ADF cells MeSADP treatment, through the selective $P2Y_1R$ activation, didn't induce any changes in A_1R expression levels, thus showing on the contrary an increase in the

selective A_1R antagonist DPCPX affinity, towards A_1R binding sites. Moreover, our results showed MeSADP induced a significant impairment of agonist-stimulated A_1R-G protein coupling and A_1R functional coupling to adenylyl cyclase; anyway, MeSADP didn't result able to directly bind to A_1R binding sites and to interfere with G_i pathway.

In ADF cells, lines of evidence indicate the existence of an A_1-$P2Y_1$ receptor cross-talk and interaction at membrane level, thus suggesting a short-term heterologous $P2Y_1R$-mediated A_1R desensitization.

These data are partially in disagree with those reported by Yoshioka and co-workers (2001) which have demonstrated that the co-expression of A_1 and $P2Y_1$ receptors induced a decrease both in A_1R density and in A_1R agonist/antagonist affinities and the $P2Y_1R$ agonist ADPβs resulted active in displacing [^{3}H]NECA from A_1R binding sites. Moreover, the agonist-stimulated A_1R functional impairment at G protein coupling level was also reported in the A_1R-$P2Y_1R$ co-transfected cell model by Yoshioka and co-workers (2001) and resembled our previous data obtained in hippocampal rat membranes (*see Chapter 2*).

In ADF cells, indications about a A_1R signalling pathway activation by the $P2Y_1R$ agonist didn't come out. In fact, the $P2Y_1R$ agonist MeSADP didn't result able to directly bind to A_1R binding sites either to activate G_i-cAMP pathway, as Yoshioka and co-workers (2001) reported.

Such a difference could be explained by considering that, in one side, our model is a native glial cell system, which expresses constitutively both the purinergic receptors, and, on the other side, we stimulated $P2Y_1Rs$, while Yoshioka and co-workers worked in basal unstimulated conditions.

In ADF cells the $P2Y_1$ receptor activation increased A_1R antagonist binding in one side but in the other side decreased agonist-stimulated A_1R responsiveness. Therefore, agonists and antagonists show different characteristics in ligand binding to GPCRs: while antagonists bind equally well to inactive receptor conformation (R) and to a spontaneously active form (R*), the agonists preferably bind to R* (Barrington *et al.*, 1989; Lorenzen *et al.*, 1996). Our results suggest TNF-alpha could induce a conformational change of A_1R, thus reducing the A_1R affinity for activation.

Considering that ATP and $P2Y_1Rs$ have been reported to inhibit the release of glutamate (Mendoza-Fernandez *et al.*, 2000; Koizumi *et al.*, 2003; Rodrigues *et al.*, 2005) and thus to interact in addition with A_1Rs (Masino *et al.*, 2002; Fredholm *et al.*, 2003), we can

speculate the A_1-$P2Y_1$ receptor interaction may be used to fine-tuning the purinergic signalling, thus leading in particular to the inhibition of neurotransmission (Nakata *et al*., 2004).

Extracellular purines have been proposed to be an activity-dependent signalling molecule that regulates glia-glia and glia-neuron communications (Franke *et al*., 2006). Astroglial cells may play a far more active roll in neurotransmission than earlier believed (Anderson *et al*., 2003), thus contributing to damage or promoting neurogenesis and regeneration in CNS. Moreover receptor cross-talk/heterodimerization may be a crucial event to understand biological systems involved in diseases pathogenesis and to develop strategical therapies nevertheless to set novel drug targets. Based on these considerations, we envisage that the characterization of A_1R cross-talk with $P2Y_1R$ in astroglial cells may have important functional implications.

The functional role and the explanation for an A_1-$P2Y_1$ receptor cross-talk are complicated by the availability of their endogenous ligands as well by the nucleotide different regulation in physiological or pathological conditions (Braun *et al*., 1998).

3.5 References

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Chapter 4:

Short-term TNF-alpha treatment induced A_{2B} adenosine receptor desensitization in human astroglial cells

4.1 Abstract

Long-term glial cell treatment with the pro-inflammatory cytokine TNF-alpha has been demonstrated to increase the functional responsiveness of A_{2B} adenosine receptors (A_{2B}Rs), which in turn synergize with the cytokine inducing chronic astrogliosis. In the present study, we investigated the short-term effects of TNF-alpha on A_{2B}R functional responses in human astroglial cells (ADF), simulating the acute phase of cerebral damage, which is characterized by cytokine and adenosine high level release. Short-term TNF-alpha cell treatment caused A_{2B} AR phosphorylation inducing, in turn, impairment in A_{2B}R-G protein coupling and cAMP production. These effects, which appeared to be maximal at 1000 U/ml TNF-alpha concentration for three hour cell exposure, were mediated by specific kinases such as PKA and PKC, but not by PI3K. The results could indicate the A_{2B}R functional impairment as a cell defense mechanism to counteract the A_{2B} receptor-mediated effects during the acute phase of brain damage, underlying the A_{2B}R as a possible target to modulate early inflammatory responses.

4.2 Introduction

Astrocytes, which are involved both in physiological brain functions and in pathological events (Eddleston and Mucke, 1993), are the most important source of extracellular adenine-based purines in the brain (Ciccarelli *et al.*, 1999) and moreover express all the A_1, $A_{2A,}$ A_{2B} and A_3 adenosine receptors (ARs) (Peakman and Hill, 1996; Trincavelli *et al.*, 2002a, Trincavelli *et al.*, 2004). Among all these receptors, A_{2B}Rs are quite unique, since they exhibit a relatively low affinity for adenosine and seem to be activated only under hypoxic or ischemic conditions, when large amounts of adenosine are released (micromolar range) (Ribeiro *et al.*, 2003). Under these pathological conditions pro-inflammatory cytokines, a group of potent multifunctional pleiotropic proteins, are re-

leased in the brain at high levels, contributing to neurodegeneration and inflammation processes (Szeleni, 2001; Zaremba *et al.*, 2001). Evidence suggests a regulatory connection between inflammatory cytokines and the adenosine system: the former are known to be involved in ARs response regulation mechanisms (for A_1R see Biber *et al.*, 2001; for $A_{2A}R$ see Khoa *et al.*, 2001; Trincavelli *et al.*, 2002b; for $A_{2B}R$ see Rosi *et al.*, 2003; Trincavelli *et al.*, 2004), while ARs are involved in the regulation of the cytokine production (for $A_{2A}R$ see Hasko *et al.*, 2000; for $A_{2B}R$ see Zhang *et al.*, 2005; Yang *et al.*, 2006). This reciprocal functional cross-talk between ARs and cytokines plays an important role in the regulation of AR responses during pathological conditions. In particular, data from our group have demonstrated that human astroglial cell chronic exposure to TNF-alpha induces a delay in the $A_{2B}R$ desensitization process, causing up-regulation of the receptor functioning. Moreover, the $A_{2B}R$, in association with TNF-alpha, mediated chronic astrogliosis, thus suggesting this AR subtype could have a role in the long-term control of astrocytic functions (Trincavelli *et al.*, 2004).

Following ischemia and trauma, TNF-alpha is quickly synthesized (2-6 hours) in the central nervous system by resident machrophages, astrocytes and microglia (Yu and Lau, 2000; Tehranian *et al.*, 2002). Converging lines of evidence support the notion that the cytokine role in driving the short-term inflammatory response (Wang and Shuaib, 2002). In this context, to understand how the cytokines regulate the functional responsiveness of adenosine neuromodulator systems in the acute phase of cerebral damage represents an important goal to develop new strategic therapeutic intervention.

The aim of the present work was to investigate the $A_{2B}R$ modulation in ADF cells following short-term TNF-alpha exposure (3 hours) and to dissect the intracellular kinase pathways involved in TNF-alpha-mediated regulation of $A_{2B}R$ functioning.

4.3 Materials and methods

4.3.1 Materials

[^{35}S]GTPγS (specific activity 1250 Ci/mmol) was purchased from Amersham Biosciences (Freiburg, Germany). ADA was from Roche Diagnostics GmbH (Mannheim, Germany). Cell culture media were from Cambrex Bio-Science (Verviers, Belgium). Electrophoresis reagents were purchased from Bio-Rad (Hercules, CA, USA).

NECA and protease inhibitors were obtained from Sigma Chemical Co (St. Louis, MO, USA). MRS 1220 was purchased from Tocris Cookson (Bristol, United Kingdom) while SCH 58261 was a gift from Schoering-Plough. TNF-alpha (TNF-α) was purchased from Li StarFish (Milan, Italy) and protein kinases inhibitors from Calbiochem (EMD Biosciences, affiliate of Merck KgaA, Darmstadt, Germany).

Protein A Sepharose^{TM} was from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Anti-A_{2B}R antibody (rabbit) was supplied by Alpha Diagnostic (San Antonio, TX, USA), while the anti-phosphothreonine antibody (rabbit) was from Chemicon International (Temecula, CA, USA). Anti-phospho-ERK ½ kinases antibody (mouse) and secondary antibodies (anti-rabbit and -mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL Western blotting detection reagents were supplied by Amersham Biosciences (Freiburg, Germany).

4.3.2 Cell culture and treatments

ADF cells were grown adherently and maintained in culture in standard conditions, as previously described (*pag. 62*).

For experiments, when the cells were grown to sub-confluence, the complete medium was replaced with fresh RPMI1640 or DMEM F-12 (only for cAMP assay) medium containing the different compounds for cell treatments.

Aliquots of cells were treated without (Control) or with increasing TNF-alpha concentrations (10-1000 U/ml) for different times (5 min-12 hours) (TNF-α) at 37°C and then harvested for [^{35}S]GTPγS assay, cAMP assay and immunoblotting analysis.

Before being treated with TNF-alpha (1000 U/ml) for 3 hours, aliquots of cells were pre-incubated for 15 minutes with H89 dihydrochloride (1 μM) or Bisindolylmaleimide I (GF109203X) (1 μM) or wortmannin (500 nM) as inhibitors of Protein Kinase A (PKA), Protein Kinase C (PKC) or Phosphatidylinositol 3-Kinase (PI3K) respectively

(Penn *et al*., 1999; Schulte and Fredholm, 2003; Nakanishi *et al*., 1992). Aliquots of cells were also pre-incubated with PKA and PKC inhibitors (1 μM) together.

4.3.3 [^{35}S]GTPγS Binding Assay on ADF cell membranes

After different treatments, ADF cell membrane fractions were prepared and assayed according to the method previously described (*pag. 64*) with minor modifications.

A_{2B}R coupling to G proteins was evaluated assessing the ability of the AR agonist NECA to stimulate [^{35}S]GTPγS binding, in the presence of selective adenosine antagonists DPCPX, SCH 58261 and MRS 1220 to block A_1, A_{2A} and A_3 receptors, respectively.

Briefly, after pre-incubation for 15' at 30°C with ADA (2 U/ml), aliquots of membrane fractions (10 μg) were incubated for 60 minutes at 25°C in binding buffer A (25 mM Hepes, 100 mM NaCl, 5 mM $MgCl_2$, EDTA 1 mM, pH 7,4 containing DTT 1 mM, BSA 0.1%) containing 5 μM GDP and 0.3-0.5 nM [^{35}S]GTPγS. Cell membranes were stimulated with 1 μM NECA, in the presence of selective adenosine antagonists DPCPX, SCH 58261 and MRS 1220 (all used at 100 nM concentration).

ADA was added to assay to eliminate the interference of endogenous adenosine in the basal [^{35}S]GTPγS binding. Non-specific binding was determined in presence of 10 μM GTPγS and it resulted less than 10% of total binding. Binding reactions were terminated by rapid filtration under vacuum through Whatman GF/C glass fiber filters (Millipore Corporation): the filters were washed three times with 3 ml of binding buffer A and then counted in a scintillation cocktail. The concentration-dependent increase in specific [^{35}S]GTPγS bindings by agonists was expressed as a percent increase above the basal unstimulated binding (fixed as 100%). All the experiments were performed in duplicate. For test-compounds dissolved in DMSO, the final solvent concentration never exceed 2%.

4.3.4 cAMP Assay on ADF cells

Two days before the experiment, cells (50000 cells/well) were seeded in 24-well plate, to have sub-confluence conditions in 48 hours (10^5 cells/well). On the day of the cAMP assay, ADF cells were treated with or without TNF-alpha and protein kinases inhibitors and then placed in fresh DMEM F-12 medium with 2 U/ml ADA and 10 μM phosphodiesterases inhibitor Ro 201724 for 15 minutes at 37°C. Then NECA (1 μM)-

mediated cAMP production (incubated 15 min.), in presence of the A_{2A} AR antagonist SCH 58261 (100 nM) (incubated 5 min. before NECA), was evaluated. Further, control experiments were performed stimulating cells in the presence of the selective A_{2B}R antagonist MRS 1706 (10 nM).

The competitive cAMP enzyme immunoassay system kit (Sigma–Aldrich, St. Louis, MO, USA) was used, following the manufacture's instruction as previously described (*pag. 65*).

4.3.5 A_{2B}R Immunoprecipitation and Immunoblotting

Control and TNF-alpha treated cells were processed by immunoblot following the method previously described (*Chapter 3*, pag. 65) with minor modifications.

ADF cells, lysed in RIPA buffer and equalized by protein assay (1 mg proteins), were immunoprecipitated with anti-A_{2B}R antibody (3 μg/sample) and Protein A Sepharose. Immune-complexes, after being resuspended in Laemmli solution and boiled for 5 minutes, were resolved by SDS-PAGE (12%), transferred to nitrocellulose membranes and treated overnight at 4°C with primary antibody against A_{2B}R (1:1000: 1 μg/ml) or against phospho-threonine (1:500: 0.5 μg/ml). Membranes were then incubated with corresponding peroxidase-linked secondary antibody (rabbit), washed and developed using the ECL Western blotting detection reagents. Unspecific immunoprecipitation was assessed using non-immune rabbit IgG (3 μg, same amount of antibody).

Stripping protocol was applied: after washing, nitrocellulose membranes were incubated in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 (stripping buffer) at 50°C for 45 minutes. Membranes were then washed two times and incubation with a different primary antibody started.

Western blot band intensity was quantified by densitometric scanning and by a gel documentation system (ImageJ Program, National Institutes of Health, USA). Phospho-threonine immunoreactive bands were normalized by corresponding A_{2B}R immunoreactive bands and represented as arbitrary units (optical density, OD).

For ERK ½ kinase study, Control and TNF-alpha (1000 U/ml, 3 hours)-treated ADF cells were stimulated with 100 nM NECA for different times (0-60 min) or with different NECA concentrations (10nM-1μM) for 15 min. The ERK ½ kinase phosphorylation was assayed on cell lysates by immunoblotting, with the specific antibody recognizing the dually phosphorylated p-42 and p-44 kDa MAP kinases (1:500: 0.4 μg/ml).

4.3.6 Data analysis

Agonist dose–response curves were analyzed by the non-linear regression curve-fitting computer program GraphPad PRISM Version 4.00 (GraphPad Software, San Diego, CA, USA) and the EC_{50} values were derived. All data are reported as mean±SEM of three different experiments (at least) performed in duplicate. All data were statistically analyzed with One-Way ANOVA (Tukey's multiple comparison test) by the GraphPad PRISM. Significance refers to results where $P < 0.05$ was obtained.

4.4 Results

4.4.1 A_{2B}R-G protein coupling in control and short-term TNF-alpha treated cells

As a first step, we investigated the effect of short-term TNF-alpha cell exposure on the A_{2B}R-G protein coupling efficacy. In time dependence experiments, we demonstrated TNF-alpha cell treatment induced a time-dependent decrease in A_{2B}R-G protein coupling (**Fig. 1A**), with a maximal effect after 3 hour incubation (# $P<0.01$ TNF-alpha 3 hours vs. Control). Moreover, following 3 hours cell exposure, the TNF-alpha-mediated impairment of receptor-G protein activation occurred in a concentration dependent manner (**Fig. 1B**). TNF-alpha cell treatments did not induce any significant change in the basal [^{35}S]GTPγS binding (*data not shown*), thus demonstrating no effects on the G protein functional state.

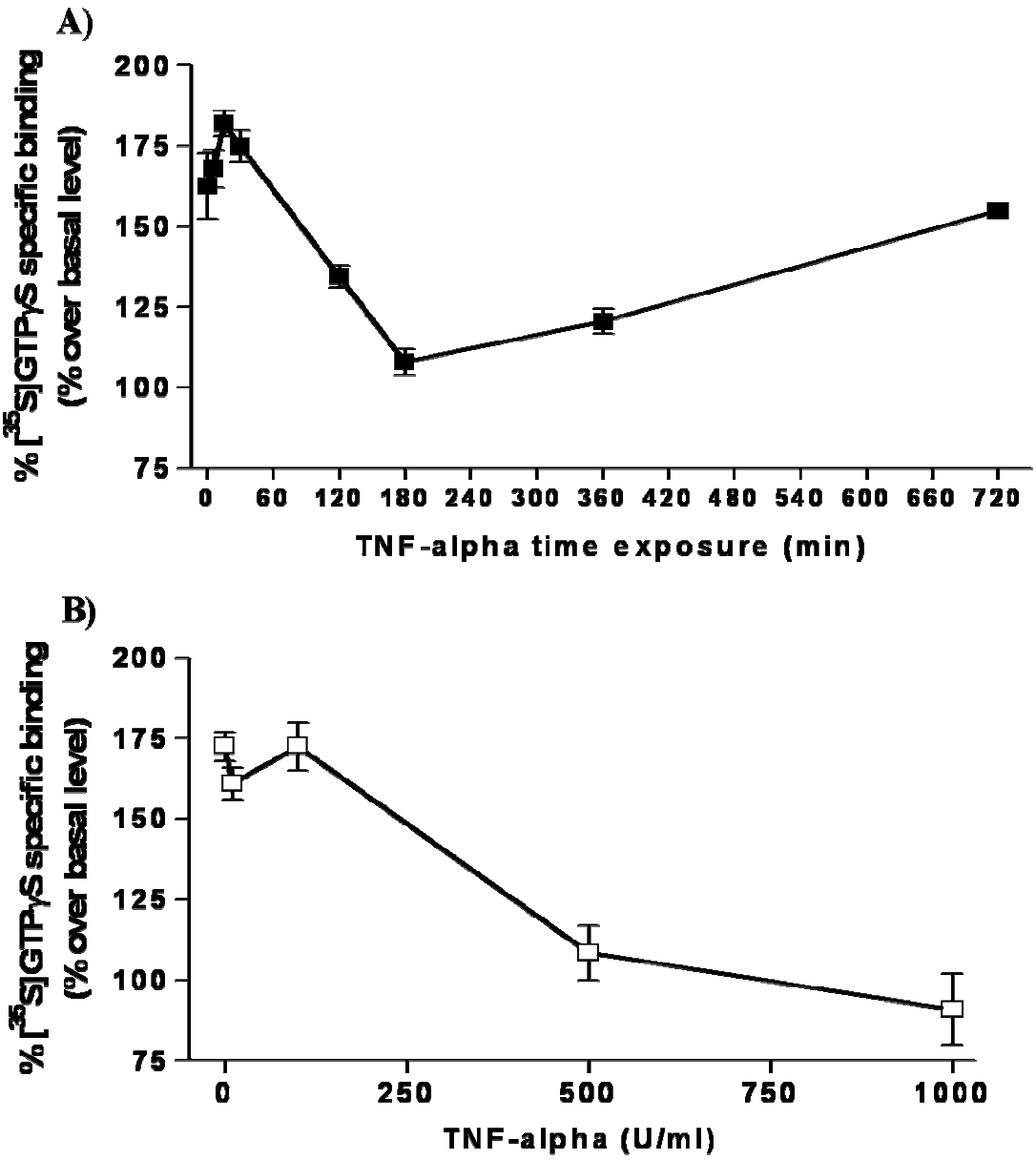

Fig. 1: TNF-alpha-mediated regulation of the A_{2B}R-G protein coupling: time (A) and concentration (B) dependence. The ability of 1 μM NECA (in the presence of A_1, A_{2A} and A_3 antagonists) to stimulate [^{35}S]GTPγS binding was evaluated in ADF cell membranes, following cell treatment without or with 1000U/ml TNF-alpha for different times (5 min-12 hours, A) or following cell treatment with different TNF-alpha concentrations (10-1000 U/ml) for 3 hours (B). Data, reported as mean±SEM (n=5), are expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%.

To dissect the possible intracellular pathways involved in the TNF-alpha mediated A_{2B}R regulation, the effect of the selective PKA, PKC and PI3K inhibitors on A_{2B}R-G protein coupling was assessed (**Fig. 2**).

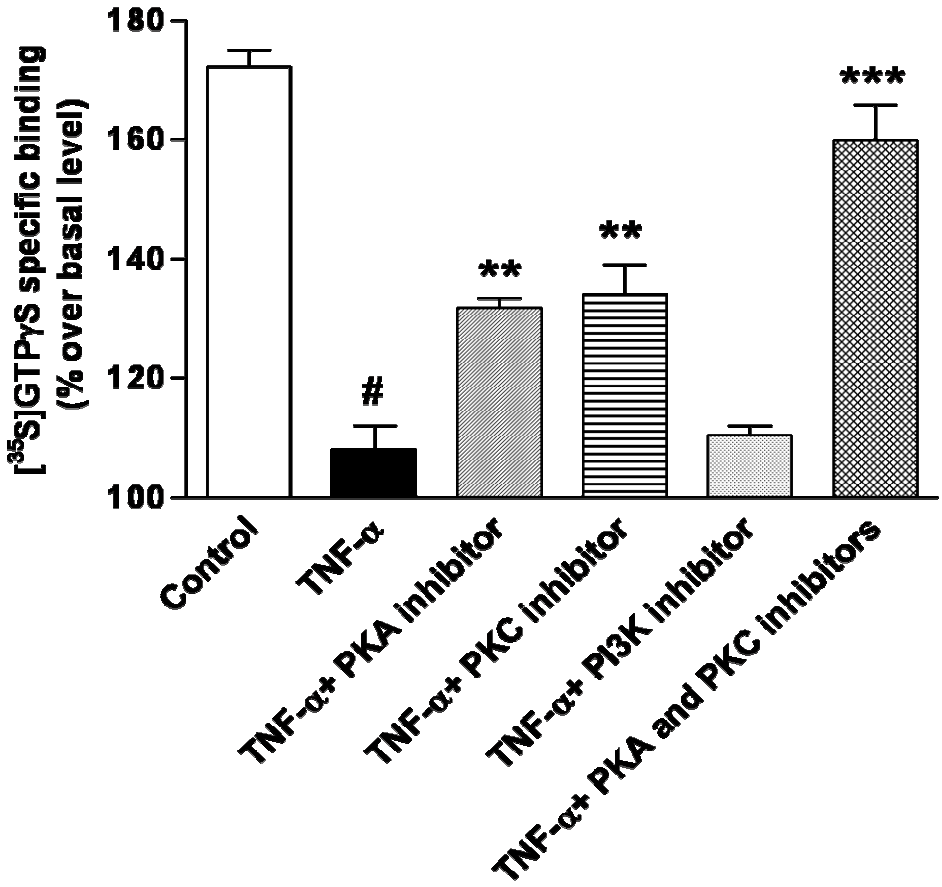

Fig. 2: Effects of intracellular kinase inhibitors on the TNF-alpha mediated A_{2B}R-G protein uncoupling. ADF cells were pre-incubated without or with PKA inhibitor (H89, 1 μM) or PKC inhibitor (GF109203X, 1 μM) or PI3K inhibitor (Wortmannin, 500 nM) or PKA and PKC inhibitors (1 μM) together, for 15 min. Cells were then treated with or without TNF-alpha 1000 U/ml for 3 hours and then the ability of 1 μM NECA (in the presence of A_1, A_{2A} and A_3 antagonists) to stimulate [^{35}S]GTPγS binding was assessed. Data, reported as mean±SEM (n=5), are expressed as percentage of basal specific [^{35}S]GTPγS binding, set to 100%.
# P<0.01 vs. Control; ** P<0.01 and *** P<0.001 vs. TNF-alpha (One-way ANOVA).

Cell pre-exposure to the PKA or PKC inhibitor was able to counteract TNF-alpha (1000 U/ml for 3 hours) effects, by inducing a partial recovery in the agonist effectiveness in stimulating GTPγS binding (** P<0.01 TNF-alpha+PKA inhibitor or TNF-alpha+PKC inhibitor vs. TNF-alpha alone). Moreover, the simultaneous cell pre-incubation with the PKA and PKC inhibitors completely restored the A_{2B}R/G protein coupling efficacy up to control level (*** P<0.001 TNF-alpha+PKA and PKC inhibitors vs. TNF-alpha

alone; P>0.05 TNF-alpha+PKA and PKC inhibitors vs. Control; **Fig. 2**). These data suggested both PKA and PKC intracellular kinases were involved in the TNF-alpha mediated regulation of A_{2B}R-G protein coupling.

4.4.2 A_{2B}R functional responsiveness in control and short-term TNF-alpha treated cells

As a second step, we evaluated the time and concentration dependence of TNF-alpha effects on the A_{2B}R functional responsiveness. The A_{2B}R responsiveness was quantified evaluating the ability of the agonist NECA, in presence of the A_{2A}R antagonist SCH 58261 (100 nM), to stimulate intracellular cAMP production in control and treated cells (Trincavelli *et al.*, 2004). Cell pre-incubation with TNF-alpha (100 U/ml) for different times induced a significant decrease in the A_{2B}R functional responsiveness (**Fig. 3A**), with a maximal effect following 3 hours incubation (# P<0.01 TNF-alpha 3 hours vs. Control). The TNF-alpha mediated impairment of A_{2B}R response occurred in a concentration dependent manner, with a maximal effect at 1000 U/ml cytokine following 3 hours treatment (**Fig. 3B**).

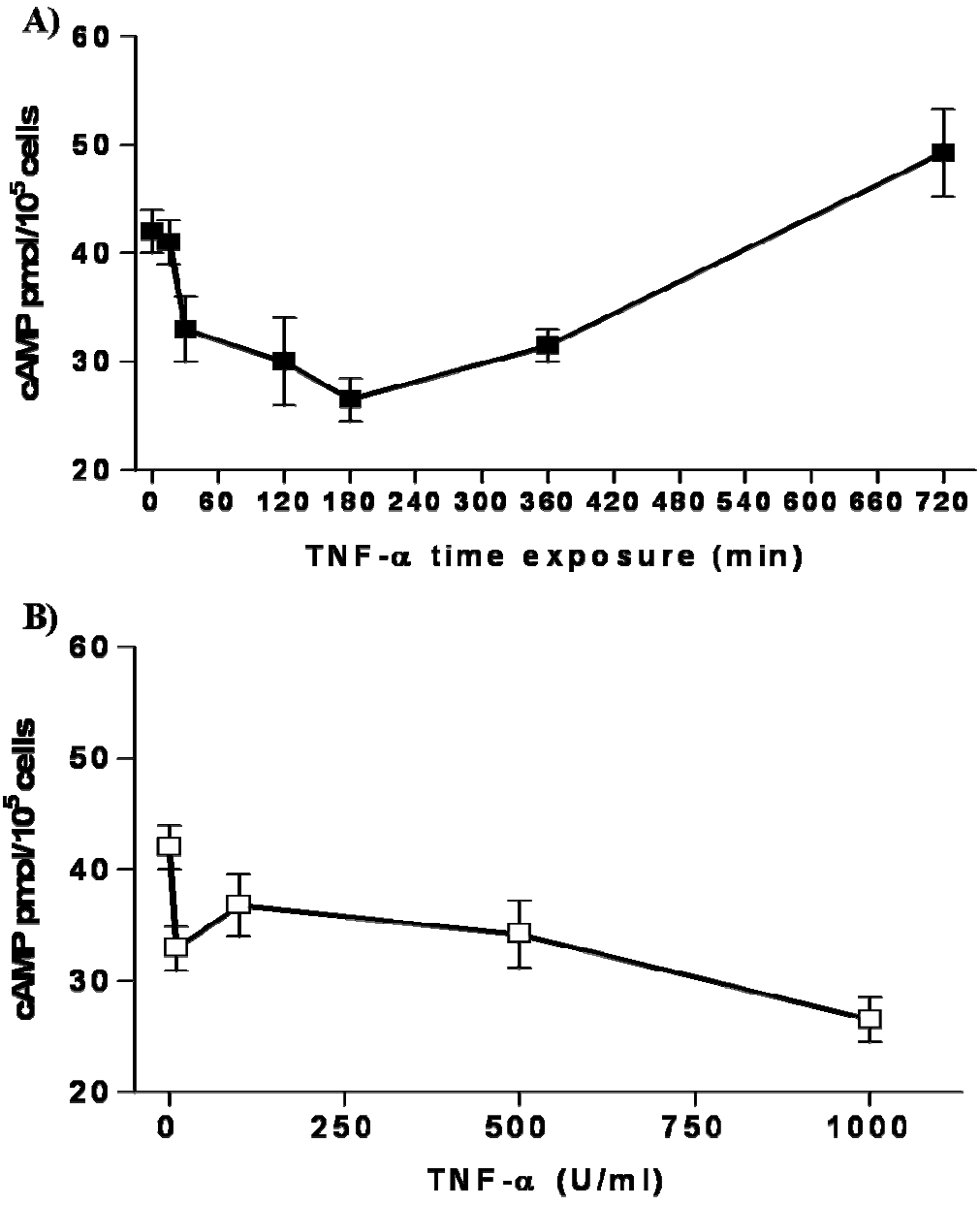

Fig. 3: TNF-alpha effect on the A_{2B}R-mediated cAMP accumulation: time (A) and concentration dependence (B). The ability of 1 µM NECA (in the presence of 100 nM SCH 58261) to stimulate cAMP accumulation was evaluated in ADF cells following cell treatment without or with 1000U/ml TNF-alpha for different times (15 min-12 hours, A) or following cell treatment with different TNF-alpha concentrations (10-1000 U/ml) for 3 hours (B). Data, reported as mean±SEM (n=3), are expressed as cAMP levels (pmol/10^5 cells).

Moreover, the effect of the PKA, PKC and PI3K inhibitors on the short-term TNF-alpha-mediated $A_{2B}R$ desensitization was assessed. Data in **Fig. 4** showed that cell pre-incubation with PKA or PKC inhibitors counteracted the TNF-alpha effects, with a 75.0 % and 67.3 % recovery of the agonist-mediated-mediated cAMP accumulation, respectively ($P<0.01$ TNF-alpha+PKA inhibitor and TNF-alpha+PKC inhibitor vs. TNF-alpha alone). The cell pre-exposure to PKA and PKC inhibitors together induced a nearly complete recovery of the $A_{2B}R$ functional responsiveness ($P>0.05$ TNF-alpha+PKA and PKC inhibitors vs. Control). On the contrary, PI3K inhibitor was not able to restore the $A_{2B}R$ functional responsiveness at level of second messenger system ($P>0.05$ TNF-alpha+ PI3K inhibitor vs. TNF-alpha alone).

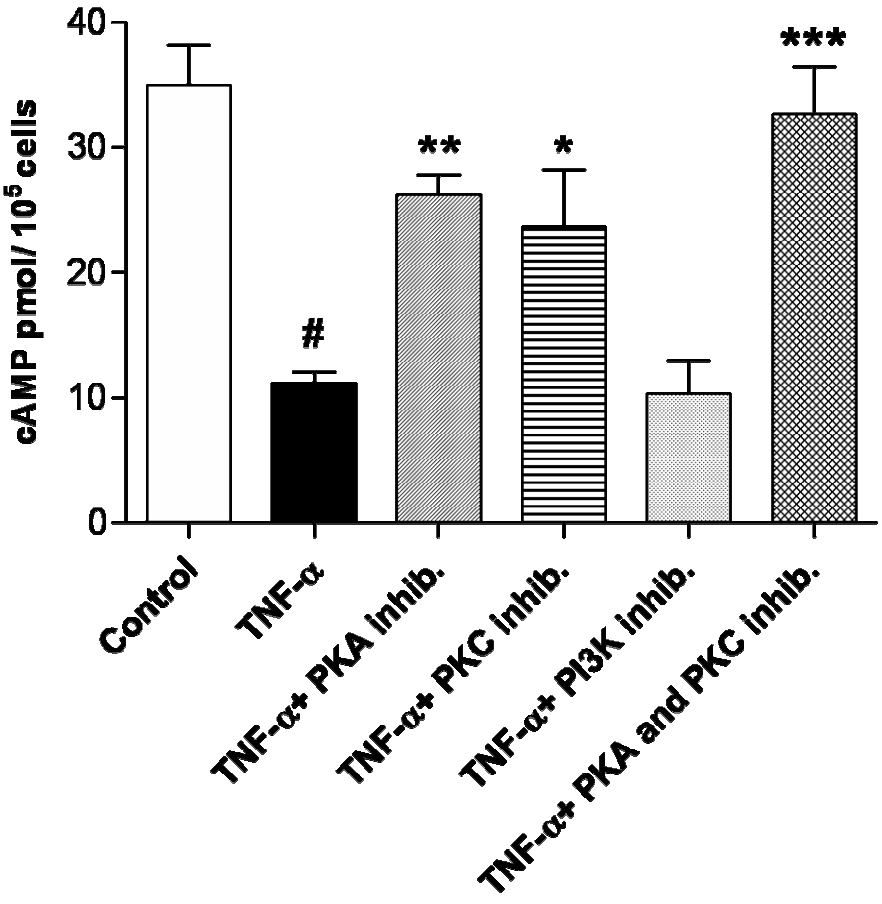

Fig. 4: Effects of intracellular kinase inhibitors on the TNF-alpha-mediated $A_{2B}R$ desensitization. ADF cells were pre-incubated without or with PKA inhibitor (H89, 1 μM), PKC inhibitor (GF109203X, 1 μM), PI3K inhibitor (Wortmannin, 500 nM) or PKA and PKC inhibitors (1 μM) together, for 15 min. Cells were then treated with or without TNF-alpha 1000 U/ml for 3 hours and the ability of 1 μM NECA (in the presence of 100 nM SCH 58261) to stimulate cAMP accumulation was

evaluated. Data, reported as mean±SEM (n=3), are expressed as cAMP (pmol/10^5 cells).
# P<0.01 vs. Control; * P<0.05, ** P<0.01 and *** P<0.001 vs. TNF-alpha (One-way ANOVA).

4.4.3 A_{2B} R expression and phosphorylation levels in control and short-term TNF-alpha treated cells

To investigate the molecular mechanisms involved in the TNF-alpha mediated A_{2B}R desensitization, A_{2B} receptor expression and phosphorylation levels were evaluated following 1000 U/ml cytokine cell treatment; in particular, we focused on the phosphorylation status of threonine residues, which are primarily involved in GPCR receptor desensitization (Palmer and Stiles, 2000; Ferguson, 2001).

As a first step, we investigated the TNF-alpha time-dependent effects on A_{2B}R protein expression and threonine-phosphorylation levels (**Fig. 5A-B**). ADF cells were treated with medium alone or with TNF-alpha (1000 U/ml) for different times and then A_{2B}R was immunoprecipitated using an antibody against A_{2B}R and processed by immunoblotting assay: the immunoprecipitates were resolved by SDS electrophoresis, transferred to nitrocellulose membranes and probed before with the anti-A_{2B}R antibody and then with the anti-phosphothreonine antibody. The A_{2B}R expression levels were not affected by the different time TNF-alpha treatments (*data not shown*), in agreement with data already published (Trincavelli *et al.*, 2004). On the contrary, TNF-alpha induced a time-dependent increase in the basal A_{2B}R phosphorylation levels, with a maximal effect following 3 hour cell exposure (P<0.01 all columns vs. TNF-alpha 3hours; **Fig. 5B**). These results suggest that TNF-alpha induced A_{2B}R desensitization: thus the cytokine affected the receptor regulatory processes by increasing the basal receptor phosphorylation.

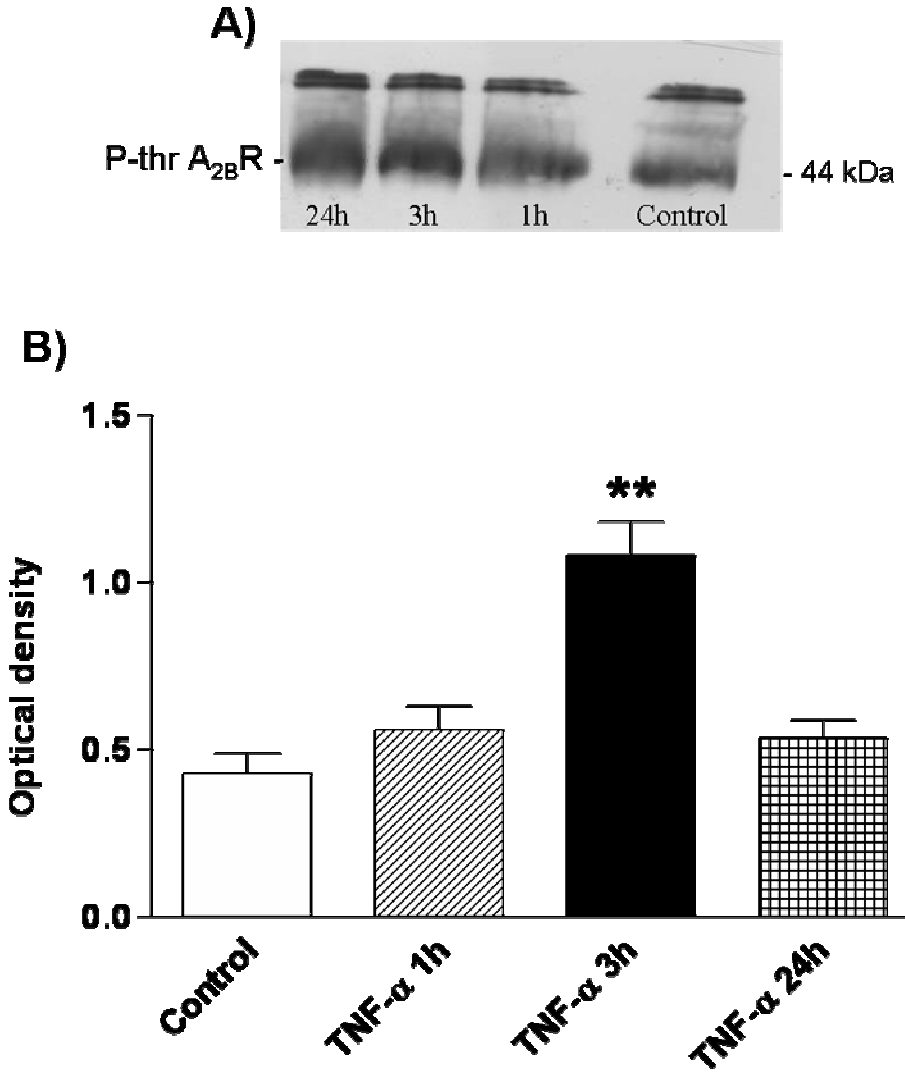

Fig. 5: TNF-alpha time-dependent effects on the $A_{2B}R$ threonine-phosphorylation levels. A) ADF cells were treated with medium alone (Control) or with TNF-alpha (1000 U/ml) for different time (1 hour; 3 hours; 24 hours). Cells were then lysed and an amount of 1 mg of proteins was immunoprecipitated using a polyclonal antibody against $A_{2B}R$. The immunoprecipitates were probed with an anti-phosphothreonine antibody to detect the threonine receptor phosphorylation levels. Phosphothreonine immunoreactive bands (at around 44 kDa) were quantified by densitometric scanning and normalized by the corresponding $A_{2B}R$ immunoreactive bands. B) Graph bar represents the normalized data (mean±SEM; n=3) obtained by the densitometric scanning of immunoreactive bands normalized by corresponding $A_{2B}R$ immunoreactive bands. ** $P<0.01$ all columns vs. TNF-alpha 3hours (One-way ANOVA).

As shown in **Fig. 6**, the TNF-alpha-mediated $A_{2B}R$ phosphorylation was significantly prevented by the PKA (optical density= 0.4±0.19; P<0.01 vs. TNF-alpha alone) and PKC (optical density= 0.52±0.11; P<0.01 vs. TNF-alpha alone) inhibitors, whereas PI3K did not seem to be involved in the regulation of receptor phosphorylation (optical density= 1.21±0.11; P>0.05 vs. TNF-alpha alone). Cell pre-incubation with PKA and PKC inhibitors together induced a nearly complete inhibition of the TNF-alpha-mediated $A_{2B}R$ phosphorylation (optical density= 0.46±0.08; P>0.05 vs. Control and P<0.01 vs. TNF-alpha alone). Moreover, A_{2B} AR expression levels were not affected by the different protein kinase inhibitor treatments (*data not shown*).

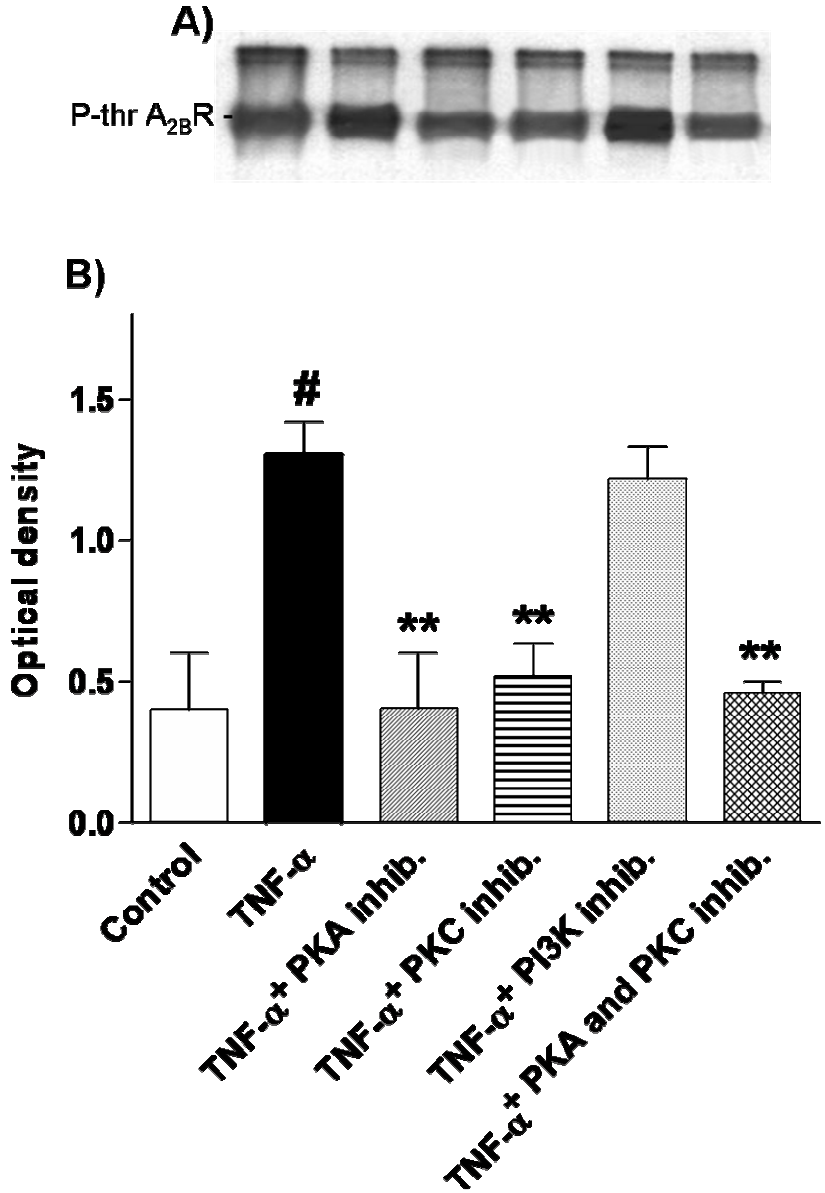

Fig. 6: Effects of intracellular kinase inhibitors on the TNF-alpha mediated $A_{2B}R$ threonine-phosphorylation levels. Cells were pretreated without or with PKC inhibitor (GF109203X, 1 μM) or PKA inhibitor (H89, 1

μM) or PI3K inhibitor (Wortmannin, 500 nM) or PKA and PKC inhibitors (1 μM) together for 15 min and then stimulated with TNF-alpha (1000U/ml) for 3 hours. **A**) The immunoprecipitate samples, after being probed with the anti-$A_{2B}R$ antibody, were subjected to analysis with the anti-phosphothreonine antibody to detect the extent of threonine receptor phosphorylation levels. **B**) Graph bar represents the normalized data (mean±SEM; n=3) of the phospho-threonine immunoreactive bands quantified by densitometric scanning and normalized by the corresponding $A_{2B}R$ immunoreactive bands. # $P<0.01$ vs. Control; ** $P<0.01$ vs. TNF-alpha (One-way ANOVA).

4.4.4 A_{2B} AR-mediated ERK ½ phosphorylation in control and short-term TNF-alpha treated cells

In order to investigate whether the impairment of $A_{2B}R$ responsiveness, induced by short-term TNF-alpha exposure, could induce any significant changes in the $A_{2B}R$-activated intracellular phosphorylative pathways, we evaluated the effect of 1000 U/ml TNF-alpha for 3 hours on the $A_{2B}R$-mediated ERK ½ phosphorylation.

Data obtained from the immunoblotting assays demonstrated that, in Control cells, the agonist NECA (100 nM) induced an ERK ½ phosphorylation with a transient and monophasic kinetics and a maximal effect following 15 minutes (**Fig. 7A**). Moreover, the ERK ½ activation occurred in a concentration dependent manner, with a maximal response at nanomolar NECA concentration (**Fig. 7B**).

When ADF cells were pre-incubated with TNF-alpha (1000 U/ml) for 3 hours, a significant decrease in the maximal NECA $A_{2B}R$-mediated ERK ½ phosphorylation was detected, thus suggesting the cytokine impaired the $A_{2B}R$-mediated intracellular phosphorylative pathways (* $P<0.05$ Control 15' vs. TNF-alpha 15', One-way ANOVA) (**Fig. 7A**). On the contrary, no significantly changes were induced on kinetic ERK ½ activation, mediated by $A_{2B}Rs$, following cytokine treatment (**Fig. 7B**).

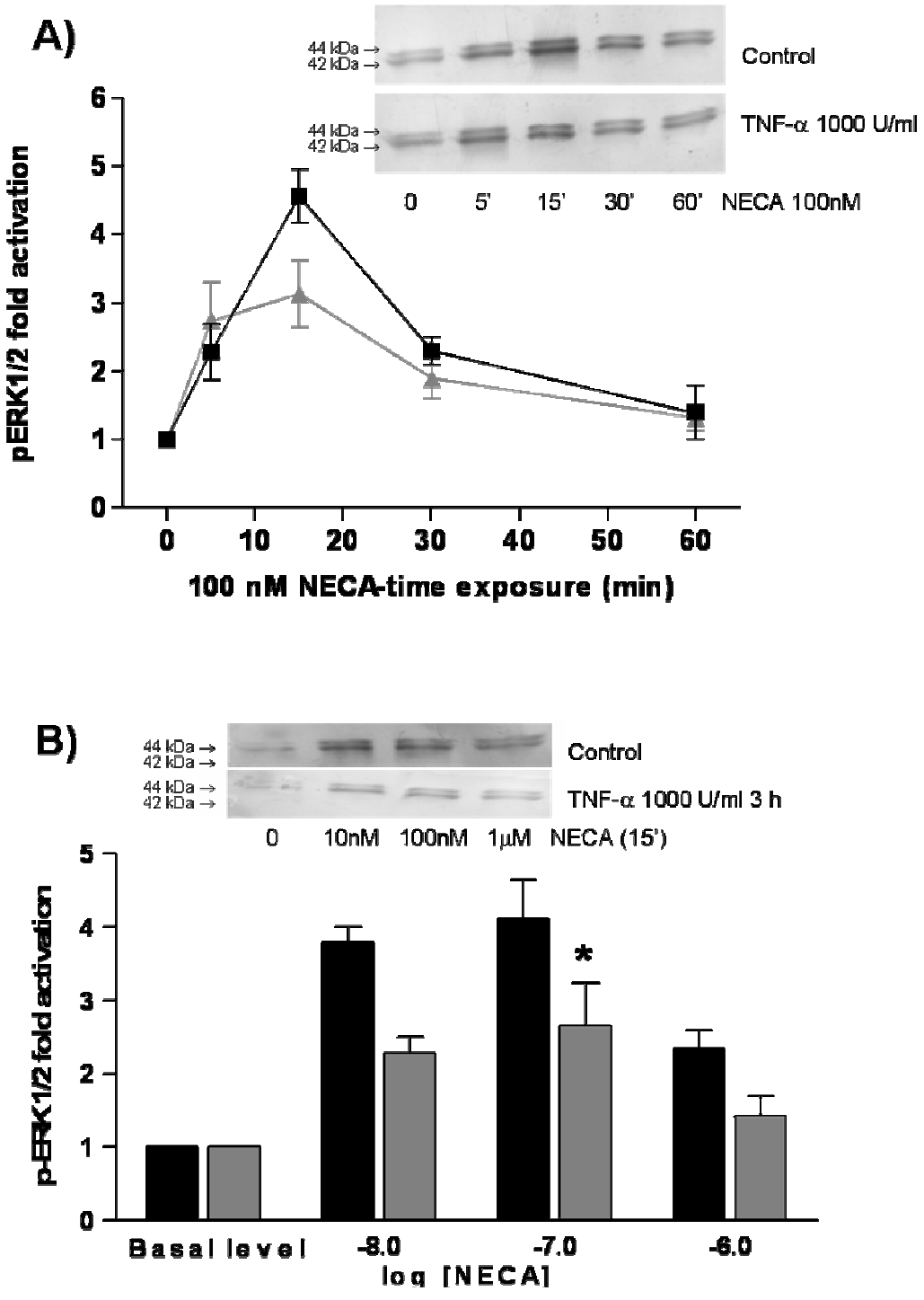

Fig. 7: TNF-alpha maximal effects on the A_{2B}R-mediated ERK ½ phosphorylation: time (**A**) and concentration (**B**) dependence. ADF cells, pretreated with medium alone (Control, ■ *black*) or with 1000 U/ml TNF-α (▲, *grey*) for 3 hours, were stimulated with 100 nM NECA for different times (0-60 min, **A**) or with different NECA concentrations (10nM-1μM) for 15 min (**B**). The ERK ½ kinase phosphorylation level was determined by immunoblotting with an antibody recognizing the dually phosphorylated p-42 and p-44 kDa MAP kinases. The immunoreactivity

was quantified by densitometry and data were normalized against the basal phosphorylation level (set to 1). Data are expressed as mean±SEM (n=3).
* P<0.05 TNF-α 15' vs. Control 15' (One-way ANOVA).

4.5 Discussion

The presence and the selective functional responsiveness of A_{2B}Rs in ADF cells had been previously characterized and demonstrated in ADF cells (Trincavelli *et al*., 2004). Furthermore it had been also assayed that all NECA-stimulated responses, in the presence of $A_{1\text{-}2A\text{-}3}$R antagonists, were abolished by the cell pre-incubation with the selective A_{2B}R antagonist MRS 1706; so these responses were mediated by A_{2B}R subtype in ADF cells.

The ADF cell short-term exposure to the pro-inflammatory cytokine TNF-alpha induced impairment of the A_{2B}R responsiveness, causing, in particular, a reduction of the agonist effectiveness in promoting the receptor-G protein coupling and in stimulating the adenylyl cyclase pathway as well the intracellular phosphorylative ERK ½ pathways, with a maximal effect following 3 hour cytokine incubation. These effects appeared to be also associated with an increase in the receptor basal phosphorylation levels, which may be responsible for the observed functional effects. These results suggested TNF-alpha was able to induce a short-term A_{2B}R desensitization.

Because of GPCR functional alterations could be ascribed to changes of intracellular signalling proteins involved in heterologous and/or homologous GPCR regulations, including different protein kinases (Vazquez-Prado *et al.*, 2003), we investigated the PKA, PKC and PI3K involvement in the TNF-alpha mediated effects on A_{2B}Rs. All these kinases, besides representing a converging point for different intracellular phosphorylative pathways, are activated both by the cytokine TNF-alpha and by A_{2B}Rs (Linden *et al.*, 1999; Baud and Karin 2001; MacEwan *et al.*, 2002; Zhang *et al.*, 2002; Queiroz *et al.*, 2004). Moreover, several data suggest a role for these proteins in the feed-back regulation of several GPCRs through different mechanisms, such as a direct effect on the receptor itself and/or an effect on the G protein activity (Casas-Gonzalez *et al.*, 2000; Jo *et al.*, 2002; Tan *et al.*, 2003). Among these protein kinases, while PI3K didn't result to be involved in the TNF-alpha mediated A_{2B}R regulation, PKA and PKC

mediated the TNF-alpha effects on $A_{2B}R$ phosphorylation, receptor G protein uncoupling and impairment in adenylyl cyclase pathway.

In line with our findings, it has been recently demonstrated that cytokines regulate the β_2 adrenergic receptor responsiveness via PKA pathway through the modulation of different targets including the receptor itself, adenylyl cyclase (Guo *et al.*, 2005) and G_s protein expression (Chapman *et al.*, 2005); these data suggest cytokines may modulate the GPCR functioning by acting both at receptor and at downstream signalling level. In a similar way, PKC has been involved in the control of the β adrenergic receptor functioning through receptor phosphorylation, G_s expression and intracellular phosphorylation regulation (Levesque and Crooke, 1998). In our system, TNF-alpha did not appear to modify either G protein activation state or basal adenylyl cyclase activity: its effects mainly occurred at A_{2B} receptor level. This difference could be justified by the different time of cytokine cell exposure: in fact TNF-alpha effects at the level of downstream signalling have been detected following long-term cell treatment. IFN gamma, for example, has been demonstrated to regulate $A_{2B}R$ functional responses by affecting the adenylyl cyclase activity following 12 hours cell treatment (Kolachala *et al.*, 2005). In summary PKA and PKC, which represent the intracellular pathways involved in AR-mediated control of the cytokine production, are, in turn, involved in the control of the A_{2B} receptor responses in the acute phase of TNF-alpha release.

Comparing the present data with those previously published by our laboratory, a significant increase in the $A_{2B}R$ functional responses was detected when TNF-alpha cell treatment was prolonged up to 24 hours (Trincavelli *et al.*, 2004): it might be suggested a dualistic and opposite effect of TNF-alpha on the $A_{2B}R$ functioning after short- and long-term cytokine cell exposure. These data parallel with those obtained in astroglioma (Fredholm and Altiok, 1994) and endothelial cells (Nguyen *et al.*, 2003), thus demonstrating the $A_{2B}R$-mediated effects are differently modified in a time dependent manner by inflammatory mediators and underlying the AR system as a promising target to modulate cerebral damage progression.

In response to injuries, resident CNS cells generate pro-inflammatory cytokines, which may contribute to acute and chronic brain disease pathogenesis through the recruitment of immune cells and the activation of glial cells (for review see Lucas *et al.*, 2006). However, cytokines may display a dualistic role, with detrimental acute effects but also beneficial effects in long-term repair and recovery. In particular, TNF-alpha, synthesized by macrophages, astrocytes and microglia with a rapid kinetic following cerebral

trauma or ischemia (up to 2 hours; Yu and Lau, 2000; Yin *et al.*, 2003; Vitarbo *et al.*, 2004), has been demonstrated to be pro-inflammatory during the acute phase of the inflammatory response and immunosuppressive during the chronic phase (Wang and Shuaib, 2002).

Zhong *et al.* (2005) have showed a synergic effect between A_{2B}Rs and hypoxia in activating human lung fibroblasts, thus suggesting the adenosine system/inflammatory mediator interaction as a common mechanism in both central and peripheral tissues. In particular in astroglial cells, a bi-directional functional cross-talk between cytokines and A_{2B}R adenosine system has been described as an important mechanism to regulate the cerebral damage progression (Fredholm and Altiok, 1994; Rosi *et al.*, 2003; Trincavelli *et al.*, 2004).

The A_{2B}R has been implicated both in the stimulation (Feoktistov *et al.*, 2002; Zhong *et al.*, 2004; Zhang *et al.*, 2005) and inhibition (Kreckler *et al.*, 2006; Yang *et al.*, 2006) of cytokine release, which in turn can exacerbate or reduce inflammatory processes. Moreover inflammatory signals other than TNF-alpha have been shown to regulate A_{2B}R signalling: for example LPS (Nemeth *et al.*, 2003) and IFN-γ (Xaus *et al.*, 1999), which was recently shown to elevate A_{2B} receptor expression in macrophages.

Since the A_{2B}R results involved in inflammatory processes, even if with a still enigmatic role (Linden, 2006), the TNF-alpha-mediated activity reduction of A_{2B}R may represent a potential *feed-back* mechanism to control the inflammatory effects induced by neurotoxic compounds and pro-inflammatory cytokines, released in the acute phase of brain damage. On the contrary, in the chronic phase of brain injury, TNF-alpha, by a significant up-regulation of A_{2B}R responsiveness, contributes to the reactive astrogliosis (Trincavelli *et al.*, 2004), thus suggesting A_{2B}Rs as a novel target for neuroinflammatory-neurodegenerative diseases.

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Charter 5:
General conclusions

5.1 Conclusions

5.1.1 A_1R-$P2Y_1R$

In the present work, we investigated the localization/co-localization of purinergic A_1 and $P2Y_1$ receptors and subsequently their functional interactions, both in rat hippocampus, which is considered as a damage sensitive brain area, and in human astroglial cells, which are important in CNS maintenance and damage progression.
The results have demonstrated:

- There is a significantly high expression of both A_1 and $P2Y_1$ receptors on synaptic and astroglia membranes in hippocampus. The receptors showed also a functional interaction at membrane G protein level: $P2Y_1R$ activation induced an impairment of A_1R-G protein coupling, while A_1R activation increased the $P2Y_1R$ functional coupling to G protein.
- In ADF astroglial cells, the $P2Y_1R$ activation by its agonist MeSADP induced an increase of A_1R antagonist affinity, an impairment of A_1R-G protein coupling and a decrease of A_1R-stimulated adenylate cyclase activity, thus suggesting a heterologous A_1R desensitisation induced by the $P2Y_1R$ activation.

A_1 and $P2Y_1$ receptors cellular co-localization, interaction and functional heterodimerization may help to clarify the patho-physiological functions of ATP and adenosine in CNS, regarding the possible alterations of P1-P2 receptor regulation processes during pathological conditions.

5.1.2 A_{2B}R-cytokine

For cytokines, equally immunoregulators and modulators of neural functions, a bidirectional interaction with CNS neurotrasmitters has been proposed, because cytokines can modulate the action and survival of neuronal and glial cells but their production is controlled just by neurotrasmitters, produced by those cells (Szelenyi, 2001).

In the present work we investigated the functional cross-talk between the inflammatory cytokine TNF-α, in human astroglial cells and A_{2B} receptors.

The results have demonstrated:

- ADF cell preincubation with TNF-α induced a significant concentration and time-dependent decrease of A_{2B}R-mediated responsiveness, with a maximal effect following 3 hours incubation; moreover, the impairment of A_{2B}R functioning occurred in a concentration dependent manner, with a maximal effect at 1000 U/ml cytokine concentration. Moreover TNF-α did not induce any significant changes in A_{2B} receptor levels.
- Short-term (3 hours) ADF cell exposure to TNF-α induced A_{2B}R responsiveness impairment, in particular reduction in the efficacy of the adenosine agonist to promote receptor-G protein coupling, to stimulate adenylyl cyclase pathway and to activate ERK ½ phosphorylation. These effects, associated with an increase in the basal receptor phosphorylation levels which may be responsible for the functional effects, resulted mediated by the intracellular kinase PKA and PKC.

A main finding of the present work is that the proinflammatory cytokine TNF-α selectively modulated A_{2B}Rs responsiveness in human ADF cells. Results confirm that shedding light on adenosine receptor-cytokine cross-talk might be a useful tool to clarify the patho-physiological role of A_{2B}R in response to brain damage.

5.2 Future perspectives

In the CNS the metabolic stress associated with hypoxia, ischemia, trauma and excessive neuronal firing elicits large increases in the concentration of ATP and adenosine, which has an important role in controlling subsequent tissue damage. Although the actions of extracellular adenosine are mainly protective, it is an "*imperfect endogenous*

neuroprotective agent" because, in same scenarios, adenosine receptor stimulation further aggravates damage (Picano and Abbracchio, 2000).

Anyhow, the up-regulation of both adenosine A_1 and A_{2B} receptors has been showed to play an important role in the protective effects of short cerebral ischemic preconditioning in hippocampus; in particular for A_1R the increase occurred in neurons while for $A_{2B}R$ in astrocytes (Zhou *et al*., 2004). In the same way, the activation of both A_1 and A_{2B} receptors seems required for the ischemic preconditioning protection in the heart, during early minutes of reperfusion (Solenkova *et al*., 2006).

Furthermore, it has been recently suggested a mechanism whereby astrocytes can sense the severity of damage in the CNS via ATP release from damaged cells and can modulate the TNF-α mediated inflammatory response depending on the extracellular ATP concentration and corresponding type of astrocyte ATP/P2 receptor activated (Kucher and Neary, 2005).

The interactions and the reciprocal regulation between P1 and P2 purinergic receptors, which also interact as well with cytokines, both in physiological and pathological conditions, are a clear example of increasing biological complexity. It is no chance that P2 receptor system has been proposed as a "combinatorial receptor web": the dynamic architecture demonstrates economic efficiency and involves a process of "fine-tuning", a mechanism which endorses the dynamic nature of all biological reactions (Volontè *et al*., 2006).

At the present it is becoming clear that, in biological systems, what matters most is not only the single response to a single mediator but above all the final effect of the interaction and the reciprocal regulation between different receptor systems and signalling pathways.

Despite intensive efforts, relatively few adenosine and purinergic receptor ligands have made into clinical trials; in fact it is only now that the full potential for drug development is becoming clear (Fredholm *et al*., 2001). Thus, adenosine receptors are and remain an attractive target for drug development.

5.3 References

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