Neuromodulatory and Neuroprotective Roles of Synaptic P2 Receptors

Papel Neuromodulador e Neuroprotector dos Receptores P2 Sinápticos

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Abreviations List

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

2-MeSATP – 2-methylthio-ADP

5-HT – 5-hydroxytryptamine

ACh – Acetylcholine

ADP - Adenosine-5´-diphosphate

AMP - Adenosine-5´-monophosphate

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

AP – Alkaline phoshatase

ATP – Adenosine-5´-triphosphate

Aβ – β-amyloid

BBG – Brilliant blue G

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

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

cAMP- Ciclic adenosine-5´-monophosphate

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

CNS – Central nervous system

D-AP5 – D-(-)-2-Amino-5-phosphonopentanoic acid

DMEM - Dulbecco’s Modified Eagle’s Medium

DMSO - Dimethylsulphoxide

DNA – Desoxiribonucleic acid

DPCPX – 1,3-dipropyl-8-cyclopentyladenosine

DTT – Dithiothreitol

EC_{50} – Concetration of a ligand eliciting 50% of the maximal response

Ecto-5’-NT – Ecto-5’-nucleotidase

EDTA – Ethylenediaminetetraacetic acid

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

E-NPP – Ecto-nucleotide pyrophosphatase

E-NTPDases – Ecto-nucleoside triphosphate diphosphohydrolases

Extra – Extrasynaptic

FCS – Foetal calf serum

GABA – γ-aminobutyric acid

GFAP – Glial fibrillary acidic protein

HEK293 – Human embrionic kidney 293 cells

HEPES – N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)

HPLC – High performance liquid cromatography

IC_{50} - Concentration of a ligand that causes 50% of the maximal inhibition

Ins415 - P1, P3-di(uridine 5'-) triphosphate

Ins45973 - P1-(urinine 5'-), P4-(inosine 5'-) tetraphosphate

IP3 – 1, 4, 5 – inositol triphosphate

K_D – Dissociation constant

MAP-2 – Microtubules-associated protein 2

MAPK – Mitogen-activated protein kinase

mEPSP – miniature excitatory postsynaptic potentials

mIPSC – miniature inhibitory postsynaptic current

mRNA – Messenger ribonucleic acid

MRS2179 - 2´deoxy-N^6-methyladenosine-3´,5´-bisphosphate

nAChR – Nicotinic acetylcholine receptor

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

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

P2R – P2 receptors

P2XR – Ionotropic P2X receptors

P2YR – Metabotropic P2Y receptors

PBS – Phosphate buffered saline medium

PKC - Protein Kinase C

PLC - Phospholipase C

PMSF – Phenylmethanesulfonylfluoride

Post – Postsynaptic fraction

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

Pre – Presynaptic fraction

RB2 – Reactive Blue 2

RNA – Ribonucleic acid

RT – Room temperature

SCH 58261 – (5-amino-7-(3-fenilpropil)-2-(2-funil)pirazol[4,3-e]-1,2,4-triazolo[1,5-c]pirimidina)

SDS – Sodium dodecyl sulfate

TBS – Tris buffered saline medium

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

TES – N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid sodium salt

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

UDP – Uridine-5´-diphosphate

UTP – Uridine-5´-triphosphate

VOCCs – Voltage-operated calcium channels

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

α,β-MeATP – α,β-MethyleneATP

β,γ-ImATP – β,γ-ImidoATP

Publications

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Abstract

In the central nervous system (CNS), ATP is released in a vesicular manner from nerve terminals in a frequency-dependent manner. Once in the extracelular milieu ATP can activate either metabotropic P2Y (P2YR) or ionotropic P2X receptors (P2XR), which are widely expressed and distributed in the brain, namely in the hippocampus. However the role of central P2 receptors remains ill defined. By analogy with the well stablished neurotransmitter role of ATP in the peripheral nervous system, it has been assumed that ATP would also be a fast neurotransmitter in CNS. However, an ATPergic contribution to synaptic transmission could only been demonstrated in few central synapses. This led to the hypothesis that ATP acts principally as a neuromodulator in the brain, in particular with a presynaptic locus of action. This hypothesis would reconcile the heavy expression and the robust density of P2 receptors in central neurons, with the difficulty of ascribing a clear role for ATP as a fast neurotransmitter in the brain.

To test this hypothesis purified nerve terminals were used (which allows isolating presynaptic effects) to assess whether P2 receptors could presynaptically control the evoked release of neurotransmitters. It is shown that ATP analogues biphasically modulate the evoked release of glutamate from purified nerve terminals of the rat hippocampus, the facilitation being mediated by $P2X_{1,2/3,3}$ (antagonized by NF023) and the inhibition by $P2Y_{1,2,4}$ (antagonized by reactive blue 2, MRS2179 and mimicked by Ins47953) receptors. The combination of single cell RT-PCR analysis of rat hippocampal pyramidal neurons, Western blot analysis of purified presynaptic active zone fraction and immunocytochemical analysis of glutamatergic terminals further re-enforced that the P2 receptors expressed in glutamatergic neurons, located in the active zone and in glutamatergic terminals were precisely $P2X_{1,2,3}$ and $P2Y_{1,2,4}$. Thus, this study demonstrates that P2 receptors are present and act presynaptically as modulatory system capable to control neurotransmitter release.

The identification and characterisation of a neuromodulatory role of synaptic P2 receptors, together with the known massive release of ATP into the extracelular space upon a noxious insult led to the investigation of a possible pathophysiological role of P2 receptors in the most common chronic neurodegenerative disease Alzheimer's disease that is believed to be initially due to subtle alterations of cortical and hippocampal synaptic efficacy prior to frank neuronal degeneration. Synapses seem to be the primordial target to the toxic Aβ peptides leading to a synaptic failure that underlies the mnemonic impairment. Thus, a major lead for the development of novel therapeutics strategies for AD is the search of mechanisms able to prevent this early synaptotoxicity caused by $A\beta_{1-42}$, which led to the investigation of a possible involvement of P2 receptors. Pharmacological blockade of $P2Y_1$ receptors in rat hippocampal neurons prevented the neuronal death caused by the exposure to $A\beta_{1-42}$. Interestingly, the blockade of $P2Y_1$ receptors also prevents the early synaptotoxicity caused by $A\beta_{1-42}$ that

precedes the subsequent neuronal death. This protective role of $P2Y_1$ receptors was also observed *in vivo*. In rodents, the *intracerebroventricular* administration of $A\beta_{1-42}$ caused a decrease of the density of nerve terminals in the hippocampus and a mnemonic impairment in the absence of hippocampal neuronal death, which was not present in mice deficient in $P2Y_1$ receptor. Furthermore, $A\beta_{1-42}$ administration increased $P2Y_1$ receptors density selectively at the synaptic level. These results demonstrate that the synaptic $P2Y_1$ receptors are involved and mandatory in order for Aβ peptides to cause their neurotoxic effects. This prompts $P2Y_1$ receptor antagonists as potential candidates to arrest the evolution of Alzheimer´s disease, in particular at the early onset of this neurodegenerative disease.

It was also concluded that presynaptic P2 receptors functionally interact with other neuromodulatory systems, namely that operated by nACh receptors. Both immunocytochemical analysis and microfluorimetric $[Ca^{2+}]_i$ measurements showed that P2XR and nAChR are co-localised in a subset of rat hippocampal nerve terminals and neurochemical studies allowed defining pharmacologically a functional interaction between P2X and nACh in the control of neurotransmitter release. This is mostly concluded by the observation of a cross-antagonism between P2X and nACh receptors in the control of hippocampal glutamate release, which involves $P2X_1$, $P2X_{2/3}$, $P2X_3$ receptors and α3β2 receptors.

This promiscuous role of P2X receptors was further re-enforced by the existence of a hybrid $P2X_2$/NR2B receptor explored by investigating a possible interaction between P2X and N-methyl-D-aspartate (NMDA) receptors. However, this study fitted into a broader objective to gather data supporting the existence of hybrid ionotropic receptors composed by subunits from different families of receptors. NR2B binds glutamate but cannot form a channel alone. However, in HEK293 cells the co-expression of $P2X_2$ rescued NR2B function. Furthermore, it is shown that the $[Ca^{2+}]$ transients elicited by NMDA receptors in cells co-expressing $P2X_2$ and NR2B subunits are insensitive to Mg^{2+}, are independent of glycine and are abolished by the presence of PPADS and D-AP5, selective antagonists of P2 and NMDA receptors, respectively. Co-immunoprecipitation assays also showed a physical interaction between $P2X_2$ and NR2B, both in transfected HEK293 cells and also in mouse brain homogenate. These results provide for the first time evidences for the existence of hybrid $P2X_2$/NR2B receptors composed by subunits from different families of receptors, which constitutes a new concept of receptor signalling.

Overall, this thesis provides evidence supporting a presynaptic neuromodulatory role for ATP P2 receptors in the brain, in particular in the hippocampus. ATP biphasically modulates the release of neurotransmitters through P2Y and P2X receptors. These P2X receptors are promiscuous proteins that interact with and control other ionotropic receptors such as nACh and NMDA receptors. Albeit the physiological role of P2 receptors remains to be more throughfully explored, it was found that $P2Y_1$ receptors blockade prevented neuronal damage and memory

loss in models of Alzheimer's disease. This indicates a pathophysiological role of ATP, prompting $P2Y_1$ receptors antagonists as potential candidates to arrest the evolution of Alzheimer's disease.

Resumo

No sistema nervoso central (SNC), o ATP é libertado exocitoticamente de uma forma dependente da frequência. Extracelularmente, o ATP pode activar quer receptores metabotrópicos P2Y, quer receptores ionotrópicos P2X, que são receptores abundantemente expressos no cérebro, nomeadamente no hipocampo. No entanto, o papel dos receptores P2 no SNC permanece por determinar. Por analogia com o papel bem definido do ATP como neurotransmissor no sistema nervoso periférico, assumiu-se que o ATP podia ter também um papel como neurotransmissor no SNC. Contudo, uma contribuição do ATP para a transmissão sináptica apenas foi demonstrada em algumas sinapses. Esta observação conduziu à hipótese de que o ATP deverá actuar principalmente como neuromodulador no cérebro, em particular ao nível pré-sináptico.

Para testar esta hipótese utilizou-se como modelo experimental terminais nervosos purificados, os quais permitem isolar os efeitos pré-sinápticos, para testar se a activação dos receptores P2 controla pré-sinapticamente a libertação estimulada de glutamato de terminais nervosos de hipocampo de rato. É demonstrado que análogos do ATP modulam de um modo bifásico a libertação estimulada de glutamato de terminais nervosos de hipocampo de rato, sendo a facilitação mediada pelos receptores $P2X_{1,2/3,3}$ (efeito antagonizado pelo NF023) e a inibição pelos receptors $P2Y_{1,2,4}$ (efeito antagonizado pelo reactive blue 2 e MRS2179 e mimetizado por Ins47953). A combinação da análise por RT-PCR de célula única de neurónios piramidais de hipocampo de rato, da análise por *Western blot* de uma fracção contendo as proteínas pré-sinápticas presentes na zona activa e da análise por imunocitoquímica dos terminais glutamatérgicos de hipocampo de rato revelou que os receptores P2 expressos pelos neurónios glutamatérgicos, localizados na zona activa e em terminais glutamatérgicos são precisamente $P2X_{1,2,3}$ e $P2Y_{1,2,4}$. Estes resultados demonstram que os receptores P2 estão localizados pré-sinapticamente e a sua activação modula a libertação de neurotransmissores.

A identificação e caracterização de um papel neuromodulador dos receptores P2 sinápticos, conjuntamente com o facto de ocorrer uma libertação elevada de ATP para o espaço extracelular após um evento citotóxico levou ao estudo de um possível envolvimento dos receptores P2 na doença neurodegenerativa crónica mais comum, a doença de Alzheimer, cuja etiopatologia é caracterizada inicialmente por alterações ao nível sináptico no córtex e hipocampo, que ocorre antes da observação de morte neuronal. As sinapses aparentam ser os alvos primordiais dos péptidos Aβ, os quais se presume serem os principais agentes tóxicos na doença de Alzheimer, causando um mau funcionamento das sinapses o qual é responsável pelo déficit cognitivo. Deste modo, a principal estratégia no desenvolvimento de terapias para a doença de Alzheimer consiste na descoberta de mecanismos capazes de prevenir a sinaptotoxicidade inicial causada pelo péptido $A\beta_{1-42}$, o que levou ao estudo de um possível envolvimento dos receptores P2 sinápticos. O bloqueio farmacológico dos receptores $P2Y_1$ em

neurónios de hipocampo de rato não só preveniu a morte neuronal causada pela presença de $A\beta_{1-42}$, bem como a sinaptoxicidade que precede essa morte neuronal. Este efeito protector foi também observado *in vivo*. Em roedores, a administração *intracerebroventricular* de $A\beta_{1-42}$ provocou uma diminuição na densidade de terminais nervosos no hipocampo e causou um déficit mnemónico, não tendo sido observado morte neuronal. Estes efeitos resultantes da administração de $A\beta_{1-42}$ não foram observados em ratinhos aos quais foi removido geneticamente o receptor $P2Y_1$. Para além disto, a administração de $A\beta_{1-42}$ quer a ratos, quer a ratinhos levou a um aumento da densidade dos receptores $P2Y_1$ localizados nas sinapses. Estes resultados demonstram que os receptores $P2Y_1$ sinápticos estão envolvidos e são essenciais para a acção neurotóxica dos péptidos $A\beta$. Estes resultados indicam que os antagonistas dos receptores $P2Y_1$ são potenciais candidatos para impedir a evolução da doença de Alzheimer, em particular numa fase inicial do desenvolvimento desta doença neurodegenerativa.

Também é demonstrado que os receptores P2 pré-sinápticos interactuam funcionalmente com outros sistemas neuromoduladores, particularmente com o sistema neuromodulador operado pelos receptores nicotínicos para a acetilcolina (nACh). Quer a análise por imunocitoquímica, quer a monitorização microfluorimétrica de $[Ca^{2+}]_i$ demonstraram que os receptores P2X e nACh estão simultaneamente presentes numa subpopulação de terminais nervosos de hipocampo de rato e estudos neuroquímicos permitiram definir farmacologicamente a existência de uma interacção funcional entre os receptores P2X e nACh no controlo da libertação de neurotransmissores. Esta conclusão é suportada essencialmente pela observação de um antagonismo cruzado entre os receptores P2X e nACh no controlo da libertação de glutamato de terminais nervosos de hipocampo de rato, envolvendo os receptores $P2X_1$, $P2X_{2/3}$, $P2X_3$ e $\alpha3\beta2$.

Este papel promíscuo dos receptores P2 foi reforçado pela demonstração da existência de um receptor híbrido $P2X_2$/NR2B no âmbito do estudo de uma possível interacção entre os receptores P2X e N-metil-D-aspartato (NMDA) para o glutamato. Contudo, este estudo estava inserido num objectivo mais vasto de testar a existência de receptores ionotrópicos híbridos compostos por subunidades de diferentes famílias de receptores. A subunidade NR2B tem um centro de ligação ao glutamato, mas não é capaz por si só de formar um canal. Todavia, em células HEK293, a co-expressão de $P2X_2$ recupera a função NR2B. Para além disto foi observado que os transientes de $[Ca^{2+}]_i$ induzidos pela aplicação de NMDA em células às quais se induziu a co-expressão das subunidades $P2X_2$ e NR2B são insensíveis a Mg^{2+}, são independentes de glicina e são prevenidos na presença de PPADS e D-AP5, antagonistas selectivos dos receptores P2 e NMDA, respectivamente. Estudos de co-imunoprecipitação demonstraram ainda a existência de uma associação física entre $P2X_2$ e NR2B, quer em células HEK293 transfectadas, quer em cérebro de ratinho. Estes resultados demonstram pela

primeira vez a existência de receptores $P2X_2$/NR2B híbridos compostos por subunidades de diferentes famílias de receptores, o que constitui um novo conceito de receptor.

Resumindo, esta tese apresenta evidências experimentais que indicam que os receptores P2 para o ATP têm essencialmente um papel neuromodulador ao nível pré-sináptico no cérebro, em particular no hipocampo. O ATP modula de um modo bifásico a libertação de neurotransmissores através da activação dos receptores P2Y e P2X. Estes receptores P2X são proteínas promíscuas que interactuam e controlam outros receptores ionotrópicos tais como receptores nACh e NMDA. Embora sejam necessários mais estudos para uma melhor compreensão do papel fisiológico dos receptores P2, também foi observado que o bloqueio dos receptores $P2Y_1$ preveniu o dano neuronal e a perda de memória em modelos da doença de Alzheimer. Estes resultados demonstram um papel patofisiológico do ATP, indicando os antagonistas dos receptores $P2Y_1$ como potenciais candidatos para impedir a evolução da doença de Alzheimer.

INTRODUCTION

The nervous system is basically an inter-cellular network presenting a high degree of complexity due to the diversity of the elements that compose it. The interaction between these elements is essentially restricted to specialized junctions (Ramon y Cajal, 1911) called synapses, where it is processed the chemically-mediated communication between neurons or between a neuron and an effector cell, denominated synaptic transmission. The synapse is a central element in brain function, because it is the only place of electrical discontinuity in neuronal networks that mostly relies on chemical transmission. Synaptic transmission is mediated by molecules denominated neurotransmitters that are released from the presynaptic cell, usually triggered by the arrival of electric stimuli called action potential to the nerve terminals, which then deliver the signal to the postsynaptic cell through the activation of membrane receptors. Neuromodulators are another class of neuroactive substances that, in contrast to neurotransmitters, do not convey information, but rather modulate the processing of transmission of that information.

1. Extracellular ATP

Adenosine 5´-triphosphate (ATP) is a multifunctional nucleotide discovered in 1929 (Lohmann, 1929), that is most important as a "molecular currency" of intracellular energy transfer (Lipman, 1941). ATP belongs to the purines' family, since it is composed by a purine (adenine), attached to a pentose (ribose), to which three phosphate groups are attached (Figure 1). Virtually all living organisms use ATP to transfer energy from energy generator to energy consumer systems, and thus ATP is involved in nearly all cellular activities. Furthermore, as an adenine nucleotide, it is also used as a building block of nucleic acids, the genetic material of the cell. Thus, due to these pivotal roles of intracellular ATP in cell life, the scenario of ATP being released out of the cell to the extracellular milieu, to function as an extracellular signalling molecule, might seem a non-sense. However, already in 1929, a seminal paper by Drury and Szent-Györgyi described the potent actions of exogenously added purine nucleotides and nucleosides, ATP and adenosine, on the heart and blood vessels. In the following years, further papers were published reporting effects of extracellular ATP in several systems/tissues (reviewed in Burnstock, 2006). However, it was only in 1972, when ATP was proposed by Geoffrey Burnstock to be a neurotransmitter in non-adrenergic, non-cholinergic nerves in the gut and bladder (Burnstock, 1972), that the concept of purinergic signalling started.

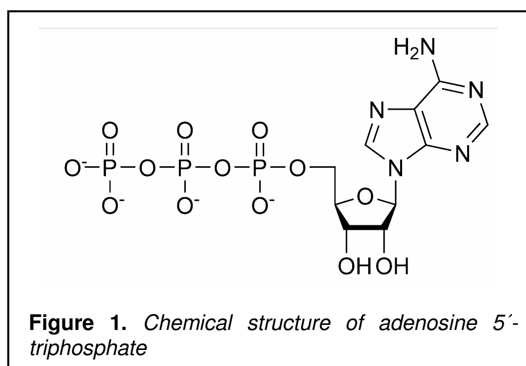


Figure 1. *Chemical structure of adenosine 5´-triphosphate*

In agreement with a role of ATP as an extracelular signaling molecule, the majority of the cells are endowed with ecto-enzymes capable to metabolize ATP in the extracellular space, denominated ecto-nucleotidases. Much is now known about the ecto-nucleotidases that breakdown the ATP released (Zimmermann, 2000). Several enzymes families are involved in the conversion of ATP into its metabolites ADP, AMP and adenosine: ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), of which NTPDase 1, 2, 3 and 8 are extracellular; ecto-nucleotide pyrophosphatase (E-NPP), which has 3 subtypes; alkaline phosphatases (AP) and ecto-5´-nucleotidase (Ecto-5'-NT). The catabolic actions of these enzymes are illustrated in figure 2.

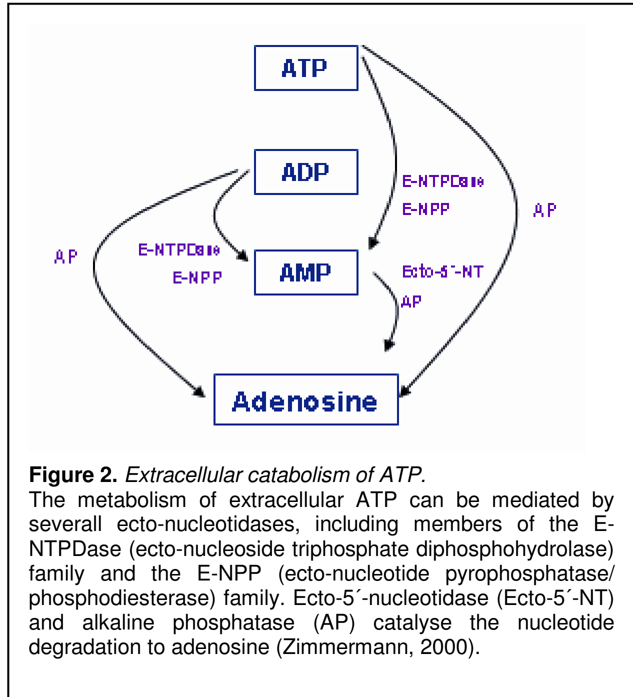


Figure 2. *Extracellular catabolism of ATP.*
The metabolism of extracellular ATP can be mediated by severall ecto-nucleotidases, including members of the E-NTPDase (ecto-nucleoside triphosphate diphosphohydrolase) family and the E-NPP (ecto-nucleotide pyrophosphatase/ phosphodiesterase) family. Ecto-5´-nucleotidase (Ecto-5´-NT) and alkaline phosphatase (AP) catalyse the nucleotide degradation to adenosine (Zimmermann, 2000).

Furthermore, it is also known now that the release of ATP via damaged cell membranes from injured or dying cells is not the only source of extracellular ATP. In fact there are multiple mechanisms discussed, by which adenine nucleotides can be released into the extracellular milieu. ATP is an ubiquitous intracellular metabolite, and thus every cell is a potential source of ATP. There is a large gradient for ATP efflux, transport or secretion out of the cells. Intracellular ATP concentrations are in the millimolar range, whereas extracellular ATP concentrations-estimates range from nanomolar to micromolar (Agteresch *et al.*, 1999; Schwiebert, 2000). Thus, besides the ATP leak via the damaged cell membrane of injured and dying cells, it was reported that ATP may cross intact cellular membranes of healthy cells by transmembrane transport using plasma membrane ATP transporters or channels, such as connexin hemichannels (Cotrina *et al.*, 2000), osmotic transporters linked to anion channels (Abdipranoto *et al.*, 2003; Darby *et al.*, 2003), or ATP-binding cassette (ABC) transporters (Schwiebert, 1999; Ballerini *et al.*, 2002). There is also compelling evidence for exocytotic vesicular release of ATP from neurons (Pankratov *et al.*, 2006), and from non-neuronal cells through intracellular granules (Gordon, 1986; Coco *et al.*, 2003), both mediated by Ca^{2+}-dependent membrane/vesicle fusion. It is now recognized that ATP release from many cells is a physiological as well as a pathophysiological phenomena (Bodin and Burnstock, 2001).

Besides the existence of mechanisms of release of ATP and of extracellular enzymatic pathways to metabolize extracellular ATP, the purinergic signalling concept implies for the existence of membrane receptors designed to respond to purines, in order to transduce the signal into the intracellular space. In the early nineties, when purinergic receptors were cloned and molecularly identified, the purinergic signalling was finally consolidated.

There is now strong molecular, cellular and systems-level evidence for extracellular ATP signalling during physiological processes, placing ATP signalling at centre stage as widespread facet of cell sensing in diverse organisms. Apparently, evolution has assured that ATP is indispensable inside cells, and as a signalling molecule between them.

2. Purinergic receptors

In the extracellular space, ATP can produce its biological effects *per se* through the activation of P2 receptors or through its metabolites ADP, which also activates some P2 receptors, and adenosine, through the activation of P1 receptors, both formed by the extracellular catabolism mediated by ecto-nucleotidases (Figure 2). This division in P1 receptors, at which adenosine is the principal natural ligand, and P2 receptors recognizing ATP and ADP was based in the relative potencies and affinities of ATP, ADP, AMP and adenosine, but mainly by the selective antagonism of the effects of adenosine by methylxanthines, such as theophiline or caffeine, observed by Satin and Rall (1970). Nowadays, this major division remains a fundamental part of purine receptor classification, although P1 and P2 receptors are now characterised primarily according to their distinct molecular structures, supported by evidence of different effectors systems and pharmacological profiles (for review see Ralevic and Burnstock, 1998).

2.1 Adenosine P1 receptors

The adenosine (P1) receptor family comprises A_1, A_{2A}, A_{2B} and A_3 adenosine receptors, identified by convergent molecular, biochemical and pharmacological data (Table 1).

The existence of A_1 and A_2 receptors was first described by van Calker *et al.* in 1979, showing that activation of these receptors by adenosine inhibited, via A_1, or facilitated, via A_2, adenylate cyclase activity. A_2 receptors are further subdivided into types A_{2A} and A_{2B}, which was originally based on the recognition that adenosine-mediated stimulation of adenylate cyclase in rat brain was affected via receptors with distinct affinities (Daly *et al.*, 1983), latter termed A_{2A} and A_{2B} (Bruns *et al.*, 1986). The existence of these receptors, predicted from extensive pharmacological data, was definitively confirmed by their cloning and sequencing (Furlong *et al.*, 1992; Mahan *et al.*, 1991; Stehle *et al.*, 1992). The fourth receptor A_3 was more unexpected (Zhou *et al.*, 1992). By now these four adenosine receptors have been cloned from several

mammalian and non-mammalian species, and their functional characterisation in heterologous expression systems, largely confirms the data gathered in native tissue (Fredholm *et al*., 2001).

Table 1. *Some properties of the adenosine P1 receptors (Fredholm et al., 2001 and 2003).*

	A_1	A_{2A}	A_{2B}	A_3
G protein-coupling	G_i, G_o	G_s, G_{olf}	G_s, G_q	G_i
Effects	↓cAMP ↓Ca^{2+}-currents ↑IP_3 ↑MAP Kinase ↑K^+	↑cAMP ↑MAP Kinase ↑PKC	↑cAMP ↑IP_3 ↑/↓MAP Kinase	↓cAMP ↑IP_3 ↑MAP Kinase
Selective agonists	CPA, CCPA CHA	CGS21680 HE-NECA CV-1808, CV-1674	None	Cl-IB-MECA
Selective Antagonists	DPCPX	SCH58261 ZM241385	MRS1754	MRS1220, MRS1191

All adenosine receptors couple to G proteins and, in common with other G protein-coupled receptors, they have seven putative transmembrane domains, the N-terminal lying on the extracellular side and the C-terminal on the cytoplasmic side of the membrane. With respect to signal transduction, adenosine receptors have been traditionally divided into two broad groups, A_1 and A_3, negatively coupled to adenylate cyclase via G_i protein, and A_{2A} and A_{2B}, positively coupled to adenylate cyclase via G_s protein (Fredholm *et al*., 2000). However, it should be kept in mind that this division based on G-protein coupling is being questioned because all adenosine receptors have been shown to couple to different G proteins operating via different signal transducing pathways (see Cunha, 2005).

The existence of selective pharmacological tools (Table 1) has greatly improved the knowledge on adenosine receptors function in the last decade, and several physiological and pathophysiological roles have been ascribed to the different adenosine receptors (Fredholm *et al*., 2000 and 2003).

2.2 P2 receptors

Purinergic P2 receptors are divided in two main classes, P2X and P2Y receptors, a nomenclature originaly used in a subdivision of P2 receptors based on pharmacological criteria (Burnstock and Kennedy, 1985), which upon the cloning and identification of the first P2 receptors, became the classification used nowadays, but now based on protein structure and signal transduction mechanisms: P2X receptors are ligand-gated cation channels and P2Y receptors are metabotropic receptors, belonging to the superfamily of G protein coupled

receptors with seven transmembranar domains (Abbracchio and Burnstock, 1994; Fredholm *et al.*, 1994).

2.2.1 Ionotropic P2X receptors

P2X receptors are ligand-gated cation channels, which, upon activation by the principal endogenous ligand ATP (at the micromolar range), mediate rapid (< 10 ms) opening of a non-selective cation pore, with almost equal permeability to Na^+ and K^+ and significant permeability to Ca^{2+} (North, 2002). Seven mammalian P2X receptor subunits $P2X_{1-7}$ have been cloned so far, containing 379 ($P2X_6$) to 595 ($P2X_7$) aminoacid residues (Ralevic and Burnstock, 1998). The analysis of their hydrophobicity pattern predicts two transmembrane-spanning regions, the first being involved with channel gatting and the second lining the ion pore, placing the N- and C-terminal on the intracellular compartment. The C-terminus has consensus binding-motifs for protein kinases responsible for the kinetics, permeation and desensitization of the channels (Khakh, 2001). The large extracellular loop, with ten conserved cysteine residues forming a series of disulphide bridges, contains the ATP binding sites and sites for antagonists and modulators (Khakh, 2001). Biochemical evidence indicates that P2X receptors are trimeric or hexameric combinations of P2X subunits (Nicke *et al.*, 1998). Thus, based on gene and protein sequence and in the predicted channel structures, P2X receptors constitute another family of mammalian ligand-gated cation channels, in addition to the nicotinic-receptor like family and glutamate-receptor like family (Khakh, 2001) (Figure 3).

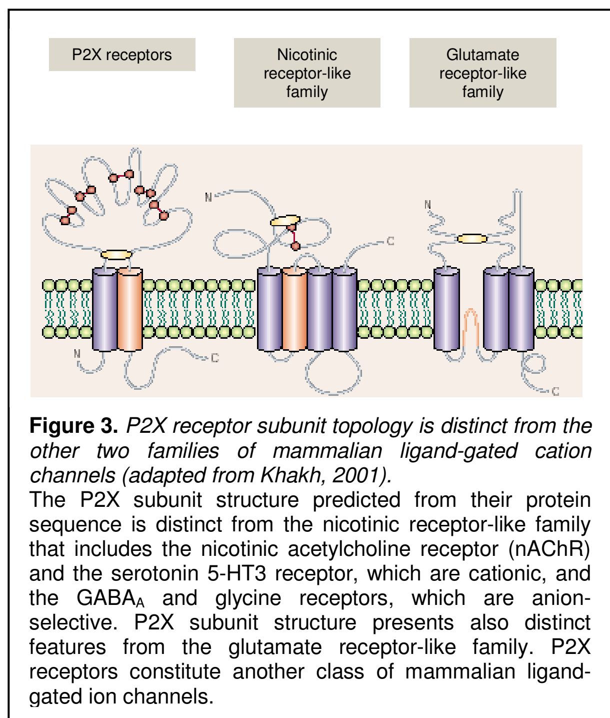


Figure 3. *P2X receptor subunit topology is distinct from the other two families of mammalian ligand-gated cation channels (adapted from Khakh, 2001).*
The P2X subunit structure predicted from their protein sequence is distinct from the nicotinic receptor-like family that includes the nicotinic acetylcholine receptor (nAChR) and the serotonin 5-HT3 receptor, which are cationic, and the $GABA_A$ and glycine receptors, which are anion-selective. P2X subunit structure presents also distinct features from the glutamate receptor-like family. P2X receptors constitute another class of mammalian ligand-gated ion channels.

Functional and biochemical data gathered by studies on recombinant receptors established several functional homomultimers and heteromultimers P2X receptors. Seven homomeric $P2X_{1-7}$ receptors have been functionally characterised, although there are some doubts if the homomeric assembly of $P2X_6$ subunits actually forms a functional homomultimer receptor, since it has been reported only in a small fraction of transfection experiments (North and Surprenant, 2000). Also, several heteromultimers have already been functionally characterised: $P2X_{2/3}$ (Radford *et al.*, 1997), $P2X_{4/6}$ (Lê *et al.*, 1998a), $P2X_{1/5}$ (Lê *et al.*, 1999), $P2X_{2/6}$ (King *et al.*,

2000) and more recently $P2X_{1/2}$ (Brown *et al.*, 2002) and $P2X_{1/4}$ (Nicke *et al.*, 2005). However, more heteromeric P2X receptors may come into play, since Torres *et al.* (1998) demonstrated biochemically that more pairs of subunits are potentially able to assemble. As illustrated in table 2, with the exception of $P2X_7$, all the other subunits can also form heteromeric channels.

The P2X receptors so far described can be divided in three different groups according to their kinetic and pharmacological profiles. The $P2X_1$ and $P2X_3$ receptors present high sensitivity to α,β-MeATP (EC_{50} ~ 1 µM) and to ATP (EC_{50} ~ 1 µM), in comparison to other P2X receptors, as summarized in table 3. Another particular feature of both $P2X_1$ and $P2X_3$ is their rapid desensitization during agonist application (1-2 s) (North and Surprenant, 2000; North, 2002).

The second class of P2X receptors includes $P2X_2$, $P2X_4$ and $P2X_5$. They show lower sensitivity for ATP (EC_{50} ~ 10 µM) and a very low sensitivity to α,β-MeATP (> 100 µM), and they display a slow desensitization kinetics within 1-2 s time-scale. There are no selective ligands for these receptors (Table 3). However, $P2X_2$ receptors are the only P2X receptor at which the response to ATP is increased by acidification of the extracelullar solution, which together with the potentiation of $P2X_2$ receptor-mediated current by low micromolar concentrations of both zinc and copper, allows distinguish $P2X_2$ from $P2X_4$ receptors-mediated responses (North and Surprenant, 2000; North, 2002).

Table 2. *Potential assembly of P2X receptor subunits (Torres et al., 1998).*

	$P2X_1$	**$P2X_2$**	**$P2X_3$**	**$P2X_4$**	**$P2X_5$**	**$P2X_6$**	**$P2X_7$**
$P2X_1$	+	+	+	+	+	+	-
$P2X_2$		+	+	-	+	+	-
$P2X_3$			+	-	+	-	-
$P2X_4$				+	+	+	-
$P2X_5$					+	+	-
$P2X_6$						-	-
$P2X_7$							+

The third group of P2X receptors is represented only by the $P2X_7$ receptor. This receptor has very low sensitivity for ATP (EC_{50} > 300 µM) and shows little or no desensitization. In fact the prolonged activation of $P2X_7$ receptor leads to a change of its permeability to organic cations and small peptides (Surprenant *et al.*, 1996), a phenomena also reported for $P2X_2$ and $P2X_4$ (Khakh *et al.*, 1999; Virginio *et al.*, 1999). One particular pharmacological characteristic of $P2X_7$ receptors is the higher potency of the ATP analogue BzATP, in comparison to ATP. BzATP is not selective for $P2X_7$ receptors and other P2X receptors are also activated by BzATP, but at these P2X receptors, ATP and BzATP are equipotent or less potent than ATP (North and Surprenant, 2000). Another particular characteristic of $P2X_7$ receptors, often used to discriminate $P2X_7$ receptor function, is its sensitivity to Mg^{2+}, which at the micromolar range, causes a decrease in the amplitude of the current mediated by $P2X_7$ receptor (Surprenant *et al.*, 1996).

In relation to the heteromeric receptors so far described, interestingly, it seems that there is a rule concerning the heteromeric receptors containing $P2X_1$ or $P2X_3$ subunits in relation to their pharmacological profile and kinetics. These heteromeric receptors present a similar pharmacological profile of the homomeric $P2X_1$ and $P2X_3$ receptors, sharing a high sensitivity to α,β-MeATP (EC_{50} ~ 1 μM), and a slow desensitization kinetics characterisitic of their P2X subunit partner. The discovery of further functional heteromeric receptors will answer if this is in fact a rule or just a coincidence.

Table 3. *Agonist and antagonist potencies at cloned P2X receptors.*
The values presented are EC_{50} for agonists and IC_{50} for antagonists at micromolar concentration, unless otherwise stated. $P2X_{2/6}$, $P2X_{1/2}$ and $P2X_{1/4}$ are not presented due to the lack of data reported for these receptors. However, the data provided so far for $P2X_{1/2}$ and $P2X_{1/4}$ receptors indicates for a similar pharmacological profile of homomeric $P2X_1$ and $P2X_3$ receptors (North and Surprenant, 2000; North, 2002; Jiang *et al.*, 2000; Braun *et al.*, 2001).

		$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_7$	$P2X_{2/3}$	$P2X_{1/5}$	$P2X_{4/6}$
Agonists	**ATP**	1	10	1	10	10	100	1	1	10
	α,β-MeATP	1-3	>100	1	>>100	>100	>>300	1	5	30
	BzATP	3	30	-	-	> 500	3	-	-	-
Antagonists	**Suramin**	1	10	3	>300	4	500	-	-	-
	PPADS	1	1	1	>300	3	50	5	-	-
	NF023	200 nM	100	1	>100	-	-	1	-	-
	TNP-ATP	6 nM	1	1 nM	15	-	>30	7 nM	200 nM	-
	Brilliant blue G	>10	>10	>10	>10	-	10 nM	>10	>10	-
	NF449	0.3 nM	47	1.8	>300	-	-	0.3	0.7 nM	-

There are not good selective pharmacological tools to discriminate between each P2X receptor subtype, not even to distinguish between P2X- from P2Y-mediated effects. The availability of selective antagonists is even more critical in order to address the functional role of endogenous ATP at P2X receptors. PPADS and suramin are the most used antagonists, but they are not selective for P2X receptors, and also block P2Y receptors. Both PPADS and suramin are not only non-selective but also non-universal, since PPADS and suramin show a very low potency at $P2X_4$ receptor (IC_{50} > 300 μM) and $P2X_7$ receptor has a very low sensitive to suramin (IC_{50} ~ 500 μM) (Table 3). Furthermore, it has been shown that suramin also blocks other ion channel receptors at concentrations similar to those that block P2X receptors (Nakazawa *et al.*, 1995; Peoples and Li, 1998). However, more useful antagonists have been developed, which present a higher selectivity for some P2X receptors: TNP-ATP (Evans *et al.*, 1995) and NF023 (Soto *et al.*, 1999) for $P2X_1$- and $P2X_3$-containing receptors, NF449 (Braun *et al.*, 2001) for $P2X_1$ and $P2X_{1/5}$ receptors and Brilliant blue G for rodent $P2X_7$ receptors (Jiang *et al.*, 2000) (Table 3).

2.2 Metabotropic P2Y receptors

P2Y receptors are metabotropic receptors for extracellular nucleotides. Eight different mammalian P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) receptors subtypes have been cloned so far (Abbracchio *et al.*, 2006). These metabotropic receptors have the general signature of seven membrane spanning regions with an extracelullar N-terminus and an intracellular C-terminus. There is a high level of sequence homology between the hydrophobic transmembrane regions of the several P2Y receptors sub-types, in particular TM3, TM6 and TM7. On the other hand, the intracellular loop and the C-terminus posses a structural diversity among the P2Y receptor subtypes, which influences the degree of coupling with $G_{q/11}$, G_S and G_i, the G proteins so far reported to be coupled to P2Y receptors (Abbracchio *et al.*, 2006). Our current knowledge indicates that each P2Y receptors binds to a single heterotrimeric G protein, tipically $G_{q/11}$, although $P2Y_{11}$ can couple to both $G_{q/11}$ and G_S, whereas $P2Y_{12,13,14}$ couple to G_i solely. $P2Y_{1,2,4,6,11}$ receptors couple to members of the $G_{q/11}$ family and stimulate phospholipase C (PLC) resulting in the formation of inositol-(1,4,5)- triphosphate (IP3) and diacylglycerol, with subsequent mobilization of Ca^{2+} from intracelullar stores. $P2Y_{12,13,14}$ couple to G_i proteins inhibiting adenylate cyclase (summarized in table 4; reviewed in Abbracchio *et al.*, 2006)). In addition, it has been reported that native P2Y receptors may activate phospholipases A_2 and D, mitogen-activated protein kinases (MAPK), tyrosine kinase and the serine-threonine kinase Akt (reviewed in Lazarowski *et al.*, 2003).

Table 4. *Characteristics of P2Y receptors: pharmacological profiles and signal transduction mechanisms* (reviewed in Lazarowski *et al.*, 2003; Burnstock and Knight, 2004; Abbracchio *et al.*, 2006; for Ins45973 and Ins415 see Shaver *et al.*, 2005).

Receptor	Agonists	Antagonists	Transduction mechanisms
$P2Y_1$	2-MeSADP=ADPβS>2-MeSATP=ADP>ATP	MRS2179, MRS2500	G_q/G_{11}; PLCβ activation
$P2Y_2$	UTP=ATP, Ins45973	Suramin,RB2	G_q/G_{11} and possibly G_i; PLCβ activation
$P2Y_4$	UTP=ATP, Ins45973	RB2, Suramin, PPADS	G_q/G_{11} and possibly G_i; PLCβ activation
$P2Y_6$	UDP>UTP>>ATP Ins415	MRS2578	G_q/G_{11}; PLCβ activation
$P2Y_{11}$	BzATP=ATPγS>ATP	Suramin,RB2	G_q/G_{11} and G_S; PLCβ activation
$P2Y_{12}$	ADP=2-MeSADP>>ATP	CT50547, Ins49266, AR-C69931MX	$G_{i/o}$; inhibition of adenylate cyclase
$P2Y_{13}$	ADP=2-MeSADP>>ATP	MRS2211	$G_{i/o}$
$P2Y_{14}$	UDPglucose=UDPgalactose	-	$G_{i/o}$

P2Y receptor subtypes show a low level of sequence homology between them (19-55% identical) and, consequently, not only show significant differences at the signal transduction

mechanisms through which they operate, but also at their pharmacological profiles. In contrast to P2X receptors, P2Y receptors not only bind to ATP, but ADP and the pyrimidine nucleotides UTP, UDP and UDP-glucose are also endogenous ligands of some P2Y receptors. $P2Y_{1,11,12,13}$ receptors are selectively activated by adenine nucleotides, being $P2Y_{11}$ solely activated by ATP and $P2Y_{1,12,13}$ activated also by ATP, but at a higher potency by ADP (Webb *et al*., 1993; Communi *et al*., 2001; Hollopeter *et al*., 2001; Lazarowski *et al*., 2003). $P2Y_{2,4}$ receptors respond in an equipotent manner to ATP and UTP in rat (Lustig *et al*., 1993; Bogdanov *et al*., 1998). $P2Y_6$ receptors are activated by UDP and at a much lesser potency by UTP and ATP (Chang *et al*., 1995). $P2Y_{14}$ receptors respond to UDP-glucose and to other sugar nucleotides (Abbracchio *et al*., 2003).

Several agonists and antagonists have been developed for the several P2Y receptors subtypes, in particular for $P2Y_1$ and $P2Y_{12}$ receptors due to their major role in platelets function (Gachet, 2006). At mammalian $P2Y_1$ receptors, 2-MeSADP is a potent agonist (Hechler *et al*., 1998) and MRS2179 (Boyer *et al*., 2002) and the recently developed MRS2500 (Hechler *et al*., 2006) are potent antagonists. In addition to $P2Y_1$ receptor, 2-MeSADP is also a high potent agonist at $P2Y_{12}$ and $P2Y_{13}$ (Hollopeter *et al*., 2001; Communi *et al*., 2001). In table 4 is summarized the current status of P2Y receptor subtype agonists and antagonists, as well as their transduction mechanisms.

3. <u>ATP in the central nervous system</u>

Since the seminal paper by Drury and Szent-Györgyi (1929), in which it was first shown extracellular ATP mediated effects, several papers reported other effects of extracellular ATP, some of them in nervous tissue (reviewed in Burnstock, 2006). The first hint of a transmitter role for ATP in the nervous system was provided by Pamela Holton in 1959, by demonstrating the release of ATP during antidromic stimulation of sensory nerves. However, the acceptance of a transmitter role of ATP in the autonomic nervous system mostly stems from the persistent work of Burnstock since 1960s, when it was proposed ATP as a neurotransmitter in non-cholinergic and non-noradrenergic nerves in the gut and bladder, raising the concept of purinergic transmission (Burnstock, 1972).

In the central nervous system (CNS), ATP is the main adenine nucleotide released from nerve terminals upon stimulation (White, 1977; Potter and White, 1977). Several lines of evidence indicate that ATP in central presynaptic terminals is accumulated and stored in the synaptic vesicle (Sperlagh and Vizi, 1996), and can be released and/or co-released via exocytosis (Pankratov *et al*., 2006), in a frequency dependent-manner (Wierasko *et al*., 1989; Cunha *et al*., 1996). Other brain compartments, such as glial cells (*e.g.* Caciagli *et al*., 1988; Queiroz *et al*., 1997; Coco *et al*., 2003) and postsynaptic structures (Inoue *et al*., 1995; Hammann *et al*., 1996) may also contribute to ATP release. Once in the extracellular milieu,

ATP acts directly through the activation of P2 receptors (Ralevic and Burnstock, 1998). In addition it can act as a substrate for ecto-protein kinases (Wierasko and Enrlich, 1994), or it can be converted through ecto-nucleotidases into adenosine (Zimmermann, 2000), with subsequent activation of P1 (adenosine) receptors, highly abundant in the brain (Fredholm *et al.*, 2005).

Likewise, the CNS also displays a robust expression of both P2X and P2Y receptors. *In situ* hybridisation and Northern blot studies indicate the expression of mRNA in the CNS of $P2X_1$ and $P2X_2$ (Collo *et al.*, 1996), $P2X_3$ (Séguela *et al.*, 1996; Vulchanova *et al.*, 1998), $P2X_4$ (Kidd *et al.*, 1995; Buell *et al.*, 1996; Soto *et al.*, 1996), $P2X_5$ and $P2X_6$ subunits (Kidd *et al.*, 1995; Collo *et al.*, 1996). The mRNA expression of $P2X_7$ subunit in the CNS was also detected, but it was reported to be restricted to glial cells (Collo *et al.*, 1997). The transcripts enconding for $P2Y_1$ (Moore *et al.*, 2001), $P2Y_2$ (Lustig *et al.*, 1993), $P2Y_4$ (Webb *et al.*, 1998), $P2Y_6$ (Chang *et al.*, 1995), $P2Y_{11}$ (Moore *et al.*, 2001), $P2Y_{13}$ (Communi *et al.*, 2001; Zhang *et al.*, 2002) and $P2Y_{14}$ receptors (Fumagalli *et al.*, 2003) were also detected in the CNS. Cellular resolution of the expression of $P2Y_{12}$ receptor mRNA was obtained by *in situ* hybridisation histochemistry of brain sections and the observations are consistent with a glial expression pattern (Hollopeter *et al.*, 2001) as observed for $P2X_7$ receptor. Immunohistochemical studies have reported the presence of all P2X receptors in several CNS structures. For instance, immunohistochemical analyses revealed a widespread distribution of $P2X_4$ (Lê *et al.,* 1998b) and $P2X_2$ receptors (Kanjhan *et al.*, 1999), the presence of $P2X_1$ receptors in the cerebellum (Loesch and Burnstock, 1998), $P2X_3$ receptors in the midbrain (Díaz-Hernandez *et al.*, 2001) and the receptors subunits $P2X_{2,4,6}$ in the cerebellum and hippocampus (Rubio and Soto, 2001). Besides its glial localization (Franke *et al.*, 2001), $P2X_7$ receptors were recently shown to be located in neurons as well (Deuchars *et al.*, 2001; Miras-Portugal *et al.*, 2003), although some studies cast some doubts on the selectivity of the most commonly used antibodies against $P2X_7$ receptors (Sim *et al.*, 2004). $P2Y_1$ receptors have been shown to be widely distributed in the human and rat brain (Moore *et al.*, 2000a; Moran-Jimenez and Matute, 2000). This widespread distribution of $P2Y_1$ in rodent brain was recently confirmed by binding assays using the radio labelled [^{32}P]-MRS2500, a new recently developed selective antagonist for $P2Y_1$ receptors (Houston *et al.*, 2006). Autoradiographic and membrane binding studies with [^{3}H]-α,β-MeATP, which mostly labels $P2X_1$- and $P2X_3$-containing receptors, reveal abundant high affinity labelling in different CNS areas (Michel and Humphrey, 1993; Schafer and Reiser, 1997; Worthington *et al.*, 1998). Similar studies with thio-labelled ATP analogues ([^{35}S] deoxyATPαS, [^{35}S] ATPγS and [^{35}S] ATPαS) have claimed to label metabotropic P2Y receptors in the CNS (Webb *et al.*, 1998; Schafer and Reiser,1997; Pintor *et al.*, 1993; Simon *et al.*, 1997). In summary, both P2X and P2Y receptors are found in the CNS in both neuronal (for review see Illes and Ribeiro, 2004) and non-neuronal elements such as astrocytes and microglia (Abbracchio and Verderio, 2006). Thus, the existence of several different sources of extracellular ATP and the widespread

distribution of P2 receptors in the CNS allow hypothesising several possible roles for extracellular ATP in the CNS.

Astrocytes are endowed with both P2X and P2Y receptors and there is a growing evidence that ATP through the activation of these P2 receptors is involved in glia-glia and glia-neuron communication (reviewed in Fields and Burnstock, 2006), as well as in astrocyte proliferation as a trophic factor (reviewed in Neary *et al.*, 2006). P2 receptors have also been implicated in chemotaxis and in the control of microglia reactivity (reviewed in Farber and Kettenman, 2006).

Concerning the neuronal P2 receptors and the neuroactive role of ATP, by analogy with the well stablished neurotransmitter role of ATP in the autonomic nervous system (Burnstock, 1990), it has been assumed that ATP would also be a fast neurotransmitter in CNS. However, evidence for the existence of ATPergic transmission has been so far demonstrated in the *medial habenula* (Edwards *et al.*, 1992), in the spinal cord (Bardoni *et al.*, 1997), in the *locus coeruleus* (Nieber *et al.*, 1997), the *nucleus tractus solitarius* (Thomas and Spyer, 2000) and in the somato-sensory cortex (Pankratov *et al.*, 2002b and 2003). In the hippocampus, one of the areas expressing the highest levels of P2 receptors, ATP mediates only a minor component of excitatory transmission in glutamatergic synapses (Pankratov *et al.*, 1998; Mori *et al.*, 2001). This marked mismatch between the robust ATP release, mRNA expression and P2 receptors density in the CNS with the lack of a clear neurotransmitter role for extracellular ATP, led to the hypothesis that, despite some few synapses, P2 receptors should mainly behave as a neuromodulatory system in the brain (Cunha and Ribeiro, 2000).

Accordingly, unlike other neurotransmitter systems, the hallmark of which is their exquisite specificity, ATP signalling is characterised by its promiscuity. As described above, ATP is not only released by the presynaptic terminal, but can be also released by postsynaptic compartments and other cells. Moreover, whereas classical neurotransmitters are regulated simply by the release and removal of transmitters from the synaptic cleft, each of the products of ATP hydrolysis can activate different types of receptors; P2 receptors bind ATP and ADP and P1 receptors bind adenosine, an ubiquitous neuromodulator in the brain (Fredholm *et al.*, 2005). Also, there are several experimental data indicating that ATP is co-released with other neurotransmitters like glutamate and GABA (Pankratov *et al.*, 2006) and acethylcholine (Richardson and Brown, 1987), suggested to be an universal feature of central synapses (Pankratov *et al.*, 2006). This places ATP in several distinct synaptic populations. Another interesting feature of P2X receptors that re-enforce the possible neuromodulatory role operated by these receptors is their promiscuity, since it has been shown that P2X receptors can interact with different ionotropic receptors, such as $GABA_A$ receptors (Boue-Grabot *et al.*, 2004; Sokolova *et al.*, 2001), 5-HT3 receptors (Boue-Grabot *et al.*, 2003) and eventually NMDA (Pankratov *et al.*, 2002a; Peoples and Li, 1998) and AMPA/kainate receptors (Zona *et al.*, 2000). This promiscuity suggests that the signalling of ATP through the activation of P2

receptors may be designed not only to modulate efector systems, but also other modulatory systems. This evidence together with the growing evidence of a pivotal role of ATP in cell-cell communication clearly suggest that ATP is designed and well suited to modulate rather to convey information in the CNS.

The scenario of ATP to function essentially as a neuromodulator, in particular through the activation of presynaptic P2Y receptors in the control of neurotransmitter release is well supported because the most extensively studied types of presynaptic receptors were the G-protein-coupled seven transmembrane domain receptors (Langer, 1997; Miller, 1999). Presynaptic metabotropic receptors are well accepted as presynaptic modulatory receptors probably due to their considerable slower speed of signalling in comparison to ionotropic receptors. With respect to ionotropic receptors, it was mostly considered that, since ionotropic receptors are designed to mediate fast signals, they should be involved in fast transmission and there were no reasons to seek other possible roles. Thus, the knowledge of the molecular characteristics of ionotropic receptors and the study of their location would be enough to predict where and when a given ionotropic receptor would be active. But this strategy has already failed in the case of two ionotropic receptors systems, the nicotinic acetylcholine receptors (nACh) and the kainate subtype of glutamate receptors. In these two systems, like for the P2 (ATP) receptor, there was a clear paradox between the abundant expression of receptor subunit mRNA and robust binding by selective ligands in the CNS and the inability to clearly identify transmission mediated by these receptors (Role and Berg, 1996; Chitajallu *et al.*, 1999). This led to the proposal that instead of being involved in fast neurotransmission, kainate and nicotinic receptors would mainly behave as neuromodulatory systems (Role and Berg, 1996; Malva *et al.*, 1998). Thus, a presynaptic locus of action of kainate and nicotinic receptors as modulators of neurotransmitter release (Malva *et al.*, 1998; Wonnacott, 1997) would reconcile a heavy expression and receptor density with their scarce involvement in fast transmission. In recent years a wealth of data has been gathered to support the localization of ligand-gated cation channels also in presynaptic nerve terminals where they function as modulatory systems (reviewed in Engelman and MacDermott, 2004). This has contributed to resolve the paradoxical findings that some ionotropic receptors were present in brain tissue although they were only episodically found to mediate synaptic transmission.

In fact, mechanistically, ATP can control neuronal function not only through the activation of metabotropic P2Y receptors by modulating for instance voltage-operated calcium channels (VOCCs) (Kulick and von Kugelgen, 2002) or other ion channels (Filippov *et al.*, 2003), through their second messenger cascades. It can also control neuronal function through the activation of ionotropic P2X receptors by modulatory actions in Ca^{2+} entry, either directly - P2X receptors are characterised by a high permeability to Ca^{2+} (Roger and Dani, 1995) - or indirectly by inducing small depolarizations modulating the triggering of sodium-dependent action potentials

and/or the open-state of VOCCs (see Hur *et al.*, 2001), which results in a modulatory action in the neurotransmitter release (see Khakh and Henderson, 2000; Engelman and MacDermott, 2004).

Thus, a presynaptic locus of action of P2 receptors as modulators of neurotransmitter release would explain the contradiction between the heavy expression and the robust P2 receptors density in neurons, with the difficulty of ascribing a clear role for ATP as a fast transmitter in the CNS.

3.1 ATP as a presynaptic neuromodulator in the central nervous system

Several studies have contributed to strenght the hypothesis that P2 receptors might be located and acting presynaptically in the brain. Immunohystochemical and electron microscopy analysis have reported that several P2X subunits are targeted to presynaptic terminals in the brain (Vulchanova *et al.*, 1997; Deuchars *et al.*, 2001; Díaz-Hernandez *et al.*, 2001 and 2002; Atkinson *et al.*, 2004) whereas a presynaptic localization of P2Y receptors in the brain was concluded from binding studies in rat brain synaptosomal membranes (Pintor *et al.*, 1993; Schäfer and Reiser, 1997; Simon *et al.*, 1997). Accordingly, several functional studies suggest that ATP may have a biphasic presynaptic neuromodulatory effect in the release of several types of neurotransmitters in different brain regions: inhibition via P2Y and facilitation via P2X receptors (reviewed in Cunha and Ribeiro, 2000), as observed in the peripheral nervous system (*e.g.* Boehm *et al.*, 1999). Unfortunately, most functional studies solely rely on the use of the limited number of P2 receptor ligands that have an insufficient selectivity to distinguish the different P2 receptor subtypes (Ralevic and Burnstock, 1998) instead of also attempting to use molecular or morphological approaches to identify the putative P2 receptors involved.

Furthermore, it has been difficult to unambiguously ascribe a clear involvement of P2 receptors in the majority of the presynaptic effects of ATP observed, because limited attempts were made to exclude the involvement of adenosine (formed upon extracellular catabolism of ATP by ecto-nucleotidases), an ubiquitous neuromodulator in the brain through the activation of P1 receptors (Fredholm *et al.*, 2005). Moreover, the use of P2 receptors antagonists does not allow distinguishing between P2 and P1 receptor-mediated responses, since most P2 receptor antagonists are inhibitors of ecto-ATPases (Ralevic and Burnstock, 1998). This is particularly critical when studying glutamatergic transmission, since adenosine efficiently inhibits excitatory transmission through the activation of A_1 receptors (Ribeiro, 1995). The catalytic efficiency of the ecto-nucleotidase pathway is such that activation of adenosine A_1 receptors can occur within milliseconds after iontophoretical application of ATP (Dunwiddie *et al.*, 1997). Furthermore, the channelling organisation between ecto-nucleotidases and adenosine A_1 receptors, *i.e.*, the ability of ATP-derived adenosine to activate A_1 receptors without equilibrating with the biophase (Cunha, 1997; Cunha *et al.*, 1998), makes it mandatory to

appropriately exclude the involvement of adenosine A_1 receptors in any presynaptic inhibitory effect of ATP (reviewed in Cunha, 2001). Facilitatory effects may also be confounded by facilitatory effects of adenosine via activation of adenosine A_{2A} receptors (Cunha, 2001; Lopes *et al.*, 2002; Almeida *et al.*, 2003).

Moreover, in studies using neurotransmitter quantification approaches in superfused slices and in microdialyses studies, it is difficult to distinguish between the direct involvement of presynaptic P2 receptor involvement from indirect circuit-mediated effects or from astrocyte-mediated effects that may lead to indirect modulation in synaptic function. For example, the activation of $P2X_7$ receptors on astrocytes mediates glutamate (Duan *et al.*, 2003) and GABA (Pannicke *et al.*, 2000) release, which are able to participate and modulate synaptic function (Newman, 2003).

P2X-receptors enhance the frequency of spontaneous fast excitatory postsynaptic currents in various brain stem nuclei (Khakh and Henderson, 1998; Kato and Shigetomi, 2001). Also, ATP increases presynaptic intracellular free calcium concentration in nerve terminals of different brain areas, in an extracellular calcium-dependent manner and, thus, apparently mediated by a P2X-like receptor (Troadec *et al.*, 1998; Pintor *et al.*, 1999, Díaz-Hernandez *et al.*, 2001). In the rat hippocampus where $P2X_2$, $P2X_4$ and $P2X_6$ mRNAs are highly expressed (Kidd *et al.*, 1995; Buell, 1996; Collo, 1996; Soto *et al.*, 1996; Kanjhan *et al.*, 1999), although immunogold studies showed that these subunits are strictly located at the excitatory postsynaptic specialization of Schaffer Collaterals in CA1 pyramidal cells (Rubio and Soto, 2001), it was latter reported a facilitatory effect mediated by presynaptic $P2X_2$-containing channels in the excitatory transmission from CA3 pyramidal cell selectively onto *stratum radiatum* interneurons (Khakh *et al.*, 2003). $P2X_7$ receptor was also shown to be targeted to central nerve terminals (Deuchars *et al.*, 2001; Miras-Portugal *et al.*, 2003), and in particular in the hippocampus it was reported to be located in mossy fibers nerve terminals in *stratum lucidum* of the CA3 region and in excitatory terminals in *dentate gyrus* (Sperlagh *et al.,* 2002; Atkinson *et al.*, 2004). Accordingly, extracellular and patch-clamp recordings in CA3 pyramidal cells of the hippocampus demonstrated that activation of presynaptic $P2X_7$ receptors depresses mossy fiber-CA3 synaptic transmission (Armstrong *et al.*, 2001). However, the involvement of presynaptic $P2X_7$ receptors on those effects remains elusive (Kukley *et al.*, 2004) and even the existence of neuronal $P2X_7$ receptors is still controversial due to the presumable lack of selectivity of the most commomly used antibodies against $P2X_7$ receptors (Sim *et al.*, 2004). Recently, it was elegantly demonstrated a tonic decrease in the frequency of mEPSP via presynaptic PTX-sensitive P2Y receptors (Zhang *et al.*, 2003). It was also observed a modulatory effect of activation of P2Y receptors on GABAergic inhibitory transmission. In particular it was reported an increase in the frequency of mIPSC via $P2Y_1$ receptor (Kawamura *et al.*, 2004) and that the activation of $P2Y_1$ receptor leads to an increase in synaptic inhibition

of the postsynaptic targets of interneurons in the hippocampus (Bowser and Khakh, 2004). However, there is still a lack of functional and molecular direct evidences that clearly identify a presynaptic localization and neuromodulatory action of P2 receptors in the brain.

AIM

1. Identification and characterisation of the neuromodulatory role of presynaptic P2 receptors in the control of glutamate release in the rat hippocampus: characterisation of the presynaptic P2 receptors involved.

2. Investigation of the role of synaptic P2 receptors in the etiopathology of Alzheimer's disease, in particular at the early synaptic failure: pathophysiological role of the neuromodulatory system operated by ATP.

3. Functional interaction between P2X and nACh receptors in the control of neurotransmitter release in the rat hippocampus: modulation of modulators as a new insight in the neuromodulatory role of ATP.

4. Identification and characterisation of putative $P2X_2$/NR2B receptors, the first heteromeric hybrid ionotropic receptor composed by subunits of distinct families of ionotropic receptors: a new concept of receptor signalling.

MATERIALS AND METHODS

1 BIOLOGICAL MODELS AND PREPARATIONS

1.1 Animals used

The animals used in the experiments presented in this thesis were male Wistar rats (6-8 weeks old) (Figure 4) obtained from Harlan Ibérica (Barcelona, Spain) and male C57BL/6 mice and $P2Y_1$ receptor knockout C57BL/6 mice (Leon *et al.*, 1999) (4-8 weeks old) that were kindly provided by Prof. Christian Gachet (INSERM U311, Strasbourg, France). The animals were handled according to European guidelines for use of experimental animals, the rats being anesthetized under halothane atmosphere, before being sacrificed by decapitation, unless otherwise stated. The primary cultures of hippocampal neurons were performed from 17-19 day-old Wistar rat embryos handled according to European guidelines as well.

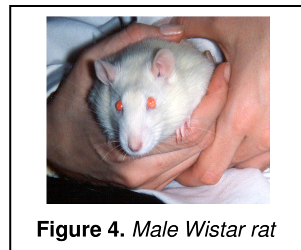


Figure 4. *Male Wistar rat*

1.1.1 Animal model of Alzheimer´s disease

The aim of the study designed to investigate the involvement of P2 receptors in neurodegeneration, in particular in the Alzheimer´s disease, was essentially to investigate the role of synaptic P2 receptors in the early events occurring in this neurodegenerative disease, such as synaptotoxicity and memory loss (see 2 of Results). Since these two early features of Alzheimer´s disease are thought to result from the increased levels of soluble Aβ, mainly $A\beta_{1-42}$ (Hardy and Selkoe, 2002; Klein *et al.*, 2004), it was used an injection of 2 nmol of $A\beta_{1-42}$ icv in rodents, to mimick the early synaptotoxicity and memory loss observed in Alzheimer´s disease, based on previously reported data (Dall'Igna *et al.*, 2007). In fact, 14 days after the single injection of 2 nmol $A\beta_{1-42}$ icv, both male Wistar rats and C57BL/6 mice presented a decrease in the density of nerve terminals and a mnemonic impairment as described in chapter 2 of Results.

After induction of avertine anesthesia (0.2 mg/ g of weight), rodents received a stereotaxic injection of 2 nmol of soluble $A\beta_{1-42}$ (American Peptides, Sunnyvale, USA) at the following coordinates: anteroposterior +3.0, dorsoventral -2.5, lateral +1.0. The control group received a stereotaxic injection of a same volume of the vehicle (Tris 0.05M, pH 7.4). The volume of 4 µl was injected at a rate of approximately 0.5 µl/ min and the cannula was left in place for an additional 3 minutes before being slowly retracted. Following a 15-day recovery period in the animal-care facility, the rats were submitted to behavioural studies (see 2.11 of Materials and Methods) to evaluate their mnemonic performance which was compared to their behavioural performance before icv injections. Only after were the several experiments performed with these animals.

1.2 Synaptosomal preparations

One of the aims of the study presented in this thesis was the investigation of the neuromodulatory role of ATP at the presynaptic level through the activation of P2 receptors. For that purpose, it was chosen the best model to study presynaptic phenomena, *i.e.* synaptosomes (Cunha, 1998). This preparation is constituted essentially by nerve terminals free of their integration in the neuronal networks (Gray and Whittaker, 1962). Synaptosomes are prepared by applying shear forces to nervous tissues (Gray and Whittaker, 1962; Dunkley *et al.*, 1988), causing the separation of the presynaptic buttons, whose membranes re-seal, forming morphologically isolated (Gray and Whittaker, 1962) and biochemically autonomous vesicles (Marchbanks *et al.*, 1967). These vesicles can then be purified by gradient centrifugation using sucrose or percoll gradients.

Synaptosomes are the most basic preparation that still presents the property of releasing neurotransmitters in a Ca^{2+}-dependent manner and they are sufficiently structured to preserve: 1) a physiological plasma membrane potential -60 to -70 mV (Scott and Nicholls, 1980); 2) a basal intracellular Ca^{2+} concentration of 0.1-0.3 µM (Adam-Vizi and Ashley, 1987); 3) a stable energetic charge and a stable energetic metabolism (Scott and Nicholls, 1980); and 4) an intact intra-terminal compartimentalization, essentially mitochondria and synaptic vesicles (Gray and Whittaker, 1962; Dunkley *et al.*, 1988). Thus, synaptosomes can be considered as "regenerated mini-cells" specialized in the release of neurotransmitters (Verhage, 1990). Furthermore, synaptosomes constitute the only preparation that allows isolating presynaptic effects (Nicholls, 1989).

The release of neurotransmitters is mostly triggered by depolarization of the presynaptic membrane that occurs when an action potential reaches the nerve terminal, which in turns causes the opening of voltage-operated Ca^{2+} channels (VOCCs), which are embedded in the presynaptic membrane, and Ca^{2+} rushes in. This influx of calcium ions at the presynaptic active zone triggers a series of events, which ultimately results in the release of the neurotransmitter from a storage vesicle into the synaptic cleft. Due to their small size, it is virtually impossible to stimulate synaptosomes electrically, because it would be necessary to apply an elevated current, which would end in a great thermal change. Thus, in order to stimulate synaptosomes, it is necessary to rely on electrochemical properties of the process of action potential conductance. Changing the electrochemical potentials of some ions allows changing the membrane potential and thus to stimulate synaptosomes (see Nicholls, 1989). The most popular strategy to depolarize synaptosomes is to elevate K^+ concentration (Scott and Nicholls, 1980). Although this strategy bypasses presynaptic Na^+-channel activation, the physiological trigger of an action potential dependent Ca^{2+}-entry due to the immediate clamping of the membrane potential in the depolarized state, is an efficient way to depolarize synaptosomes chemically. Another method to

raise extracellular K^+ concentration is to use 4-aminopyridine, which is a K^+-channel blocker (Tibbs *et al.*, 1989). An alternative method to the elevation of extracellular K^+ concentration is veratridine, which depolarizes synaptosomes by activating voltage-gated Na^+-channels (Blaunstein, 1975). However, it has some disadvantages in comparison to the elevation of extracellular K^+ concentrations, in particular the fact that, using veratridine, the increase in the intracellular Ca^{2+} concentration is much slower and continues to rise over a period of minutes, in contrast with the initial peak and partial recovery seen after elevated K^+ depolarization (Adam-Vizi and Ashley, 1987). In the present study, the chemical stimulation strategy used to depolarize synaptosomes, and thus evoke both [^{3}H]-glutamate release and a transient raise in the free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, was the elevation of the extracellular K^+ concentration. It was used an isomolar substitution of NaCl by 20 mM of KCl in the Krebs solution (see 2.1 and 2.2 of Materials and Methods).

1.2.1 Partially purified synaptosomes (P2 fraction)

For $[Ca^{2+}]_i$ measurements in synaptosomal populations, a partially purified synaptosomal fraction (P2) was isolated from hippocampi of male Wistar rats (2-month-old), essentially as described previously (Malva *et al.*, 1995). Briefly, rat hippocampi were homogenised in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, and centrifuged at 3,000 *g* for 2 min. The pellet obtained was resuspended, followed by sedimentation at the same speed. The combined supernatants were spun for 12 min at 14,000 *g*, and a P2 pellet was obtained. The upper, whiter, layer of this pellet, a mitochondrial free fraction, was resuspended in the sucrose-HEPES medium and aliquots of 0.75 mg were pelleted and kept on ice.

1.2.2 Synaptosomes purified by a 45% percoll gradient

For the release of [^{3}H]-glutamate assay, rat hippocampal nerve terminals were prepared using a combined sucrose/Percoll centrifugation protocol as previously described (Lopes *et al.*, 2002). Briefly, hippocampal tissue was homogenised in a sucrose-HEPES medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% BSA and 10 mM HEPES (pH 7.4). The homogenate was spun for 10 min 3,000 *g* at 4 ºC and the supernatant spun again at 14,000 *g* for 12 min. The pellet (P2 fraction) was resuspended in 1 ml of Percoll 45% (v/v) in Krebs-HEPES-Ringer (KHR) medium (in mM: NaCl 140, EDTA 1, KCl 5, glucose 5 and HEPES 10, pH 7.4) and spun again at 14,000 *g* for 2 min. Synaptosomes were then removed from the top layer, washed once with KHR medium and resuspended in a Krebs solution (in mM: NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 25, $MgSO_4$ 2, $CaCl_2$ 2 and glucose 10), which was gassed with a 95% O_2 and 5% CO_2 mixture, pH 7.4.

1.2.3 Synaptosomes purified by a discontinuous percoll gradient

For the immunocytochemical assays and for the microfluorimetric $[Ca^{2+}]_i$ measurement in isolated single nerve terminals, hippocampal synaptosomes were obtained through a discontinuous percoll gradient based in the method described by Dunkley *et al.* (1988), as previously described (Díaz-Hernández *et al.*, 2002). This procedure for preparation of the synaptosomes is crucial to reduce the amount of postsynaptic density material. Immunocytochemical analysis of the synaptosomes obtained with this discontinuous Percoll gradient showed that less than 1% of the synaptophysin-positive elements were labelled by an anti-PSD95 antibody, as found by others in different brain regions (Díaz-Hernández *et al.* 2002). This is particularly critical for the immunocytochemical assays in single nerve terminals, since the aim was to identify receptors located just in the presynaptic component, and thus, the synaptosomes prepared for these experiments should not have residual amounts of postsynaptic contaminant, otherwise it would be impossible to discriminate if the immunolabelling observed was from the presynaptic or the postsynaptic compartments.

Briefly, hippocampi or striata from two male Wistar rats (6-8 weeks old) were homogenised in a medium containing 0.32 M sucrose and 10 mM TES, pH 7.4. The homogenate was spun at 900 *g* for 5 min at 4℃ and the supernatant spun again at 9,500 *g* for 12 min at 4 ºC. The pellets formed were resuspended in 8 ml of 0.32 M sucrose, 10 mM TES, pH 7.4. Two milliliters of this synaptosomal suspension were placed onto 3 ml of Percoll discontinuous gradients containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM DL-dithiothreitol, and 3, 10, or 23% Percoll, pH 7.4. The gradients were centrifuged at 25,000 *g* for 10 min at 4℃. Synaptosomes equilibrating between the 10 and 23% Percoll phases, were collected and diluted in 30 ml of HEPES buffer medium (HBM: 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) prior to centrifugation at 22,000 *g* for 10 min. For microfluorimetric $[Ca^{2+}]_i$ measurements, aliquots of 0.5 mg were pelleted and kept on ice. For immunocytochemical analysis, the synaptosomes were pelleted, diluted in 5 ml HBM medium and plated in coverslips previously coated with poli-L-lysine (100 µl per coverslip).

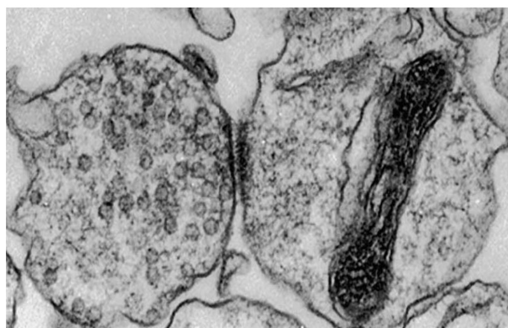


Figure 5. *Electron microscopic image of the intact synaptosomes obtained by the discontinuous sucrose gradient method, showing vesicular and mitochondrial components and intact filamentous cross-bridges at the synaptic cleft (adapted from Phillips et al., 2001).*

1.2.4 Synaptosomes purified by a discontinuous sucrose gradient

For the subcellular fractionation of hippocampal nerve terminals, hippocampal synaptosomes were obtained through a discontinuous sucrose gradient as described by Philips *et al.* (2001), with minor modifications, which is based in the known isopicnic densities of the several cellular components (Cohen *et al.*, 1977). This synaptosomal preparation is the best to perform the subcellular fractionation of nerve terminals in presynaptic active zone (Pre), postsynaptic density (Post) and non active zone fractions (Extra), since on the opposite of the synaptosomes obtained by the methods described above, in particular the synaptosomes purified by the discotinuos percoll gradient, it preserves intact the postsynaptic density, as well as the perisynaptic membranes of the postsynaptic cell, that is kept attached to the presynaptic button as depicted in figure 5.

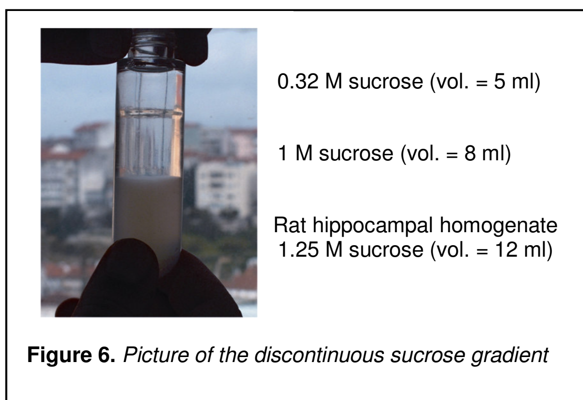


Figure 6. *Picture of the discontinuous sucrose gradient*

Briefly, hippocampi from 10 rats were homogenised at 4 ºC in 15 ml of isolation solution (0.32 M sucrose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM phenylmethyl-sulfonylfluoride, PMSF). The concentration of sucrose was raised to 1.25 M by addition of 75 ml of 2 M sucrose and 30 ml of 0.1 mM $CaCl_2$ and the suspension divided into 10 ultracentrifuge tubes. The homogenate was overlaid with 8 ml 1.0 M sucrose, 0.1 mM $CaCl_2$ and with 5 ml of homogenisation solution (Figure 6) and centrifuged at 100,000 *g* for 3 h at 4 ºC. Synaptosomes were collected at the 1.25/1.0 M sucrose interface (Figure 7), diluted 1:10 in cold 0.32 M sucrose with 0.1 mM $CaCl_2$ and pelleted (15,000 *g* for 30 min at 4 ºC). Pellets were resuspended in 1 ml of 0.32 M sucrose with 0.1 mM $CaCl_2$ and a small sample taken for Western blot analysis.

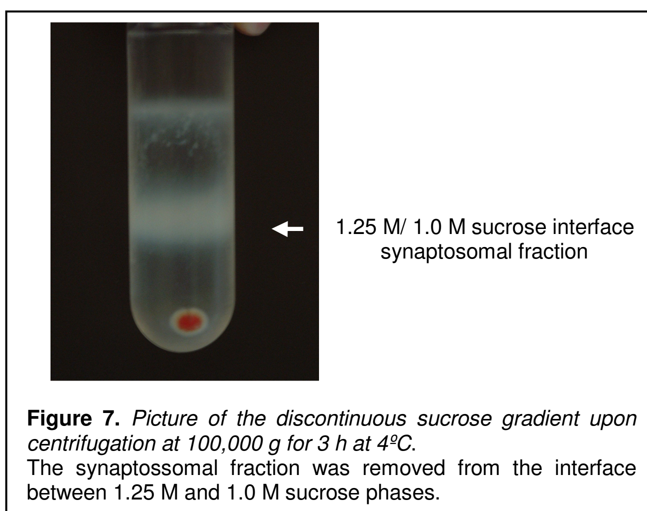


Figure 7. *Picture of the discontinuous sucrose gradient upon centrifugation at 100,000 g for 3 h at 4ºC.*
The synaptossomal fraction was removed from the interface between 1.25 M and 1.0 M sucrose phases.

1.3 Other brain fractions

1.3.1 Mouse brain homogenate

Rat or mice hippocampal and/or cortical tissue was rapidly dissected, homogenised in 2x volume of ice-cold Sucrose-HEPES buffer (0.32 M sucrose, 1 mM EGTA, 0.1 mM EDTA and 10

mM HEPES, pH 7.4) containing protease inhibitors (cocktail of protease inhibitors inhibiting a broad spectrum of serine and cysteine proteases; Roche Diagnostics GmbH, Germany) and centrifuged at 1,000 *g* for 10 min at 4 ºC. The pellet was discarded and the supernatant was centrifuged at 48,000 *g* for 40 min. The pellet obtained was resuspended in MKM buffer (KCl 150 mM, MOPS 20 mM, 1% Triton X-100, pH 7.4). The resuspended pellet was then left in a rotator at 4 ºC for 1 h to better dissolve proteins and centrifuged at 3,000 *g* for 10 min in order to pellet out crude undissolved fractions. The supernatant was stored at -20 ºC. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology, Rockford, USA).

1.3.2 Total hippocampal membranes

Rat hippocampi were homogenised in a sucrose-HEPES medium containing 0.32 M sucrose, 1 mM EDTA, 0.1% BSA and 10 mM HEPES (pH 7.4). The homogenate was centrifuged at 3,000 *g* for 10 min at 4 ºC. The supernatant was then centrifuged at 100,000 *g* for 30 min at 4 ºC. The supernatant was discarded and the pellet resuspended in 25 mM Tris and 2 mM $MgCl_2$ (pH 7.2), containing protease inhibitors (Roche Diagnostics GmbH, Germany) and stored at -20 ºC. The protein concentration was determined using the BCA protein assay reagent (Pierce Biotechnology, Rockford, USA).

1.4 Rat hippocampal neurons

Primary cultures of hippocampal neurons were obtained from 17 to 19 days embryos of Wistar rats, handled according to European guidelines, as previously described (Agostinho and Oliveira, 2003). Briefly, the neohippocampi of the embryos were dissected and placed in Ca^{2+}- and Mg^{2+}- free Krebs buffer (in mM: NaCl 120, KCl 4.8, KH_2PO_4 1.2, glucose 10, HEPES 10, pH 7.4) and 0.3% BSA. After removal of the meninges, the tissue was washed and incubated in Krebs buffer containing 0.02% trypsin and 0.004% DNAse I (Sigma, Portugal), for 10 min at 37 ºC. The digestion was stopped with Krebs buffer containing 0.05% trypsin inhibitor (type II-S) (Sigma, Portugal) and 0.004% DNAse I and the tissue was centrifuged at 140 *g* for 5 min. After washing the pellet once with Krebs buffer, the cells were dissociated mechanically through a large-bore 5 ml glass pipette. The cells were plated in poly-L-lysine (0.1 mg/ml) coated 16-mm-diameter coverslip or 6 wells dishes at densities of $5x10^4$/coverslip (cell viability and immunocytochemistry assays) or $1x10^6$/well (Western blot analysis), respectively. Hippocampal neurons were cultured in Neurobasal medium (GIBCO, BRL, Life Technologies, Scotland, UK) supplemented with 0.5 mM L-glutamine, 25 μM glutamate, 2% B27 supplement (GIBCO, BRL, Life Technologies, Scotland, UK) and 0.12 mg/ml gentamicine (Sigma, Portugal), and were grown at 37 ºC in a humidified atmosphere of 5% CO_2/ 95% O_2. In these conditions, it is obtained a near 90% pure neuronal preparation, as evaluated by the ratio of elements stained with

antibodies against a microtubule-associated protein (MAP-2, a neuronal marker) and glial fibrillary acidic protein (GFAP, an astrocytic marker) as described by Rebola *et al.* (2005).

1.5 Cell lines

The **H**uman **E**mbryonic **K**idney cell line (HEK293 cells; Figure 8) was used as a model to study P2X receptors in an isolated manner, in particular to check the selectivity of the antibodies used against the several P2X subunits (1 of Results). It was also used to study possible interactions between NMDA and P2X receptors (4 of Results). These cells are excellent heterologous expression systems, because they present endogenously the expression of a very few types of receptors, and they are highly transfectable, allowing their use to express the receptors of interest. Furthermore, since they are mammalian cell lines they ensure a post-transcriptional processing and a co-assembly of subunits and protein trafficking similar to that of native tissues. Human astrocytoma cell lines stably transfected with $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$, kindly provided by Prof. J.M. Boeynaems (University of Brussels, Brussels, Belgium), were also used to gauge the selectivity of the antibodies used against the several types of P2Y receptors.

1.5.1 Transient expression of P2X and NMDA receptors in HEK293 cells

The cDNAs encoding for each P2X receptor ($P2X_{1-7}$) and for NMDA receptor subunits NR1 and NR2B were donated by Prof. Alan North (Univ. Sheffield, UK) and Prof. John J. Woodward (University of South Carolina, South Carolina, USA), respectively. The HEK293 cells were grown in Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 10% of heat-inactivated foetal calf serum (FCS), 1% mixture of streptomycin (100 U/ml)/penicillin (200 U/ml) (Sigma, Portugal) in an humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. The day before transfection, HEK293 cells were platted in 16 mm coverslips previously coated with poly-L-Lysine (for immunocytochemical assays and for microfluorimetric $[Ca^{2+}]_i$ measurements) or in 100 mm Petri dishes (for immunoprecipitation and Western blot analysis) at a density of $2.0x10^5$ cells/coverslip and $2.0x10^6$ cells/well, respectively. For transfection of HEK293 cells in 16 mm coverslips, 0.5-2 µg of cDNA(s) was/were mixed with 7.5 µl Superfect reagent (Qiagen, USA) in 75 µl of DMEM without serum or antibiotics. After 5-10 min of incubation to allow the formation of DNA-liposome complexes, this mixture was diluted in DMEM with 10% FCS and 1% mixture of streptomycin (100 U/ml)/penicillin (200 U/ml), which was overlaid onto the cells seeded the day before that should have reached a 50-80% confluence. The cells were incubated with the transfection complexes for 2-3 h under their normal growth conditions and then the medium containing the

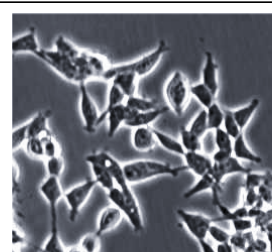


Figura 8. *HEK293 cells*

remaining complexes was removed and cell fresh growth medium (containing serum and antibiotics) was added. After 24 h-48 h, the cells were washed twice with phosphate buffer saline medium (PBS) (in mM: NaCl 137, KCl 2.7, KH_2PO_4 1.8, NaH_2PO_4 10 mM, pH 7.4) and ready to be used for fluorescence $[Ca^{2+}]_i$ measurements (2.2.2 of Materials and Methods), for immunocytochemical analysis (2.7.2 of Materials and Methods) or for patch-clamp analysis (2.10 of Materials and Methods). For immunoprecipitation and Western blot analysis, cells were harvested in a cell lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% 40 mM NaF, 1 mM Na_3VO_4, 0.1 mM DTT, 1% TritonX-100 and 20 mM HEPES (pH 7.4). After passing through an 18 gauge needle to disperse any large aggregates, the lysate was centrifuged at 16,000 *g* for 15 minutes at 4 ºC. The supernatant was stored at -20 ºC. The protein concentration was determined using the BCA protein assay reagent (Pierce Biotechnology, Rockford, USA).

1.5.2 Cell culture and membrane preparation of Human astrocytoma 1321N1 cell line stably transfected with $P2Y_{1,2,4,6,11}$

Human astrocytoma 1321N1 cells stably transfected with cDNAs encoding for $P2Y_{1,2,4,6 \text{ or } 11}$ were grown in DMEM nutrient mix supplemented with 10% FCS, 1% mixture of streptomycin (100 U/ml)/penicillin (200 U/ml) (Sigma, Portugal) and 0.5 mg/ml of geneticin G 418 sulphate (Calbiochem, UK) in an humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C, and passaged when confluent. Wild-type cells were maintained in the same medium, but without geneticin G 418. The preparation of membranes and determination of protein concentration for Western blot analysis was performed as described for HEK293 cells.

2 EXPERIMENTAL PROCEDURES

2.1 [^{3}H]-Glutamate release from rat hippocampal nerve terminals

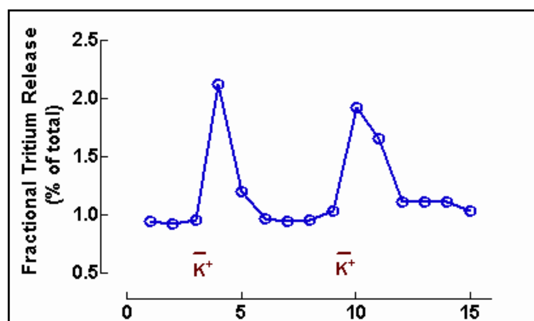


Figure 9. *Time course of [^{3}H]-glutamate release from rat hippocampal synaptosomes.*
Rat hippocampal synaptosomes were challenged with two periods of stimulation with 20 mM of K^+ for 30 s (S1 and S2), as indicated by the bars above the abscissa. The evoked tritium outflow, expressed as a percentage of the total amount of tritium, was taken as a measure of [^{3}H]-glutamate release.

The release of glutamate from rat hippocampal nerve terminals prepared using a combined sucrose/Percoll centrifugation protocol (see 1.2.2 of Materials and Methods) was performed as previously described (Lopes *et al.*, 2002). Basically, the synaptosomes were equilibrated at 37 ºC for 10 min, loaded with 0.2 µM [^{3}H]-glutamate (Amersham Biosciences, UK) for 5 min at 37 ºC, washed, layered over Whatman GF/C filters and superfused (flow rate: 0.8 ml/min) with Krebs solution (in mM: NaCl 120, KCl 3, $MgSO_4$ 1, $CaCl_2$ 2, NaH_2PO_4 1.2, glucose 10, $NaHCO_3$ 25) for 20 min before starting collection of the superfusate. The synaptosomes were stimulated with 20 mM K^+ (isomolar substitution of NaCl by KCl in the Krebs solution) at 3 and 9 min after starting sample collection (S_1 and S_2) triggering a release of tritium in a Ca^{2+}-dependent manner that is mostly [^{3}H]-glutamate, gauged by HPLC (Lopes *et al.*, 2002) (Figure 9). Tested agonists were added 2 min before S_2 onwards and their effect quantified by the modification of S_2/S_1 ratio *versus* control (*i.e.* absence of agonist), whereas antagonists were added from 10 min before starting sample collection onwards and none of the tested antagonists modified the S_2/S_1 ratio *versus* control. Radioactivity was expressed in terms of disintegrations per second per milligram of protein (Bq/mg) in each chamber (see Lopes *et al.*, 2002). The flow rate of 0.8 ml/min used does not allow tonic effects of endogenous substances released by nerve terminals to occur and thus the effects observed are due solely to the drugs applied.

2.2 Intracellular calcium measurements

All the measurements of $[Ca^{2+}]_i$ were performed using a fluorescent probe selective for Ca^{2+}, Fura-2 ($C_{44}H_{47}N_3O_{24}$; molecular weight of 1000.86 Da). This molecule is an analogous of the Ca^{2+}-chelating molecule EGTA, with the addition of a chemical group with fluorescent properties, as depicted in figure 10 (Grynkiewickz *et al.*, 1985).

Fura-2 molecule has a dissociation constant (K_D) for Ca^{2+} near the basal $[Ca^{2+}]_i$ of a mammalian cell (100 nM) and it is highly selective for Ca^{2+} in comparison to the other abundant dication Mg^{2+}. However, the Fura-2 binding to Ca^{2+} is disturbed in the presence of physiological

levels of Mg^{2+}. The K_D of Fura-2 for Ca^{2+} is 135 nM in Mg^{2+}-free medium, whereas it is 224 nM in the presence of 1 mM Mg^{2+} (values provided by the supplier), the conditions used in these studies.

Fura-2 presents a high molar extinction coeficient (ε) (30,800 $cm^{-1}M^{-1}$; value provided by the supplier) and thus there is a high fluorescence intensity per molecule of Fura-2, which permits measurements at intracellular concentrations of dye unlikely to cause significant Ca^{2+} buffering or damping of Ca^{2+} transients.

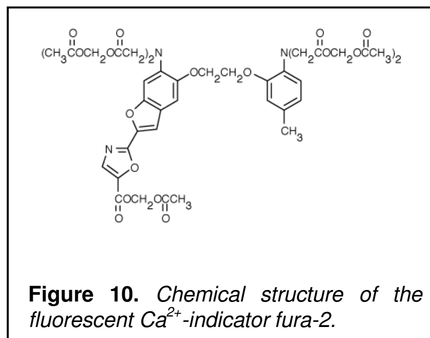


Figure 10. *Chemical structure of the fluorescent Ca^{2+}-indicator fura-2.*

The plasmatic membranes are not permeable to the charged molecule Fura-2. Thus, it is used Fura-2 acetoxymethyl ester (Fura-2AM) that is the result of the modification of the carboxylic acids of Fura-2 into acetoxymethyl esters (AM) groups, which makes it permeable to the membrane (Oakes *et al.*, 1988). Once inside the cell, the lipophilic blocking groups are cleaved by non-specific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound.

Fura-2 is a ratiometric calcium indicator, since the maximum absorption wavelength of 370 nm decreases to 335 nm after the binding to Ca^{2+}, with an isobestic point slightly above 350 nm (Figure 11). The best fluorescence Ca^{2+}-dependent signals were obtained using two wavelengths of excitation, 380 nm for Ca^{2+}-free Fura-2 and 340 nm for Fura-2-Ca^{2+}. The ratio of the fluorescence intensities (F) obtained at 340 nm (F_{340}) by the fluorescence obtained at 380 nm (F_{380}), detected at an emission wavelength of 510 nm, gives an indirect measurement of $[Ca^{2+}]_i$ transients. However, from these fluorescence intensities it is possible to estimate $[Ca^{2+}]_i$ using the equation described by Grynkiewickz *et al.*, (1985):

$$\left[Ca^{2+}\right]=K_D\,\frac{(R-R_{min})}{(R_{\text{máx}}-R)}\,\beta$$

where R represents the ratio F_{340}/F_{380}, R_{min} the ratio of the fluorescence intensities in the absence of Ca^{2+} (F_{340min}/F_{380min}) and the R_{max} at Ca^{2+}-saturating conditions (F_{340max}/F_{380max}). As described above, the K_D for Fura-2 in physiological conditions (1 mM Mg^{2+}) is 224 nM and the β constant is the ratio of the intensities F_{380min} and F_{380max} (F_{380min}/F_{380max}). The use of ratiometric Ca^{2+}-indicators is very advantageous, because it minimizes undesirable factors such as photobleaching or a non uniform distribution of the probe, and it allows the user to identify apparent Ca^{2+}-transients that result from artefacts.

2.2.1 In rat hippocampal nerve terminals

Intracellular calcium measurements were performed in a partially purified synaptosomal fraction (P2) from the rat hippocampus (see 1.2.1 of Materials and methods), essentially as described by Malva *et al.* 1995. Synaptosomes (3 mg/ml) were incubated with 5 µM Fura-2AM (Molecular Probes, Alfagene, Portugal) and 0.02% Pluronic F-127 (Sigma, Portugal) in an incubation medium (in mM: NaCl 132, KCl 1, $MgCl_2$ 1, $CaCl_2$ 0.1, H_3PO_4 1.2, glucose 10 and HEPES-Na 10, pH 7.4) with 0.1% fatty acid-free BSA for 20 min at 25 ºC. After this loading period, synaptosomes were pelleted, the non-hydrolysed probe was removed and the synaptosomes were placed in the same medium plus 1.2 mM $CaCl_2$. Synaptosomes were pre-incubated with the antagonists 5 min before starting recording at 37 ºC and agonists were applied 50 s after starting recording and 150 s before stimulation with 20 mM KCl (Figure 12). The fluorescence was monitored at 37 ºC, using a computer-assisted Spex Fluoromax spectrofluorometer, at 510 nm emission and double excitation at 340 nm and 380 nm, using 5 nm slits. The calibration was made using 2.5 µM ionomycin (1.2 mM $CaCl_2$; R_{max}), at 400 s, and 24 mM EGTA (R_{min}), at 500 s (Figure 12). The fluorescence intensities were converted into $[Ca^{2+}]_i$-values by using the calibration equation for double excitation wavelength measurements, taking the dissociation constant of the Fura-2/Ca^{2+} complex as 224 nM as described by Grynkiewicz *et al.* (1985). Evoked $[Ca^{2+}]_i$ transients were determined by subtracting the $[Ca^{2+}]_i$ basal value immediately before stimulation from the $[Ca^{2+}]_i$ value at 24 s after stimulation (peak values).

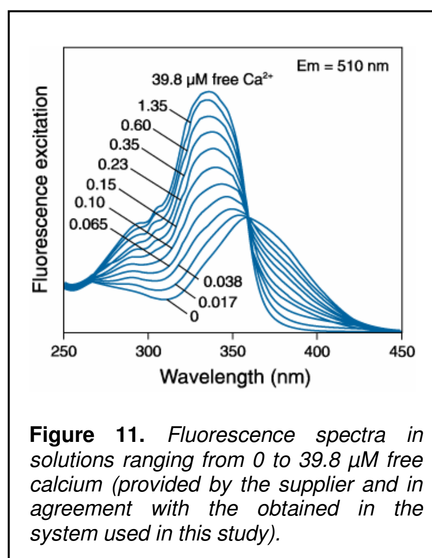


Figure 11. *Fluorescence spectra in solutions ranging from 0 to 39.8 µM free calcium (provided by the supplier and in agreement with the obtained in the system used in this study).*

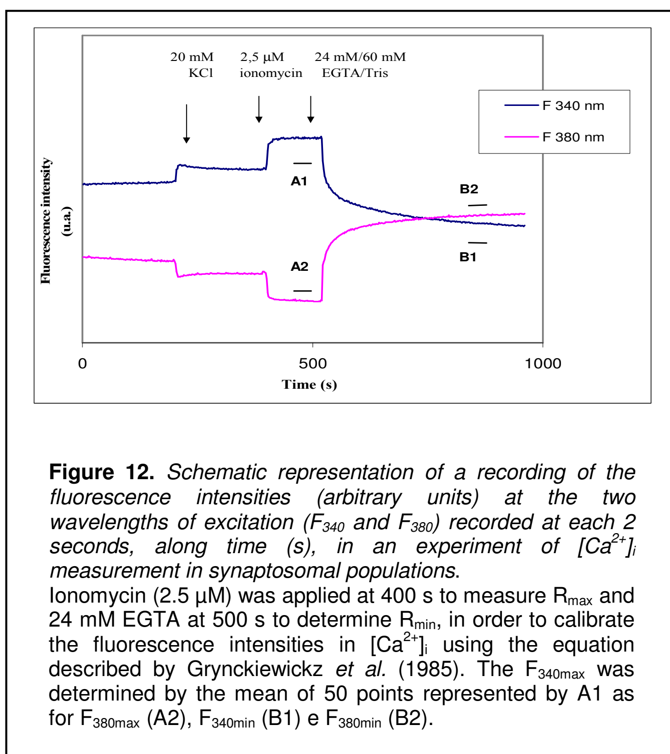


Figure 12. *Schematic representation of a recording of the fluorescence intensities (arbitrary units) at the two wavelengths of excitation (F_{340} and F_{380}) recorded at each 2 seconds, along time (s), in an experiment of $[Ca^{2+}]_i$ measurement in synaptosomal populations.*
Ionomycin (2.5 µM) was applied at 400 s to measure R_{max} and 24 mM EGTA at 500 s to determine R_{min}, in order to calibrate the fluorescence intensities in $[Ca^{2+}]_i$ using the equation described by Grynckiewickz *et al.* (1985). The F_{340max} was determined by the mean of 50 points represented by A1 as for F_{380max} (A2), F_{340min} (B1) e F_{380min} (B2).

2.2.2 Microfluorimetric $[Ca^{2+}]_i$ measurements in HEK293 cells

After 24h or 48h of transfection with cDNAs encoding for $P2X_2$, NR2B and/or NR1, the transfected HEK cells were incubated with 5 µM of Fura-2AM in Krebs medium (in mM: NaCl 160 mM, KCl 2.5, $CaCl_2$ 1.8, glucose 10, HEPES 10, $MgCl_2$ 2, pH 7.2) for 40 min in an humidified atmosphere of 95% O_2 and 5% CO_2 at 37°C. In the experiments performed to study NMDA receptors, it was used the same Krebs medium, but without Mg^{2+}. The cells were then washed twice in Krebs fresh medium to remove remaining Fura-2AM and mounted in a small superfusion chamber (Warner Instruments, Izasa, Portugal) in the stage of a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera (Roper Scientific, Tucson, USA) that allowed the acquisition of 12-bit images at a rate of 1 Hz using MetaFluor 5.0 software. The exposure time was 750 ms for each wavelength and the changing time was < 5 ms. Time course data represents the ratio of the average of fluorescence intensity obtained at the excitation wavelength of 340 nm, by the obtained at 380 nm, measured from an elliptical region defining each studied cell. Tested drugs were added through the superfusion solution (Krebs medium).

2.2.3 Microfluorimetric $[Ca^{2+}]_i$ measurements in individual nerve terminals

After preparation of hippocampal synaptosomes as described in 2.1.3 of Materials and methods, synaptosomal pellets containing 0.5 mg of protein were resuspended in 1 ml of Elliot´s medium (in mM: NaCl 122, KCl 3.1, KH_2PO_4 0.4, $NaHCO_3$ 5, $MgSO_4$ 1.2, glucose 10 and TES buffer 20, pH 7.4). Five minutes after synaptosomal resuspension, $CaCl_2$ (1.33 mM) and Fura-2AM (5 µM, from Molecular Probes, Alfagene, Portugal) were added. Following incubation for 1 hour, the synaptosomes were adhered to grided coverslips pre-treated with poly-L-lysine during a 45 min period. The coverslips were washed with PBS and mounted in a small superfusion chamber in the stage of a Nikon TE-200 microscope (Nikon, Tokyo, Japan). Synaptosomes were imaged through a 100x objective (Nikon, 1.3 numerical aperture) and illuminated with a 280 nm UV light using a bandpass filter (Omega Optical Inc., Brattleboro, USA). Emitted light was isolated with a dichroic mirror (430 nm) and a bandpass filter (510 nm). A Hamamatsu C-4880-80 multiformat CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) allowed the acquisition of 12-bit images using Kalcium PC software (Kinetic Imaging Inc., Liverpool, UK) at a rate of 1.06 Hz. The exposure time was 822 ms for each wavelength and the changing time was < 5 ms. Time course data represent the average light intensity from an elliptical region defined inside each studied nerve terminal and was corrected for photobleaching as previously described (Díaz-Hernández *et al.*, 2002). Tested drugs were added through the superfusion solution and a maximum of 3 agonists could be added to the same preparation. One min after the last application of an agonist, a pulse of K^+ (30 mM) was applied to verify the integrity of the synaptosomes. One min after, a mixture EGTA (5 mM) and Tris (30 mM) was applied to eliminate extracellular Ca^{2+} followed by 20 µl of Triton X-100

(0.2%) to obtain the F_{min}. This was followed by the addition of 30 µl of a 15 mM Ca^{2+} solution to obtain the F_{max}. Once this calibration was obtained, F_{min} and F_{max} were calculated and applied to Grynkiewicz's equation to transform fluorescence intensities into Ca^{2+} concentrations (Grynkiewicz *et al.*, 1985). After the imagiological study, the synaptosomes were fixed with 4% paraformaldehyde to carry out the immunocytochemical characterisation of the recorded synaptosomes (see 2.7.1 of Materials and methods), identified using the grid-labelled coverslip (Eppendorf, Germany).

2.3 Single cell RT-PCR analysis of P2 receptor expression in laser-dissected hippocampal pyramidal neurons

Coronal sections (6 and 10 µm) were obtained from frozen rat brains (between -4.52 and – 2.80 mm from bregma). These sections were dehydrated in 100% ethanol and rehydrated gradually (100-50% ethanol), stained with Nissl stain and rehydrated again with xylene. CA1 or CA3 pyramidal cell bodies were micro-dissected with laser with a microscope Arcturus LCM 210 PixCell II with a spot size of 7.5 µm (Lopes *et al.*, 2003). The total RNA was then extracted according to StrataPrep Total RNA micropep kit instructions from Stratagene and subjected to reverse transcription and cDNA amplification using a Three Primer End Amplification Polymerase Chain Reaction protocol, as previously described (Richardson *et al.*, 2000). Purification of cDNA was achieved using QIA Quick PCR purification Kit (Quiagen). Once obtained, samples of the pre-amplified cDNA were subjected to 45 rounds of PCR in 20 µl of 45 mM Tris-HCl (pH 8.1), 12.5% (w/v) sucrose, 12 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$ and 0.5 mM deoxynucleotide triphosphates, with 100 ng of the forward and reverse primers for each P2 receptor cDNA, detailed in table 5. The cycling conditions were 2.5 min at 92 ºC (denaturation), 1.5 min at 60 ºC (annealing) and 1 min at 72 ºC (extension). After amplification, the products were separated on 2% agarose gels and visualized using ethidium bromide. In all experiments, positive controls for the primers used were electrophoresed in parallel to the gene-specific assays. These routinely contained cDNA derived from 1 ng of whole-brain total RNA.

2.4 Subcellular fractionation of nerve terminals

The solubilization of extrasynaptic, presynaptic active zone and postsynaptic fractions from rat hippocampal synaptosomes was performed according to the method previously described (Phillips *et al.*, 2001), with minor modifications. It was previously confirmed that this subsynaptic fractionation method allows an over 90% effective separation of active zone (SNAP25), postsynaptic density (PSD95) and non-active zone fraction (synaptophysin) markers and can be used to access the subsynaptic distribution of receptors (Pinheiro *et al.*, 2003; Rebola *et al.*, 2003).

The synaptosomal suspension prepared as described in 1.2.4 of Materials and Methods, was diluted 1:10 in cold 0.1 mM $CaCl_2$ and an equal volume of 2x solubilization buffer (2% Triton X-100, 40 mM Tris, pH 6.0) was added to the suspension. The membranes were incubated for 30 min on ice with mild agitation and the insoluble material (synaptic junctions) was pelleted (40,000 *g* for 30 min at 4 ºC). The supernatant (extrasynaptic fraction) was decanted and proteins precipitated with 6 volumes of acetone at -20 ºC and recovered by centrifugation (18,000 *g* for 30 min at -15 ºC). The synaptic junctions pellet was washed in pH 6.0 solubilization buffer, resuspended in 10 ml of 1% Triton X-100 and 20 mM Tris (pH 8.0), incubated for 30 min on ice with mild agitation, centrifuged (40,000 *g* for 30 min at 4 ºC) and the supernatant (presynaptic active zone fraction) processed as above. PMSF (1 mM) was added to the suspension in all extraction steps. The pellets from the supernatants and the final insoluble pellet (postsynaptic density fraction) were solubilized in 5% SDS and the protein concentration was measured using the BCA protein assay reagent (Pierce Biotechnology, Rockford, USA).

Table 5. . *PCR primers used for detection of P2 receptor mRNA in single hippocampal pyramidal neurons of both CA3 and CA1 areas.*

Name		Primers		PCR product
		Forward (5´-3´)	Reverse (5´-3´)	(bp)
Ribosomal protein L11		TTCTATGTGGTGCTGGGTAGG	TTGCCTCCTCTTTGCTGATT	187
Glutamate decarboxylase		ATCTTGCTTCAGTAGCCTTTGC	TGTCTTCAAAAACACTTGTGGG	220
Glial Fibrillary acidic protein		CTGGGCAGGGTACAGG	AAACAGGATGGACGCTTAAA	222
Intronic marker		GCCTGCATTCATCTTCATCTGC	AAAGGTGGAACTCGCCCGTTT	189
NMDA receptor subunit 1		TAGTGGCAGTGCTTCAGGG	GTGACACCGGACTGGGGAG	178
ATP (P2) receptors	$rP2X_1$	GTTCAGCATGAAGACAGGCA	GAGTATAGATGTGTGAGGGGCC	136
	$rP2X_2$	TGTGACTGGGAAACAGAAACC	AGGAGATGGCAGGGAACC	114
	$rP2X_3$	AAGAAGGGGCTGCTATTTCTGC	AGGCATGCAAGGGGTAAAG	126
	$rP2X_4$	CTGGTGTGCTGTTGGCTG	ACCTGAGAGAGCCTCCTTCC	152
	$rP2X_5$	ACTTAGGGAAGAGCAAACTCCC	AGCAAGAGCTGAACTGCACA	156
	$rP2X_6$	AGGCTAGGGTGAAAGCAACA	GCAGGAATATCAGGTTCTTTGG	201
	$rP2X_7$	1-TAAAGTTTGGATGTGGCTTGG 2-AGACAAACAAAGTCACCCGG 3-AGGAGCCCCTTATCAGCTCT	1-TCTGTGTGGTGTGTGGTGTGTG 2-GGTATACACCTGCCGGTCTGG 3-CATTGGTGTACTTGTCGTCC	156 400 711
	$rP2Y_1$	CCCTAACTATGATGCAGCTT	GCTGCATCTTATCACCCT	151
	$rP2Y_2$	GCCACCTGACTCCCATGCAA	CCGCTGAGCTAAATCCC	167
	$rP2Y_4$	GGGGACAAGTATCGAAACCA	GCCCCTGCAGTTAGTTCCCTT	208
	$rP2Y_6$	GACACCTGTGTTTCGGGGAC	CCTCTACAGGAGGGGCCTT	259
	$hP2Y_{11}$	ACTGGTGGTTGAGTTCCTGG	TCAGGTGGGAGAAGCTGAGT	410
	$rP2Y_{12}$	CAGAAATTCCTTGATGAGCA	ATGTGGTGATTTCCTTGGAG	175

2.5 Co-Immunoprecipitation

Transiently transfected HEK293 cells lysates (1.5.1 Materials and Methods) or mouse cortices and hippocampi homogenates (1.3.1 Materials and Methods), both at a concentration of 0.6 mg/ml, were incubated for 1 h at room temperature (RT) with constant rotation, with 200 µl of Protein A cross-linked to agarose beads per ml of sample. These pre-clear lysate/homogenate containing 10% BSA and protease inhibitors were incubated either with rabbit polyclonal IgG anti-NR2B (4 µg/ml; Chemicon, PG-Hitec, Portugal) or rabbit polyclonal IgG anti-P2X$_2$ (2 µg/ml; Alomone labs, Israel). Then 50 µl of a 50% Slurry Protein A cross-linked to agarose beads were added and the mixture was incubated for 2 h at 4 ºC with constant agitation. The beads were washed three times with MKM buffer containing KCl 150 mM, MOPS 20 mM, 1% Triton X-100 (pH 7.4) (for mouse brain homogenate) or cell lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% 40 mM NaF, 1 mM Na_3VO_4, 0.1 mM DTT, 1%TritonX-100 and 20 mM HEPES (pH 7.4) (for transiently transfected HEK293 cells lysate). Then, 50 µl of 6x diluted sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.35 M Tris, 30 % glycerol, 10% SDS, 0.6 M DTT, 0,012% bromophenol blue, pH 6.8) was added to the beads. Immune complexes were dissociated from the beads and denaturated by heating to 95 ºC for 5 min and these samples were analysed by Western blot analysis (2.6 of Materials and Methods). A negative control containing the same amount of rabbit polyclonal IgG instead of the rabbit polyclonal IgG anti-P2X$_2$ antibody was always performed in parallel in each experiment.

2.6 Western blot analysis

Western blot analysis was performed as previously described (Rebola *et al.*, 2003), based in Laemli *et al.*, (1970). Briefly, after determining the amount of protein by the BCA, each sample that was analysed was added to 1/6 volume of 6x SDS-PAGE sample buffer (0.35 M Tris, 30 % glycerol, 10% SDS, 0.6 M DTT, 0,012% bromophenol blue, pH 6.8) and boiled at 95 ºC for 5 min. These diluted samples and the prestained molecular weight markers (BioRad, Portugal) were separated by SDS-PAGE electrophoresis (7.5-12% resolving gels with a 4% concentrating gel) under reducing conditions at 80-160 mV and electro-transferred to polyvinylidene difluoride membranes (0.45 µm from Amersham, Biosciences, Carnaxide, Portugal). After blocking for 1 h at RT with 5% milk in Tris-buffered saline (Tris 20 mM, NaCl 140 mM , pH 7.6) containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4°C with the primary antibody diluted in TBS-T with 5% milk (the primary antibodies used are described in table 6). After three washing periods of 15 min with TBS-T-5% milk, the membranes were incubated with the appropriate alkaline phosphatase-tagged secondary antibody (dilution 1:5,000-20,000; see 2.12.2) diluted in TBS-T-5% milk for 90 min at RT. After three 15-min washes in TBS-T containing 0.5% milk, the membranes were incubated with Enhanced Chemi-Fluorescence substrate (Amersham Biosciences, Carnaxide, Portugal) for 5 min and then analyzed with

Molecular Imager VersaDoc MP imaging systems with the help of Quantity one software (BioRad, Portugal).

2.7 Immunocytochemistry

2.7.1 In synaptosomes

For immunocytochemical analysis of single nerve terminals, hippocampal synaptosomes were obtained through a discontinuous Percoll gradient, as described in 1.2.3 of Materials and Methods. These hippocampal synaptosomes were placed onto coverslips previously coated with poly-L-lysine, fixed with 4% paraformaldehyde for 15 min and washed twice with PBS medium. The synaptosomes were permeabilized in PBS with 0.2% Triton X-100 for 10 min and then blocked for 1 h in PBS with 3% BSA and 5% normal rat serum. The synaptosomes were then washed twice with PBS and incubated with primary antibodies diluted in PBS with 3% BSA for 1 h at RT. The antibodies used and the dilutions used are described in table 6. The synaptosomes were then washed three times with PBS with 3% BSA and incubated for 1 h at RT with the respective AlexaFluor secondary antibodies (1:200-1:1,000; Molecular Probes, Alfagene, Portugal), in order to detect the primary antibodies bound. Whenever occurring, to avoid recognition of the goat anti-guinea pig, goat anti-rabbit and goat anti-mouse antibodies by the donkey anti-goat antibody, first it was applied the donkey anti-goat and, after washing with PBS, we applied the other secondary antibodies. After washing and mounting on slides with Vectashield mounting medium (Vector Laboratories, Burlingame, USA), the preparations were visualized in a Zeiss Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analysed with MetaFluor 5.0 software. Each coverslip was analyzed by counting 3 different fields and in each field a total amount of 500 individualized elements.

2.7.2 In rat hippocampal neurons and HEK293 cells

For the immunofluorescence experiments in cells, either transfected HEK cells or rat hippocampal neurons, the cells were rinsed with PBS, fixed in 4% paraformaldehyde and 0.09 M sucrose for 15 min, and washed again with PBS. Cells were subsequently permeabilized with PBS containing 0.2% Triton X-100 for 5 minutes. Blocking was done using PBS containing 3% BSA for 30 min at RT. Then the cells were incubated with the primary antibodies (table 6) diluted in PBS 3% BSA for 1h at RT. After washing three times in PBS 3% BSA, the bound primary antibodies were detected using AlexaFluor secondary antibodies (1:200-1:1,000; Molecular Probes, Alfagene, Portugal) diluted in PBS 3% BSA for 1h at RT. Then coverslips were rinsed in PBS and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, USA). Confocal microscope observations were made with a LeicaSPII confocal microscope. Fluorescence microscope observations were made in a Zeiss Axiovert 200

inverted fluorescence microscope equipped with a cooled CCD camera and analysed with MetaFluor 5.0 software.

2.8 FluoroJade-C stainning

After anaesthesia with 50 μg/g weight of sodium pentobarbital, the animals were opened ventrally leaving the heart exposed. The right atrium was cutted and 10 ml of saline solution (0.9%NaCl + 4% sucrose) was perfused at the left ventricle. After it, it was done the perfusion of 4% paraformaldehyde in 0.9% NaCl and 4% sucrose. Then, the brain was removed to small container with 4% paraformaldehyde and left overnight at 4 ºC. Subsequently, the brains were left for 48 h in a saline solution of 0.9% NaCl and 20% sucrose. The brain was then frozen in dry ice and 20 μm coronal sections were prepared using a cryostat apparatus (Leica). The brain sections were mounted with distilled water onto slides previously coated with 2% gelatine with chromium and potassium sulphate. Then the slides were immersed in 0.1 % NaOH in 80% ethanol for 5 min, in 70% ethanol for 1 min and in distilled water for 2 min. The slides were then transferred to a solution of 0.06% potassium permanganate for 10 min and were gently shaken on a rotating platform. The slides were rinsed with distilled water for 1-2 min and were then transferred to the 0.0001 % FluoroJade-C (Sigma, Portugal) staining solution (in 0.1 % acetic acid), where they were gently agitated for 10 min. After the staining, the sections were rinsed three times with distilled water. The excess water was removed and the sections dried with a hot air gun. After it, the slides were immersed in xylene and then covered with a coverslip with DPX Mountant for histology (Sigma, Portugal). The FluoroJade-C staining of the sections was visualized in an Axiovert 200 inverted fluorescence microscope equipped with a cooled CCD camera and analysed with MetaFluor 5.0 software.

2.9 Drugs treatments and neuronal death evaluation

$A\beta_{1-42}$-induced neuronal damage was evaluated after culturing rat hippocampal neurons for 5 days. $A\beta_{1-42}$ (0.5 μM) was directly added to the medium and incubated for 12-48 hours. To test the ability of any drug (PPADS, Reactive Blue 2, NF023 or MRS2179) to modify the toxic effects of $A\beta_{1-42}$, the drugs were added 15 minutes before addition of $A\beta_{1-42}$ onwards.

In order to evaluate dendritic atrophy or synaptotoxicity caused by $A\beta_{1-42}$, after 12-48 hours incubation with $A\beta_{1-42}$, cells were fixed in 4% paraformaldehyde and 0.09 M sucrose for 15 min. Then, it was carried out immunocytochemical analysis as described in 2.7.2 of Materials and Methods, for MAP-2 (dendritic marker) and for synaptophysin (synaptic marker), respectively (see 2.12.2 for information of the antibodies used).

Cell viability assays were performed by double-labeling (3 min incubation) with the fluorescent probes Syto-13 (4 μM) and propidium iodide (PI, 4 μg/ml) (Molecular Probes,

Alfagene, Portugal) followed by fluorescence microscopy cell counting. Viable neurons present nuclei homogenously labeled with syto-13 (green fluorescent nuclei). In contrast, putative apoptotic neurons show condensed and fragmented nuclei labeled with Syto-13 (primary apoptosis) or with Syto-13 plus PI (secondary apoptosis) and necrotic neurons present intact nuclei labeled with PI (red fluorescent nuclei). Each experiment was repeated using different cell cultures in duplicate, and cell counting was carried out in at least six fields per coverslip, with a total of approximately 300 cells.

In these experiments, synthetic $A\beta_{1-42}$ was incubated for seven days at 37 ºC to allow its oligomerization, but not the formation of aggregated insoluble material, as evaluated by electron microscopy and Congo Red birefringence (Lorenzo and Yankner, 1994).

2.10 ***Whole cell patch-clamp recordings in HEK293 cells***

Electrophysiological experiments in transiently transfected HEK293 cells were carried out 24–48 h after transfection (see 1.5.1 of Materials and Methods). Membrane currents were recorded at a given membrane potential under the whole-cell configuration of the patch-clamp technique using a List EPC-7 amplifier. Series resistance was compensated by 50-60%. Patch electrodes were fabricated from borosilicate glass (World Precision Instruments, Germany) with a resistance of 2–5 MΩ. Pippettes were filled with (in mM): 140 cesium methanesulfonate, 5 CsCl, 0.5 CaCl2, 5 MgCl2, 10 EGTA, and 10 HEPES, pH 7.4. Currents were filtered (two-pole Butterworth filter, -12 dB/octave) and transferred into a personal computer at a sampling rate of 1-5 kHz for analysis using pCLAMP software (Axon Instruments, Foster City, CA). The cells were rapidly perfused using a linear array of eight glass tubes placed 200–300 µm from the cell body. Krebs solution (in mM: NaCl 160 mM, KCl 2.5, $CaCl_2$ 1.8, Glucose 10, HEPES 10, $MgCl_2$ 2, pH 7.2) with and without agonist and/or antagonist, flowed from adjacent barrels, and solution changes were achieved by displacing the whole perfusion array laterally using a motorized device controlled by a personal computer (Paternain *et al.*, 2003). Whenever tested, antagonists were superfused for 1 min prior to the addition of the agonists.

2.11 ***Behavioural studies***

To study spatial learning and memory in rats, several mazes have been developed. These maze studies in animals have helped uncover general principles about learning and memory. Nowadays, the studies on these mazes are used to determine whether different treatments or conditions affect learning and memory in rats. In the present work, it was used the Y-maze to evaluate the mnemonic performance in the animals injected with $A\beta_{1-42}$ (1.1.1 Materials and methods). In order to check if the animals had any impairment at the locomotors level or at their

exploratory behaviour, which would affect their performance in the Y-maze, the animals were always submitted to the open-field maze.

2.11.1 Open-field

Locomotor activity was monitored in an open field arena (30 cm along, 30 cm wide and 40 cm high, divided in 9 squares of 10 cm wide) and the exploratory behaviour of the rats was evaluated by counting the total number of line crossings over a period of 5 minutes. An animal was considered to be completely within a quadrant of the maze when all parts of the body, excepting the tail, have crossed the threshold into that quadrant. In parallel, the number of rearings was also counted.

2.11.2 Y-maze

Spatial memory was evaluated by recording spontaneous alternation behaviour in a Y-maze as described in Sakaguchi *et al*. (2006). The maze was made of black painted acrylic, each arm was 50 cm long, 40 cm high and 10 cm wide, and they converged at equal angles of 120º. Each mouse, new to the maze, was placed in one arm and allowed to explore the Y-maze for a period of 8 min. Arm choices, including returns to the same arm, were recorded visually, and three consecutive choices of three different arms were counted as an alternation. Thus, the percentage of alternation was determined by dividing the total number of alternations by the total number of choices minus 2. Mice that completed only 10 arm entries or less within 8 min were excluded from further analysis. The total number of entries in all the arms of the maze, during the 8 min trial, was used to evaluate the locomotors activity of the animals.

2.12 Reagents

2.12.1 Drugs

Adenosine 5´-triphosphate disodium salt (ATP), 1,3-dipropyl-8-cyclopentyladenosine (DPCPX), α,β-methyleneATP (α,β-MeATP), β,γ-ImidoATP (β,γ-ImATP), suramin, Brilliant Blue G (BBG), epibatidine, *d*-tubocurarine, ifenprodil and pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) (PPADS) were from Sigma (Portugal), 8-(benzamido)naphthalene-1,3,5-trisulfonate (NF023), 2´deoxy-N^6-methyladenosine-3´,5´-bisphosphate (MRS2179), Reactive Blue 2 (RB2), 2´-3´-O-(4-benzoylbenzoyl)adenosine 5´-triphosphate (Bz-ATP), 2',3'-O-(2,4,6-trinitrophenyl) adenosine-5'-triphosphate (TNP-ATP), 2-methylthio-ADP, D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5), and 4-(2-[7-amino-2-(2-furyl)]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl) phenol (ZM241385) were purchased to Tocris (UK), P1,P3-di(uridine 5'-) triphosphate (INS415) and P1-(urinine 5'-),P4-(inosine 5'-) tetraphosphate (INS45973) were provided by J.L.Boyer (Inspire Pharmaceuticals, North Carolina, USA). All drug stock solutions were made in water at a concentration of 10 mM and diluted directly into the superfusion or batch solution to the

appropriate final concentration. [^{3}H]glutamate (specific activity 45 Ci/mmol) was from Amersham (UK). $A\beta_{1-42}$ peptides were purchased to American Peptides (Sunnyvale, USA)

2.12.2 Antibodies

All the primary antibodies used are described in table 6. The secondary antibodies used for Western blot analysis were alkaline phosphatase-tagged goat anti-mouse, and goat anti-rabbit purchased to Amersham Biosciences (Carnaxide, Portugal), and goat anti-guinea pig, rabbit anti-goat and goat anti-rat purchased to Santa Cruz Biotechnology (Frilabo, Portugal). For immunocytochemistry, the secondary detection was perfomed using AlexaFluor secondary antibodies purchased to Molecular Probes (Alfagene, Portugal).

Table 6. *Primary antibodies used throughout the studies presented.*

Antibodies	Supplier	Host	Type	$[Antibody]_{initial}$ (mg/ml)	Dilution
$P2X_1$	Alomone labs	Rabbit	Polyclonal	0.6	1:500
$P2X_2$	Alomone labs	Rabbit	Polyclonal	0.2	1:1 000
$P2X_2$	Santa Cruz Biotechnology	Goat	Polyclonal	0.2	1:500
$P2X_3$	Santa Cruz Biotechnology	Goat	Polyclonal	0.2	1:500
$P2X_4$	Alomone labs	Rabbit	Polyclonal	0.3	1:500
$P2X_5$	Santa Cruz Biotechnology	Goat	Polyclonal	0.2	1:200
$P2X_6$	Santa Cruz Biotechnology	Goat	Polyclonal	0.2	1:200
$P2X_7$	Alomone labs	Rabbit	Polyclonal	0.3	1:5 000
$P2Y_1$	Santa Cruz Biotechnology	Goat	Polyclonal	0.2	1:500
$P2Y_2$	Alomone labs	Rabbit	Polyclonal	0.6	1:500
$P2Y_4$	Alomone labs	Rabbit	Polyclonal	0.3	1:1 000
$P2Y_6$	Santa Cruz Biotechnology	Goat	Polyclonal	0.2	1:500
$P2Y_{11}$	Zymed	Rabbit	Polyclonal	0.3	1:500
$P2Y_{12}$	Alomone labs	Rabbit	Polyclonal	0.8	1:200
NR1	Chemicon	Goat	IgG1	0.5	1:1000
NR2B	Chemicon	Rabbit	Polyclonal	0.2	1:500
α3	Sigma	Mouse	IgG2A	5	1:1000
α4	Sigma	Mouse	IgG1	1	1:1000
Synaptophysin	Sigma	Mouse	IgG1	-	1:5 000
MAP-2	Sigma	Rabbit	IgG1	2	1:1000
SNAP-25	Sigma	Mouse	IgG1	-	1:5 000
PSD-95	Chemicon	Rabbit	Polyclonal	-	1:1 000
Sintaxin	Chemicon	Rabbit	Polyclonal	1	1:5 000
vGluT1	Chemicon	Guinea-pig	Polyclonal	-	1:5 000
vGluT2	Chemicon	Guinea-pig	Polyclonal	-	1:5 000
vGAT	Calbiochem	Guinea-pig	Polyclonal	-	1:1 000
Dopamine β-hydroxylase	Affiniti	Rabbit	Polyclonal	-	1:500

2.13 Data presentation

Whenever possible, the data is presented as mean ± SEM of *n* experiments. To test the significance of the effect of a drug *versus* control, an unpaired Student's *t* test was generally used considering a statistical difference for a $p<0.05$. For viability assays of rat hippocampal neurons, it was used one-way ANOVA followed by Newman-Keuls test considering a statistical difference for a $p<0.05$. For open-field and Y-maze tests Kruskal-Wallis test followed by Dunn's test was used, considering a statistical difference for a $p<0.05$.

RESULTS

1 DUAL PRESYNAPTIC CONTROL BY ATP OF GLUTAMATE RELEASE VIA FACILITATORY $P2X_1$, $P2X_{2/3}$ AND $P2X_3$, AND INHIBITORY $P2Y_1$, $P2Y_2$ AND/OR $P2Y_4$ RECEPTORS IN THE RAT HIPPOCAMPUS

Stimulation of nerve terminals triggers a release of ATP (Sperlágh and Vizi, 1996; Pankratov *et al.*, 2006), which can act directly as an extracellular signalling molecule through activation of P2 (ATP) receptors (Ralevic and Burnstock, 1998) or be converted through ecto-nucleotidases into adenosine (Zimmermann, 2000), with subsequent activation of P1 (adenosine) receptors (Fredholm *et al.*, 2005). The P2 receptor family comprises seven ionotropic P2X receptors subunits ($P2X_{1-7}$), forming either homomeric and heteromeric receptors (North, 2002), and eight metabotropic P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) (Lazarowski *et al.*, 2003). The brain displays a robust mRNA expression, an intense binding and immunoreactivity for both P2X and P2Y receptors in neuronal and non-neuronal elements, although the role of central P2 receptors remains ill defined (reviewed in Cunha and Ribeiro, 2000). Since an ATPergic contribution to synaptic transmission has only been demonstrated in few central synapses (reviewed in Cunha and Ribeiro, 2000; Mori *et al.*, 2001; Pankratov *et al.*, 2002b, 2003), it was proposed that P2 receptors act principally as presynaptic modulators in the brain (Cunha and Ribeiro, 2000). This view is supported by the observed localization of several P2X subunits (reviewed in Cunha and Ribeiro, 2000; Deuchars *et al.*, 2001; Díaz-Hernández *et al.*, 2001) and P2Y receptors in brain nerve terminals (Schäfer and Reiser, 1997; Simon *et al.*, 1997) and by functional studies indicating a biphasic P2 receptor modulation of the release of several types of neurotransmitters in different brain regions: inhibition via P2Y and facilitation via P2X receptors (reviewed in Cunha and Ribeiro, 2000). However, it is difficult to unambiguously ascribe P2 receptor mediated effects to presynaptic modulation since most functional studies rely solely on the use of the limited number of P2 receptor ligands that have an insufficient selectivity (Ralevic and Burnstock, 1998), and few attempts have been made to complement the studies with molecular or morphological approaches to identify the putative P2 receptors involved. Similarly, limited attempts are made to exclude the involvement of adenosine (formed upon extracellular catabolism of ATP by ecto-nucleotidases) P1 receptors (*e.g.* Cunha *et al.*, 1998; Dunwiddie *et al.*, 1997), which is particularly critical when studying glutamatergic transmission, since adenosine efficiently inhibits excitatory transmission (Fredholm *et al.*, 2005). Finally, it is difficult to unequivocally ascribe the modulation of transmitter release to a presynaptic mechanism, especially when using slices or microdialysis, due to indirect circuit- or astrocyte-mediated effects that lead to indirect modulation of synaptic function (*e.g.* Zhang *et al.*, 2003).

Accordingly, purified nerve terminals were used (which allows isolating presynaptic effects, see Raiteri and Raiteri, 2000) to assess whether P2 receptors could presynaptically control the

evoked release of glutamate. In parallel, functional and morphological approaches were used to identify the receptor subtypes mediating these effects and to demonstrate their presence in nerve terminals. This combined use of molecular biological, immunological and pharmacological approaches, enabled the demonstration that ATP presynaptically modulates the evoked release of glutamate in a biphasic manner, through the activation of presynaptic facilitatory $P2X_1$, $P2X_{2/3}$ and $P2X_3$ and inhibitory $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors.

1.1 ATP modulates the evoked release of glutamate in a biphasic manner

To assess the effect of P2 receptor activation on the evoked release of glutamate from rat hippocampal nerve terminals, it was tested two stable ATP analogues, β,γ-ImATP (a non-selective P2 receptor agonist) and α,β-MeATP (a selective agonist of P2 receptors containing $P2X_1$ and $P2X_3$ subunits), because they are poor substrates of both the ecto-nucleotidases catabolism and ecto-protein kinases (Ralevic and Burnstock, 1998). The chemical stimulation with 20 mM K^+ for 30 s of the superfused hippocampal synaptosomes that had previously been loaded with $[^3H]$-glutamate, triggered a Ca^{2+}-dependent release of $[^3H]$-glutamate that represented 90% of the total tritium released (gauged by HPLC), which was considered to represent the evoked release of glutamate (see Lopes *et al.*, 2002). Two periods of chemical stimulation (S1 and S2), separated by a 6 min interval, produced a similar evoked release of glutamate (Figure 13A) with an S2/S1 ratio of 0.88±0.03 (n=16). β,γ-ImATP (present only in S2) caused a concentration-dependent biphasic effect in the evoked release of glutamate: at the lower concentrations tested, 10 μM and 30 μM, β,γ-ImATP inhibited glutamate release by 42.9±7.1% (n=4) and 20.0±4.1% (n=4) respectively, whereas 60 μM and 100 μM β,γ-ImATP facilitated glutamate release by 46.9±8.7% (n=4) and 17.6±4.0% (n=4) (Figure 1B). When α,β-MeATP was tested, only facilitatory effects were observed in the concentration range tested (10-100 μM), with a maximal facilitatory effect of 31.4±5.8% (n= 4) observed at 60 μM of α,β-MeATP (Figure 13B).

1.2 The biphasic effects of ATP are mediated by P2 receptors

The next necessary step was to test if these effects of ATP analogues on glutamate release were mediated through activation of P2 receptors or through adenosine (P1) receptors after extracellular catabolism of ATP analogues by the ecto-nucleotidase pathway (see Cunha *et al.*, 1998; Dunwiddie *et al.*, 1997). For that purpose, it was tested the ability of P2 and P1 receptor antagonists to modify the effects of β,γ-ImATP and α,β-MeATP. Neither the adenosine A_{2A} receptor antagonist, ZM241385 (50 nM), nor the adenosine A_1 receptor antagonist, DPCPX (50 nM), significantly (p>0.05) modified the modulatory effects of α,β-MeATP or β,γ-ImATP (Figures 13C and 13D). In contrast, these effects were abolished by the P2 receptor antagonists,

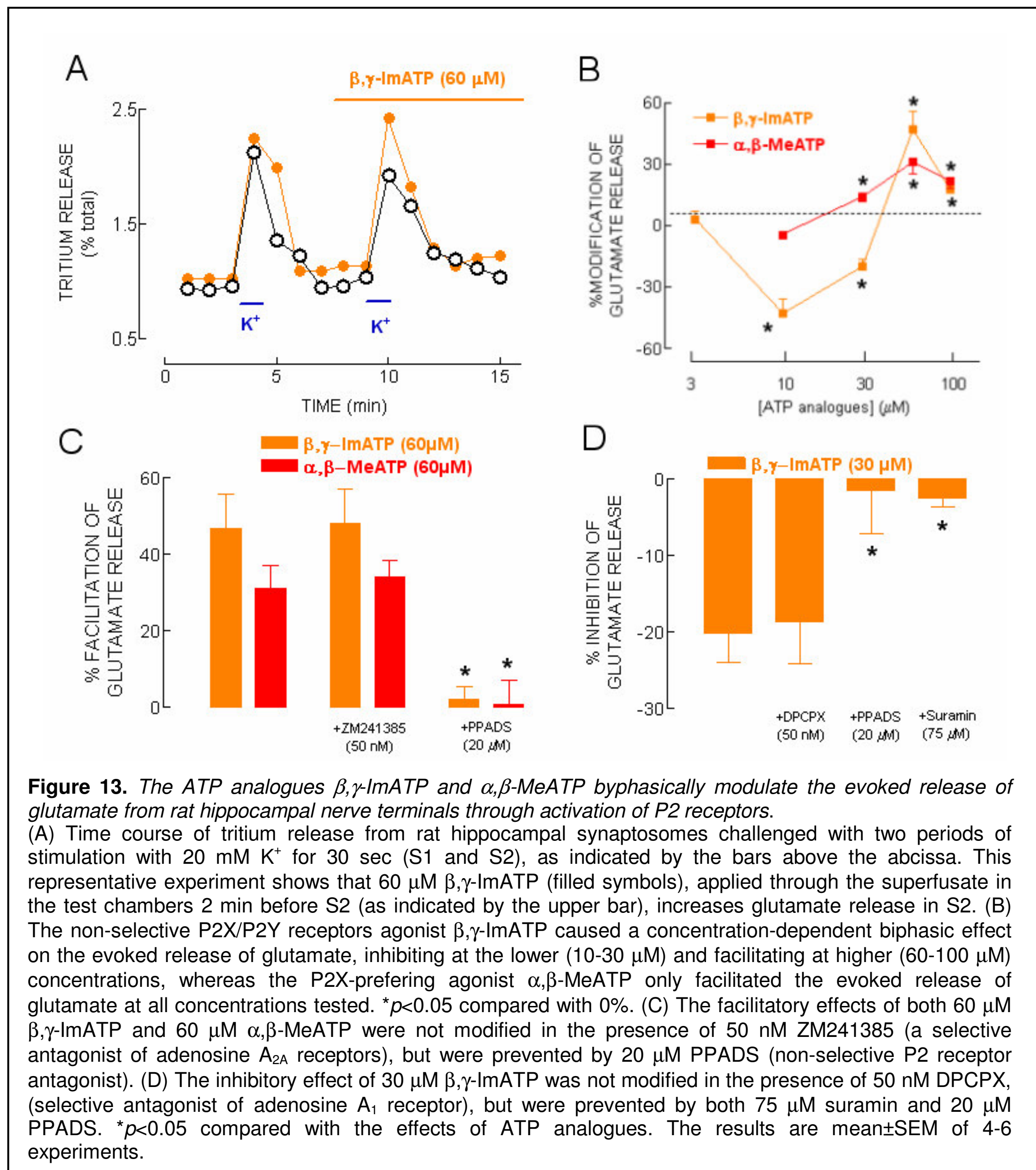


Figure 13. *The ATP analogues β,γ-ImATP and α,β-MeATP byphasically modulate the evoked release of glutamate from rat hippocampal nerve terminals through activation of P2 receptors.*
(A) Time course of tritium release from rat hippocampal synaptosomes challenged with two periods of stimulation with 20 mM K^+ for 30 sec (S1 and S2), as indicated by the bars above the abcissa. This representative experiment shows that 60 μM β,γ-ImATP (filled symbols), applied through the superfusate in the test chambers 2 min before S2 (as indicated by the upper bar), increases glutamate release in S2. (B) The non-selective P2X/P2Y receptors agonist β,γ-ImATP caused a concentration-dependent biphasic effect on the evoked release of glutamate, inhibiting at the lower (10-30 μM) and facilitating at higher (60-100 μM) concentrations, whereas the P2X-prefering agonist α,β-MeATP only facilitated the evoked release of glutamate at all concentrations tested. *p<0.05 compared with 0%. (C) The facilitatory effects of both 60 μM β,γ-ImATP and 60 μM α,β-MeATP were not modified in the presence of 50 nM ZM241385 (a selective antagonist of adenosine A_{2A} receptors), but were prevented by 20 μM PPADS (non-selective P2 receptor antagonist). (D) The inhibitory effect of 30 μM β,γ-ImATP was not modified in the presence of 50 nM DPCPX, (selective antagonist of adenosine A_1 receptor), but were prevented by both 75 μM suramin and 20 μM PPADS. *p<0.05 compared with the effects of ATP analogues. The results are mean±SEM of 4-6 experiments.

PPADS (20 μM) or suramin (75 μM) (Figures 13C and 13D). These results indicate that both the facilitatory effect of α,β-MeATP and the facilitatory and inhibitory effects of β,γ-ImATP on the evoked release of glutamate were mediated through the activation of P2 rather than P1 receptors. It should be noted that DPCPX, ZM241385, PPADS or suramin alone did not cause any effect on the evoked release of glutamate nor on the S2/S1 ratio, in agreement with the expected absence of tonic effects of endogenously released modulators in superfused synaptosomes. This is probably because any released substance was effectively washed out by superfusion using a flow rate of 0.8 ml/min (see Raiteri and Raiteri, 2000).

1.3 The inhibitory effects of ATP are mediated by P2Y receptors whereas the facilitatory effects are mediated by P2X receptors

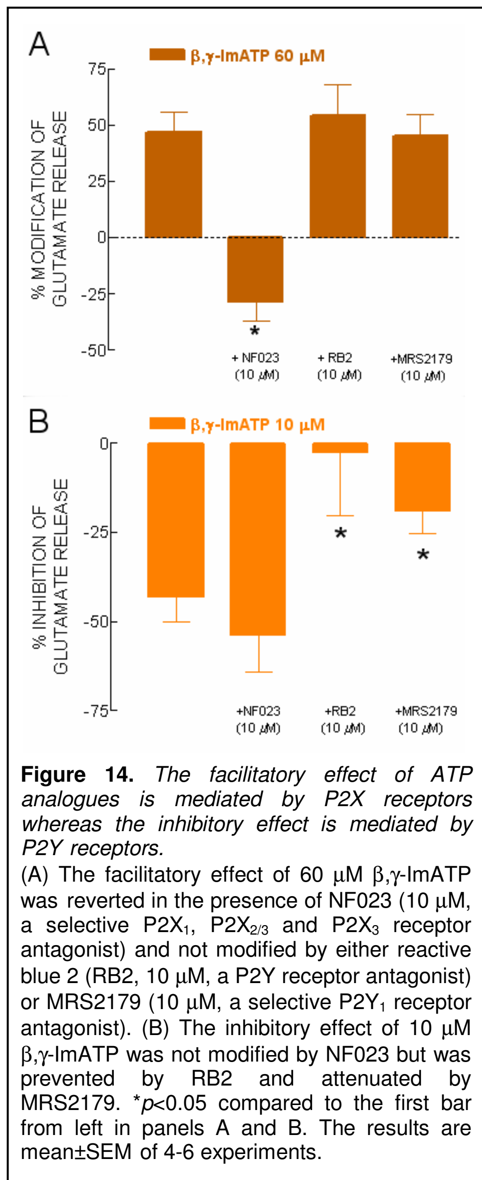


Figure 14. *The facilitatory effect of ATP analogues is mediated by P2X receptors whereas the inhibitory effect is mediated by P2Y receptors.*
(A) The facilitatory effect of 60 µM β,γ-ImATP was reverted in the presence of NF023 (10 µM, a selective $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptor antagonist) and not modified by either reactive blue 2 (RB2, 10 µM, a P2Y receptor antagonist) or MRS2179 (10 µM, a selective $P2Y_1$ receptor antagonist). (B) The inhibitory effect of 10 µM β,γ-ImATP was not modified by NF023 but was prevented by RB2 and attenuated by MRS2179. **p*<0.05 compared to the first bar from left in panels A and B. The results are mean±SEM of 4-6 experiments.

As illustrated in Figure 14A, the facilitatory effect of 60 µM β,γ-ImATP was reversed ($p<0.05$) in the presence of the selective $P2X_{1,2/3,3}$ receptor antagonist NF023 (10 µM) (Soto *et al.*, 1999), whereas it was not modified (*p*>0.05) by either the P2Y receptor antagonist Reactive Blue 2 (RB2, 10 µM) or the selective $P2Y_1$ receptor antagonist MRS2179 (10 µM) (Boyer *et al.*, 2002). Furthermore, in the presence of another $P2X_{1-3}$ selective antagonist, TNP-ATP (100 nM) (North, 2002), the facilitation by α,β-MeATP (60 µM) of the evoked release of glutamate was abolished (3.4±5.5%, n=5, *p*<0.05). On the other hand, the inhibition caused by 10 µM β,γ-ImATP was not modified (*p*>0.05) by NF023 (10 µM), was attenuated (*p*<0.05) by MRS2179 (10 µM) and prevented (*p*<0.05) by RB2 (10 µM) (Figure 14B). This suggests that P2X receptors were responsible for the facilitatory effects whereas P2Y receptors mediate the inhibitory effect of ATP analogues on the evoked release of glutamate.

1.4 Activation of P2X and P2Y receptors also biphasically modulate the evoked $[Ca^{2+}]_i$ transients

The release of neurotransmitters is triggered by the influx of Ca^{2+} in the active zone of nerve terminals and the presynaptic modulation of neurotransmitter release often involves the control of this Ca^{2+}-entry, either directly (through ionotropic receptors) or indirectly through modulation of calcium channels (Khakh and Henderson, 2000). Thus, it was tested the effect of 30 µM and 60 µM of β,γ-ImATP on the evoked calcium transients, concentrations that had caused inhibitory and facilitatory (respectively) effects on the evoked release of glutamate. Hippocampal nerve terminals were stimulated with 20 mM K^+ (as in the previous experiments) resulting in an increase in $[Ca^{2+}]_i$ of 331±10 nM over a basal value of 212±6 nM. To exclude the involvement of

P1 receptors, all experiments were carried out in the presence of ZM241385 (50 nM) and DPCPX (50 nM). As for the control of glutamate release, β,γ-ImATP modulated $[Ca^{2+}]_i$ transients in a biphasic manner (Figure 15A): β,γ-ImATP (60 µM) facilitated $[Ca^{2+}]_i$ transients by 14.3±1.7% (n=6) and β,γ-ImATP (30 µM) inhibited $[Ca^{2+}]_i$ transients by 11.0±2.6% (n=6). In spite of their low amplitude, which is expected based on the exponential relation between $[Ca^{2+}]_i$ transients and neurotransmitter release (Augustine, 2001), it is possible to pharmacologically characterise the receptors involved, as previously carried out for other presynaptic receptors (e.g. Díaz-Hernández *et al.*, 2001; Malva *et al.*, 1995). Both the facilitatory and inhibitory effects of β,γ-ImATP were prevented by PPADS (20 µM), indicating the involvement of P2 receptors. Furthermore, the facilitatory effect

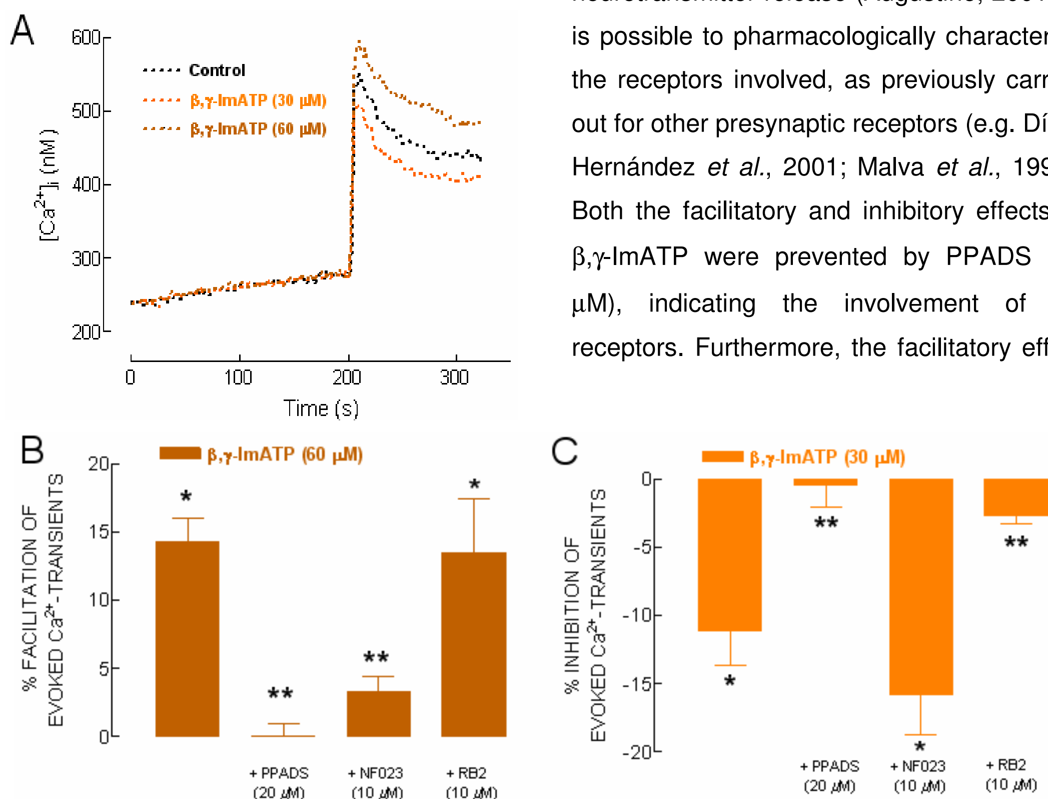


Figure 15. *β,γ-ImATP modulates the evoked $[Ca^{2+}]_i$ transients in a biphasic manner through inhibitory P2Y and facilitatory P2X receptors in rat hippocampal nerve terminals.*
(A) Representative experiment of the increase and decrease of the K^+ (20 mM)-evoked $[Ca^{2+}]_i$ caused by β,γ-ImATP at 60 µM and 30 µM, respectively. All experiments were performed in the presence of 50 nM ZM241385 and 50 nM DPCPX to exclude P1 receptor-mediated effects. (B) The facilitatory effect of 60 µM β,γ-ImATP was prevented by PPADS (20 µM, a non-selective P2 receptor antagonist) and by NF023 (10 µM, a selective antagonist of $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors) and not modified by Reactive Blue 2 (RB2, 10 µM, a preferring antagonist of P2Y receptors). (C) The inhibitory effect of 30 µM β,γ-ImATP was not modified by NF023 but was prevented by PPADS and attenuated by RB2. The percentage variations of $[Ca^{2+}]_i$ are relative to control values (B and C). * $p<0.05$ compared with 0%. ** $p<0.05$ compared with first bar from left in B and C. The results are mean±SEM of 4-6 experiments.

of 60 µM β,γ-ImATP was attenuated ($p<0.05$) by NF023 (10 µM) but not by 10 µM RB2 (Figure 15B), whereas the inhibitory effect of 30 µM β,γ-ImATP was attenuated by 10 µM RB2 ($p<0.05$), but not by 10 µM NF023 (Figure 15C). This re-enforces the idea that the presynaptic biphasic effects of ATP analogues are mediated by inhibitory P2Y and facilitatory P2X receptors.

1.5 $P2X_7$ receptor does not presynaptically control glutamate release

It was recently described that the $P2X_7$ receptor is targeted to glutamatergic nerve terminals in different brain regions (*e.g.* Deuchars *et al.* 2001; Miras-Portugal *et al.*, 2003), including the hippocampus (Sperlagh *et al.*, 2002; Armstrong *et al.*, 2002). However, some studies cast doubts on the selectivity of the most commonly used antibodies against $P2X_7$ receptors (Sim *et al.*, 2004) and it has been shown that purported $P2X_7$ receptor agonists might also indirectly activate adenosine A_1 receptors (Kukley *et al.*, 2004). We report that Bz-ATP (5 µM, a concentration selective for $P2X_7$ or $P2X_1$ receptor activation, see North, 2002) facilitated by 48.5±11.2% (n=4) the evoked release of glutamate (Figure 16). This facilitatory effect of BzATP was not modified (p>0.05) by Brillant Blue G (200 nM, 51.4±11.5% facilitation, n=4) but was prevented (p<0.05) by suramin (75 µM, 5.7±7.6% inhibition, n=4), indicating the involvement of $P2X_1$ receptors and ruling out the involvement of $P2X_7$ receptors that are sensitive to Brillant Blue G (especially in the rat) and insensitive to suramin (North, 2002) (Figure 16).

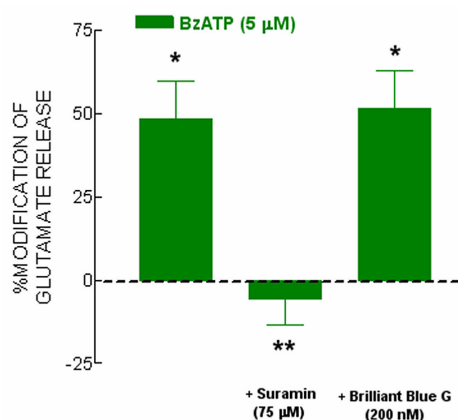


Figure 16. *$P2X_7$ is not involved in the presynaptic facilitatory effect of ATP in the rat hippocampus.* BzATP (5 µM, a selective $P2X_1$ and $P2X_7$ receptor agonist) facilitated the evoked release of glutamate from rat hippocampal nerve terminals, which was prevented in the presence of suramin (75 µM, a $P2X_{1,2,3,5,6}$ but not $P2X_7$ receptor antagonist), but not in the presence of Brilliant Blue G (200 nM, selective antagonist for P2X7). *p<0.05 vs. 0% and **p<0.05 vs. BzATP effect. The results are mean±SEM of 4 experiments.

1.6 Pharmacological identification of P2Y receptors inhibiting glutamate release

The ability of MRS2179 to attenuate (but not abolish like RB2) the inhibition of glutamate release by ATP analogues indicates that $P2Y_1$ receptors as well as other P2Y receptors are involved. Accordingly, a $P2Y_{2,4}$ receptor agonist synthesized by Inspire Pharmaceuticals, INS45973 (0.5 µM), inhibited glutamate release by 26.2±6.0% (n=4), whereas a selective $P2Y_6$ receptor agonist, INS415 (2 µM), was devoid of effects (1.5±7.7%, n=4) (Shaver *et al.*, 2005) (Figure 17A). Furthermore, the $P2Y_{1/12/13}$-selective agonist, 2-MeSADP (1 µM), inhibited by 34.7±6.4% (n=5, p<0.05) the evoked release of glutamate, an effect that was abolished (p<0.05) in the presence of 10 µM MRS2179 (3.8±6.1% inhibition, n=6) (Figure 17B). These results suggest that, in addition to $P2Y_1$ receptors, $P2Y_2$ and/or $P2Y_4$ receptors, but not $P2Y_6$, $P2Y_{12}$ or $P2Y_{13}$ receptors, are also involved in the inhibitory control of the evoked release of glutamate from rat hippocampal nerve terminals.

1.7 Identification of P2 receptors expressed in single hippocampal pyramidal neurons

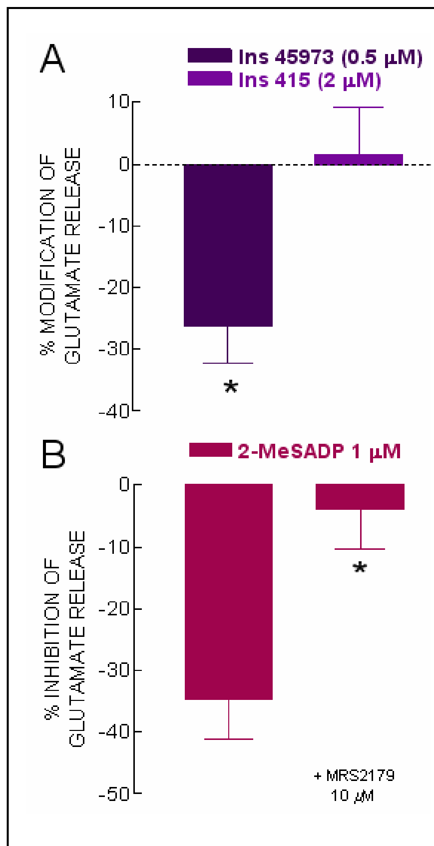


Figure 17. *$P2Y_2$ and/or $P2Y_4$ receptors but not $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptors activation inhibit the evoked release of glutamate from rat hippocampal nerve terminals.* (A) INS45973 (0.5 µM, a selective agonist of $P2Y_2$ and/or $P2Y_4$ receptors) inhibited the evoked release of glutamate, whereas INS415 (2 µM, a selective agonist of $P2Y_6$ receptors) was devoid of effects. * $p<0.05$ compared with 0% in A. The results are mean±SEM of 4 experiments. (B) 2-MeSADP (1 µM, a selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor agonist) inhibited the evoked release of glutamate from rat hippocampal nerve terminals, which was prevented in the presence of MRS2179 (10 µM, a $P2Y_1$ receptor antagonist). *$p<0.05$ compared with 2-MeSADP effect in B. The results are mean±SEM of 5-6 experiments

The general lack of subtype-selective P2 receptors ligands limits the pharmacological characterisation of the P2 receptors involved in the control of glutamate release. Thus, it was defined which receptors are expressed in single pyramidal neurons (*i.e.* glutamatergic neurons) to short-list the candidate P2 receptors likely to be involved. It was first confirmed the quality of the mRNA extracted from single pyramidal neurons by the lack of PCR product corresponding to an intronic marker (lane A of Figure 18A), which was present when analysing genomic DNA (1 ng) (lane B of Figure 18A). It was then selected for analysis 12 laser-dissected pyramidal neurons for which the recovered cDNA obeyed the criteria expected for glutamatergic neurons, *i.e.* presence of PCR products corresponding to a house keeping gene (ribosomal protein L11) and to the NR1 subunit of NMDA receptors and absence of PCR products corresponding to glutamate decarboxylase (GAD, a marker of GABAergic neurons) and to glial fibrillary acidic protein (GFAP, a marker of glial cells) (Figure 18B). In these 12 samples, it was detected PCR products corresponding mainly to $P2X_3$ (6 out of 12) but also $P2X_4$ (3 out of 12), $P2X_1$ and $P2X_2$ (2 out of 12) receptor subunits. It was not detected in any of the neurons, transcripts for $P2X_5$, $P2X_6$ and $P2X_7$, in spite of our care to use 3 different sets of primers (see table 5 in 2.3 Materials and methods). In relation to P2Y receptors, it was detected PCR products corresponding predominantly to $P2Y_1$ (9 out of 12), but also for $P2Y_2$ (6 out of 12), $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ (2 out of 12).

Combining these molecular biology data with the pharmacology data, $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors emerge as the leading candidates to mediate P2 receptor facilitation and $P2Y_1$,

$P2Y_2$ and eventually $P2Y_4$ receptors stand as the most likely candidates to mediate P2 receptor inhibition, although $P2Y_{11}$ receptor should also be considered.

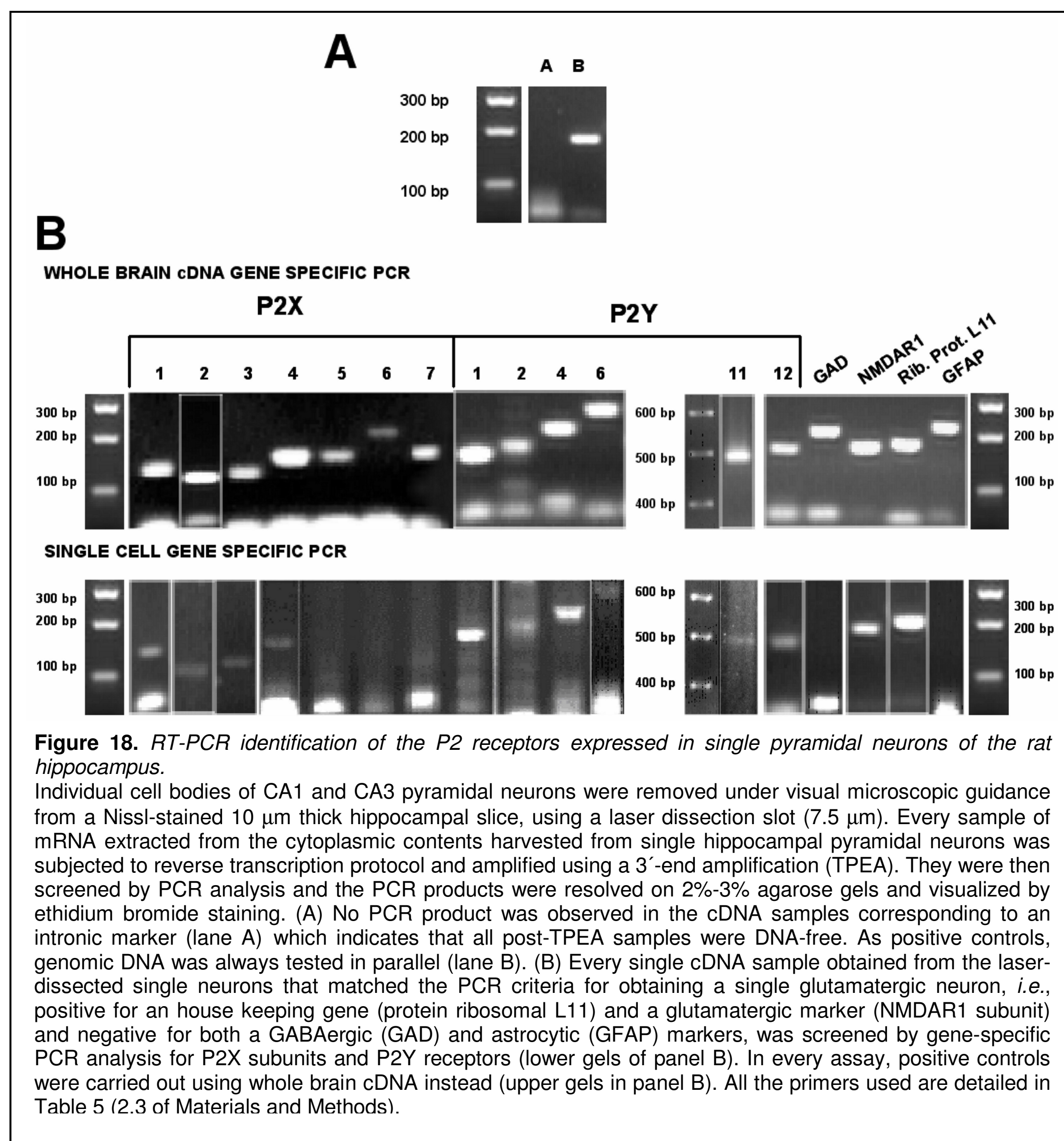
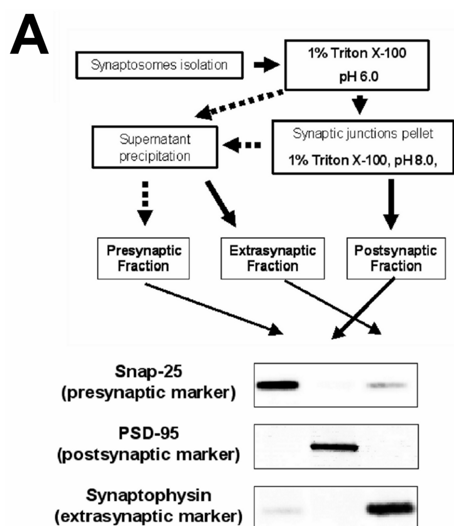


Figure 18. *RT-PCR identification of the P2 receptors expressed in single pyramidal neurons of the rat hippocampus.*
Individual cell bodies of CA1 and CA3 pyramidal neurons were removed under visual microscopic guidance from a Nissl-stained 10 µm thick hippocampal slice, using a laser dissection slot (7.5 µm). Every sample of mRNA extracted from the cytoplasmic contents harvested from single hippocampal pyramidal neurons was subjected to reverse transcription protocol and amplified using a 3´-end amplification (TPEA). They were then screened by PCR analysis and the PCR products were resolved on 2%-3% agarose gels and visualized by ethidium bromide staining. (A) No PCR product was observed in the cDNA samples corresponding to an intronic marker (lane A) which indicates that all post-TPEA samples were DNA-free. As positive controls, genomic DNA was always tested in parallel (lane B). (B) Every single cDNA sample obtained from the laser-dissected single neurons that matched the PCR criteria for obtaining a single glutamatergic neuron, *i.e.*, positive for an house keeping gene (protein ribosomal L11) and a glutamatergic marker (NMDAR1 subunit) and negative for both a GABAergic (GAD) and astrocytic (GFAP) markers, was screened by gene-specific PCR analysis for P2X subunits and P2Y receptors (lower gels of panel B). In every assay, positive controls were carried out using whole brain cDNA instead (upper gels in panel B). All the primers used are detailed in Table 5 (2.3 of Materials and Methods).

1.8 Identification of P2X and P2Y receptors located in the presynaptic active zone

The ability of P2X receptors to facilitate and P2Y receptors to inhibit the evoked release of glutamate allows the prediction that the candidate receptors to mediate these effects should be located in the active zone of rat hippocampal nerve terminals. Thus, it was used a recently described fractionation procedure to purify the presynaptic active zone (Phillips *et al.*, 2001) to test by Western blot analysis which P2 receptors were present there. This technique for separation of the presynaptic active zone from the postsynaptic density and from other presynaptic proteins not located in synapses was previously validated, and it allows an over 90% efficiency of separation of these fractions (see Figure 19A), and it has been confirmed its

usefulness to evaluate the subsynaptic distribution of both ionotropic (Pinheiro *et al.*, 2003) and metabotropic receptors (Rebola *et al.*, 2003). The most abundant P2X receptor subunits enriched in the presynaptic active zone fraction are $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_6$ (Figure 19B). $P2X_1$, $P2X_5$ and $P2X_6$ are also abundantly located in the postsynaptic density and $P2X_3$, $P2X_4$ and $P2X_6$ are abundantly located extrasynaptically in nerve terminals. Concerning the tested P2Y receptors, they are all mainly located in the postsynaptic density, except the $P2Y_{11}$ receptor that has a predominant extrasynaptic localization (Figure 19C). The most abundant P2Y receptors in the presynaptic active zone were $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors, whereas $P2Y_6$ and $P2Y_{12}$ were nearly exclusively found in the postsynaptic density. The relative sub-synaptic distribution of all P2X subunits and P2Y receptors in presynaptic, postsynaptic and extrasynaptic fractions is detailed in table 7.



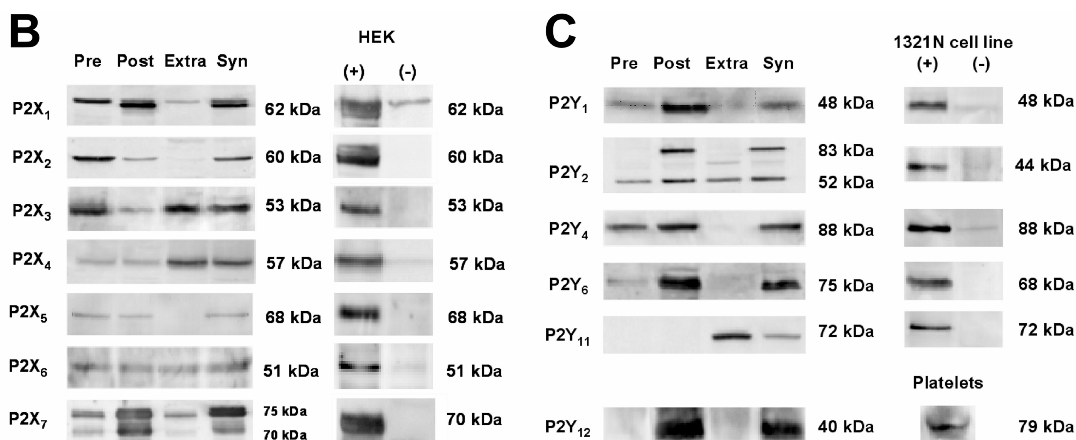


Figure 19. *Sub-synaptic distribution of P2X and P2Y receptors in the hippocampus.*
(A) Selective antibodies for each P2X and P2Y receptors were tested by Western blot analysis in a fraction enriched in the presynaptic active zone (Pre), in the postsynaptic density (Post), in nerve terminals outside the active zone (Extra) and in the initial synaptosomal fraction (syn) from which fractionation began. These fractions were over 90% pure, as illustrated by the ability to recover the immunoreactivity for SNAP25 in the presynaptic active zone fraction, PSD95 in the postsynaptic density fraction and synaptophysin (a protein located in synaptic vesicles) in the extrasynaptic fraction. In B and C are presented Western blots in these fractions evaluating the sub-synaptic distribution of the immunoreactivity of the antibodies selective for each P2X subunit and for each P2Y receptor tested (20-120 μg of protein of each fraction were applied to SDS-PAGE gels). In order to gauge the selectivity of the antibodies used, their immunoreactivity were tested in membranes of HEK cells transiently transfected with cDNAs encoding for each P2X subunit (B) and of 1321N Human astrocytoma cell line stably transfected with each P2Y receptor (C). For $P2Y_{12}$ receptor, rat platelet membranes were used as a positive control. Each blot is representative of at least 3 blots from different groups of animals with similar results. For each fractionation procedure, Western blot analysis for the markers of each fraction was performed as illustrated in (A), in order to assess the efficiency of each fractionation.

1.9 Identification of P2X and P2Y receptors located in hippocampal glutamatergic terminals

Since the fractionation procedure used above does not allow purification of only the synaptic fractions from glutamatergic terminals, it was performed a complementary double immunocytochemistry study in single nerve terminals aimed to identify the P2X and P2Y receptors located in particular in glutamatergic terminals, identified as the terminals labelled with antibodies against vesicular glutamate transporters type 1 and type 2 (vGluT1 and vGluT2) (see Figure 20A as an example for $P2X_3$ of the strategy used to identify its presence in glutamatergic terminals). vGluT1 and/or vGluT2 immunopositive nerve terminals comprised 38.6±0.9% (n=3) in the total population of hippocampal nerve terminals, identified as synaptophysin immunoreactive elements. As presented in Figure 20B, the P2X receptor subunits most frequently located in glutamatergic terminals were $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ receptor subunits (filled bars in Figure 20B). The P2Y receptors most frequently located in glutamatergic terminals were $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors (open bars in Figure 20B).

Table 7. *Relative sub-synaptic distribution of $P2X_{1-7}$ subunits and $P2Y_{1,2,4,6,11,12}$ receptors in the presynaptic, postsynaptic and extrasynaptic fractions.*
The data shows relative average percentage of immunoreactivity (mean±SEM) measured by densitometry of 3 different Western blots from different synaptosomal preparations from different animals.

	Subsynaptic fraction		
P2 Receptor	Presynaptic	Postsynaptic	Extrasynaptic
$P2X_1$	43.3±2.8	57.3±3.1	3.1±1.0
$P2X_2$	75.1±1.4	24.0±1.4	0.9±0.1
$P2X_3$	47.0±1.4	14.6±0.7	38.4±1.5
$P2X_4$	22.6±1.2	17.1±2.0	60.2±1.2
$P2X_5$	55.4±1.7	44.1±1.8	0.5±0.2
$P2X_6$	35.4±1.6	33.9±2.4	30.4±0.8
$P2X_7$	22.4±4.8	72.8±4.9	4.8±0.6
$P2Y_1$	28.5±1.5	70.3±1.3	1.2±0.2
$P2Y_2$	23.1±1.5	53.0±0.8	23.9±0.9
$P2Y_4$	44.3±2.6	55.0±2.5	0.7±0.2
$P2Y_6$	5.0±0.7	94.5±.6	0.4±0.1
$P2Y_{11}$	0.8±0.3	0.5±0.2	98.7±0.5
$P2Y_{12}$	0.6±0.2	98.6±0.4	0.8±0.3

Crossing this double immunocytochemistry data with the data obtained in the sub-synaptic fractionation of the presynaptic active zone, it can be concluded that $P2X_1$, $P2X_2$ and $P2X_3$ receptor subunits and $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors are the most abundantly located in the presynaptic active zone membrane and also the most frequently found in glutamatergic terminals. These receptors are precisely those that are most expressed in pyramidal neurons and the leading candidates based on the pharmacological characterisation of the evoked release of glutamate.

1.10 Discussion

The present study first shows that ATP can biphasically modulate (*i.e.* both facilitate and inhibit) the evoked release of glutamate from rat hippocampal nerve terminals through the

activation of P2X and P2Y receptors, respectively. However, the main finding is the identification of the presynaptic P2X and P2Y receptors that are responsible for these inhibitory and facilitatory effects. By combining the data obtained in pharmacological studies, single cell PCR, Western blot upon sub-synaptic fractionation and double immunocytochemistry in single nerve terminals, it can be concluded that the facilitatory effects are due to $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors and the inhibitory effects are mediated by $P2Y_1$, $P2Y_2$ and/or $P2Y_4$ receptors.

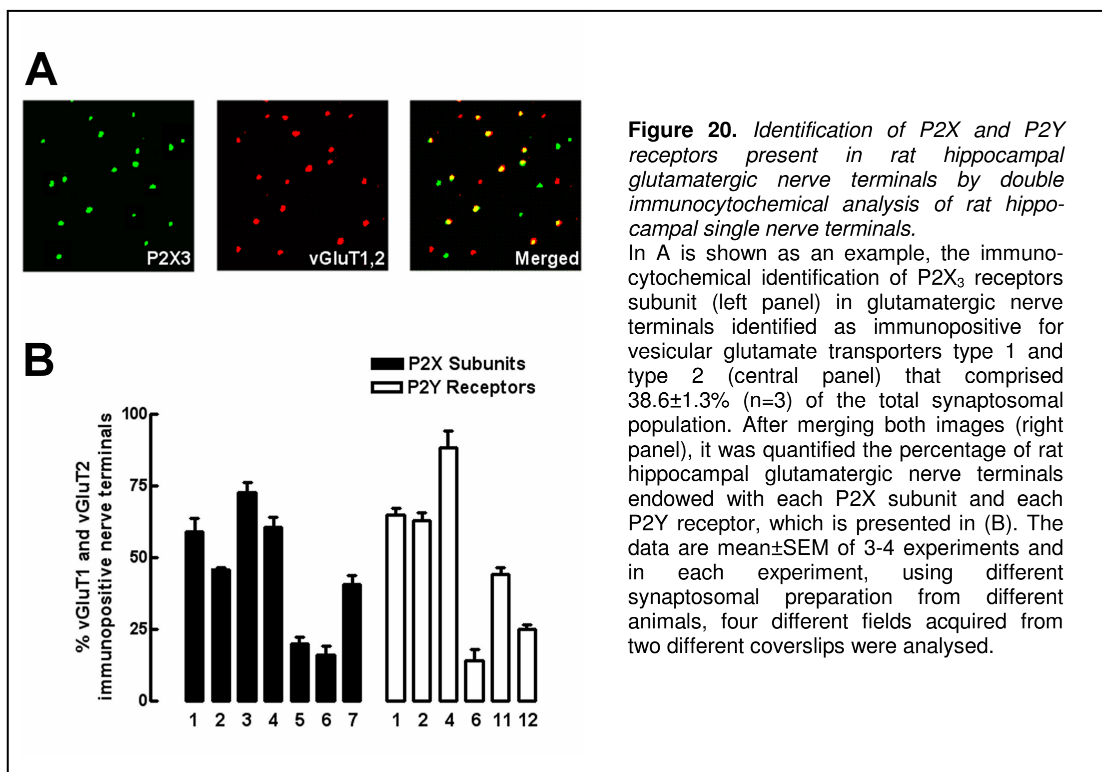


Figure 20. *Identification of P2X and P2Y receptors present in rat hippocampal glutamatergic nerve terminals by double immunocytochemical analysis of rat hippocampal single nerve terminals.*
In A is shown as an example, the immunocytochemical identification of $P2X_3$ receptors subunit (left panel) in glutamatergic nerve terminals identified as immunopositive for vesicular glutamate transporters type 1 and type 2 (central panel) that comprised 38.6±1.3% (n=3) of the total synaptosomal population. After merging both images (right panel), it was quantified the percentage of rat hippocampal glutamatergic nerve terminals endowed with each P2X subunit and each P2Y receptor, which is presented in (B). The data are mean±SEM of 3-4 experiments and in each experiment, using different synaptosomal preparation from different animals, four different fields acquired from two different coverslips were analysed.

These conclusions are based on the observation that the ATP analogue, β,γ-ImATP, modulated the evoked release of glutamate from rat hippocampal nerve terminals in a concentration-dependent biphasic manner, inhibiting at the lower concentrations tested (10-30 μM) and facilitating at concentration of 60-100 μM. These biphasic effects are mediated through P2, but not adenosine P1 receptor activation because the selective antagonists of adenosine A_1 or A_{2A} receptors (DPCPX or ZM241385) failed to modify the effects of β,γ-ImATP, which were prevented by the P2 receptor antagonists, PPADS and suramin. The pharmacological characterisation was developed to show that the facilitatory effects are mediated by P2X receptors and the inhibitory effects by P2Y receptors since: 1) α,β-MeATP, an agonist of $P2X_1$- and $P2X_3$-containing receptors, only facilitated glutamate release at all concentrations tested (10-100 μM); 2) the selective antagonists of $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors, NF023 and TNP-ATP, prevented the facilitatory (but not the inhibitory) effect of ATP analogues; 3) the selective P2Y and $P2Y_1$ antagonists, RB2 and MRS2179, prevented and attenuated respectively, the inhibitory

(but not the facilitatory) effect of ATP analogues. This is re-enforced by the fact that a similar pharmacological profile was obtained both for the release of glutamate and for calcium transients in the same hippocampal nerve terminals. Furthermore, apart from $P2Y_1$ receptors, we could define the involvement of $P2Y_2$ and $P2Y_4$ receptors and exclude $P2Y_6$ receptors, based on the use of selective agonists provided by Inspire Pharmaceuticals, disregard the involvement of $P2Y_{12}$ and $P2Y_{13}$ receptors, based on the ability of MRS2179 to abolish the inhibition caused by 2-methylthio-ADP, and rule out the involvement of $P2X_7$ receptors.

The lack of selective pharmacological tools to further narrow the candidate P2 receptors led us to use alternative approaches to identify the P2X and P2Y receptors presynaptically controlling glutamate release. For that purpose, we used three different molecular approaches from the mRNA to the protein level. The rationale was the following: 1) the receptors should be expressed in hippocampal glutamatergic neurons; 2) they should be located in the active zone; 3) they should be located in glutamatergic terminals. Only the receptor candidates fulfilling these 3 criteria, which also matched the pharmacological characterisation should be claimed to be involved in the control of glutamate release. By single cell RT-PCR, we detected mRNA expression in glutamatergic neurons from CA1 and CA3 areas for $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ subunits and $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ receptors. Looking at the subsynaptic distribution at the protein level, we found that $P2X_1$, $P2X_2$ and $P2X_3$ were enriched in the presynaptic active zone, while $P2Y_1$, $P2Y_2$ and $P2Y_4$ were the only tested P2Y receptors located at the presynaptic active zone. Restricting the analysis to the glutamatergic nerve terminals, double immunocytochemistry revealed that $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$ were present in a higher percentage of glutamatergic nerve terminals (identified as vGluT1/2-positive) whereas $P2Y_1$, $P2Y_2$ and $P2Y_4$ were the most frequently found P2Y receptors in glutamatergic terminals. Thus, combining the data obtained in these molecular studies, the P2 receptors that are expressed in glutamatergic cells, targeted to nerve terminals and located in the presynaptic active zone are $P2X_1$, $P2X_2$ and $P2X_3$ subunits and $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors. This is in total agreement with the pharmacological characterisation, thus confirming the involvement of $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors in the facilitation and of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in the inhibition of hippocampal glutamate release.

This study constitutes the first attempt to thoroughly identify which P2 receptors control glutamate release using combined functional and molecular approaches, which yielded remarkably coincident conclusions. The main conclusion that P2 receptors presynaptically control the release of glutamate, together with the morphological data indicating that a substantial proportion of glutamatergic terminals are endowed with different subtypes of P2 receptors (both P2X and P2Y) confirms the hypothesis that P2 receptors play a major neuromodulatory role at the presynaptic level controlling the release of neurotransmitters rather than acting postsynaptically (see Cunha and Ribeiro, 2000). This provides a logical explanation

to reconcile the robust expression and density of P2 receptors in the hippocampus with the discrete contribution of P2 receptors to synaptic transmission (Pankratov *et al.*, 1998; Mori *et al.*, 2001). It was also interesting to conclude that receptors for the same ligand (ATP) but with opposite effects were present in hippocampal glutamatergic terminals, as reported to occur in glutamatergic synapses of the rat medial habenula nucleus (Price *et al.*, 2003). In particular, β,γ-ImATP caused biphasic effects on glutamate release over a narrow concentration-range, in a manner analogous to that observed to occur for the biphasic P2X/P2Y receptor modulation of noradrenaline release from the rat vas deferens (Queiroz *et al.*, 2003). It remains to be determined if this prevalence of P2X receptor-mediated facilitation over P2Y receptor-mediated inhibition of hippocampal glutamate release with increasing concentrations of ATP analogues results from: 1) a greater efficiency of glutamatergic P2X compared to P2Y receptors; 2) a greater number of glutamatergic terminals equipped with a greater density of P2X compared to P2Y receptors; 3) an interaction between P2X and P2Y receptors so that the former would down-regulate the later. The possible involvement of $P2X_1$ and/or $P2X_3$ receptors in this predominant P2X receptor-mediated facilitation of hippocampal glutamate release at higher concentrations of ATP analogues might also seem surprising in view of their fast desensitising kinetics observed in heterologous expression systems or when located postsynaptically (North, 2002). However, like for $P2X_{1-3}$ (present study; Díaz-Hernández *et al.*, 2001; Queiroz *et al.*, 2003) the activation of other presynaptic ionotropic receptors, such as kainate or nicotinic acetylcholine receptors (Khakh and Henderson, 2000), also causes long-lasting non-desensitising presynaptic changes, in clear contrast with their fast desensitising properties when located outside nerve terminals, for reasons still to be unravelled.

Finally, it still remains to be defined in what physiological conditions might this P2 receptor modulation of glutamate release play a significant role. In fact, most attempts to define a possible role for P2 receptor modulation of excitatory synaptic transmission in hippocampal slices essentially concluded that all the effects of exogenously added ATP or ATP analogues were due to adenosine P1 receptor activation (Dunwiddie *et al.*, 1997; Cunha *et al.*, 1998; Mendoza-Fernandez *et al.*, 2000; Masino *et al.*, 2002). This is in agreement with the efficiency of ecto-nucleotidases in these synapses, which are able to convert ATP (and ATP analogues) into adenosine in a channelling manner (Cunha *et al.*, 1998) within milliseconds (Dunwiddie *et al.*, 1997), making it possible to conceive a scenario where exogenously added ATP might never reach synaptic P2 receptors before being degraded by ecto-nucleotidases (Zimmermann, 2000). It should also be pointed out that these electrophysiological studies were all carried out in the same group of glutamatergic synapses connecting the Schaffer fibers to CA1 pyramidal neurons. When studying other synapses, it was found that $P2X_2$ receptors controlled glutamatergic transmission onto interneurons of the CA1 region (Khakh *et al.*, 2003), $P2Y_1$ receptors controlled interneuron excitability (Kawamura *et al.*, 2004), whereas in mossy fiber/CA3 pyramid synapses,

a mixed P2/P1 modulation by exogenously added ATP was concluded (Kukley *et al.*, 2004). Thus, it largely remains to be tested if P2 receptors might exert a more important control of glutamate release in other glutamatergic synapses in the hippocampus. A credible alternative might be that the neuromodulatory role of P2 receptors in glutamatergic synapses might not be related to conditions of low frequency stimulation, where one would expect calcium influx through P2X receptors to be most effective, but might only come into play during bursts of actions potentials, where one would expect this P2 receptor-mediated calcium entry to be over-shadowed by the massive calcium influx and the effect of other facilitating factor. Accordingly, the most consistent modulatory role of P2 receptors has been found in the control of long-term potentiation (O´Kane and Stone 2000; Pankratov *et al.*, 2002a; Almeida *et al.*, 2003). This is also in agreement with the finding that the release of ATP in hippocampal preparations increases disproportionally with increasing frequencies of nerve stimulation (Wieraszko *et al.*, 1989; Cunha *et al.*, 1996).

In conclusion, the present study provided coincident functional and molecular evidences showing that P2 receptors biphasically modulate the release of glutamate from hippocampal nerve terminals, thus explaining the discrepancy between P2 receptor expression and detected functional activity in this area of the brain. The precise physiological conditions under which this modulation becomes functionally important remain to be determined.

2 PHARMACOLOGICAL BLOCKADE OR GENETIC DELETION OF $P2Y_1$ RECEPTORS PREVENTS SYNAPTOTOXICITY AND MEMORY IMPAIRMENT CAUSED BY $A\beta_{1-42}$ ADMINISTRATION

Alzheimer's disease (AD) is the most common chronic neurodegenerative disease, characterised clinically by an atrophy of hippocampal regions and a progressive cognitive impairment (Selkoe, 2001). Although the neuropathological hallmarks of AD are the presence of neurofibrillar tangles and the accumulation of senile plaques resulting from β-amyloid peptide (Aβ) aggregation (Selkoe, 2001), the measures that correlate better with memory dysfunction in AD are the levels of soluble Aβ, mainly $A\beta_{1-42}$ - produced by β-secretase cleavage of amyloid β protein precursor (APP), favoured over α-secretase activity by particular missense mutations in APP, leading to an overproduction of these toxic Aβ peptides - and a decreased density of nerve terminals in cortical areas (reviewed in Selkoe, 2002; Coleman *et al.*, 2004). In fact, this early memory loss in AD associated with a synaptic failure is thought to result from the increased levels of Aβ oligomers rather than fibrils (Hardy and Selkoe, 2002; Klein *et al.*, 2004). Synapses seem to be the primordial target to the toxic Aβ peptides leading to a synaptic failure that underlies the mnemonic impairment (Selkoe, 2002; Hardy and Selkoe, 2002; Klein *et al.*, 2004). Thus a major lead for the development of novel therapeutic strategies for AD might be to explore mechanisms able to prevent this early synaptotoxicity caused by $A\beta_{1-42}$.

ATP is one of the most abundant metabolites in eukaryotic cells. Thus, whenever there is a noxious insult, ATP is expected to leak from damaged cells or be released by any other mechanism and thereby may reach high concentrations in the extracellular space. In fact, there are several reported observations showing that there is a massive release of ATP to the extracellular milieu that can reach up to the milimolar range either by exocytose, or via transmembrane transport, or as a result of cell damage, upon both acute and chronic injury/disorder (reviewed in Franke and Illes, 2006), and it has been shown that at high extracellular concentrations, ATP *per se* is neurotoxic (Volonté *et al.*, 2003; Amadio *et al.*, 2005). Furthermore, most effects of ATP are mediated through the activation of both types of P2 receptors, either through the ionotropic P2X receptors or through the metabotropic P2Y receptors, that has been shown to be widely expressed and functional under normal and pathophysiological conditions and can mediate and aggravate toxic signalling in many CNS neurons, because it has been reported neuroprotective effects of P2 receptors antagonists against several brain injuries/disorders (Volonté *et al.*, 2003; Franke and Illes, 2006). Moreover, the observations of changes in the expression of P2 receptors in different cell types and tissues under various pathological conditions support for the existence of pathophysiological implications of P2 receptor functions (Franke and Illes, 2006).

The involvement of P2 receptors was already described under acute pathological conditions, such as ischemia/hypoxia, and under chronic pathological conditions, such as pain, epilepsia, toxic influence of several drugs, retinal diseases and also Alzheimer´s disease (reviewed in Franke and Illes, 2006). In respect to Alzheimer's disease, although very few data is available, the reported observations give some hints for a possible involvement of P2 receptors in this neurodegenerative disease. The most prominent finding is that ATP increases the vulnerability of neurons to Aβ peptide-induced cell death (Haughney and Matson, 2003). Re-enforcing a possible role of P2 receptors in Alzheimer's disease, it was observed up-regulation of $P2X_7$ receptors around β-amyloid plaques in a transgenic mouse model of AD (Parvathenani *et al.*, 2003) and another study revealed that $P2Y_1$ receptors immunolabelling was concentrated at a number of characteristic AD structures, such as neurofibrillary tangles, neuritic plaques and neuropil threads in the hippocampus and enthorinal cortex, in comparison with the control human brain (Moore *et al.*, 2000a, 2000b).

Moreover, the growing evidence that Alzheimer's disease begins with subtle alterations of hippocampal synaptic efficacy prior to frank neuronal degeneration (Selkoe, 2002; Coleman *et al.*, 2004) indicates that the best therapeutic strategies for AD that should be developed might be to explore mechanisms able to prevent the early synaptotoxicity caused by $A\beta_{1-42}$. Several P2X and P2Y receptors are targeted to hippocampal nerve terminals, where they perform essentially a neuromodulatory role of the synaptic function (see 1 of Results; Rodrigues *et al.*, 2005). Thus P2 receptors may be potentially suitable molecular targets to control in particular the initial synaptotoxicity observed in Alzheimer's disease. Thus, this study was designed to gauge a possible involvement of P2 receptors in the neurodegeneration and in particular the early synaptotoxicity and memory loss associated to this synaptic failure caused by exposure to Aβ peptides.

2.1 Blockade of $P2Y_1$ receptors abrogates $A\beta_{1-42}$-induced neuronal death

The aim of this study was not only to evaluate if P2 receptors were involved in the neuronal death, but mainly in the early events, such as synaptoptoxicity and memory loss, caused by $A\beta_{1-42}$ oligomers. Thus, at the cellular level, the aim was to evaluate if P2 receptors were involved in the neuronal death caused by $A\beta_{1-42}$, and particularly in the synaptotoxicity and dendritic atrophy, that was suggested to precede the subsequent neuronal death (Selkoe, 2002; Coleman *et al.*, 2004). For that purpose, an *in vitro* model using rat hippocampal neurons was developed, in order to be possible to study P2 receptors involvement in the early toxic events and neuronal death caused by $A\beta_{1-42}$, in a differential manner. An *in vitro* model of $A\beta_{1-42}$ toxicity with those particular features was achieved challenging rat hippocampal neurons with 0.5 µM of $A\beta_{1-42}$, which caused a decrease in the number of viable neurons upon 48 h incubation (82.0 ± 0.5% of viable cells, n=5) in comparison to the obtained in control conditions (95.8±0.5%, n=5;

$p<0.05$) (Figure 21A). However, 12 h upon incubation with 0.5 µM $A\beta_{1-42}$, when no significant neuronal death was yet detected, it was possible to observe already a synaptotoxicity and a dendritic atrophy of rat hippocampal neurons, evaluated by immunocytochemical analysis of synaptophysin (synaptic marker) and MAP-2 (microtubule associated protein 2; dendritic marker), respectively, as illustrated in figure 21B.

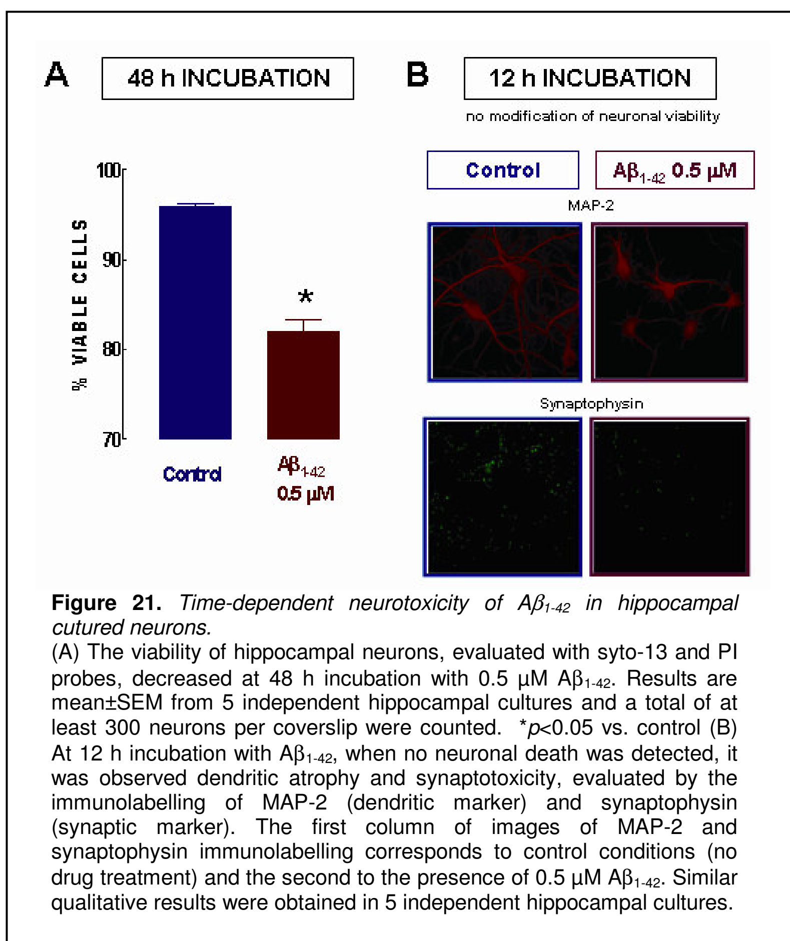


Figure 21. *Time-dependent neurotoxicity of $A\beta_{1-42}$ in hippocampal cutured neurons.*
(A) The viability of hippocampal neurons, evaluated with syto-13 and PI probes, decreased at 48 h incubation with 0.5 µM $A\beta_{1-42}$. Results are mean±SEM from 5 independent hippocampal cultures and a total of at least 300 neurons per coverslip were counted. *$p<0.05$ vs. control (B) At 12 h incubation with $A\beta_{1-42}$, when no neuronal death was detected, it was observed dendritic atrophy and synaptotoxicity, evaluated by the immunolabelling of MAP-2 (dendritic marker) and synaptophysin (synaptic marker). The first column of images of MAP-2 and synaptophysin immunolabelling corresponds to control conditions (no drug treatment) and the second to the presence of 0.5 µM $A\beta_{1-42}$. Similar qualitative results were obtained in 5 independent hippocampal cultures.

Thus, it was first evaluated the possible involvement of P2 receptors in the decrease of neuronal viability caused by the exposure of rat hippocampal neurons to 0.5 µM $A\beta_{1-42}$ for 48 h. The presence of PPADS (10 µM), a generic P2 receptors antagonist, prevented ($p<0.05$) the neuronal death caused by 0.5 µM $A\beta_{1-42}$ (Figure 22A). In order to discriminate if it was the blockade of P2X or of P2Y receptors that was affording the neuroprotection observed in the presence of PPADS (10 µM), two antagonists that better discriminate between P2X and P2Y receptors, NF023 (10 µM), a selective antagonist for $P2X_1$, $P2X_{2/3}$ and $P2X_3$ (Soto *et al.*, 1999), and RB2 (10 µM), that at this concentration is a preferring antagonist for P2Y receptors, were probed. NF023 (10 µM) did not cause any modification in the decreased neuronal viability caused by $A\beta_{1-42}$ ($p>0.05$), whereas RB2 (10 µM) mimicked the effect observed in the presence of PPADS (10 µM), preventing ($p<0.05$) the modification of neuronal viability caused by 0.5 µM $A\beta_{1-42}$ (Figure 22B). These results indicate that it is the blockade of P2Y receptors and not of P2X receptors that is responsible for the neuroprotection observed in the presence of PPADS (10 µM). Next, in order to discriminate which P2Y receptors would be involve in the $A\beta_{1-42}$ neurotoxicity, the effect of the selective antagonist for $P2Y_1$ receptors, MRS2179 (10 µM), was tested. Notably, the blockade of $P2Y_1$ receptors with

MRS2179 (10 µM) prevented the decrease of the number of viable neurons caused by the exposure to 0.5 µM $A\beta_{1\text{-}42}$ (Figure 22C), indicating that $P2Y_1$ receptor is involved in the neuronal death caused by $A\beta_{1\text{-}42}$. It should be noted that none of the antagonists tested alone significantly modified neuronal viability (Figure 22).

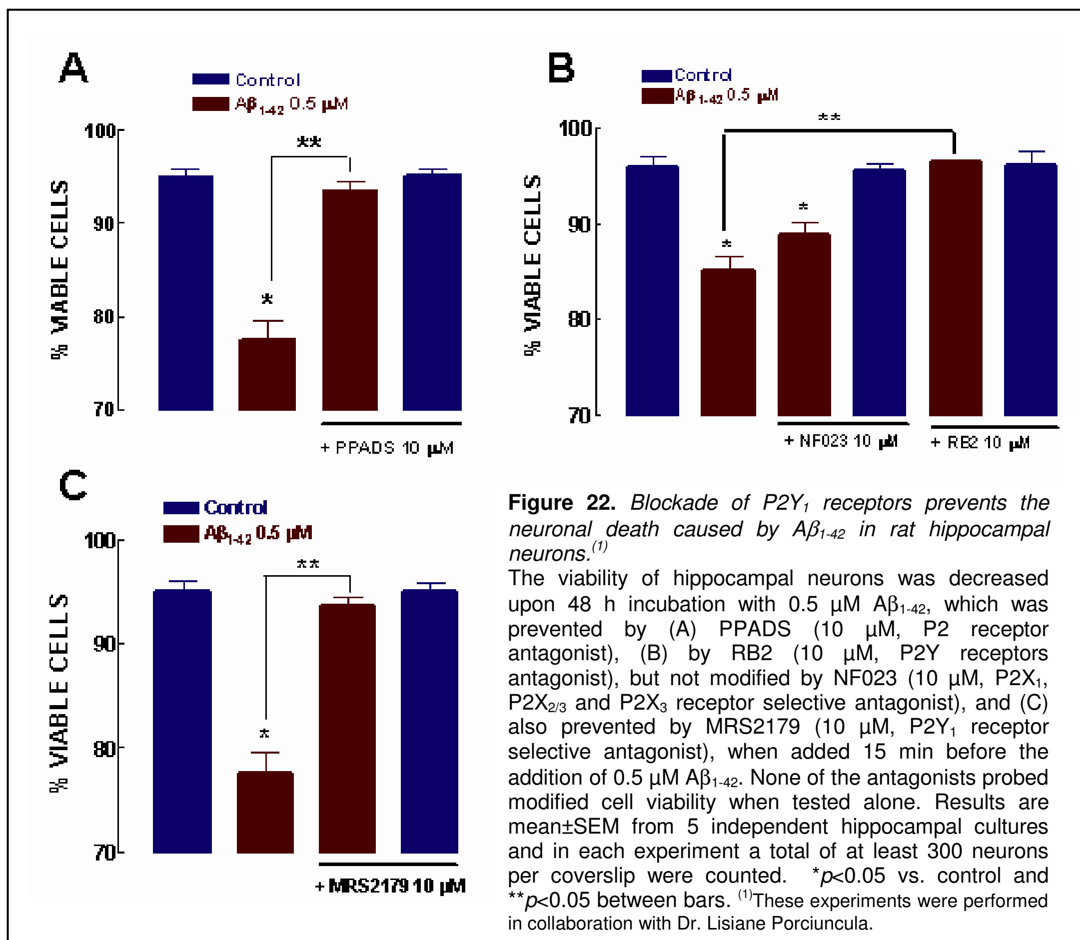


Figure 22. *Blockade of $P2Y_1$ receptors prevents the neuronal death caused by $A\beta_{1\text{-}42}$ in rat hippocampal neurons.*[(1)]
The viability of hippocampal neurons was decreased upon 48 h incubation with 0.5 µM $A\beta_{1\text{-}42}$, which was prevented by (A) PPADS (10 µM, P2 receptor antagonist), (B) by RB2 (10 µM, P2Y receptors antagonist), but not modified by NF023 (10 µM, $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptor selective antagonist), and (C) also prevented by MRS2179 (10 µM, $P2Y_1$ receptor selective antagonist), when added 15 min before the addition of 0.5 µM $A\beta_{1\text{-}42}$. None of the antagonists probed modified cell viability when tested alone. Results are mean±SEM from 5 independent hippocampal cultures and in each experiment a total of at least 300 neurons per coverslip were counted. $^*p<0.05$ vs. control and $^{**}p<0.05$ between bars. [(1)]These experiments were performed in collaboration with Dr. Lisiane Porciuncula.

2.2 Blockade of $P2Y_1$ receptor prevents the sinaptotoxicity and dendritic atrophy caused by $A\beta_{1\text{-}42}$ in rat hippocampal neurons

Next it was evaluated if the blockade of $P2Y_1$ receptors was also able to prevent the early toxic events synaptotoxicity and dendritic atrophy observed to precede neuronal death, as illustrated in figure 21 and also as previously postulated (Selkoe, 2002). In fact, immunocytochemical analysis of hippocampal neurons double-labelled for MAP-2 and synaptophysin revealed that the blockade of $P2Y_1$ receptors with MRS2179 (10 µM) attenuated both synaptic loss and dendritic retraction caused by 0.5 µM $A\beta_{1\text{-}42}$, as illustrated in figure 23. Thus, $P2Y_1$ receptor blockade not only prevents neuronal death, but also presents a neuroprotective effect in the synaptotoxicity caused by $A\beta_{1\text{-}42}$ that precedes the subsequent neuronal death.

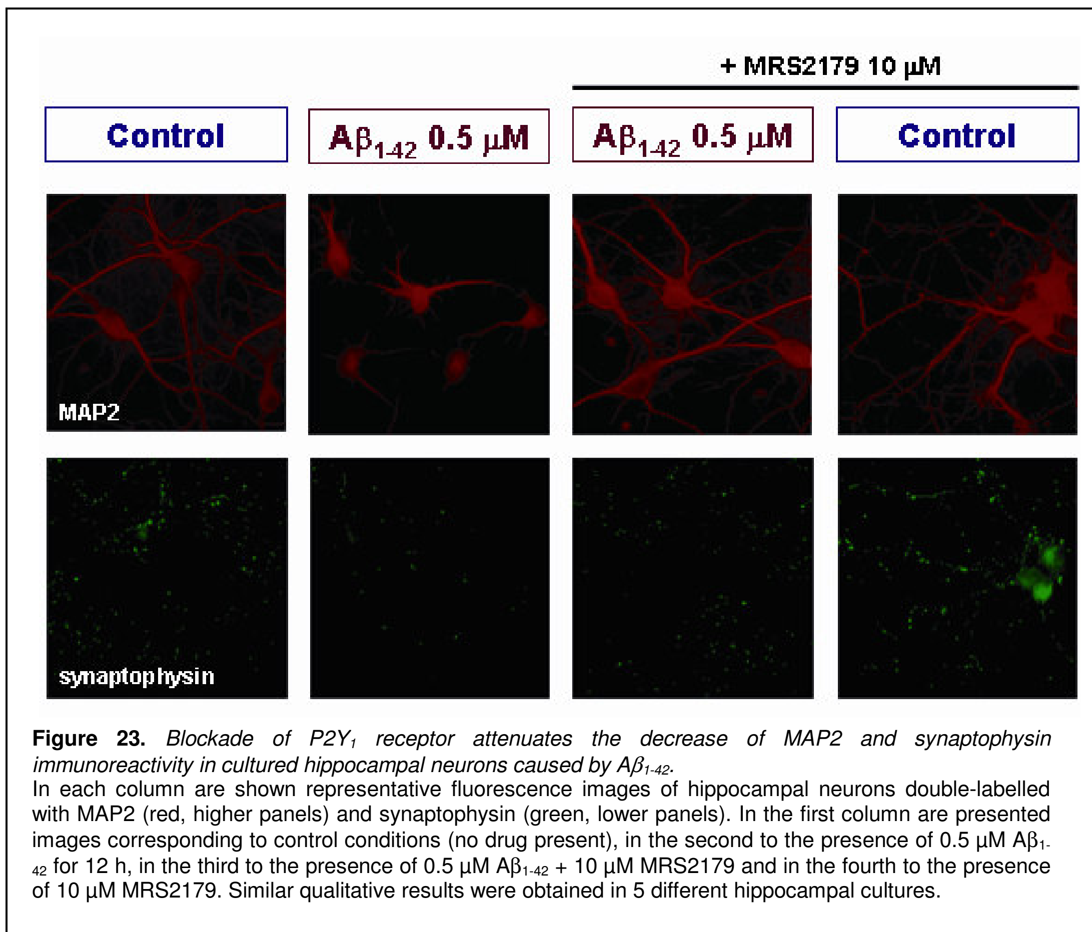


Figure 23. *Blockade of $P2Y_1$ receptor attenuates the decrease of MAP2 and synaptophysin immunoreactivity in cultured hippocampal neurons caused by $A\beta_{1-42}$.*
In each column are shown representative fluorescence images of hippocampal neurons double-labelled with MAP2 (red, higher panels) and synaptophysin (green, lower panels). In the first column are presented images corresponding to control conditions (no drug present), in the second to the presence of 0.5 µM $A\beta_{1-42}$ for 12 h, in the third to the presence of 0.5 µM $A\beta_{1-42}$ + 10 µM MRS2179 and in the fourth to the presence of 10 µM MRS2179. Similar qualitative results were obtained in 5 different hippocampal cultures.

2.3 $P2Y_1$ receptors are expressed in rat hippocampal neurons and are targeted to nerve terminals

Since it was observed that $P2Y_1$ receptors are involved in the neurotoxic actions of $A\beta_{1-42}$ on rat hippocampal neurons, it was necessary to confirm their localization in rat hippocampal neurons. For that purpose, immunochemical analysis of $P2Y_1$ receptors in rat hippocampal neurons was performed by both immunocytochemistry and Western blot analysis. As expected, rat hippocampal membranes presented immunoreactivity for $P2Y_1$ receptors revealed by Western blot analysis (Figure 24B). This was found to be located in neuronal elements, since by double-labelling immunocytochemistry, it was possible to observe co-localization of $P2Y_1$ receptors with the dendritic/neuronal marker MAP-2 (Figure 24A). Furthermore, it was also observed that some of the immunoreactivity detected for $P2Y_1$ receptors was found to be co-located with synaptophysin immunolabelling (Figure 24A), which shows that $P2Y_1$ receptors are targeted to nerve terminals in rat hippocampal neurons, like the observed in adult brain tissue

(1 of Results; Rodrigues *et al.*, 2005). This re-enforces the involvement of $P2Y_1$ receptors in the synaptotoxicity caused by $A\beta_{1-42}$.

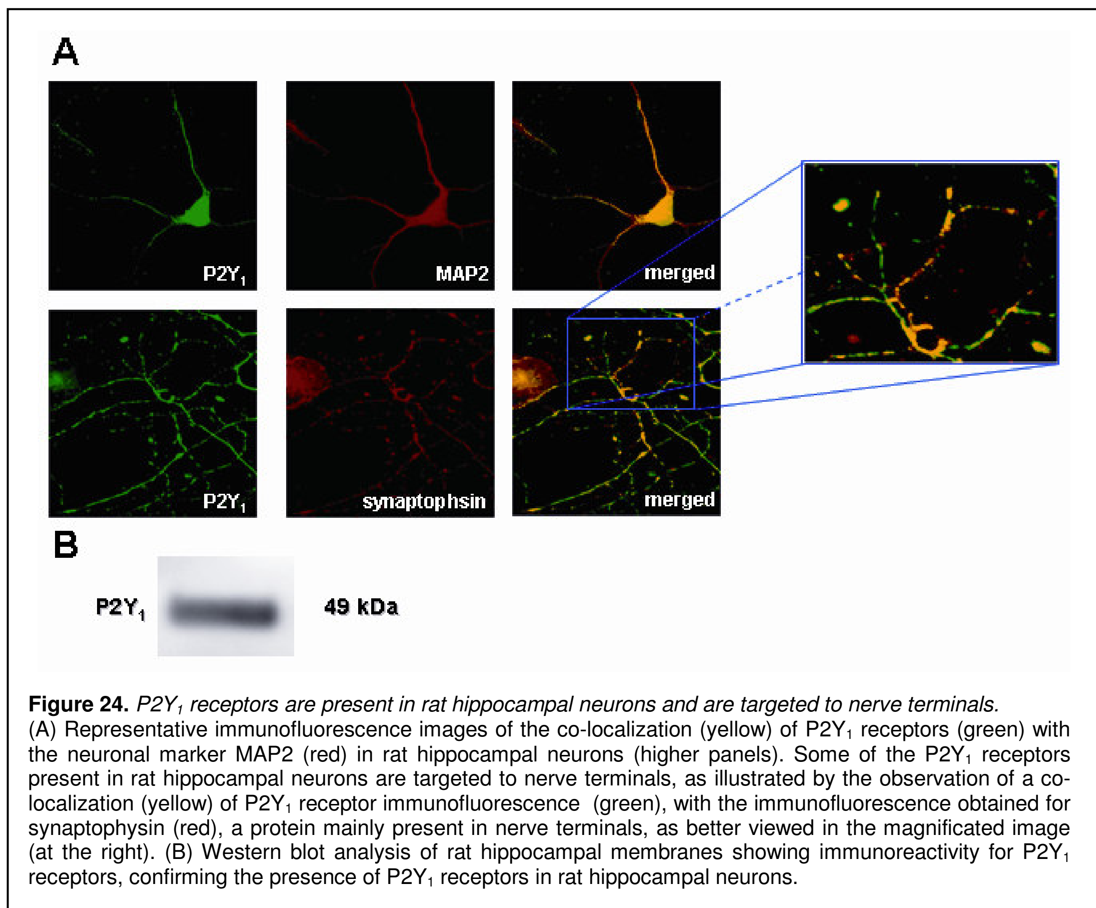


Figure 24. *$P2Y_1$ receptors are present in rat hippocampal neurons and are targeted to nerve terminals.* (A) Representative immunofluorescence images of the co-localization (yellow) of $P2Y_1$ receptors (green) with the neuronal marker MAP2 (red) in rat hippocampal neurons (higher panels). Some of the $P2Y_1$ receptors present in rat hippocampal neurons are targeted to nerve terminals, as illustrated by the observation of a co-localization (yellow) of $P2Y_1$ receptor immunofluorescence (green), with the immunofluorescence obtained for synaptophysin (red), a protein mainly present in nerve terminals, as better viewed in the magnificated image (at the right). (B) Western blot analysis of rat hippocampal membranes showing immunoreactivity for $P2Y_1$ receptors, confirming the presence of $P2Y_1$ receptors in rat hippocampal neurons.

2.4 $A\beta_{1-42}$ exposure increases the density of $P2Y_1$ receptors in rat hippocampal neurons

Very often, the prolonged activation of receptors leads to their desensitization, making them poor molecular targets (see Burrone and Murthy, 2001). Thus, it was evaluated if the exposure to $A\beta_{1-42}$ caused any modification at the density of $P2Y_1$ receptors. As illustrated in figure 25, the membranes of the hippocampal neurons exposed to 0.5 µM $A\beta_{1-42}$ for 48 h presented a higher density of $P2Y_1$ receptors, in comparison to the control (129.0 ± 5.6% of the immunoreactivity obtained in the control that was set as 100 %; n=4; $p<0.05$). This result demonstrates that there is no desensitization of $P2Y_1$ receptors and it shows that the exposure to $A\beta_{1-42}$ actually increases the density of $P2Y_1$ receptors. Thus, upon $A\beta_{1-42}$ exposure, there is an up-regulation of $P2Y_1$ receptors, which supports a scenario where $P2Y_1$ receptors may be over-functioning

and may become deleterious to cells in the realm of $A\beta_{1-42}$ neurotoxicity. This would agree with the neuroprotective role observed with the blockade of $P2Y_1$ receptors.

2.5 Increased density of synaptic $P2Y_1$ receptors in an animal model of Alzheimer´s disease

The results obtained in the *in vitro* model of $A\beta_{1-42}$ neurotoxicity presented above, clearly show that the pharmacological blockade of $P2Y_1$ receptors prevents not only the neuronal death, but also the synaptotoxicity and dendritic atrophy that precedes that neuronal death. Furthermore, it was observed that the exposure to $A\beta_{1-42}$ induces an increase in the density of $P2Y_1$ receptors, suggesting that there is an over and abnormal function of $P2Y_1$ receptors induced by $A\beta_{1-42}$, which should be deleterious to neurons, which is in agreement with the neuroprotection observed upon the blockade of $P2Y_1$ receptors.

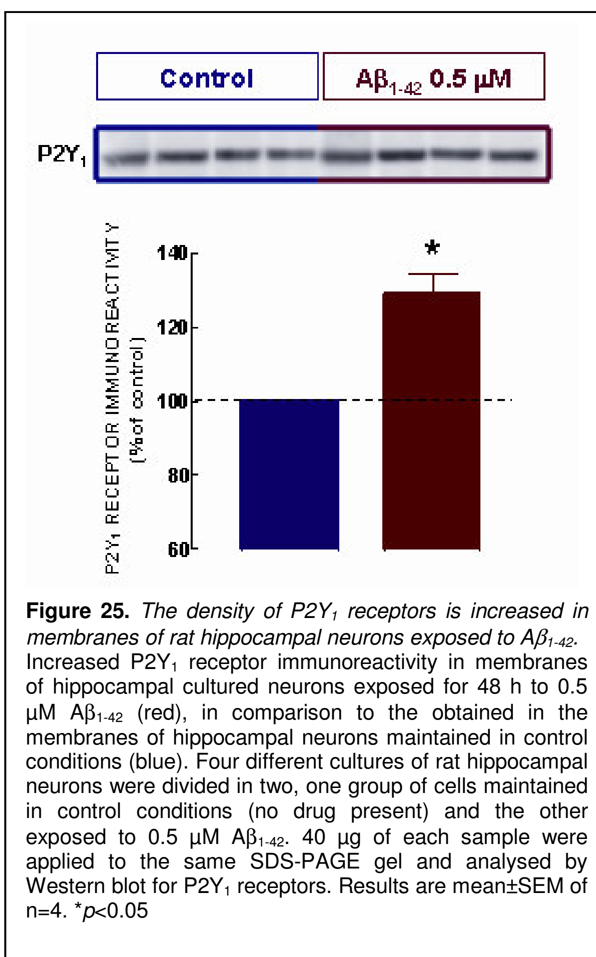


Figure 25. *The density of $P2Y_1$ receptors is increased in membranes of rat hippocampal neurons exposed to $A\beta_{1-42}$.* Increased $P2Y_1$ receptor immunoreactivity in membranes of hippocampal cultured neurons exposed for 48 h to 0.5 µM $A\beta_{1-42}$ (red), in comparison to the obtained in the membranes of hippocampal neurons maintained in control conditions (blue). Four different cultures of rat hippocampal neurons were divided in two, one group of cells maintained in control conditions (no drug present) and the other exposed to 0.5 µM $A\beta_{1-42}$. 40 µg of each sample were applied to the same SDS-PAGE gel and analysed by Western blot for $P2Y_1$ receptors. Results are mean±SEM of n=4. *$p<0.05$

The next logical question needed to be answered was if these observations made *in vitro* had some parallel in an *in vivo* model, in particular if blocking $P2Y_1$ receptors is also effective to counteract the early synaptotoxicity also *in vivo,* and the early memory loss postulated to be due to a synaptic failure in AD (Selkoe, 2002; Coleman *et al.*, 2004). To address this question it was needed to be developed an animal model that would mimic AD, in particular the early intertwined events synaptic loss and memory loss. Both the early synaptotoxicity and memory loss, the early occurring events in AD aimed to be studied in this work, are thought to result from the increased levels of Aβ peptides, mainly $A\beta_{1-42}$ (Selkoe, 2002; Coleman *et al.*, 2004). Thus, 2 nmol of soluble $A\beta_{1-42}$ were injected icv in young adult male Wistar rats (6-8 weeks), as described in 1.1.1 of Materials and Methods, and 14 days later it was observed a mnemonic impairment ($p<0.05$), gauged by Y-maze test (Figure 26B), and a lower density of hippocampal nerve terminals ($p<0.05$), indirectly measured by the

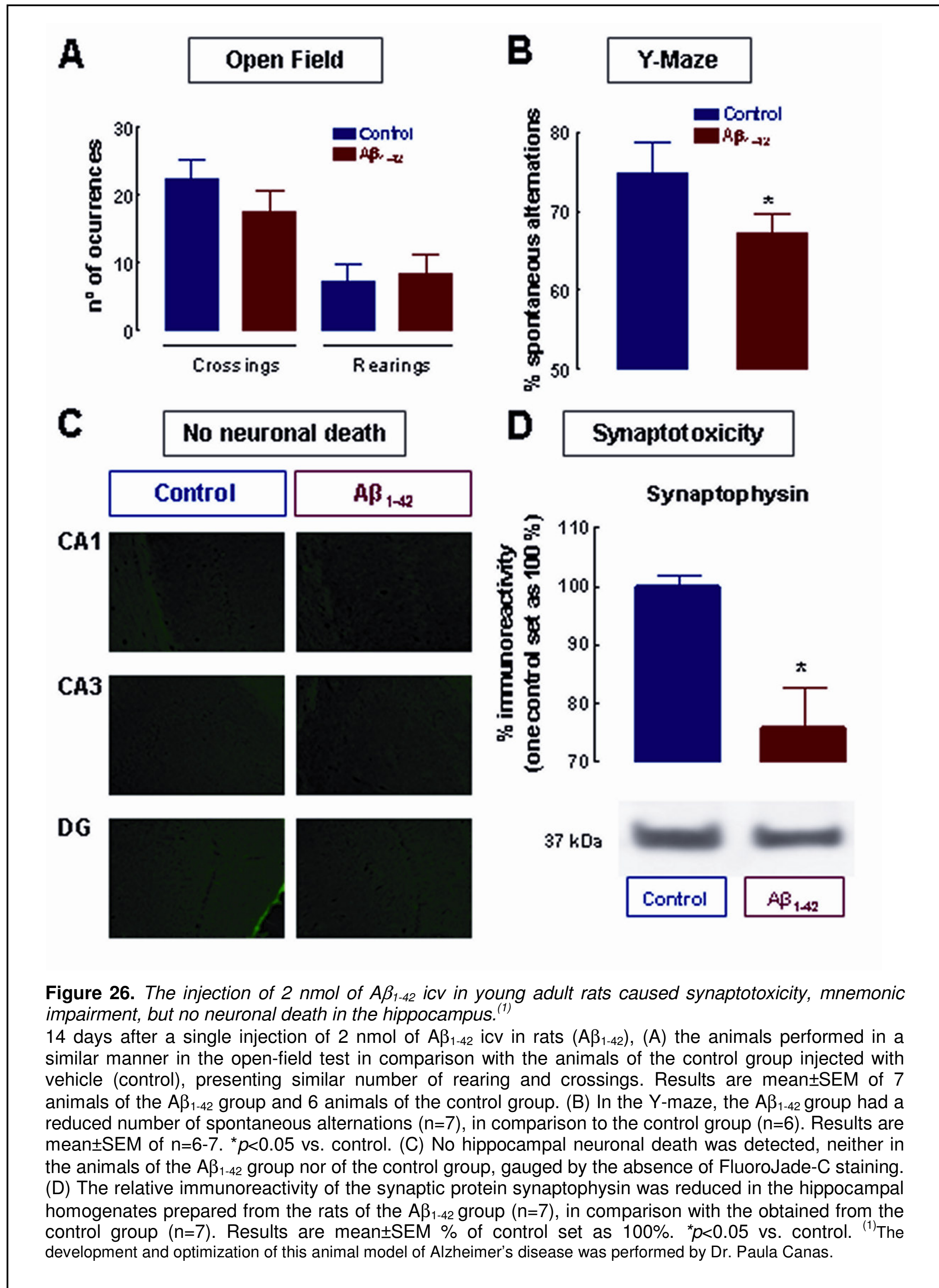


Figure 26. *The injection of 2 nmol of $A\beta_{1\text{-}42}$ icv in young adult rats caused synaptotoxicity, mnemonic impairment, but no neuronal death in the hippocampus.*[(1)]
14 days after a single injection of 2 nmol of $A\beta_{1\text{-}42}$ icv in rats ($A\beta_{1\text{-}42}$), (A) the animals performed in a similar manner in the open-field test in comparison with the animals of the control group injected with vehicle (control), presenting similar number of rearing and crossings. Results are mean±SEM of 7 animals of the $A\beta_{1\text{-}42}$ group and 6 animals of the control group. (B) In the Y-maze, the $A\beta_{1\text{-}42}$ group had a reduced number of spontaneous alternations (n=7), in comparison to the control group (n=6). Results are mean±SEM of n=6-7. *$p<0.05$ vs. control. (C) No hippocampal neuronal death was detected, neither in the animals of the $A\beta_{1\text{-}42}$ group nor of the control group, gauged by the absence of FluoroJade-C staining. (D) The relative immunoreactivity of the synaptic protein synaptophysin was reduced in the hippocampal homogenates prepared from the rats of the $A\beta_{1\text{-}42}$ group (n=7), in comparison with the obtained from the control group (n=7). Results are mean±SEM % of control set as 100%. *$p<0.05$ vs. control. [(1)]The development and optimization of this animal model of Alzheimer's disease was performed by Dr. Paula Canas.

reduction of the relative immunoreactivity obtained for synaptic proteins (Figure 26D). Importantly, no hippocampal neuronal death was observed (Figure 26C). It should be noted that the injection of 2 nmol of soluble $A\beta_{1\text{-}42}$ *icv* did not modifiy locomotor activity (Figure 26A), which

shows that the worse performance of the animals injected with $A\beta_{1-42}$ in the Y-maze was not due to a locomotor-derived artefact.

The first question that was addressed was if the up-regulation of $P2Y_1$ receptors observed in rat hippocampal neurons upon exposure to $A\beta_{1-42}$ also occurs in the whole animal *in vivo*. For that purpose, it was evaluated by Western blot analysis the immunoreactivity of $P2Y_1$ receptors in total hippocampal membranes and nerve terminals membranes of the hippocampi of rats

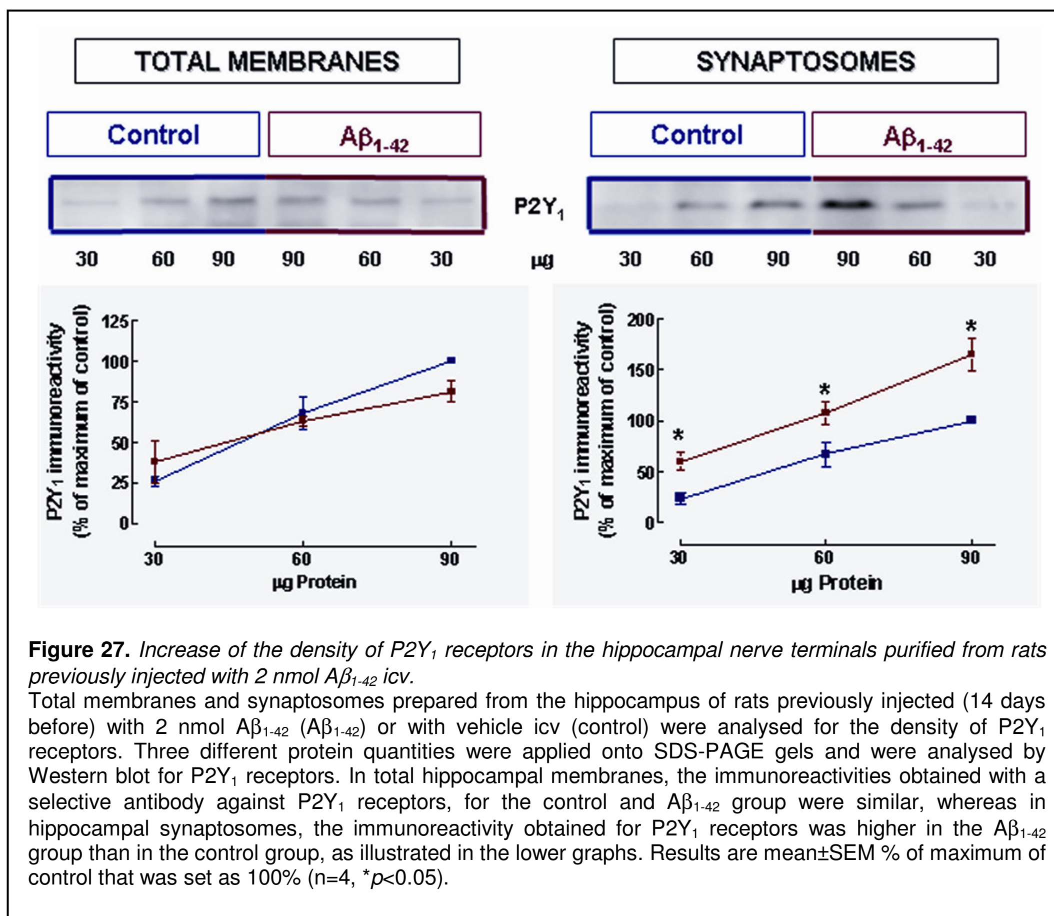


Figure 27. *Increase of the density of $P2Y_1$ receptors in the hippocampal nerve terminals purified from rats previously injected with 2 nmol $A\beta_{1-42}$ icv.*
Total membranes and synaptosomes prepared from the hippocampus of rats previously injected (14 days before) with 2 nmol $A\beta_{1-42}$ ($A\beta_{1-42}$) or with vehicle icv (control) were analysed for the density of $P2Y_1$ receptors. Three different protein quantities were applied onto SDS-PAGE gels and were analysed by Western blot for $P2Y_1$ receptors. In total hippocampal membranes, the immunoreactivities obtained with a selective antibody against $P2Y_1$ receptors, for the control and $A\beta_{1-42}$ group were similar, whereas in hippocampal synaptosomes, the immunoreactivity obtained for $P2Y_1$ receptors was higher in the $A\beta_{1-42}$ group than in the control group, as illustrated in the lower graphs. Results are mean±SEM % of maximum of control that was set as 100% (n=4, **p*<0.05).

previously (14 days before) injected with 2 nmol of $A\beta_{1-42}$ icv. It should be noted that in every experiments performed, all the animals used had to present the phenotype described in figure 26, *i.e.*, mnemonic deficit, synaptotoxicity and no neuronal death 14 days after the injection with $A\beta_{1-42}$. As illustrated in figure 27, it was observed a higher density of $P2Y_1$ receptors in hippocampal nerve terminals purified from the animals injected with $A\beta_{1-42}$, whereas in total hippocampal membranes there was no significant change (p>0.05) in comparison to the obtained for the control animals. Thus, these results show that, as observed in the *in vitro* model of $A\beta_{1-42}$ neurotoxicity, also *in vivo,* $A\beta_{1-42}$ administration induces an increase in the density of $P2Y_1$ receptors. Furthermore, it clearly shows that this up-regulation occurs selectively at the synaptic level, which strongly suggests that the over-functioning suggested by

the up-regulation of $P2Y_1$ receptors should occur at the synapses. This indicates that $P2Y_1$ receptors should be involved also *in vivo* in the synaptotoxicity and subsequently in the memory loss caused by $A\beta_{1-42}$

2.6 *Genetic deletion of $P2Y_1$ receptor prevents synaptotoxicity caused by $A\beta_{1-42}$*

Taking advantage of a $P2Y_1R$ KO mouse (Leon *et al*., 1999), it was evaluated if the removal of $P2Y_1$ receptor function, imposed by the genetic deletion of $P2Y_1$ receptor, was also able to prevent synaptotoxicity caused by $A\beta_{1-42}$ *in vivo*. The same protocol of induction of the early events of Alzheimer's disease used for rats was applied for wild-type and $P2Y_1R$ KO C57BL/6 mice. As illustrated in figure 28, 4 groups of animals were analysed: 1) control (+/+) (wild-type mice injected with vehicle), 2) $A\beta_{1-42}$ (+/+) (wild-type mice injected with 2 nmol $A\beta_{1-42}$ icv), 3) control (-/-) ($P2Y_1R$ KO mice injected with vehicle) and 4) $A\beta_{1-42}$ (-/-)($P2Y_1R$ KO mice injected with 2 nmol $A\beta_{1-42}$ icv). In wild-type mice, 14 days after the injection of 2 nmol $A\beta_{1-42}$ icv, it was observed a reduction ($p<0.05$) in the relative immunoreactivity for the synaptic proteins synaptophysin and SNAP-25 in the hippocampus. This shows that, as observed in rats, also in mice $A\beta_{1-42}$ induced a detectable reduction in the density of hippocampal nerve terminals (Figure 29). On the other hand, in the $P2Y_1R$ KO mice injected with 2 nmol of $A\beta_{1-42}$, it was not detected any significant ($p>0.05$) modification of the relative immunoreactivity for synaptophysin and SNAP-25 in comparison to $P2Y_1R$ KO mice injected with vehicle (Figure 29). The relative immunoreactivity obtained in hippocampal tissue of $P2Y_1R$ KO mice for both synaptic proteins was similar ($p>0.05$) to that obtained for the wild-type mice. These data clearly show that the removal of $P2Y_1$ receptors prevents the synaptotoxicity caused by $A\beta_{1-42}$ also *in vivo*. It should be noticed that, as observed in rats, 14 days after the injection of $A\beta_{1-42}$, it was not detected any hippocampal neuronal death, *i.e*, absence of FluoroJade-C staining, in either wild-type or $P2Y_1R$ KO mice, as illustrated in figure 30.

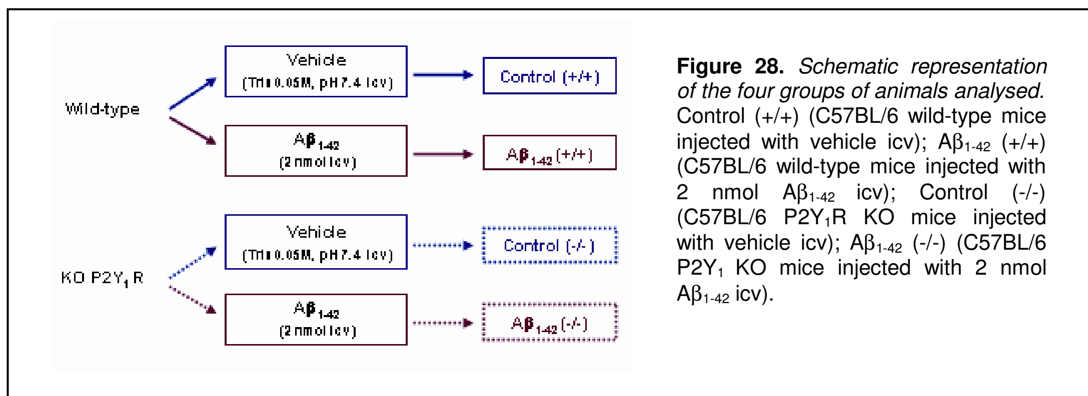


Figure 28. *Schematic representation of the four groups of animals analysed.* Control (+/+) (C57BL/6 wild-type mice injected with vehicle icv); $A\beta_{1-42}$ (+/+) (C57BL/6 wild-type mice injected with 2 nmol $A\beta_{1-42}$ icv); Control (-/-) (C57BL/6 $P2Y_1R$ KO mice injected with vehicle icv); $A\beta_{1-42}$ (-/-) (C57BL/6 $P2Y_1$ KO mice injected with 2 nmol $A\beta_{1-42}$ icv).

Taken together, these results clearly show that $P2Y_1$ receptors play a pivotal role in the early toxicity caused by $A\beta_{1-42}$, because the removal of $P2Y_1$ receptors function, either by the

pharmacological blockade or by the genetic deletion of $P2Y_1$ receptors, prevented the early synaptotoxicity caused by $A\beta_{1-42}$.

2.7 *Increased density of the synaptically located $P2Y_1$ receptors in a mice model of of Alzheimer´s disease*

The previous results indicate that $P2Y_1$ receptors are necessary to enable $A\beta_{1-42}$-induced neurotoxicity, which together with the observation of an up-regulation of $P2Y_1$ receptors upon the exposure to $A\beta_{1-42}$, in particular at the synaptic level, strongly suggests that this increase in the density of synaptic $P2Y_1$ receptors, is a mandatory step of the pathway by which $A\beta_{1-42}$ peptides cause synaptotoxicity/neurotoxicity. Thus, to re-enforce this suggestion, it was evaluated if there was an increase in the density of $P2Y_1$ receptors in hippocampal nerve terminals in these C57BL/6 mice as well, in which the genetic removal of $P2Y_1$ receptors

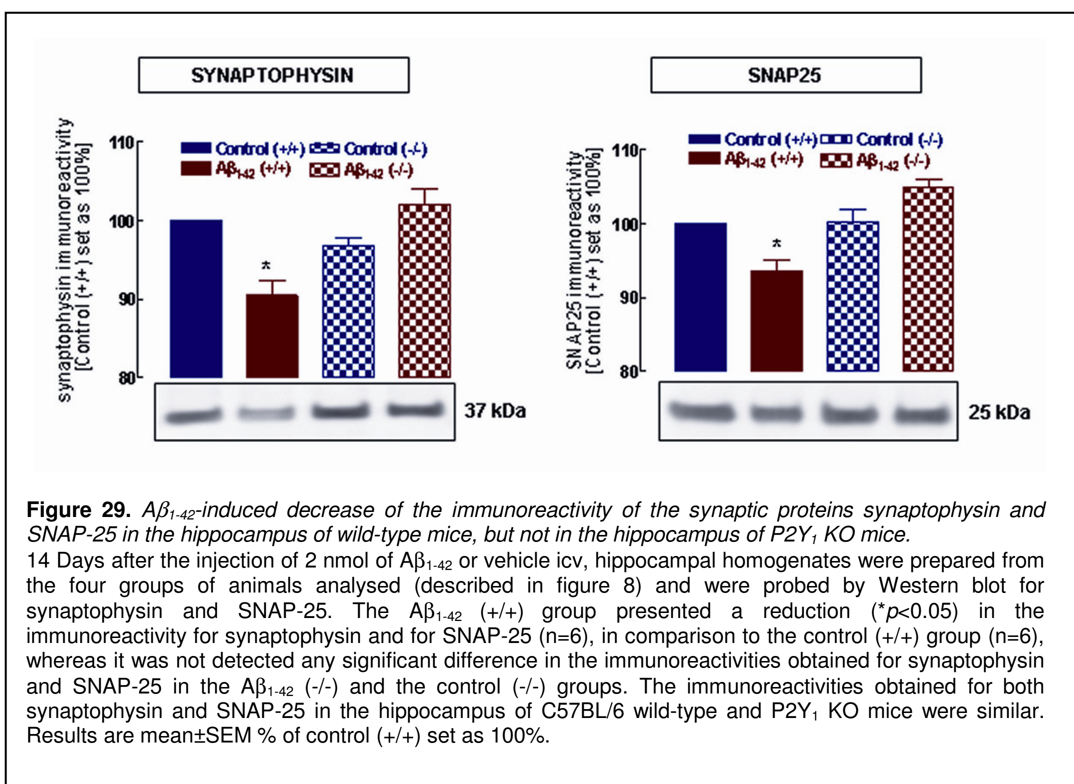


Figure 29. *$A\beta_{1-42}$-induced decrease of the immunoreactivity of the synaptic proteins synaptophysin and SNAP-25 in the hippocampus of wild-type mice, but not in the hippocampus of $P2Y_1$ KO mice.*
14 Days after the injection of 2 nmol of $A\beta_{1-42}$ or vehicle icv, hippocampal homogenates were prepared from the four groups of animals analysed (described in figure 8) and were probed by Western blot for synaptophysin and SNAP-25. The $A\beta_{1-42}$ (+/+) group presented a reduction (*$p<0.05$) in the immunoreactivity for synaptophysin and for SNAP-25 (n=6), in comparison to the control (+/+) group (n=6), whereas it was not detected any significant difference in the immunoreactivities obtained for synaptophysin and SNAP-25 in the $A\beta_{1-42}$ (-/-) and the control (-/-) groups. The immunoreactivities obtained for both synaptophysin and SNAP-25 in the hippocampus of C57BL/6 wild-type and $P2Y_1$ KO mice were similar. Results are mean±SEM % of control (+/+) set as 100%.

prevented the synaptotoxicity caused by $A\beta_{1-42}$. In fact, as observed in rats, there was an increase ($p<0.05$) in the relative immunoreactivity of $P2Y_1$ receptors in the hippocampal nerve terminals of the wild-type mice injected with $A\beta_{1-42}$ icv (Figure 31).

2.8 Genetic deletion of $P2Y_1$ receptor prevents memory loss caused by $A\beta_{1-42}$

Memory loss in AD seems to be associated with a synaptic loss (Selkoe, 2002; Coleman *et al.*, 2004) and since it was observed that either the pharmacological blockade or the genetic

deletion of $P2Y_1$ receptors prevented the synaptotoxicity caused by $A\beta_{1-42}$, the next logical step was to investigate if the $A\beta_{1-42}$-induced memory impairment could be also prevented by the removal of $P2Y_1$ receptors function.

14 Days after the injection of 2 nmol $A\beta_{1-42}$ icv in C57BL/6 wild-type mice ($A\beta_{1-42}$ (+/+)), it was observed a mnemonic impairment ($p<0.05$; Y-maze) in comparison to the performace of the control (+/+) group (Figure 32B), as observed in rats (Figure 26B). On the other hand, $P2Y_1R$ KO mice injected with 2 nmol $A\beta_{1-42}$ icv presented a similar ($p>0.05$) number of spontaneous alternations in comparison with the obtained for the control animals (control (-/-)) (Figure 32B). This means that $A\beta_{1-42}$ was no longer able to provoke a mnemonic impairment in mice lacking $P2Y_1$ receptors. It should be pointed out that it was confirmed that the injection of $A\beta_{1-42}$ did not produce any modification of the locomotor activity either in wild-type or in $P2Y_1$ KO mice (Figure 32A). Thus, these data clearly show that $P2Y_1$ receptors is not only involved in the synaptic loss, but also in the memory loss caused by $A\beta_{1-42}$, as expected considering that synaptic failure and memory loss are intertwined phenomena in Alzheimer's disease.

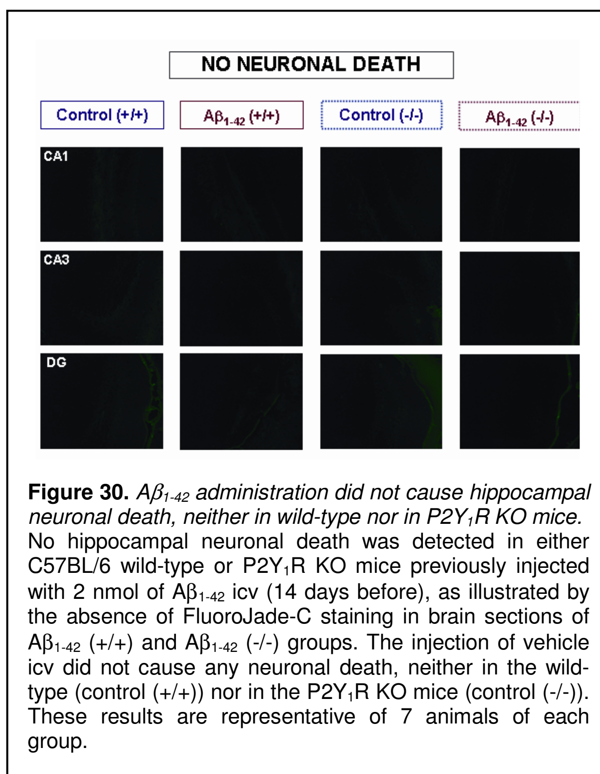


Figure 30. *$A\beta_{1-42}$ administration did not cause hippocampal neuronal death, neither in wild-type nor in $P2Y_1R$ KO mice.* No hippocampal neuronal death was detected in either C57BL/6 wild-type or $P2Y_1R$ KO mice previously injected with 2 nmol of $A\beta_{1-42}$ icv (14 days before), as illustrated by the absence of FluoroJade-C staining in brain sections of $A\beta_{1-42}$ (+/+) and $A\beta_{1-42}$ (-/-) groups. The injection of vehicle icv did not cause any neuronal death, neither in the wild-type (control (+/+)) nor in the $P2Y_1R$ KO mice (control (-/-)). These results are representative of 7 animals of each group.

2.9 Discussion

The present study shows that the pharmacological blockade of $P2Y_1$ receptors prevents the neuronal death of hippocampal neurons exposed to $A\beta_{1-42}$. Furthermore, the time-course of neurotoxicity triggered by $A\beta_{1-42}$ in the cultured neurons indicates that there is a synaptotoxicity and dendritic atrophy that precedes the loss of neuronal viability and both of these early $A\beta_{1-42}$-provoked events were abrogated by the blockade of $P2Y_1$ receptors, shown to be present in rat hippocampal neurons and targeted to nerve terminals. This role of $P2Y_1$ receptors is further strengthen by the present observations *in vitro*, that it has a parallel role in the whole animal, *in vivo*. The injection of 2 nmol of $A\beta_{1-42}$ icv, both in rats and mice, caused a decrease in the density of nerve terminals in the hippocampus, a brain structure involved in learning and

memory processes (Lopes da Silva *et al.*, 1990), and a mnemonic impairment, with no hippocampal neuronal death, screened 14 days after the injection. In contrast, in mice deficient in $P2Y_1$ receptor ($P2Y_1R$ KO C57BL/6 mice; Leon *et al.*, 1999), $A\beta_{1\text{-}42}$ was no longer able to provoke synaptic and memory loss. Thus, the genetic deletion of $P2Y_1$ receptors prevented the synaptotoxicity caused by $A\beta_{1\text{-}42}$ *in vivo*, in agreement with the data obtained by the pharmacological blockade of $P2Y_1R$ in the *in vitro* model of $A\beta_{1\text{-}42}$ neurotoxicity. Finally, in this study it is clearly demonstrated that $A\beta_{1\text{-}42}$ causes also an up-regulation of $P2Y_1$ receptors, an observation made both *in vitro* and *in vivo*. Furthermore, it was shown that this increase in the density of $P2Y_1$ receptors occurs selectively at nerve terminals. This result not only re-enforces the involvement of $P2Y_1$ receptor in the $A\beta_{1\text{-}42}$-induced toxicity, but also indicates that the $P2Y_1$ receptors involved in the $A\beta_{1\text{-}42}$ toxicity should be the $P2Y_1$ receptors located at synapses. Thus, this study provides the first molecular, cellular, morphological and behavioural data demonstrating that $P2Y_1$ receptors are mandatory in the $A\beta_{1\text{-}42}$-induced neurotoxic effects, making $P2Y_1$ receptor a potential suitable molecular target to arrest Alzheimeir´s disease, in particular at the early onset of this neurodegenerative disease.

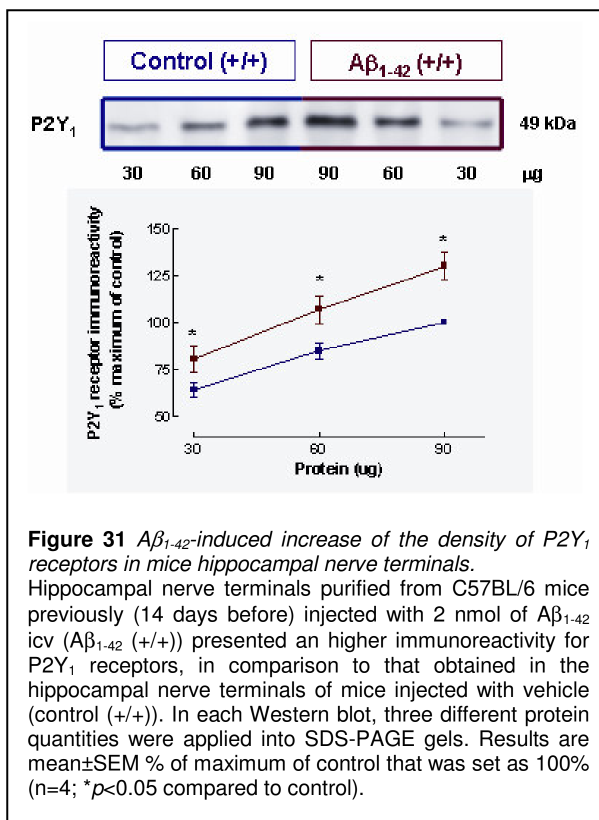


Figure 31 *$A\beta_{1\text{-}42}$-induced increase of the density of $P2Y_1$ receptors in mice hippocampal nerve terminals.* Hippocampal nerve terminals purified from C57BL/6 mice previously (14 days before) injected with 2 nmol of $A\beta_{1\text{-}42}$ icv ($A\beta_{1\text{-}42}$ (+/+)) presented an higher immunoreactivity for $P2Y_1$ receptors, in comparison to that obtained in the hippocampal nerve terminals of mice injected with vehicle (control (+/+)). In each Western blot, three different protein quantities were applied into SDS-PAGE gels. Results are mean±SEM % of maximum of control that was set as 100% (n=4; *$p<0.05$ compared to control).

Current evidence favours the idea that soluble Aβ species, of which $A\beta_{1\text{-}42}$ is predominant, play a prominent role in pathogenesis of AD (reviewed in; Klein *et al.*, 2004). One avenue of research hopefully allowing interfering with AD is to study the mechanisms of Aβ-induced neuronal failure (Coleman *et al.*, 2004; Hardy and Selkoe, 2002). There is a growing evidence that the Aβ-induced neurotoxicity may be primarily a synaptic dysfunction which then spreads to include a pattern of neuronal death (reviewed in Selkoe, 2002; Coleman *et al.*, 2004). In fact, this synaptic dysfunction appears to be an early event, preceeding other characteristic neuropathological features, both in patients (*e.g.* Terry *et al.*, 1991), as well as in APP transgenic models (Hsia *et al.*, 1999; Mucke *et al.*, 2000). Also in the present study, it was observed that $A\beta_{1\text{-}42}$ induced a decrease in the immunoreactivity of presynaptic proteins at a time when no neuronal death could be measured, both *in vitro* and *in vivo*. It obviously remains

to be demonstrated if the early synaptotoxicity triggered by $A\beta_{1-42}$ is the primary cause that leads to a delayed neuronal death or if $A\beta_{1-42}$ may also induce neuronal death independently of its synaptotoxic effects, as occurs in non-neuronal cells (*e.g.* Keil *et al.*, 2004). However, the

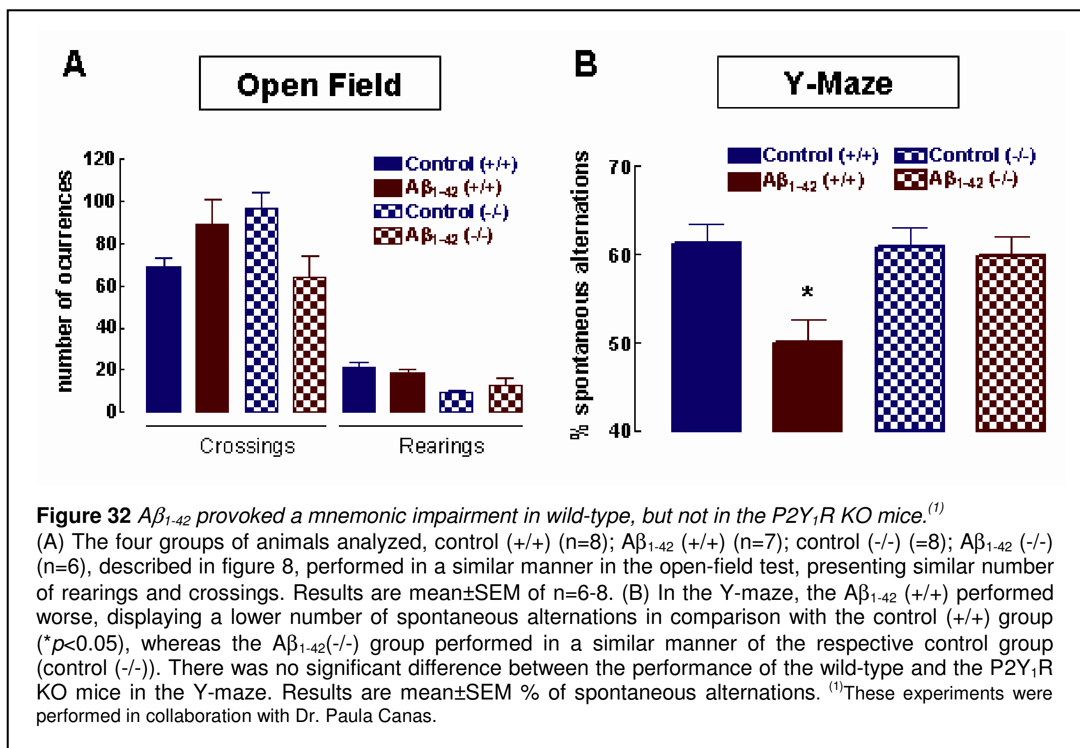


Figure 32 *$A\beta_{1-42}$ provoked a mnemonic impairment in wild-type, but not in the $P2Y_1R$ KO mice.*[(1)]
(A) The four groups of animals analyzed, control (+/+) (n=8); $A\beta_{1-42}$ (+/+) (n=7); control (-/-) (=8); $A\beta_{1-42}$ (-/-) (n=6), described in figure 8, performed in a similar manner in the open-field test, presenting similar number of rearings and crossings. Results are mean±SEM of n=6-8. (B) In the Y-maze, the $A\beta_{1-42}$ (+/+) performed worse, displaying a lower number of spontaneous alternations in comparison with the control (+/+) group (*$p<0.05$), whereas the $A\beta_{1-42}$(-/-) group performed in a similar manner of the respective control group (control (-/-)). There was no significant difference between the performance of the wild-type and the $P2Y_1R$ KO mice in the Y-maze. Results are mean±SEM % of spontaneous alternations. [(1)]These experiments were performed in collaboration with Dr. Paula Canas.

observed ability of an antagonist of $P2Y_1$ receptors, which has been shown to be located at nerve terminals, to prevent both $A\beta_{1-42}$-induced synaptotoxicity, as well as latter neuronal death in rat hippocampal neurons, indicates that manipulations that preserve synapses are sufficient to abrogate the delayed neuronal death.

Furthermore, it has been proposed that there is a correlation between synaptic loss and dementia in AD (Selkoe, 2002). In fact *in vivo* studies concluded that cortical and hippocampal synaptic loss is an excellent correlate of dementia (*e.g.* DeKosky and Scheff, 1990; Terry *et al.*,1991), which is accompanied by a decrease of the levels of presynaptic proteins, in particular synaptophysin (*e.g.* Terry *et al.*, 1991; Heinonen *et al.*, 1995; Sze *et al.*, 1997; Callahan *et al.*, 1999; Masliah *et al.*, 2001). This is confirmed with the present data since the injection of $A\beta_{1-42}$ in rodents caused a synaptic loss and a mnemonic impairment, at a time when no neuronal death was observed. Furthermore, in mice deficient in $P2Y_1$ receptors, in which $A\beta_{1-42}$ was no longer able to cause synaptotoxicity, no mnemonic impairment was observed. Thus, one possible therapeutical approach to arrest AD should involve strategies to prevent this early synaptotoxicity and therefore cognitive impairment. Accordingly, the data gathered in this study shows that the blockade of $P2Y_1$ receptors function prevents the early

synaptotoxicity and mnemonic impairment, which prompts $P2Y_1$ receptors antagonists as potential candidates to arrest the early onset of Alzheimer's disease.

The mechanism(s) underlying the involvement of $P2Y_1$ receptors in the neurodegeneration caused by $A\beta_{1-42}$ remains to be elucidated. From the known functions so far ascribed to $P2Y_1$ receptors in the brain, several possible mechanisms can be involved. For instance, due to $P2Y_1$ receptors role in the control of microglia function (Farber and Kettenmann, 2006), one possible mechanism by which $P2Y_1$ receptors are involved in the $A\beta_{1-42}$-induced neurotoxicity may be at the control of neuroinflamation ocurring in AD, as suggested for $P2X_7$ receptor (Rampe *et al.*, 2004). However, although this mechanism may also be relevant *in vivo*, the neuroprotection afforded by $P2Y_1$ receptors antagonists in a neuronal preparation free of microglia elements indicates the involvement of neuronal $P2Y_1$ receptors. Furthermore, as described above, there are several evidences indicating that the initial events of AD might be a dysfunction at the synaptic level. The synaptic localization of $P2Y_1$ receptors, where they perform essentially a neuromodulatory role (1 of Results; Rodrigues *et al.*, 2005), suggests that may be the $P2Y_1$ receptors located at the nerve terminals that might be involved in $A\beta_{1-42}$-induced neurotoxicity. This is further indicated by the observation that it is synaptic $P2Y_1$ receptors that are up-regulated after $A\beta_{1-42}$ treatment. However, further studies should now be developed in order to allow the comprehension of the mechanisms underlying these phenomenological observations of a robust neuroprotection afforded by $P2Y_1$ receptors antagonists.

In conclusion, the results obtained demonstrate that extracellular ATP, through the activation of $P2Y_1$ receptors, in particular through the $P2Y_1$ receptors targeted to the nerve terminals, is involved and mandatory in order for $A\beta$ peptides, believed to be the molecular agents primarily responsible for AD, to cause their neurotoxic effects. Accordingly, the abrogation of the function of $P2Y_1$ receptors prevents the early synaptotoxicity and memory loss caused by the administration of $A\beta_{1-42}$ in rodents, which prompts $P2Y_1$ receptors antagonists as potential candidates to arrest the evolution of Alzheimer´s disease, in particular at the early onset of this neurodegenerative disease.

3 FUNCTIONAL INTERACTION BETWEEN PRESYNAPTIC $P2X_{1-3}$ AND α3* NICOTINIC RECEPTORS IN THE CONTROL OF NEUROTRANSMITTER RELEASE IN THE RAT HIPPOCAMPUS

Fast synaptic transmission in the central nervous system is mainly conveyed by the activation of post-synaptically located transmitter gated ion-channels. In recent years a wealth of data has been gathered to support the localization of ionotropic receptors also in presynaptic nerve terminals where they function as modulatory systems (reviewed in Engelman and MacDermott, 2004). This has contributed to resolve the paradoxical findings that some ionotropic receptors were present in brain tissue although they were only episodically found to mediate synaptic transmission, as typified by P2X receptors (see 1 of Results; Rodrigues *et al.*, 2005). Another class of ionotropic receptors that also have been shown to be located presynaptically to modulate neurotransmitter release are the nicotinic receptors (nAChRs) (Role and Berg, 1996).

The nACh receptors are heterogeneous cationic channels that constitute one of the two types of receptors whose activation is triggered by the endogenous ligand acetylcholine (the other type are the muscarinic acetylcholine receptors). Pharmacologically, the nAChRs are distinguished by their ability to be activated by nicotine. Nicotinic receptors are a widely expressed multigene family, comprising ten α, four β, one ε, one δ and one γ subunits (Lukas *et al.*, 1999). Nicotinic receptors are present in many tissues and can be divided in nAChR of the neuronal type, which includes the receptors located both in the central nervous system and in the peripheral nervous system, and the muscle-type, including those located at neuromuscular junctions (Gotti *et al.*, 2006). Neuronal nAChRs differ from those located at neuromuscular junctions, since they do not have γ, ε, δ, α1 and β1 subunits in their constitution. Also in contrast to their muscle counter-parts, neuronal nACh receptors are pentameric combinations of the subunits α2-10 and β2-4, sharing a structural homology with GABA, glycine (MacDermott *et al.*, 1999), and 5-HT3 (Karlin and Akabas, 1995) receptors, assembling to a general 2α3β stoichiometry, with the possibility of more than one α subunit subtype (Conroy and Berg, 1995). However, the subunits α7, α8, α9 are also able to form functional homomeric receptors, but still obeying to a pentameric structure. These hetero- or homo-pentameric assembly of several combinations of subunits results in a large number of different receptors with distinct pharmacological and biophysical properties and locations in the brain. In the CNS, where nACh receptors are principally located at presynaptic sites, although they are also found postsynaptically (reviewed by Gotti *et al.*, 2006), these receptor channels mediate chemical interneuronal communication, regulates processes such as transmitter release, cell excitability and neuronal integration, which are crucial for network operation and play key roles in higher brain functions such as attention, memory, reward, nociception and neuronal development

(Marubio and Changeux, 2000; Rezvani and Levin, 2001; Mansvelder and McGehee 2002). These functions are achieved by mediating fast synaptic transmission (Jones and Yakel, 1999) and by auto- and/or paracrine-regulation of neurotransmitter release (reviewed in Engelman and MacDermott, 2004).

In the realm of their neuromodulatory role at the presynaptic level, the function of these two different classes of ionotropic receptors, both P2X and nACh receptors, may be inter-twinned in view of the observations that: 1) acetylcholine and ATP are co-released from brain cholinergic nerve terminals (Richardson and Brown, 1987); 2) both types of ionotropic receptors can co-localise in nerve terminals (Diáz-Hernández *et al.*, 2002); 3) there is a physical proximity and a cross-inhibition between both receptors when heterologously expressed in cultured cells (Khakh *et al.*, 2000, 2005); 4) in peripheral preparations both electrophysiological (Barajas-Lopez *et al.*, 1998; Nakazawa, 1994; Searl *et al.*, 1998; Zhou and Galligan, 1998) and neurochemical studies (Allgaier *et al.*, 1995; Salgado *et al.*, 2000) revealed a functional interaction between both receptor types. However, does this functional interaction occur in native brain preparations?

Recently, Almeida *et al.* (2007) have reported an enrichment of both P2X and nACh receptors in nerve terminals membranes of the rat hippocampus in comparison to total membranes, by performing binding assays. It also described a binding interaction of P2X receptors and nACh receptors in rat hippocampal nerve terminals, since it observed a partial displacement of the binding of nACh ligands by P2X receptor ligands and *vice-versa*. This data, together with the reported previously by Khakh *et al.* (2005), provide a strong indication that there might be a sufficiently closed proximity and/or interaction between putatively P2X and nACh receptors in native brain tissue, in this particular in rat hippocampal nerve terminals, to allow the existence of a cross-displacement between ligands for the two types of receptors. Almeida *et al*. (2007) also demonstrated that this close interaction has a functional parallel, namely in the control of noradrenaline. It was previously suggested that the release of noradrenaline from hippocampal preparations might be controlled by both P2 receptors (Koch et al., 1997) and by nAChR receptors (Serchen *et al.,* 1997). Almeida *et al*. (2007) re-enforced these observations since it observed that the activation of either P2X receptors or nACh receptors controlled [^{3}H]-noradrenaline release from superfused rat hippocampal synaptosomes, which were enriched in the two types of receptors, both in a facilitatory manner. Furthermore, it observed a pharmacological antagonism between P2X and nAChRs in the control of the evoked release of noradrenaline from superfused hippocampal synaptosomes, showing that in fact there is a functional interaction between these two types of ionotropic receptors in native brain tissue, in this particular, in the rat hippocampus at a presynaptic level in the control of noradrenaline release.

3.1. Functional co-expression of P2X and nACh receptors in rat hippocampal noradrenergic nerve terminals

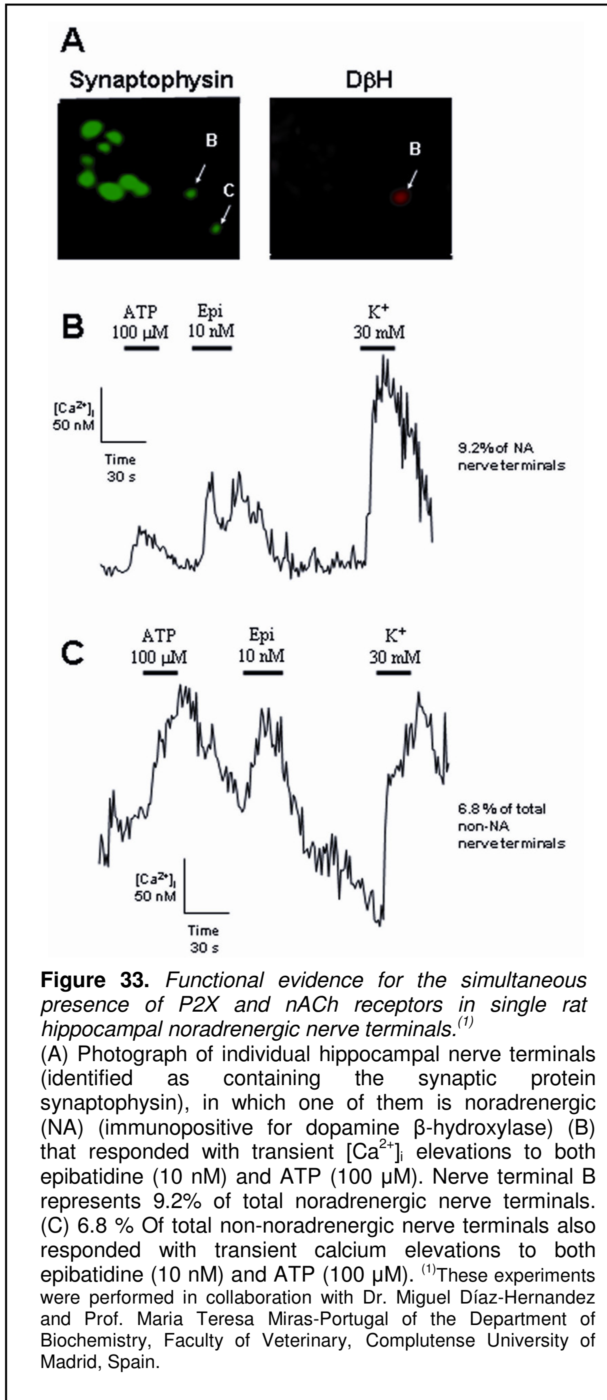


Figure 33. *Functional evidence for the simultaneous presence of P2X and nACh receptors in single rat hippocampal noradrenergic nerve terminals.*[(1)]
(A) Photograph of individual hippocampal nerve terminals (identified as containing the synaptic protein synaptophysin), in which one of them is noradrenergic (NA) (immunopositive for dopamine β-hydroxylase) (B) that responded with transient $[Ca^{2+}]_i$ elevations to both epibatidine (10 nM) and ATP (100 µM). Nerve terminal B represents 9.2% of total noradrenergic nerve terminals. (C) 6.8 % Of total non-noradrenergic nerve terminals also responded with transient calcium elevations to both epibatidine (10 nM) and ATP (100 µM). [(1)]These experiments were performed in collaboration with Dr. Miguel Díaz-Hernandez and Prof. Maria Teresa Miras-Portugal of the Department of Biochemistry, Faculty of Veterinary, Complutense University of Madrid, Spain.

If P2X and nACh receptors tightly interact to modulate the evoked release of noradrenaline, they should be simultaneously present at the same noradrenergic nerve terminals. Thus, to re-enforce this contention, it was important to confirm that it would be possible to record functional responses of both P2X and nACh receptors in the same noradrenergic nerve terminals. Taking advantage of the fact that both P2X and nACh receptors are highly permeable to calcium (Rogers and Dani 1995), it was performed microfluorimetric $[Ca^{2+}]_i$ measurements in single isolated noradrenergic nerve terminals, in which calcium transients triggered by ATP (100 µM) and epibatidine (10 nM) were recorded in individual noradrenergic synaptosomes. As illustrated in figure 33, it was possible to identify in single nerve terminals, calcium transients triggered by epibatidine (10 nM) and ATP (100 µM) and then confirm that the recorded element was immunopositive for dopamine-β–hydroxylase (DβH) (Figure 33A and 33B). Thus, this data clearly shows that there are rat hippocampal noradrenergic nerve terminals endowed with functional P2X and nACh receptors.

3.2. Identification of the P2X and nAChR subunits present in the active zone of hippocampal nerve terminals

The next step was to define the molecular subunits of P2X and nAChRs present in the active zone of nerve nerve terminals of the rat hippocampus and thus able to be involved in the presynaptic control of noradrenaline release, in order to narrow the possible candidates involved in the functional interaction between P2X and nACh receptors observed in the control of noradrenaline release. For that purpose, the fractionation procedure allowing the purification of the presynaptic active zone (Phillips *et al.*, 2001) as described in 2.4 of Materials and Methods, was used once again, in order to identify by Western blot analysis which P2X and nACh receptors were present there. This technique has previously been validated and shown to allow an over 90% efficiency of separation of the presynaptic active zone from the postsynaptic density and from other presynaptic proteins not located in synapses (Pinheiro *et al.*, 2003; Rebola *et al.*, 2003). In accordance with the results described in chapter 1 of Results (Rodrigues *et al.*, 2005), it was confirmed the presence of $P2X_{1\text{-}3}$ subunits in the specialized component of the nerve terminals, *i.e.* the active zone, where receptors controlling the release of neurotransmitters are expected to be located (Figure 34). With respect to nACh receptors, the pharmacological data described by Almeida *et al.* (2007) strongly suggested the involvement of nACh receptors containing β2 and α3 and/or α4, excluding the involvement of receptors containing α7. Thus, it was analysed the subsynaptic distribution of α3 and α4 subunits and it was observed that α3 was highly enriched in the active zone, whereas α4 subunit was virtually absent in the active zone and most abundantly located in the post-synaptic density fraction (Figure 34).

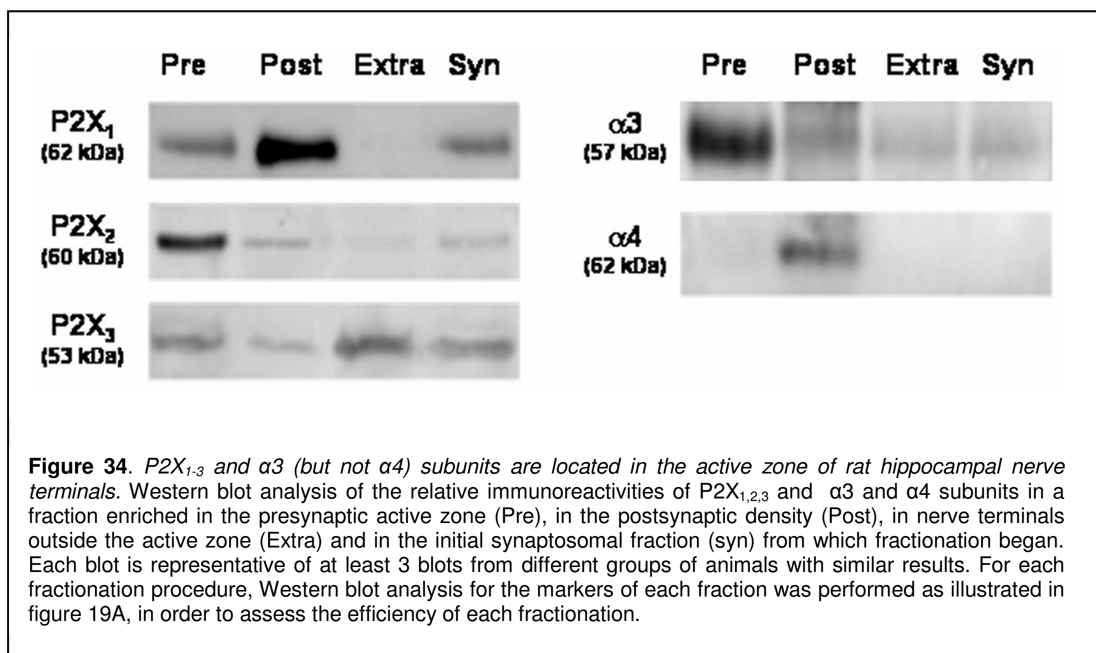


Figure 34. *$P2X_{1\text{-}3}$ and α3 (but not α4) subunits are located in the active zone of rat hippocampal nerve terminals.* Western blot analysis of the relative immunoreactivities of $P2X_{1,2,3}$ and α3 and α4 subunits in a fraction enriched in the presynaptic active zone (Pre), in the postsynaptic density (Post), in nerve terminals outside the active zone (Extra) and in the initial synaptosomal fraction (syn) from which fractionation began. Each blot is representative of at least 3 blots from different groups of animals with similar results. For each fractionation procedure, Western blot analysis for the markers of each fraction was performed as illustrated in figure 19A, in order to assess the efficiency of each fractionation.

Since the fractionation procedure used above does not allow purification of only the synaptic fractions from noradrenergic terminals, it was performed a complementary triple immunocytochemistry study in hippocampal single nerve terminals aimed to identify if there was a co-localization of $P2X_{1-3}$ and α3 nAChR subunits in particular in noradrenergic terminals, identified as the terminals labelled with antibodies against dopamine-β-hydroxylase (DβH). As illustrated in figure 35, there was a co-localization of $P2X_1$, $P2X_2$ or $P2X_3$ and α3 nAChR subunits in near 50% of the noradrenergic terminals, which comprised 3.4±0.9% (n=3) of the total population of hippocampal nerve terminals, identified as synaptophysin immunoreactive elements. In accordance with the absence of α4 subunits in the presynaptic active zone of hippocampal nerve terminals, it was confirmed that there was no measurable immunoreactivity of α4 subunits in hippocampal nerve terminals, although it was present in striatal nerve terminals (Figure 36), in accordance with its known presynaptic neuromodulatory effect in this particular brain area (see Luetje, 2004).

3.3. Functional interaction between P2X and nACh receptors in the control of hippocampal glutamate release

In microfluorimetric $[Ca^{2+}]_i$ measurements in single nerve terminals (3.1 of Results) it was observed that the functional co-expression of P2X and nACh receptors was not a particular feature of the rat hippocampal noradrenergic nerve terminals population, since it was recorded $[Ca^{2+}]_i$ transients for ATP (100 µM) and epibatidine (10 nM) applications in nerve terminals (immunopositive elements for the synaptic marker synaptophysin), that did not present immunoreactivity for DβH (Figure 33C). This suggests that the functional interaction between P2X and nACh receptors at the presynaptic level should not be restricted to the control of noradrenaline release, being more likely a general mechanism in the presynaptic control of neurotransmitter release operated by both ATP and ACh in the rat hippocampus. In order to gauge this hypothesis, since glutamate release in the hippocampus is controlled by both nACh (*e.g.* McGehee *et al.*, 1996) and P2X (see 1 of Results; Rodrigues *et al.*, 2005) receptors, it was investigated if there was a functional interaction between these two presynaptic ionotropic receptors in the control of glutamate release in the rat hippocampus.

The modulation of neurotransmitter release from superfused synaptosomes allows unambiguously defining that a modulatory system is presynaptic. Using this approach, it was confirmed (see 1 of Results; Rodrigues *et al.*, 2005) that the P2X receptor agonist, α,β-MeATP (60 µM) enhanced by 35.2±6.0% (n=6) the evoked release of glutamate from hippocampal nerve terminals. This effect was prevented ($p<0.05$) by the P2 receptor antagonist PPADS (20 µM) (Figure 37).

Electrophysiological recordings in rat hippocampal slices had previously shown that nAChRs also facilitated glutamatergic transmission, an effect ascribed to presynaptic receptors

(*e.g.* McGehee *et al.*, 1995). Accordingly, it was observed that the nAChR agonist, epibatidine (100 nM) facilitated by 27.2±5.3% (n=6) glutamate release from rat hippocampal nerve terminals (Figure 37). This effect was prevented (p<0.05) by the nAChR antagonist, *d*-tubocurarine (1 μM) (Figure 37). This unambiguously shows that nAChRs presynaptically facilitate the evoked release of glutamate in the hippocampus.

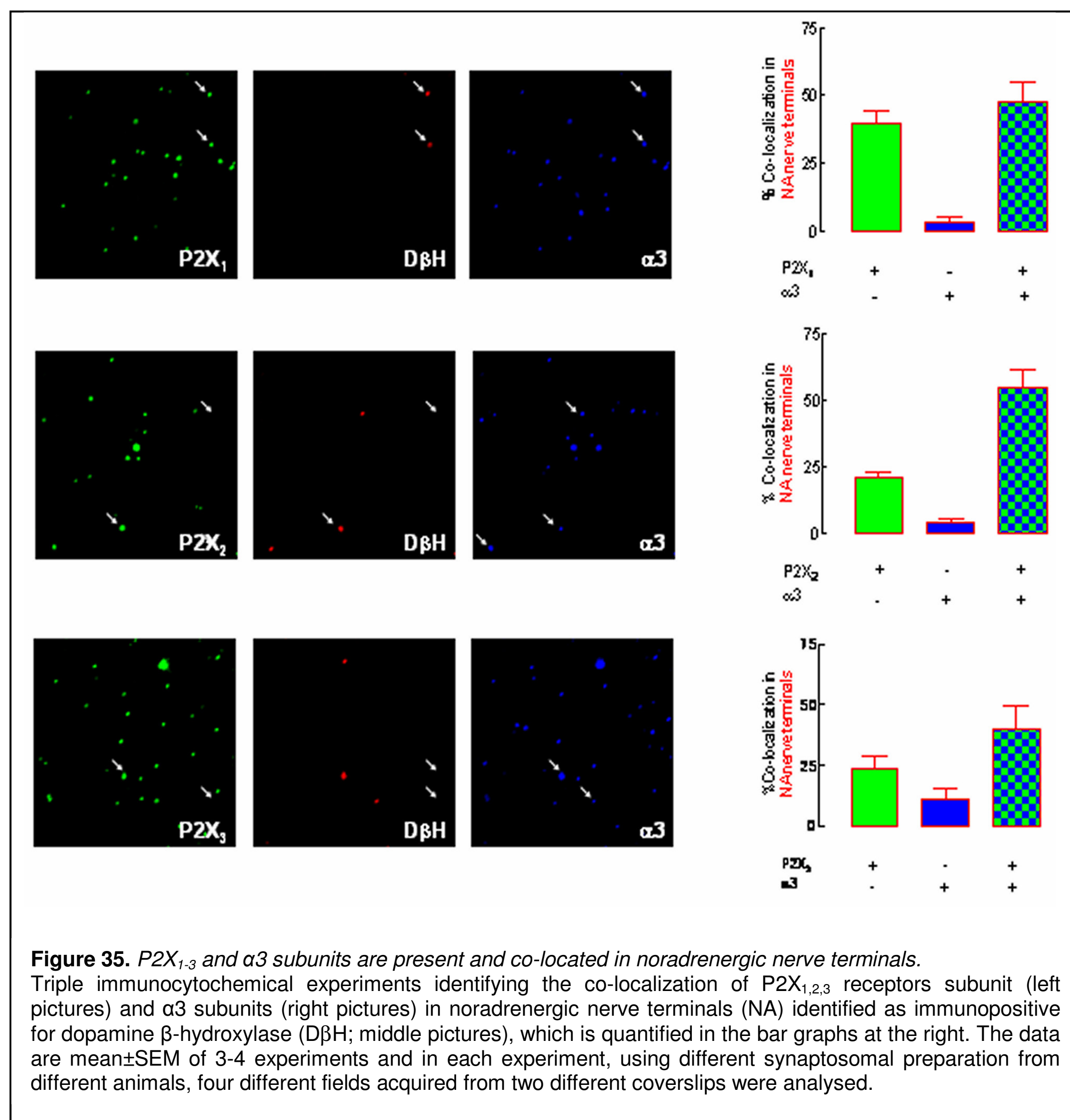


Figure 35. *$P2X_{1-3}$ and α3 subunits are present and co-located in noradrenergic nerve terminals.*
Triple immunocytochemical experiments identifying the co-localization of $P2X_{1,2,3}$ receptors subunit (left pictures) and α3 subunits (right pictures) in noradrenergic nerve terminals (NA) identified as immunopositive for dopamine β-hydroxylase (DβH; middle pictures), which is quantified in the bar graphs at the right. The data are mean±SEM of 3-4 experiments and in each experiment, using different synaptosomal preparation from different animals, four different fields acquired from two different coverslips were analysed.

In order to explore if there is a functional interaction between P2X and nACh receptors also in the control of glutamate release in the rat hippocampus, it was investigated if there was also a cross-antagonism between these two receptors in the control of hippocampal glutamate release. As illustrated in figure 37, *d*-tubocurarine (1 μM) prevented (p<0.05) the facilitatory effect of α,β-MeATP (60 μM, n=6). Conversely, the facilitatory effect of epibatidine (100 nM) was attenuated by near 50% by PPADS (20 μM), although it does not reach a statistical

significancy (p>0.05). This shows a cross-antagonistic interaction between nAChRs and P2XRs also in the control of glutamate release from rat hippocampal nerve terminals.

3.4. Discussion

Together with the data reported by Almeida *et al.* (2007), the present results provide the first demonstration in a native brain preparation of the co-localization and functional interaction between presynaptic P2X and nACh receptors in the control of noradrenaline release in the rat hippocampus. Furthermore, the results presented indicate that this functional interaction is selective for a particular subunit combination of these two groups of ionotropic receptors. The pharmacological data reported by Almeida *et al.* (2007), in the control of noradrenaline release, already has indicated the involvement of P2X receptors containing the subunits $P2X_{1\text{-}3}$ and nACh receptors containing β_2, α_3 and/or α_4, rulling out α_7 subunit involvement. The evaluation of the immunoreactivity of these subunits in the presynaptic active zone of rat hippocampal nerve terminals and the immunochemical analysis of rat hippocampal noradrenergic nerve terminals

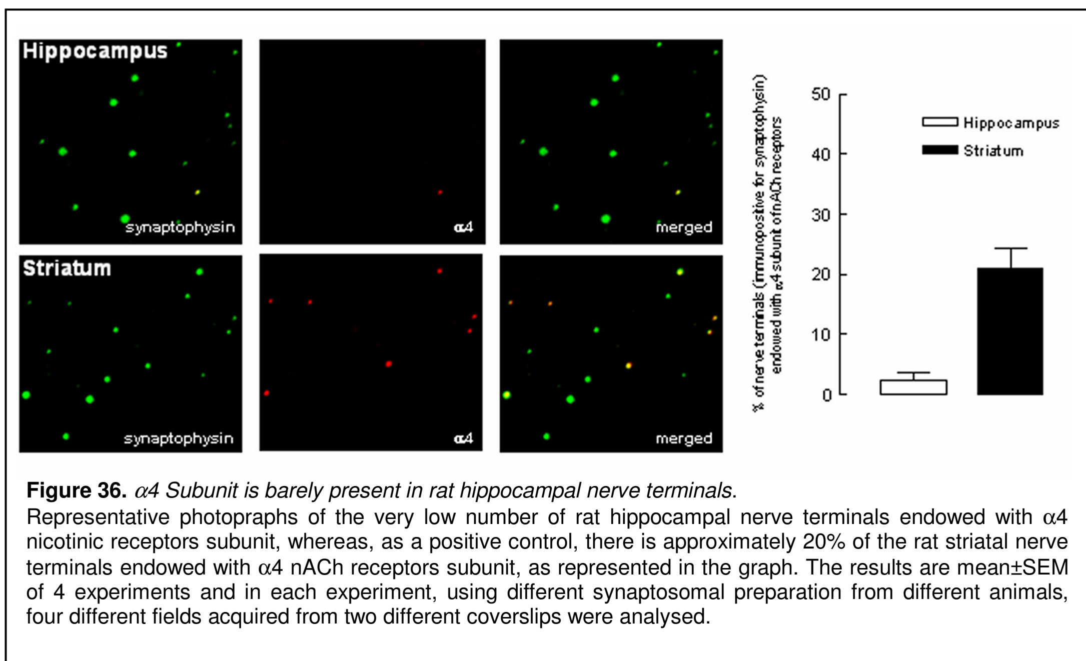


Figure 36. *α4 Subunit is barely present in rat hippocampal nerve terminals.*
Representative photopraphs of the very low number of rat hippocampal nerve terminals endowed with α4 nicotinic receptors subunit, whereas, as a positive control, there is approximately 20% of the rat striatal nerve terminals endowed with α4 nACh receptors subunit, as represented in the graph. The results are mean±SEM of 4 experiments and in each experiment, using different synaptosomal preparation from different animals, four different fields acquired from two different coverslips were analysed.

also ruled out receptors containing α4 subunit, only detected at the postsynaptic density, indicating for the involvement of P2X receptors containing $P2X_{1\text{-}3}$ subunits, in a manner analogous to that previously found to occur in glutamatergic nerve terminals of the rat hippocampus (1 of Results; Rodrigues *et al.*, 2005) and $\alpha_3\beta_2$ nACh receptors - α_3 was solely present in the presynaptic active zone - in the functional interaction observed at the presynaptic level in the control of noradrenaline release. The involvement of the subunit β_2 is in agreement with the predominant expression of β_2 subunits in the cell bodies of noradrenergic neurons in

the locus coeruleus (Lena *et al.*, 1999). In fact, Almeida *et al.* (2007) observed in experiments carried out in transfected oocytes, a cross-inhibition of the current amplitudes of $P2X_2$ and $\alpha_3\beta_2$ nACh receptors when co-activated, which was not observed between $P2X_2$ and $\alpha_4\beta_2$ nACh receptors. Moreover, Almeida *et al.* (2007) also reported a physical interaction between $P2X_2$ and α_3 and β_2 but no with α_4, observed by co-immunoprecipitation assays, both in heterologous expression system and in native brain tissue, in particular in rat hippocampal membranes. This data not only re-enforces the existence of a particular subunit combination, confirming the involvement of P2X receptors containing $P2X_{1-3}$ and $\alpha_3\beta_2$ nACh receptors, but the fact that there is a tight physical interaction between P2X and nACh receptors also provides a molecular rationale for their tight functional interaction in nerve terminals of the rat hippocampus.

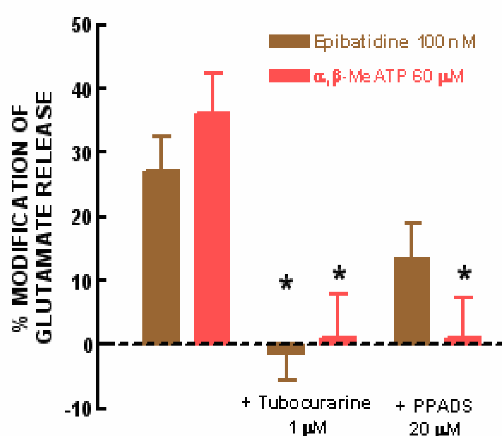


Figure 37. *Interaction between P2XRs and nAChRs in the control of glutamate release from hippocampal synaptosomes.*
Epibatidine (100 nM) and α,β-MeATP (60 µM) facilitated the evoked release of glutamate from superfused rat hippocampal synaptosomes. The facilitatory effect of α,β-MeATP (60 µM) was prevented both in the presence of *d*-tubocurarine (1 µM; nAChRs antagonist) and of PPADS (20 µM; P2Rs antagonist), whereas the facilitatory effect of epibatidine (100 nM) was prevented by *d*-tubocurarine (1 µM), but not modified by PPADS (20 µM). The data is presented as mean±SEM of 6 experiments. $^*p<0.05$ in comparison to the effects of epibatidine (100 nM) or α,β-MeATP (60 µM).

Furthermore, it was observed that this functional interaction between P2X and nACh receptors occurs not only in the control of noradrenaline release (Almeida *et al.*, 2007) but also in the control of glutamate release (Figure 37). This strongly indicates that this functional interaction between these two types of presynaptic ionotropic receptors seems to be a general mechanism of the presynaptic control of neurotransmitter release operated by both ATP and Ach. This is re-enforced by a previous observation that a population of cholinergic nerve terminals of the rat midbrain also presents functional responses to both ATP and ACh (Díaz-Hernández *et al.*, 2002). Also in this preparation it was shown recently that it seems that there is also a functional interaction between P2X and nACh receptors in that cholinergic nerve terminals population (Díaz-Hernández *et al.*, 2006). Finally, this data also shows that some potential interactions found in heterologous expression systems might be levant for one of the main physiological role of P2X and nACh receptors, *i.e.* the presynaptic modulation of neurotransmitter release and synaptic strength (Cunha and Ribeiro, 2000; Engelman and MacDermott, 2004; Role and Berg, 1996; Wonnacott, 1997). Overall, gathering the data presented here with the reported by

Almeida *et al.* (2007), these results indicate that P2X and nACh receptors can aggregate at the plasma membrane (imagiological data) forming hetero-oligomeric complexes (co-immunoprecipitation data) where the binding sites of one receptor affect the binding sites of the other receptor (radioligand binding data) and consequently lead to a functional interaction between presynaptic P2X and nACh receptors (neurochemical) in nerve terminals of the hippocampus, in the control of neurotransmitter release.

The present conclusion that P2X and nACh receptors tightly interact in native brain preparations, namely in nerve terminals, is in agreement with previous findings in peripheral preparations such as phrenic motor nerve endings (Salgado *et al.*, 1999) or different ganglionar preparations (Allgaier *et al.*, 1995; Barajas-Lopez *et al.*, 1998; Nakazawa, 1994; Searl *et al.*, 1998; Zhou and Galligan, 1998). Each of these studies investigating the functional interaction between P2X and nACh receptors found that their co-activation resulted in mutually occlusive responses (Barajas-Lopez *et al.*, 1998; Khakh *et al.*, 2000; Salgado *et al.*, 1999; Searl *et al.*, 1998; Zhou and Galligan, 1998), although the particular subunit composition of the P2X and nACh receptors involved was not determined. The most parsimonious explanation to interpret this cross-antagonism would be the formation of dimers. In accordance with these ideas, it has previously been shown that P2X receptors directly interact with other ionotropic receptors, such as the $GABA_A$ receptor (Boue-Grabot *et al.*, 2004) or the 5-HT3 receptor (Boue-Grabot *et al.*, 2003). Accordingly, a previous study carried out in heterologous expression systems also reported a physical proximity between P2X and nACh receptors subunits (Khakh *et al.*, 2005) to explain their functional cross-antagonism (Khakh *et al.*, 2000). However, the results presented here strongly indicate for an interaction involving $\alpha_3\beta_2$ and not $\alpha_4\beta_2$ nACh receptor as described by Khakh *et al.* (2005). This contention is supported by the lack of α_4, but presence of α_3 subunit immunoreactivity in the active zone and noradrenergic nerve terminals and is also in agreement with the prominent involvement of α_3-containing receptors in ganglionar transmission (Galligan and North, 2004), where P2XR/nAChR interactions have also been observed (Allgaier *et al.*, 1995; Barajas-Lopez *et al.*, 1998; Nakazawa, 1994; Searl *et al.*, 1998; Zhou and Galligan, 1998). Also, Almeida *et al.* (2007) reported less-than additive responses just between $\alpha_3\beta_2$ and $P2X_2$ receptors and not between $\alpha_4\beta_2$ and $P2X_2$ receptors in oocytes and found a physical association not only in heterologous expression systems, but also in rat hippocampal membranes, between $\alpha_3\beta_2$ and $P2X_2$ receptors, but not between $\alpha_4\beta_2$ and $P2X_2$ receptors, which actually provides an elegant internal control. Furthermore, α3 was predominantly located in the presynaptic active zone, whereas α4 was just detected at the post synaptic density, and accordingly, only a very small fraction of hippocampal nerve terminals were endowed with α4. Thus, it appears that the interaction between presynaptic nACh receptors and P2X receptors in nerve terminals preferentially involves α_3- rather than α_4-containing nACh receptors.

The physiological relevance of this P2XR/nAChR interaction is still to be understood. Based on the known co-release of ATP and ACh (Richardson and Brown, 1987; Silinsky and Redman, 1996; Zhang *et al.*, 2000), one possibility would be that this 'dimer' might act as a coincident detector to burst the probability of neurotransmitter release. The underlying hypothesis would be that neither P2X nor nACh receptors *per se* would be able to place synapses in an ON state, which could only be achieved by the simultaneous activation of the two receptors. However, given the prominent role of nACh receptors in controlling the release of different neurotransmitters (Engelman and MacDermott, 2004; Wonnacott, 1997) and to regulate synaptic efficiency and plasticity (Sher *et al.*, 2004) as well as excitotoxicity/neuroprotection (Dajas-Bailador and Wonnacott, 2004; Quick and Lester, 2002), it seems more likely that this P2XR/nAChR interaction might be designed to curtail nAChR-mediated responses. In fact, the shaping of nACh receptors signalling through 'desensitization' of this receptor may be a decisive factor to determine a proper physiological signalling of nACh receptors (see Giniatullin *et al.*, 2005; Quick and Lester, 2002). In particular, the facilitatory role of presynaptic nACh receptor, triggering and/or bolstering the release of neurotransmitters, is associated with the efficiency of nACh receptors to increase intracellular calcium levels (Engelman and MacDermott, 2004; Wonnacott, 1997). But it would be desirable to shut down this nAChR-mediated facilitatory effect on increasing firing frequencies to prevent excessive calcium inflow into terminals. Since the P2XR/nAChR interaction leads to a partial occlusion of the presynaptic nAChR-mediated actions, this means that the co-activation of both receptors actually offsets the nicotinic response. Notably, ATP release from hippocampal preparations increases disporportionally with increasing frequencies of stimulation (Cunha *et al.*, 1996; Wieraszko *et al.*, 1989). All together these observations raise the hypothesis that the P2XR/nAChR device would be well suited to allow nAChRs to function at moderate firing frequencies (where ATP release is low) and to blunt nicotinic responses at higher frequencies (when ATP release is disproportionally larger). This possibility that the role of P2X receptors may be to curtail excessive activation of nACh receptors, which still requires further experimental confirmation, adds to several report showing that P2X receptors can interact with different ionotropic receptors, such as $GABA_A$ receptors (Boue-Grabot *et al.*, 2004; Sokolova *et al.*, 2001), 5-HT3 receptors (Boue-Grabot *et al.*, 2003) and eventually NMDA (Pankratov *et al.*, 2002a; Peoples and Li, 1998) and AMPA/kainate receptors (Zona *et al.*, 2000). Since in all case the putative activation of P2X receptors leads to a decrease response of the other paired ionotropic receptor, it can be tentatively concluded that P2X receptors may be promiscuous receptors designed to act as a general modulatory system to curtail excessive activation of other ionotropic receptors.

4 $P2X_2$/NR2B: THE FIRST HYBRID IONOTROPIC RECEPTOR

Fast communication in the nervous system is critical for information processing. Fast signalling is achieved by activation of ligand-gated ion channels believed to be independent functionally and physically. However, as discussed in the previous chapter, in the recent years gathered data indicates that there is a tight interaction and cross-talk between fast signalling systems. Interestingly, these interactions between ligand-gated ion channels so far described have a common "character": the ATP-gated cation channels, *i.e.*, P2X receptors. It was already demonstrated a functional interaction between P2X and nACh (Khakh *et al.*, 2000; Almeida *et al.*, 2007; see previous chapter), 5-HT3 (Boué-Grabot *et al.*, 2003) and also with the ligand-gated anion channel $GABA_A$ receptor (Boué-Grabot *et al.*, 2004), all members of the nicotinic ligand-gated cation channels superfamily. This promiscuity that P2X receptors seems to present makes sense and gains relevance by the fact that ATP can be released and/or co-released with different other neurotransmitters such as glutamate (Pankratov *et al.*, 2006), GABA (Jo and Schlichter, 1999) and acetylcholine (Richardson and Brown, 1987). This places ATP in several distinct types of synapses where different transmitters/modulators are operating.

As described above, so far it was demonstrated a functional interaction between P2X and ligand-gated ion channels belonging to the nicotinic ligand-gated cation channels superfamily. However, a functional interaction between P2X and ionotropic glutamate receptors (iGluR) is also likely to occur. Pankratov *et al.* (2002a) reported data indicating for a possible interaction between P2X receptors and NMDA receptors that constitutes one of the three receptor families of ionotropic glutamate receptors so far described. Furthermore, a possible interaction between ionotropic signalling operated by ATP and glutamate is feasible, because it also fulfils the premises that led to the investigation of the ionotropic-ionotropic interactions so far described: 1) ATP and glutamate are co-released from glutamatergic nerve terminals (Pankratov *et al.*, 2006) and 2) both functional and immunocytochemical data indicates that P2X and NMDA receptors have an overlapping subcellular localization in the CNS (*e.g.* Pankratov *et al.*, 1998; Rubio and Soto, 2001).

Ionotropic glutamate receptors are integral membrane proteins responsible for mediating information transfer at most excitatory synapses in the brain. NMDA receptor is one of the three major types of iGluRs named after the agonists that were originally identified to activate them selectively: *N*-methyl-D-aspartate (NMDA), α-amino-3-hidroxy-5-methyl-4-isoazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptors. The different and characteristical structural features of ionotropic glutamate receptors (Hollmann and Heinemann, 1994) in comparison to the ligand-gated ion channels of the nicotinic receptor-like family (Ortells and Lunt, 1995) or to P2X receptors (Khakh, 2001), place them into a single

large superfamily (Figure 3). NMDA receptor is a heteromeric ligand-gated cation channel formed by tetrameric assemblies of seven subunits, NR1, NR2A-D and NR3A and B, which are all products of separate genes (reviewed in Kew and Kemp, 2005). The NR1 subunit is an ubiquitous and necessary component of functional NMDA receptor channels that may be comprised by NR1 and at least one NR2 subunit or NR1 and both NR2 and NR3 subunits (Kew and Kemp, 2005). In the rodent and human brain NR2A and NR2B are the predominat subunits expressed in the forebrain. NR2C and D and NR3 subunits show a more restricted spatio-temporal distribution (Kew and Kemp, 2005). The NMDA receptor is unique amongst ligand-gated ion channels in its requirement for two obligatory co-agonists, binding at glycine and glutamate binding sites localised on the NR1 and NR2 subunits, respectively (Kew and Kemp, 2005). Electrophysiological studies have demonstrated that the activation of NMDA receptor s requires occupation of two independent glycine sites and two independent glutamate sites (Benveniste and Mayer, 1991; Clements and Westbrook, 1991). Thus, the minimal requirement for a functional NMDA receptor is likely to be a tetramer composed of two NR1 and two NR2 subunits, which is re-enforced by recent reported data showing that the tetrameric structure of NMDA receptors are likely composed of pairs or dimmers, of dimmers (*e.g.* an NR1 dimer in combination with a NR2X dimmer) (Schorge and Colquhoun, 2003; Mayer and Armstrong, 2004). Another particular feature of NMDA receptors is their well-known blockage by Mg^{2+} ions in a voltage-dependent manner (Mayer *et al.*, 1984).

In respect to their cellular and subcellular distribution in neurons, NMDA receptors are concentrated at postsynaptic sites, although some appear to be presynaptic (Liu *et al.*, 1994). More recently, it was identified the presence of NMDA receptors also in astrocytes (Schipke *et al.*, 2001), to which it was ascribed a role in neuron to glia signalling (Lalo *et al.*, 2006). In fact, besides their co-release and function at the same synapses, which may indicate for a possible cross-talk in the signalling mediated by this two neuroactive substances, there is growing evidence that ATP and glutamate are the main extracellular molecules involved in the essential interaction between the electrically excitable neuronal network and electrically nonexcitable astroglia (Fellin *et al.*, 2006), performing it in an intertwined manner. Furthermore, as shown in the chapter 1 of Results, P2X receptors are also found in both presynaptic and postsynaptic compartments and they are also functionally expressed in astrocytes (Abbracchio and Verderio, 2006). Thus, these premises raise the possibility of an interaction between P2X receptors and NMDA receptors.

The interactions between P2X receptors and the members belonging to the nicotinic receptor-like superfamily so far described are all characterised by a physical interaction that leads to an activity-dependent cross inhibition. Thus, although it was expected that this interactions might behave as detectors of coincidence, this activity-dependent cross inhibition rather suggests that these interactions are designed for ATP to negatively control the excess of

function of other fast signalling systems and not to potentiate it. This is in agreement with the preferential release of ATP at high frequency stimulations (see previous chapter). However, from the point of view of the cell, the concept of detectors of coincidence is very advantageous, because it would provide the cell with a more fine-tune sensing system, and thus their existence is likely to occur, although probably involving a different kind of receptor complex. This led to the hypothesis of the existence of ionotropic receptors composed by subunits of different families of receptors. Basically, this hypothesis postulates for the existence of receptors sensitive to more than one signalling molecule, in which the co-activation by their agonists would result in a synergistic response. Although at a first look it may seem a non-sense or non-likely to occur, based on their distinct subunit topologies (Figure 3), the existence of these kind of receptors would be on one hand an advantage from the bioenergetical point of view to cells and it also would exponentially increase the capability of cells to sense and to interact with their extracellular environment. This is more in agreement with the high degree of complexity presented by biological systems, being the nervous system an unequivocal example.

Thus, the investigation of a possible interaction between P2X and NMDA receptors fits into a broader objective to gather data supporting the hypothesis of the existence of such hybrid ionotropic receptors. This hypothesis was investigated in heterologous expression systems expressing the $P2X_2$ and NR2B subunits. The choice of $P2X_2$ was based on the fact that the previous works reporting interactions between P2X receptors and other ionotropic receptors always involved $P2X_2$ receptors. The NR2B was chosen over NR2A subunits due to their more widespread cellular and subcellular distribution, which suggests for a modulatory role in addition to their predominat effector role in synaptic transmission/plasticity (Loftis and Janowsky, 2003). The present study provides both functional and molecular evidences for the existence of a hybrid $P2X_2$/NR2B ionotropic receptor, thus providing for the first time evidences for the existence of ionotropic receptors composed by subunits from different families of receptors, which constitutes a new concept of receptor.

4.1 Co-expression of $P2X_2$ rescue NR2B function

The heterologous expression system used was HEK293 cells. Taking advantage of the fact that both P2X receptors and NMDA receptors are highly permeable to Ca^{2+}, microfluorimetric $[Ca^{2+}]_i$ measurements in HEK293 cells were performed to identify and characterise a possible interaction between $P2X_2$ and NR2B. It was previously reported that HEK293 cells were endowed with functional P2Y receptors (Fischer *et al.*, 2005), but not with P2X receptors (Worthington *et al.*, 1999). To clearly exclude a possible endogenous expression of any functional P2X receptors, which would hamper the use of these cells as an heterologous expression system to study an interaction between $P2X_2$ and NR2B, it was verified if in fact HEK293 cells are only endowed endogenously with functional P2Y and not with P2X receptors,

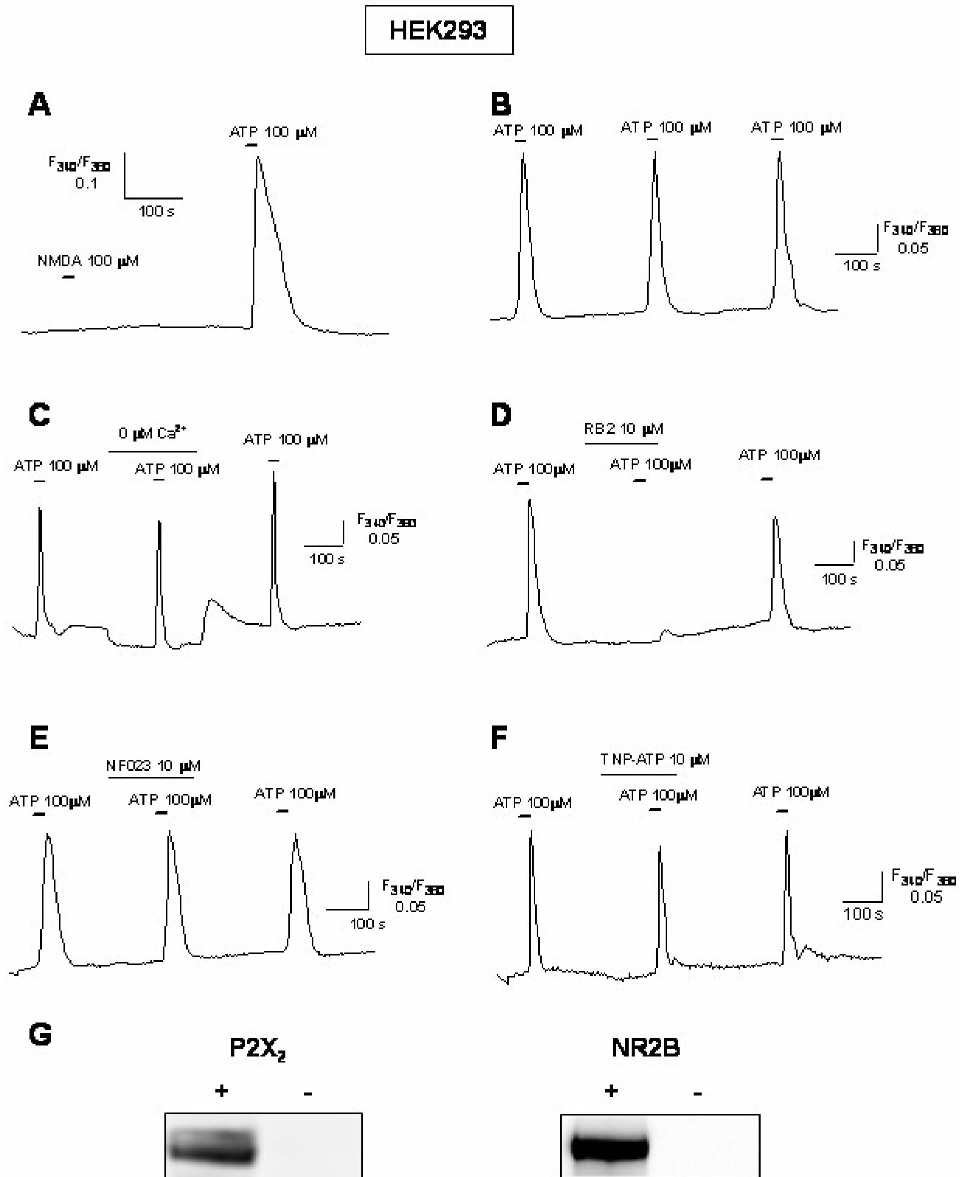


Figure 38. *HEK293 cells are endowed endogenously with functional P2Y receptors, but not with P2X receptors.*
(A) ATP (100 μM) elicit $[Ca^{2+}]_i$ raise in HEK293 cells and (B) sequential ATP applications induce consecutive $[Ca^{2+}]_i$ transients of similar amplitudes. (C) This $[Ca^{2+}]_i$ raise triggered by ATP (100 μM) is independent of extracelular Ca^{2+} and (D) is blocked by RB2 (10 μM; a preferring P2Y receptor antagonist), but not modified neither by (E) NF023 (10 μM) (F) nor by TNP-ATP (10 μM), selective antagonists for some P2X receptors. (G) Western blot analysis revealed the absence of immunoreactivity for both $P2X_2$ and NR2B in membranes of non-transfected HEK293 cells, in contrast with immunoreactivity detected in HEK293 cells transfected with cDNAs enconding for $P2X_2$ and for NR2B subunits. Each graph presented is representative of more than 100 cells analysed.

as previously reported. In fact, challenging HEK293 cells with ATP (100 μM) caused a raise in $[Ca^{2+}]_i$ in all cells analysed (Figure 38A). Sequential pulses of ATP at a supramaximal

concentration of 100 μM, measured in this experimental model, induced $[Ca^{2+}]_i$ transients with similar amplitudes (Figure 38B). The observations that this $[Ca^{2+}]_i$ transients were independent of extracellular Ca^{2+} (Figure 38C), blocked by RB2 (10 μM; a preferring P2Y receptor antagonist) (Figure 38D), but not modified by selective antagonists for some P2X receptors, NF023 (10 μM) and TNP-ATP (10 μM) (Figure 38E and F), strongly indicate that in fact the HEK293 cells are endowed with functional P2Y receptors coupled to PLC and consequently to Ca^{2+}-mobilisation from intracellular Ca^{2+} stores, but not with P2X receptors. Furthermore, Western blot analysis revealed the absence of immunoreactivity for P2X$_2$ subunit in membranes of HEK293 cells, in comparison to the obtained in HEK293 cells transfected with P2X$_2$ subunit (Figure 38G). The same was observed for NR2B subunit (Figure 38G). Moreover, NMDA (100 μM) in the presence of glycine (10 μM) and in the absence of Mg^{2+} did not cause any modification in the $[Ca^{2+}]_i$ among the non-transfected cells analysed (n>100), which indicates that HEK293 cells are not endogenously equipped with functional NMDA receptors.

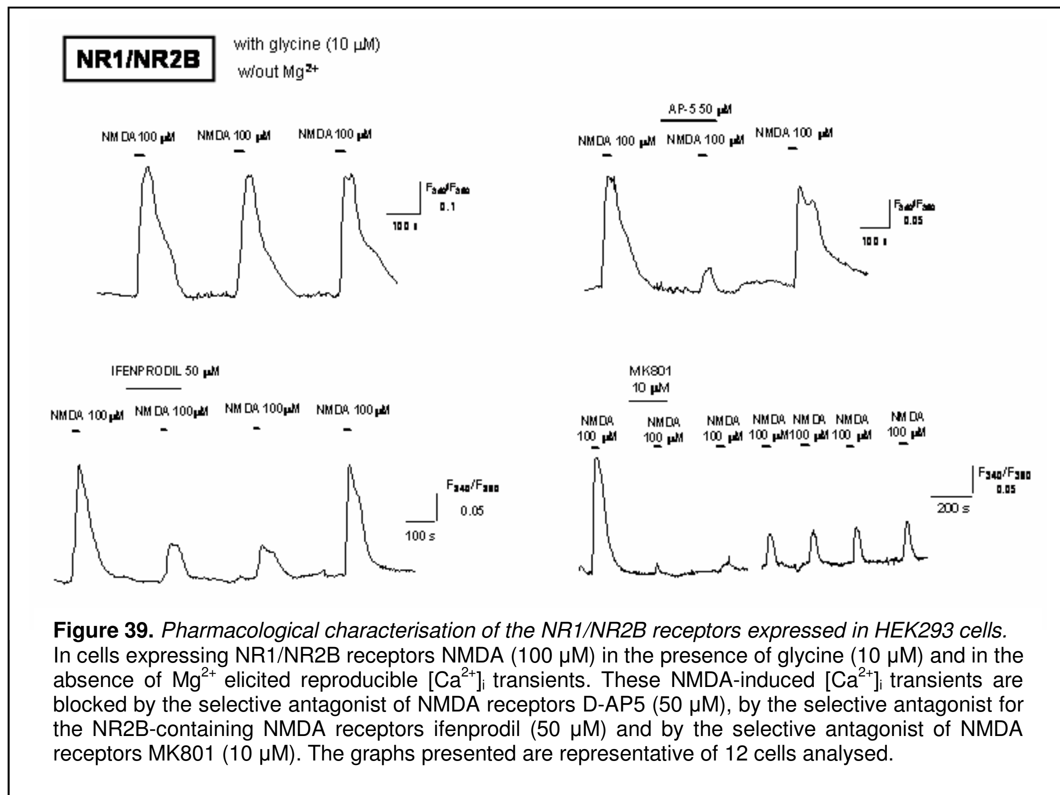


Figure 39. *Pharmacological characterisation of the NR1/NR2B receptors expressed in HEK293 cells.* In cells expressing NR1/NR2B receptors NMDA (100 μM) in the presence of glycine (10 μM) and in the absence of Mg^{2+} elicited reproducible $[Ca^{2+}]_i$ transients. These NMDA-induced $[Ca^{2+}]_i$ transients are blocked by the selective antagonist of NMDA receptors D-AP5 (50 μM), by the selective antagonist for the NR2B-containing NMDA receptors ifenprodil (50 μM) and by the selective antagonist of NMDA receptors MK801 (10 μM). The graphs presented are representative of 12 cells analysed.

HEK293 cells were then transfected with NR1/NR2B in order to characterise pharmacologically this receptor in our experimental model. NMDA at a supramaximal concentration of 100 μM, measured in this experimental model and in accordance with reported data for cloned NMDA receptors (Chen *et al.*, 2001), in the presence of glycine (10 μM) and in the absence of Mg^{2+}, elicited reproducible $[Ca^{2+}]_I$ transients (Figure 39). These NMDA-induced $[Ca^{2+}]_i$ transients were blocked by the selective antagonists of NMDA receptors D-AP5 (50 μM),

and MK801 (10 µM) and by the selective antagonist for the NMDA receptors containing NR2B subunit ifenprodil (50 µM) as expected (Figure 39).

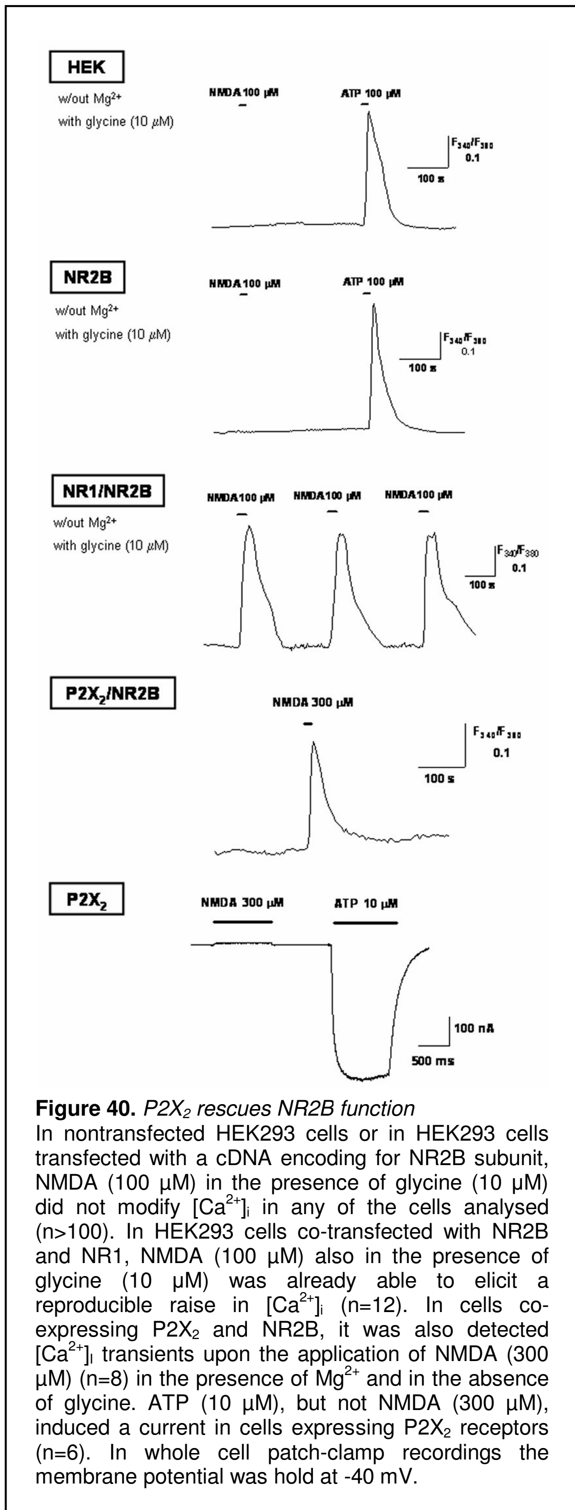


Figure 40. *$P2X_2$ rescues NR2B function*
In nontransfected HEK293 cells or in HEK293 cells transfected with a cDNA encoding for NR2B subunit, NMDA (100 µM) in the presence of glycine (10 µM) did not modify $[Ca^{2+}]_i$ in any of the cells analysed (n>100). In HEK293 cells co-transfected with NR2B and NR1, NMDA (100 µM) also in the presence of glycine (10 µM) was already able to elicit a reproducible raise in $[Ca^{2+}]_i$ (n=12). In cells co-expressing $P2X_2$ and NR2B, it was also detected $[Ca^{2+}]_i$ transients upon the application of NMDA (300 µM) (n=8) in the presence of Mg^{2+} and in the absence of glycine. ATP (10 µM), but not NMDA (300 µM), induced a current in cells expressing $P2X_2$ receptors (n=6). In whole cell patch-clamp recordings the membrane potential was hold at -40 mV.

Thus, to probe for the existence of a hybrid heteromeric receptor composed by $P2X_2$ and NR2B subunits, HEK293 cells were co-transfected with $P2X_2$ and NR2B, which alone is not able to form a functional channel, and possible functional responses were probed by monitorizing $[Ca^{2+}]_i$ transients. As illustrated in figure 40, in non-transfected HEK293 cells, NMDA (100 µM) in the presence of glycine (10 µM) was not able to elicit a detectable $[Ca^{2+}]_i$ raise confirming the absence of functional endogenous NMDA receptors in these cells. In cells transfected with just the cDNA encoding for NR2B, it was also not observed any $[Ca^{2+}]_i$ transient upon the application of NMDA (100 µM) in the presence of glycine (10 µM), which is in agreement with the fact that NR2 subunits are not able by themselves to form functional channels, despite the care of removing Mg^{2+} from the superfusion solution. Accordingly, in cells co-transfected with NR2B and NR1, NMDA (100 µM) in the presence of glycine (10 µM) was already able to induce $[Ca^{2+}]_i$ transients, confirming NR1 subunit as a necessary component of functional NMDA receptors. Impressively, the co-transfection of NR2B with $P2X_2$, in the absence of NR1 subunit, also provided cells with the capability to sense NMDA, since in these cells NMDA (300 µM) was also able to elicit $[Ca^{2+}]_i$ transients. It

should be noticed that in these cells co-expressing $P2X_2$ and NR2B, it was not observed any $[Ca^{2+}]_i$ transient upon the application of NMDA at 100 µM. However, this $[Ca^{2+}]_i$ transient caused by NMDA in cells expressing NR2B and $P2X_2$ subunits should be due to a rescue of NR2B function by co-expressing $P2X_2$ subunit, since 300 µM of NMDA did not also elicit any change in $[Ca^{2+}]_i$ either in non-transfected cells or in cells expressing solely the NR2B subunit. On the other hand, the observation of a lack of effect of NMDA at 100 µM on $[Ca^{2+}]_i$ among the cells tested, being detected NMDA-induced $[Ca^{2+}]_i$ transients only at a concentration of 300 µM, suggests that NMDA should have a lower potency at this new "molecular entity" in comparison to the classical NMDA receptors. Nevertheless, concentration dependent response curves should be performed. Importantly, it should be noticed that the NMDA-induced $[Ca^{2+}]_i$ transients in cells expressing $P2X_2$ and NR2B were insensitive to the presence of Mg^{2+} and were observed in the absence of glycine. This confirms the absence of NR1 subunit in this new "molecular entity sensitive to NMDA" and indicates that it possesses different properties from the classical NMDA receptors. As a control to clearly demonstrate that this $[Ca^{2+}]_i$ raise triggered by NMDA was not due to an hypothetical NMDA agonist binding site in $P2X_2$ receptors, by using patch-clamp analysis in the whole cell configuration in cells transfected solely with $P2X_2$, it was observed that NMDA (300 µM) did not induce any current in cells that presented a current induced by application of ATP (10 µM). This was measured by patch-clamp analysis and not by monitorization of $[Ca^{2+}]_i$ to avoid the P2Y receptor component of the $[Ca^{2+}]_i$ transients elicited by ATP in HEK293 cells.

Thus, this data clearly shows that the presence of $P2X_2$ subunit rescues the function of NR2B in the absence of NR1 subunit, which strongly indicates the existence of a functional molecular entity composed by $P2X_2$ and NR2B subunits. This supports the hypothesis proposed for the existence of hybrid ionotropic receptors composed by subunits of different families of receptors.

4.2 The $[Ca^{2+}]_i$ transients elicited by NMDA in HEK293 cells co-expressing $P2X_2$ and NR2B are abrogated by PPADS and D-AP5

If this hybrid receptor $P2X_2$/NR2B exists, it should consist in the assemblie of $P2X_2$ and NR2B subunits, and thus it should preserve the pharmacological properties of both receptors, in particular in respect to the sensitivity to their antagonists. Thus, it was tested if the $[Ca^{2+}]_i$ transients elicited by NMDA in cells co-transfected with $P2X_2$ and NR2B was blocked or attenuated by antagonists of $P2X_2$ receptors and antagonists of NR1/NR2B with a binding site at the NR2B subunit. So it was evaluated the ability of NMDA to induce $[Ca^{2+}]_i$ transients in cells co-transfected with $P2X_2$ and NR2B subunits in the presence of PPADS, a selective antagonist of P2 receptors, and in the presence of D-AP5, a competitive antagonist for the binding site of NMDA present in NR2B subunits.

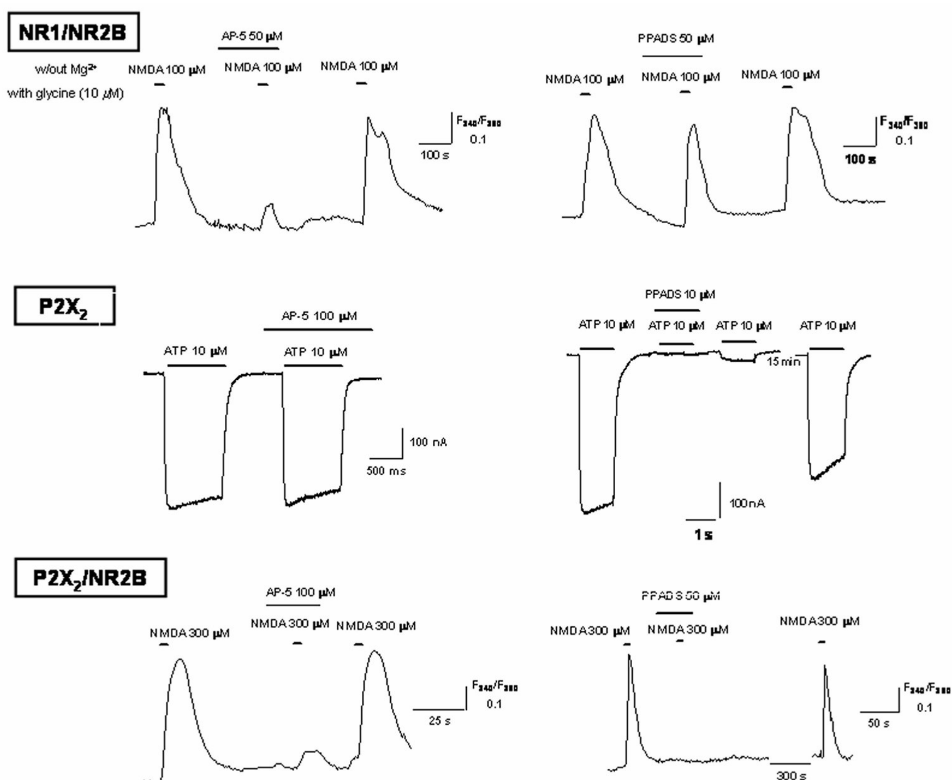


Figure 41. *The $[Ca^{2+}]_i$ transients elicited by NMDA in HEK293 cells co-expressing $P2X_2$ and NR2B are abrogated by antagonists of P2 and NR1/NR2B receptors*
D-AP5 (50-100 µM) a selective competitive antagonist of NMDA receptors for the glutamate/NMDA binding site, prevented the $[Ca^{2+}]_i$ transients induced by NMDA (100 µM) application in cells expressing NR1/NR2B receptors, did not modified the ATP-induced current in cells expressing $P2X_2$ receptors, measured by patch-clamp analysis in its whole cell configuration and not by monitorization of $[Ca^{2+}]_i$ to avoid the P2Y receptor component of the $[Ca^{2+}]_i$ transients elicited by ATP in HEK293 cells, and prevented the $[Ca^{2+}]_i$ transients caused by NMDA (300 µM) application in cells co-expressing $P2X_2$ and NR2B (left graphs). PPADS (10-50 µM), a selective antagonist of NMDA receptors, blocked the ATP-induced current in cells expressing $P2X_2$ receptors, did not produce any modification in the $[Ca^{2+}]_i$ transients induced by NMDA (100 µM) in cells expressing NR1/NR2B receptors, and abrogated the NMDA-induced $[Ca^{2+}]_i$ transients in cells co-expressing $P2X_2$ and NR2B. In whole cell patch-clamp recordings the membrane potential was hold at -40 mV. The graphs presented are representative of 4-12 cells.

As illustrated in figure 41, D-AP5 (50 µM) prevented the $[Ca^{2+}]_i$ transients induced by NMDA (100 µM) application in the presence of glycine (10 µM) and in the absence of Mg^{2+} in cells expressing NR1/NR2B receptors. D-AP5 (100 µM) did not modified the ATP-induced current in cells expressing $P2X_2$ receptors. Finally, D-AP5 (100 µM) was able to prevent the $[Ca^{2+}]_i$ transients caused by NMDA (300 µM) application in cells co-transfected with $P2X_2$ and NR2B. In the presence of the selective antagonist for P2 receptors PPADS (10-50 µM), the ATP-induced current in cells expressing $P2X_2$ receptors was blocked, whereas $[Ca^{2+}]_i$ transients induced by NMDA (100 µM) in cells expressing NR1/NR2B receptors was not modified, as expected. In cells co-transfected with $P2X_2$ and NR2B, in the presence of PPADS (50 µM)

NMDA (300 µM) was no longer able to elicit $[Ca^{2+}]_i$ transients The abrogation of the NMDA-induced $[Ca^{2+}]_i$ transients in cells co-transfected with $P2X_2$ and NR2B, with antagonists of $P2X_2$ receptors and NR1/NR2B receptors further supports for the existence of a $P2X_2$/NR2B hybrid receptor.

4.3 $P2X_2$ and NR2B are physically linked both in heterologous expression system and native brain tissue

The existence of a $P2X_2$/NR2B receptor necessary implies for a physical linkage between $P2X_2$ and NR2B. By co-immunoprecipitation assays, it was evaluated in this same experimental model, HEK293 cells co-transfected with $P2X_2$ and NR2B subunits, if it was possible to detect one of the subunits by selectively pulling-down the other subunit using selective antibodies for each subunit. As illustrated in figure 42, in the immunoprecipitation of $P2X_2$ subunit from cells co-transfected with $P2X_2$ and NR2B, it was detected by Western blot analysis the NR2B subunit. This clearly demonstrate that in this experimental model, in which it was recorded NMDA-induced $[Ca^{2+}]_i$ transients, $P2X_2$ and NR2B are physically linked. The physically linkage between $P2X_2$ and NR2B observed in cells co-transfected with $P2X_2$ and NR2B, together with the functional data previously shown clearly supports the existence of a hybrid $P2X_2$/NR2B receptor.

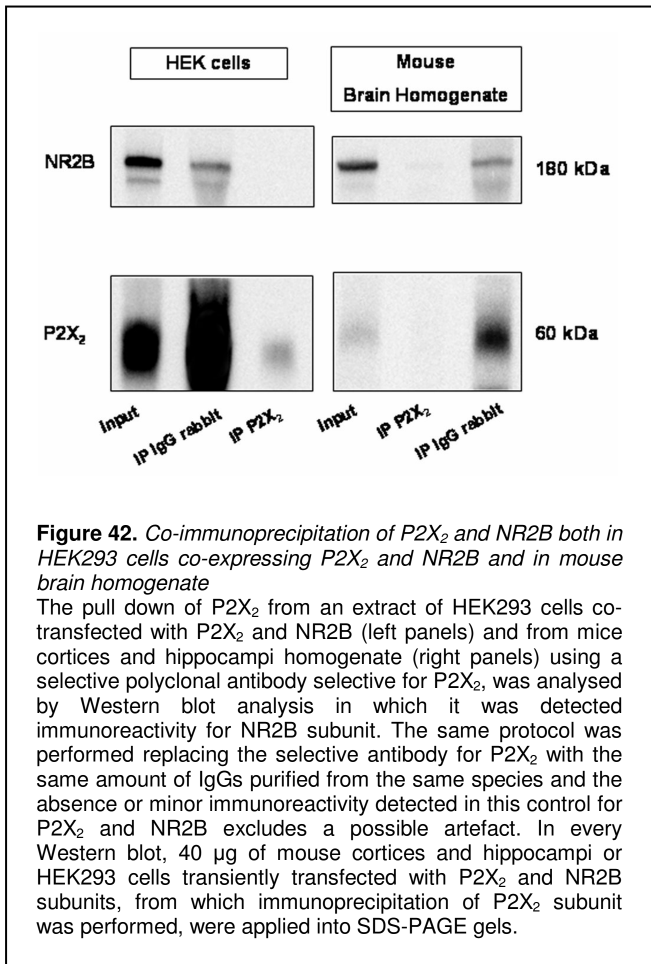


Figure 42. *Co-immunoprecipitation of $P2X_2$ and NR2B both in HEK293 cells co-expressing $P2X_2$ and NR2B and in mouse brain homogenate*
The pull down of $P2X_2$ from an extract of HEK293 cells co-transfected with $P2X_2$ and NR2B (left panels) and from mice cortices and hippocampi homogenate (right panels) using a selective polyclonal antibody selective for $P2X_2$, was analysed by Western blot analysis in which it was detected immunoreactivity for NR2B subunit. The same protocol was performed replacing the selective antibody for $P2X_2$ with the same amount of IgGs purified from the same species and the absence or minor immunoreactivity detected in this control for $P2X_2$ and NR2B excludes a possible artefact. In every Western blot, 40 µg of mouse cortices and hippocampi or HEK293 cells transiently transfected with $P2X_2$ and NR2B subunits, from which immunoprecipitation of $P2X_2$ subunit was performed, were applied into SDS-PAGE gels.

Upon these observations demonstrating the existence of a functional $P2X_2$/NR2B receptor, the question that arises is about the occurrence of this "molecular entity" in the native tissue. For that purpose, a similar co-immunoprecipitation assay was carried out now in mouse brain homogenate. This would enable gauging if $P2X_2$ and NR2B also physical interact in the brain, in order to get a clue if this hybrid receptor may occur in

native tissue as well. The evaluation of a physical interaction between NR2B and $P2X_2$ was performed in mouse cortices and hippocampi since these brain areas present the highest densities of $P2X_2$ (Collo *et al.*, 1996; Rubio and Soto, 2001) and NR2B subunits (Loftis and Janowsky, 2003). As illustrated in figure 42, in the pull-down of $P2X_2$ from an homogenate of mice cortices and hippocampi, it was detected immunoreactivity for the NR2B subunit, showing that also in the mice brain, $P2X_2$ and NR2B are physically linked. Although this result is far from demonstrating the existence of this hybrid $P2X_2$/NR2B receptor in native tissue, it indicates that it may occur. Further studies should be developed to catch this receptor in native tissue.

4.4 Discussion

The present study provides both pharmacological and molecular evidences that clearly demonstrate the assembly of $P2X_2$ and NR2B subunits into a functional hybrid receptor. Although the data gathered so far are essentially preliminary and exploratory, it supports the existence of a hybrid $P2X_2$/NR2B receptor. Thus, this study provides for the first time evidences for the existence of hybrid ionotropic receptors composed by subunits from different families of receptors, which constitutes a new concept of receptor, opening a new venue on the realm of extracellular signalling. Further studies should now be developed to better characterise both biophysically and pharmacologically this new molecular entity, to better support the existence of these receptor and also to comprehend the physiological meaning of these hybrid receptors. Furthermore, it is of most importance to demonstrate the occurrence of these receptors in native tissue, since here it is mostly provided evidence on heterologous expression systems. However, the fact that two subunits with such different structural features are able to form a functional receptor in a mammalian cell, which at a first look would be very unlikely to occur, strongly indicates that they are designed for that purpose by nature itself, strongly suggesting for their existence in native tissue. This is corroborated by the observation of a physical linkage between $P2X_2$ and NR2B in mice brain.

Several questions can be raised from this finding of the existence of these hybrid receptors. What is the physiological purpose of these receptors? Is this receptor $P2X_2$/NR2B found in this study the exception to the rule or are there other such receptors? Is this a particular feature of $P2X_2$ and NR2B subunits or may other P2X and NMDA receptors subunits be able to form such kind of receptors as well? Are there such hybrid receptors composed by subunits of other families rather than P2X and NMDA receptors? Do these hybrid receptors constitute detectors of coincidence? Many other questions can be raised, but although the answers to these questions await further studies, the existence of receptors able to sense more than one molecule indicates that they may constitute a truly detector of coincidence. Maybe the comprehension of these receptors ought to be based on safety control mechanims that cells had to evolve, *i.e.*, the binding of one of the signalling molecules it is not enough to trigger a

signal, but the existence of the two molecules in the extracellular environment at concentrations able to activate these hybrid receptors leads to an effective signal. From the point of view of the cells, the existence of such receptors behaving as detectors of coincidence would be very advantageous because they would enable cells to sense their environment in a more fine-tune manner. For instance, this might have relevance in detecting dangerous signals, in which these receptors would function as "double-gated security systems" designed for the cell to respond only in situations of true dangerous or truly disorders at their environment, and not to respond to possible false disorders, that would generate unnecessary toxic events. However, further studies are needed to confirm this rationale, since here it is just shown that NMDA is able to activate this "hybrid molecular entity", remaining to be demonstrated that also ATP is capable to activate this hybrid receptor. Also, co-activation protocols should be then performed to observe if co-activation by ATP and glutamate leads to a synergistic response in order to gauge if these receptors can really function as detectors of coincidence.

The many known roles ascribed to both NMDA receptors and P2X receptors and their known cellular and subcellular overlapping distribution described above, allows predicting several other possible physiological and/or patophysiological roles for this putative hybrid receptor $P2X_2$/NR2B. For instance, these receptors can have a major role in synaptic plasticity phenomena (see Pankratov *et al.*, 2002a) or in neuron-glia and glia-glia communication (see Fields and Burnstock, 2006). Also, these receptors may be targeted to presynaptic terminals since a presynaptic location was reported for both P2X (see 1 of Results) and NMDA receptors (Liu *et al.*, 1994), and the existence of these hybrid receptors may give highlights on the elusive role of presynaptic NMDA receptors. However, the comprehension of how these hybrid receptors work and the knowledge of their pharmacological and biophysical properties is mandatory to better predict and study their possible physiological and/or pathophysiological role. Thus, the comprehension of the physiological meaning of these hybrid receptors awaits a better characterisation of these receptors.

Although further studies are necessary, the data so far gathered in this study and presented here already reveal the existence of hybrid ionotropic $P2X_2$/NR2B receptors. This supports the hypothesis proposed for the existence of ionotropic receptors composed by subunits from different families of receptors. This constitutes a new concept of receptor and a breakthrough on extracellular signalling. In the realm of the aim of this thesis, the data gathered in this study clearly re-enforces for an essential modulatory role of ATP in the CNS.

CONCLUSION

The present work supports for a major neuromodulatory role of ATP through the activation of P2 receptors in the CNS. It was identified and characterised a direct neuromodulatory action of ATP through the activation of presynaptic P2 receptors in the control of neurotransmitter release. In particular, it was demonstrated that ATP biphasically controls the evoked release of glutamate from rat hippocampal nerve terminals in a concentration-dependent manner, being the receptors involved identified: the facilitatory effect is mediated by the ionotropic $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors whereas the inhibition is mediated by the metabotropic $P2Y_1$, $P2Y_2$ and/or $P2Y_4$ receptors. This presynaptic locus of action reconciles the robust expression and density of P2 receptors with the discrete contribution of P2 receptors to synaptic transmission (*e.g.* Pankratov *et al*., 1998). However, as discussed in chapter 1 of Results, it still remains to be defined in what physiological conditions might these P2 receptors modulation of glutamate release may come into play. Here it is shown what occurs upon P2 receptors activation, but when and in what context are they activated remains to be elucidated. For that purpose, selective pharmacological tools are needed to pave the way to study the synaptic physiology of P2 receptors. In particular, selective antagonists are needed to evaluate the effects of endogenous ATP in experimental models presenting a higher level of integration. Furthermore, it is well established that adenosine, a catabolite of ATP, is an ubiquitous neuromodulator in the brain, and is also able to control in a biphasic manner the release of neurotransmitters (Fredholm *et al*., 2005). Thus, there are two independent neuromodulatory systems operated by metabolically-linked molecules performing basically the same at the presynaptic level in the control of neurotransmitter release. Thus, one of the emergent and more fascinating issues needed to be addressed within the purinergic signalling is to understand the physiological relevance for the cells to have evolved two neuromodulatory signalling systems operated by neuroactive substances coming from the same source.

Besides this direct presynaptic neuromodulatory action, it is also demonstrated that the neuromodulatory system operated by ATP through the activation of P2 receptors is equipped with other strategies to control neuronal function. One of these strategies supported by data gathered in this study is the ability of ATP to modulate other modulatory systems. In particular it is shown a functional interaction between P2X and nACh receptors in the presynaptic control of the release of neurotransmitters. As discussed in chapter 3 and 4 of Results, it was previously shown mainly in heterologous expression systems that P2X receptors functionally interact with nACh (Khakh *et al*., 2000), GABA (Boué-Grabot *et al*., 2004) and 5-HT3 (Boué-Grabot *et al*., 2003) receptors. Here it is shown that the interaction between P2X and nACh receptors have a functional relevance in the brain, in particular in the control of neurotransmitter release.

These interactions between P2X and other ionotropic receptors are characterised by an activity-dependent cross-inhibition and by a physical interaction between the receptors. Thus, the most parsimonious explanation for the physiological relevance of these interactions is that

they are designed for ATP to negatively control the excess of function of other fast signalling systems rather than being detectors of coincidence. This is in agreement with the preferential release of ATP at high frequency stimulations (Cunha *et al.*, 1996). However, in the realm of these interactions between P2X and other ionotropic receptors, a new receptor complex was searched in order to probe for the existence of detectors of coincidence. The hypothesis gauged was the existence of receptors composed by subunits from different families of receptors and thus able to sense more than one signalling molecule, which would behave as true detectors of coincidence. Remarkably, it was gathered several evidences, for the existence of a hybrid $P2X_2$/NR2B receptor, which supports for the existence of such hybrid receptors. Although further studies are needed to better support the existence of these hybrid receptors and to understand their physiological meaning, here it is presented data revealing a new concept of receptor, which constitutes a breakthrough on extracellular signalling. Many questions arise from this novel receptor and this finding overcomes largely the study of P2X receptors, opening a new venue on the research of receptor signalling. Nevertheless, this also shows another mechanism by which P2 receptors can control neuronal function, re-enforcing that P2 receptors play a major neuromodulatory role in the CNS.

Finally, it is shown that these synaptic P2 receptors have a pathophysiological role. It is clearly demonstrated that the abrogation of $P2Y_1$ receptors function, either by pharmacological blockade or genetic deletion of $P2Y_1$ receptors, remarkably arrests the evolution of Alzheimer´s disease, in particular at the early onset preventing the early synaptic failure and memory loss observed in this pathology. Although it remains to be elucidated the mechanism(s) by which $P2Y_1$ receptors are mandatory in the etiopathology of Alzheimer's disease, these data prompt synaptic $P2Y_1$ receptors as potential suitable molecular targets to arrest the evolution of Alzheimer's disease and it also indicates a pathophysiological implication of synaptic P2 receptors function.

The known release of ATP from different cellular and subcellular compartments together with the release and/or co-release of ATP with several other neurotransmitters suggests that ATP is virtually omnipresent in the brain. P2 receptors are also highly abundant and widespread in the brain. There are progressive discoveries of many roles of P2 receptors in the several elements that compose the CNS and a growing evidence for a predominant role of ATP in intercellular communication. All these findings, together with a promiscuity that is being revealed to be a characteristic of ATP and P2 receptors, places ATP at a centre stage of extracellular signalling, suggesting for an homeostatic/modulatory role at the systems level. Thus, it seems that evolution has assured that ATP is not only indispensable inside cells, but also as a fundamental signalling molecule between them.

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