Departamento de Física

Faculdade de Ciências e Tecnologia da
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Role of P2Y1 receptors on neuronal polarity and axonal growth: implications for mossy fiber sprouting in epileptic phenomena

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Abbreviations

A1R – Adenosine A1 receptor

A2AR – Adenosine A2A receptor

A2BR – Adenosine A2B receptor

A3R – Adenosine A3 receptor

aCSF – Artificial cerebrospinal fluid

ATP – Adenosine triphosphate

AP – Alkaline phosphatase

cAMP – Cyclic adenosine monophosphate

E-5'N – Ecto-5'-nucleotidase

E-NPP – Ectonucleotide pyrophosphatase

E-NTPDases – Ectonucleoside triphosphate diphosphohydrolases

PBS – Phosphate buffered saline

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

UDP – Uridine diphosphate

UTP – Uridine triphosphate

TLE – Temporal lobe epilepsy

SE – Status epilepticus

KA – Kainate

MF – Mossy fiber

KARs – Kainate receptors

ABC - ATP-binding cassette

P2Rs – P2 receptors

P1Rs – P1 receptors

P2XRs – P2X receptors

P2YRs – P2Y receptors

Abstract

Several brain pathologies present developmental-like events in their etiology and hence can be explored from the perspective of recapitulating developmental features. One eloquent example is the abnormal axonal sprouting observed in epileptic phenomena. Our group recently found that glutamate, one of the major players during seizures, promotes axonal outgrowth through a newly found pathway, KARs-PKC-GSK3beta-CRMP2, that should be involved in MF sprouting: 1) KARs are involved in the SE-induction elicited by systemic administration of kainate (KA) and 2) blockade of CRMP2 prevents axonal sprouting upon traumatic brain injury. Since KARs are poor therapeutic targets due to the lack of selective drugs, it is now pursued the finding of other signalling systems capable of modulating axonal outgrowth through the modulation of CRMP2 function and be operational during epileptogenesis, with the ultimate goal of finding a therapeutical target to arrest MF sprouting. P2 (ATP) receptors (P2Rs) and in particular the P2Y1R fulfil these criteria. On one hand 1)ATP is preferentially released at high-frequency stimulations in the hippocampus, 2)P2Y1R is localized presynaptically and able to modulate glutamate release in the hippocampus, 3)P2Y1R density is enhanced in the hippocampus upon SE-induction by KA and 4)P2Y1R contributes to status epilepticus (SE)-induced neurodegeneration, which evidence that P2Y1R is operational during epileptogenesis. On the other hand it was recently reported that P2Y1R is present in the axonal tip of developing hippocampal neurons bolstering axonal outgrowth. More interestingly, it was shown to involve the phosphorylation of the inhibitory phosphorylation site Ser9 of GSK3β, as observed for KARs. In this study, it was found that in addition to an increase in the phosphorylation of Ser9 of GSK3β, P2Y1R activation also dephosphorylates CRMP2 at the phosphorylation site targeted by GSK3β, Thr514

. Hence, P2Y1R should promote axonal outgrowth through the desinhibition of CRMP2 by the dephosphorylation at Thr514, reflecting the upstream inhibition of GSK3β.Furthermore, it was also found that purinergic signaling can also modulate neuronal polarization as apyrase, an enzyme that catabolizes ATP and ADP into adenosine induced the formation of secondary axons This was not due to a depletion extracellular ATP and ADP as the blockade of P2Rs did not mimic the effect of apyrase, but instead it was due to activation of A2ARs. Interestingly, it was

also found that the pharmacological activation of A2ARs modifies the phosphorylation pattern of the GSK3β and CRMP2 similarly to the observed for P2Y1R, suggesting for a mechanistical convergence of both effects in the regulation of GSK3β/CRMP2 pathway.

In order to evaluate if the P2Y1R-GSK3β-CRMP2 pathway is reactivated during epileptogenesis, it was first tested the P2Y1R involvement in modifications in the phosphorylation state of GSK3β and CRMP2 observed in the hippocampus upon pilocarpine-induced SE. For that purpose we used the KA-animal model. However, the observation of almost complete cleavage of CRMP2 into the truncated form CRMP2 55 kDa in the rat hippocampus 24 h upon KA injection impaired any evaluation regarding the involvement of P2Y1R in the modifications in the phosphorylation of CRMP2. Nonetheless, it was observed that the pharmacological blockade of P2Y1R prevented the cleavage of CRMP2 by reducing calpain activity as the blockade of P2Y1R attenuated the cleavage of the calpain substrate spectrin. These observations further re-enforce the group's previous findings that the blockade of P2Y1R is neuroprotective in excitotoxic conditions through the prevention/attenuation of calpain activity.

Resumo

Algumas neuropatologias apresentam na sua etiologia mecanismos característicos do desenvolvimento neuronal e como tal, podem ser estudadas numa perspectiva de recapitulação de mecanismos subjacentes ao desenvolvimento. Um exemplo eloquente é o crescimento axonal aberrante observado em fenómenos epilépticos.

O nosso grupo recentemente descobriu que o glutamato, um dos neurotransmissores principais durante ataques epilépticos, promove o cresimento axonial através da via KARs-PKC-GSK3β-CRMP2, a qual deve estar envolvida no crescimento das fibras musgosas no hipocampo, característico da epilepsia de lobo temporal: 1) os receptores de kainato estão envolvidos na indução do estado epileptico provocado pela exposição a kainato e 2) o bloqueio da CRMP2 previne o cresimento axonal após trauma. Devido ao facto de existirem poucos fármacos selectivos para os receptores de kainato, torna-se necessário encontrar outros sistemas de sinalização que sejam capazes de modular o crescimento axonal através da modulação da função da CRMP2 e que estejam operacionais durante a epileptogénese, com o objectivo de encontrar um alvo terapêutico capaz de impedir o crescimento aberrante das fibras musgosas do hipocampo. Os receptores P2(ATP) e em particular os receptores P2Y1 preenchem estes requisitos. Por um lado 1) o ATP é libertado no hipocampo preferencialmente após estímulos de alta frequência, 2) os receptores P2Y1 encontram-se localizados pré-sinapticamente e têm a capacidade de modular a libertação de kainato no hipocampo, 3) a densidade receptores P2Y1 no hipocampo está aumentada após a indução de *status epilepticus* (SE) por kainato e 4) os receptores P2Y1 contribuem para a neurodegeneração induzida por SE,Estas observações demonstram que os receptores P2Y1R estão activos durante a epileptogenese. Por outro lado foi recentemente descrito que os receptores P2Y1 estão presentes no cone de crescimento axonial de neurónios do hipocampo em desenvolvimento e que este receptor promove o crescimento axonal. Além do mais foi mostrado que, esta indução de crescimento por parte deste receptor envolve a fosforilação inibitória do resíduo de aminoácido S9 da GSK3β, tal como observado após a activação dos receptores de kainato. Neste estudo, não só se observou igualmente um aumento na

fosforilação da S9 da GSK3β, mas também uma diminuição da fosforilação da CRMP2 no resíduo de aminoácido T514 induzida pela activação do receptor P2Y1. Esta observação sugere que os receptores P2Y1 devem promover o crescimento axonial através da desinibição da CRMP2 pela indução da desfosforilação da T514 a jusante da inibição da GSK3β. Para além disto, foi observado que a sinalização purinérgica modula também a polaridade neuronal, uma vez que, na presença de apyrase, uma enzima que cataboliza ATP e ADP em AMP favorecendo a formação de adenosina, houve um aumento dos neurónios com mais do que um axónio. Este efeito observado não foi devido à remoção do ATP e ADP extracelulares, pois o bloqueio dos receptores P2 não mimetizou os efeitos da apyrase, mas muito provavelmente devido à activação dos receptores A2A. Isto porque a activação farmacológica dos receptores A2A também induziu a formação de axónios secundários aberrantes. Uma vez mais, este efeito parece envolver a regulação da via GSK3b-CRMP2 dado que foi observado que a activação farmacológica do receptor A2A modifica o padrão de fosforilação da GSK3β e da CRMP2 de um modo semelhante à observada após a activação dos receptores P2Y1, sugerindo uma convergência mecanística de ambos os efeitos. De forma a avaliar se a via P2Y1R-GSK3β-CRMP2 é reactivada durante processos de epileptogénese, foi testado o envolvimento dos receptores P2Y1 nas modificações de fosforilação da GSK3β e da CRMP2, previamente descritas como estando presentes no hipocampo no modelo animal de epilepsia induzida por pilocarpina. Neste trabalho utilizamos o modelo de kainato e ao contrário do descrito no modelo de pilocarpina, a observação de um aumento da forma truncada de CRMP2 (55 kDA) no hipocampo do rato, 24h após a injecção de kainato, impediu qualquer avaliação sobre o envolvimento destes receptores nas modificações de fosforilação da CRMP2. No entanto, foi observado que o bloqueio farmacológico dos receptores P2Y1 preveniu a clivagem da CRMP2 através da redução da actividade da calpaina, dado que este bloqueio atenuou também clivagem do substrato da calpaina, espectrina. Estas observações vêm reenforçar as descobertas anteriores por parte do grupo sobre o papel neuroprotector do bloqueio do receptor P2Y1 em condições de excitotoxicidade, através da prevenção/atenuação da actividade da calpaina.

Introduction

Brain development is a tight controlled process, which includes a series of steps such as neurogenesis, cell migration, differentiation, neuritogenesis as well as synapse formation. A tight control of these steps is implied for the formation of a functional neuronal network and any impairment can lead to long-term deleterious effects, which range from mild cognitive impairment to severe neurological pathologies. On the other hand it is also known that several brain pathologies present developmental-like events. One eloquent example is the abnormal axonal sprouting observed in epileptic phenomena.

Epileptogenesis is a dynamic process initiated by a primary epileptogenic injury (*e.g.* traumatic brain injury, stroke, prolonged febrile seizure, status epilepticus (SE) or encephalitis) that triggers progressive structural-functional modifications, leading to recurrent spontaneous seizures. One such cardinal modification in patients and animal models of temporal lobe epilepsy (TLE) is an abnormal axonal mossy fiber sprouting, which in turn leads to synaptic reorganization in the hippocampus, more specifically in the dentate gyrus (Tauck and Nadler, 1985), creating an aberrant excitatory feedback circuit in granule cells (Zhang *et al.* 2012), contributing to the hyperexcitability underlying the seizure prone state (Nadler, 2003). The prevention of the mossy fiber sprouting has been proposed to control TLE (Koyama and Ikeaga, 2004).

Epilepsy-related neural activity is sufficient to trigger the initial formation of abnormal protrusions from MF axons in the hilar region (Ikegaya, 1999). This is followed by an abnormal retrodirective growth of MF into the inner molecular layer, most likely due to a deregulation of chemofactors necessary for the establishment of normal MF connectivity during development (see Holmaat *et al.*, 2003). Thus, a better comprehension of this phenomenon may be achieved by investigating normal developmental features. Using this rationale, we could recently identify and characterize a mechanism governing axonal outgrowth during embryonic stages that can be involved in the aberrant axonal sprouting. We found that glutamate, one of the major players during seizures, through the activation of kainate receptors (KARs), promotes axonal outgrowth and, more importantly, it can induce the formation of secondary abnormal axons in developing hippocampal neurons (Rodrigues *et al.*, manuscript under preparation). This is achieved through a newly found pathway triggered by the non-canonical signaling of KARs (Rodrigues and Lerma, 2012), involving PKC-mediated inhibition of GSK3β, leading to a disinhibition of the

microtubule-associated protein CRMP2 (Marques *et al.*, manuscript under submission), a protein crucial for axonal specification and outgrowth (Yoshimura *et al.*, 2005). In the adult brain, although the role of CRMP2 is essentially unknown, it was recently shown that the blockade of CRMP2 prevents axonal sprouting upon traumatic brain injury (Wilson *et al.*, 2012). Moreover, in the pilocarpine-animal model of TLE, a similar phosphorylation pattern of GSK3β was observed in the hippocampus (Lee *et al.*, 2012). Indeed, in an animal model of temporal lobe epilepsy, our group could also found that the known abnormal KARs activity (Mulle *et al.*, 1998) reactivates this pathway, which most likely supports the development of the characteristically hippocampal mossy fiber axonal sprouting (unpublished data). However, the potential use of KARs as therapeutically targets is hindered by the lack of selective drugs. Hence, we now pursue the finding of other signaling systems operational during epileptogenesis and capable of modulating axonal outgrowth through the modulation of CRMP2 function, with the ultimate goal of finding a therapeutically target to arrest MF sprouting.

1 - Purinergic signaling

Adenosine 5'-triphosphate (ATP) is a multifunctional nucleotide that is most important to cell life as a "molecular currency" for energy transfer. Discovered in 1929 (Lohman, 1929), this molecule belongs to the purines family, being composed by an adenine attached to a ribose molecule (adenosine) that links to a chain of three phosphate groups (Figure 1). Nowadays, it is well known that besides its role as an energetic molecule, ATP is also an extracellular signaling molecule. Already in 1929, it was reported potent actions of purine nucleotides and nucleosides, ATP and adenosine, on the heart and blood vessels (Drury and Szent-Gyorgyi, 1929) and later, further papers reported effects of extracellular ATP in several systems/tissues (reviewed in Burnstock, 2006). However, the acceptance of a purinergic/ATPergic signalling system has only started in 1972, when ATP was proposed by Geoffrey Burnstock to be a

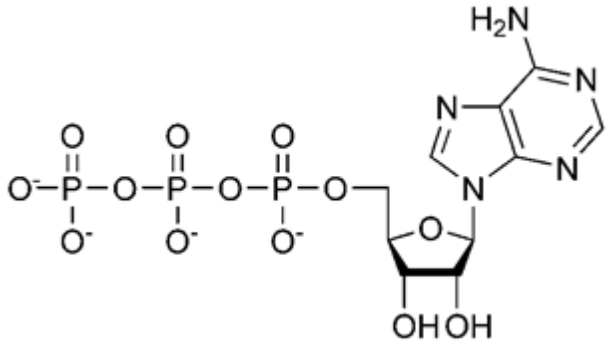

Figure 1 – ATP molecule structure

neurotransmitter in non-adrenergic, non-cholinergic nerves in the gut and bladder (Burnstock, 1972). The concept of a purinergic signalling system gain support by the discovery that the majority of cells are endowed with ecto-enzymes capable to metabolize ATP in the extracellular space, denominated ecto-nucleotidases (as reviewed in Zimmerman, 2012). Ectonucleotidases comprises several enzyme families that catabolize ATP into ADP, AMP and adenosine and are divided into several specific groups: ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase (E-NPP), alkaline phosphatases (AP) and ecto 5'-nucleotidase (Ecto-5´-NT). 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. 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 phenomenon (as reviewed in Burnstock, 2011). However, the purinergic signalling was only consolidated upon the cloning and molecular identification of purinergic receptors.

In the extracellular space, ATP can produce its biological effects *per se* through the activation of P2 receptors (P2Rs) or through its metabolites: ADP, which also activates some P2 receptors, and adenosine, through the activation of P1 receptors (P1Rs), both formed by the extracellular catabolism mediated by ecto-nucleotidases. The P1 receptor family is composed by four different adenosine receptors: A1R, A2AR, A2BR and A3R, and each subtype have its own characteristic molecular, biochemical and pharmacological properties (Table1).

All four P1Rs are metabotropic G-protein coupled receptors with seven transmembrane domains (Furlong *et al.*, 1992), and all have the enzyme adenyl cyclase as first effector, which is either stimulated or inhibited (with a consequent increase or decrease of cAMP levels) depending upon which receptor is triggered (van Calker *et al.*, 1979; Londos *et al.*, 1980). The A1R and A3R inhibit adenyl cyclase through the coupling to $G_{i1,2,3/o}$ and to $G_{i2,3}/G_{q/11}$ proteins,

respectively, whereas A2AR and A2BR stimulate adenyl cyclase through the respective coupling to $G_{S/olf}$ and $G_{S/q}$ proteins (Abbracchio *et al.*, 1995; Kull *et al.*, 2000; Fredholm *et al.*,2001).

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

Receptors	A1	A2A	A2B	A3
G-protein Coupling	Gi, Go	Gs, Golf	Gs, Gq	Gi
Effects	↓cAMP ↑IP3 ↑MAP Kinase ↑K+ ↓Ca2+-currents	↑cAMP ↑MAP Kinase ↑PKC	↑cAMP ↑IP3 ↑/↑MAP Kinase	↓cAMP ↑IP3 ↑MAP Kinase
Selective Agonists	CPA, CCPA, CHA	CGS21680 HE-NECA CV-1808, CV-1674	None	CI-IB-MECA
Selective Antagonists	DPCPX	SCH58261 ZM241385	MRS1754	MRS1220 MRS1191

P2Rs correspond to the larger purinergic receptor group and are divided into two main classes: P2X and P2Y, differing from each other in structure. P2X receptors (P2XRs) are ligand-gated cation channels and P2Y receptors (P2YRs) are metabotropic G protein coupled receptors. (Abbrachio and Burnstock, 1994; Fredholm *et al.* 1994).

Functional and biochemical data collected from recombinant receptors studies established the existence of several functional homomultimers and heteromultimers P2X receptors. Seven P2XRs subunits have been cloned: $P2X_{1,2,3,4,5,6,7}$ (Ravelic and Burnstock, 1998), all of them capable to form functional homomeric receptors. Also, several heteromultimers have already been functionally characterized: P2X2/3 (Radford *et al.*, 1997), P2X4/6 (Lê *et al.*, 1998a), P2X1/5 (Lê *et al.*, 1999), P2X2/6 (King *et al.*, 2000), P2X1/2 (Brown *et al.*, 2002) and P2X1/4 (Nicke *et al.*, 2005). However, more functional heteromers are possible (see Torres *et al.*, 1998).

As mentioned, the P2XRs are non-selective ligand-gated cation channels, activated by extracellular ATP (in the micromolar range) and present an equal permeability to sodium (Na^+) and potassium (K^+) and a significant higher permeability to calcium (Ca^{2+}). The analysis of their hydrophobicity pattern predicts that each P2X subunit has two transmembrane hydrophobic

regions. The first region is involved in the channel gating and the second region in lining the ion pore, with both terminals (-NH2 and –COOH) laying in the cytoplasm (Li *et al.*, 2008). Both terminals diverge from one receptor type to another, with the –COOH terminal binding the motifs for protein kynases responsible for the receptor kinetics, and the –NH2 terminal having an important role in the trafficking of receptors to the plasmatic membrane (as reviewed in North, 2012). The extracellular domain of these receptors has amino acid homology, containing ten cysteine residues that form a series of disulphide bridges where the ATP has its binding site (Khakh, 2001).

P2XRs can be grouped according to pharmacological and biophysical properties (Table 2). P2XRs are activated by ATP, but diadenosine polyphosphates, which present a lower potency and efficacy than ATP can also be considered a natural ligand (Wildman *et al.*, 1998). Besides ATP and diadenosine polyphosphates, the only P2XRs agonists in existence are ATP analogs such as αβmeATP and βγmeATP (metabolically more stable and do not activate any P2YR) (Coddou *et al.*, 2011).

Table 2. Grouping of P2XRs according to pharmacological and biophysical properties (Wildman *et al.*, 1998; Coddou *et al.*, 2011)

Receptors	Properties
P2X1	High sensitivity to αβmeATP and to ATP
P2X3	Rapid desensitization upon agonist application
P2X2	Lower sensitivity to ATP
P2X4	Very low sensitivity to αβmeATP
P2X5	Slow desensitization
P2X7	Lower sensitivity to ATP No desensitization

P2Y receptors (P2YRs) are metabotropic G-protein coupled receptors endogenously triggered by ATP, ADP and pyriminide nucleotides (UTP, UDP, and UDP-Glucose). To date eight different mammalian P2YRs subtypes have been cloned ($P2Y_{1,2,4,6,11,12,13,14}$). These receptors are comprised by seven transmembrane regions with an extracellular –NH2 terminal

and an intracellular –COOH terminal (Abbracchio *et al.*, 2006; Burnstock, 2007). This intracellular loop and the C-terminus present high diversity among the P2YRs subtypes, which influences the degree of coupling with $G_{q/11}$, G_s and G_i and, on the other hand, the receptor subtypes present a high homology level between them at the hydrophobic transmembrane regions, mainly at TM3, TM6 and TM7 (Abbracchio *et al.*, 2006).

While P2XRs are activated by ATP, P2YRs differ in their selectivity to ligands. As such, they can be classified regarding their natural ligands, in addition to their coupling to different G-proteins (Table 3) (Boeynaems *et al.* 2012). Several antagonists and agonists for these receptors have been developed throughout the years and are presented in Table 3.

Table 3 – P2YRs natural occurring ligands, agonists and antagonists and G-proteins (Boeynaems *et al.* 2012)

Receptors	**P2Y1**	**P2Y2**	**P2Y4**	**P2Y6**
G-protein Coupling	Gq	Gq+Gi	Gq+Gi	Gq
Natural Ligands	ADP	ATP=UTP	UTP	UDP
Agonists	2-MeSADP=ADPβS>2-MeSATP=ADP>ATP	UTP=ATP,Ins45973	UTP=ATP,Ins45973	UDP>UTP>>ATP Ins415
Antagonists	MRS2179, MRS2500	Suramin, RB2	Suramin, RB2, PPADS	MRS2578
Receptors	**P2Y11**	**P2Y12**	**P2Y13**	**P2Y14**
G-protein Coupling	Gq+Gs	Gi	Gi	Gi
Natural Ligands	ATP	ADP	ADP	UDP-Glucose
Agonists	BzATP=ATPγS>ATP	ADP=2-MeSADP>>ATP	ADP=2-MeSADP>>ATP	UDPglucose=UDPgalactose
Antagonists	Suramin, RB2	CT50547, Ins49266	MRS2211	-

The analysis of Table 3 shows a division in the P2YRs into two main groups: one corresponding to the $P2Y_{1,2,4,6,11}$Rs that use mainly G_q to activate the phospholipase Cβ (PLCβ)/ inositol-(1,4,5)-triphosphate (IP3) pathway and trigger the release of intracellular Ca^{2+} and another one corresponding to the $P2Y_{12,13,14}$Rs, which couple almost exclusively to G_i (Abbrachio *et al*, 2006).

A particular property of these receptors is the ability to link different G proteins and consequently trigger different signalling pathways. For instance, the $P2Y_{11}$R when activated by ATP leads to an increase in cAMP, IP3 and in the cytosolic Ca^{2+} whereas activation by UTP can result in Ca^{2+} mobilization without cAMP or IP3 increase (White *et al.*, 2003). Moreover, the

$P2Y_{13}R$ can simultaneously couple to G_{16} and G_i proteins and, at high ADP concentrations, to G_S proteins in a way that suggests the existence of ligand-specific conformations of the receptor (Marteau *et al.*, 2003).

2 - Purinergic Signaling in the adult brain

In the adult brain, A1Rs and A2ARs are the most predominant P1Rs, while A2BRs and A3Rs are at very low densities. A1Rs are present at a higher density in the cortex, cerebellum and hippocampus (Schindler *et al.*, 2001; Rebola *et al.*, 2003), and are observable in every cell type: neurons, astrocytes, microglia and oligodendrocytes (Gebicke-Haerter *et al.*, 1996; Biber *et al.*, 1997; Othman *et al.*, 2003). At the hippocampus, A1Rs are predominantly targeted to nerve terminals and mainly enriched post-synaptically (Tetzlaff *et al.*, 1987; Rebola *et al.*, 2003), where they decrease neuronal excitability through activation of potassium channels, decrease NMDA receptor currents and inhibit voltage-gated Ca^{2+} channels (as reviewd in de Mendonca *et al.*, 2000), but are also located at the presynaptic level (Rebola *et al.* 2003) inhibiting neurotransmitter release (Dunwiddie *et al.*, 1997).

In contrast with A1Rs, A2ARs are highly concentrated in the basal ganglia, when compared to other cerebral regions (reviewed in Cunha, 2005), predominantly at higher densities at dendritic spines and post-synaptic densities (Hettinger *et al.*, 2001; Rodrigues *et al.* 2005). But they are also expressed in cortical and hippocampal regions predominantly at the presynaptic level (Rebola *et al.* 2005), where they facilitate neurotransmitter release (Cunha *et al.*, 1998) and are involved in synaptic plasticity (*e.g.* Almeida *et al.* 2003).

As previously stated A2BRs and A3Rs present a much lower density in brain regions when compared to A1Rs and A2ARs. A2BRs are mainly present in astrocytes (Allaman *et al.* 2003) and in the median eminence, being also present in neurons and glial cells of the pituitary gland, albeit in lower levels (Daly, 1977; Fredholm *et al.*, 2005). A3Rs are present in neurons (Costenla *et al.* 2001), microglia (Hammarberg *et al.* 2003) and astrocytes, with an apparently more evident function in astrocytes (Abbracchio, 1998). Regarding brain regions A3Rs are present in the cortex, the striatum, the olfactory bulb, the nucleus accumbens, the hippocampus,

the amygdala, the hypothalamus, the thalamus and the cerebellum (Fredholm *et al.*, 2005).

P2 receptors are ubiquitously expressed in the brain, both in neuronal and glial cells (Illes and Alexandre Ribeiro, 2004; Abbracchio *et al.*, 2009). In the hippocampus, neuronal P2Rs are essentially targeted to synapses both at the pre- and postsynaptic level, where they play a neuromodulatory role through the presynaptic modulation of glutamate release (Rubio and Soto, 2001; Rodrigues *et al.*, 2005). Post-synaptically, besides a minor contribution of P2XRs to neurotransmission at Schaffer collaterals-CA1 synapses (Pankratov *et al.*, 1998), their role remains ill defined.

In astrocytes, P2Rs are expressed as well, where they modulate astrocytic function through the modulation of Ca^{2+}-homeostasis (Gallagher and Salter, 2003; Suadicani *et al.*, 2004; Bennett *et al.*, 2005). So far it is known that $P2X_{1,2,4,7}Rs$ (Lalo *et al.*, 2011) and $P2Y_{1,2,4,6,12,13}R$ (Verkhratsky *et al.*, 2009; Butt, 2011) are present in astrocytes. Indeed, the ATP through the activation of P2 receptors seems to be one of the major players in glia-glia and neuron-glia communication (Matute and Cavaliere, 2011). Besides, they also modulate in a bidirectional manner astrocyte proliferation, increasing it through the activation of P2YRs, both *in vitro* (Ciccarelli *et al.*, 1994) and *in vivo* (Hindley *et al.*, 1994; Franke *et al.*, 1999), while the activation of P2XR was found to stop the proliferation of astrocytes (Neary *et al.*, 2005 and 2008).

P2Rs are also found in the microglia as well, where they play a role in chemotaxis and in the control of microglia reactivity (Inoue, 2008).

3 - Purinergic Signaling and Neuronal Development

Purinergic signalling is an ancient signalling system (Fountain & Burnstock, 2009). This filogenetical relevance of purinergic signalling may imply for a relevant role of this signalling system in development. Indeed, both P1Rs and P2Rs and ecto-nucleotidases are expressed early on in embryogenesis. For instance, P2XRs could be identified during gastrulation (Laasberg, 1990) and P2Rs and ecto-nucleotidases are expressed in embryonic stem cells (reviewed in Zimmermann, 2011). The expression of purinergic receptors is particularly strong at mice

embryonic day 11 (E11) (Cheung *et al.*, 2003) onwards in a developmental-regulated and in some cases in a transient manner, which suggests that purinegic signalling affects stage-specific developmental processes, also suggesting that its functional significance may be even greater during development rather than in the adult nervous system (Franke & Illes, 2006; Zimmermann, 2011). However, besides a growing evidence for the involvement of purinergic signalling in neurogenesis, either modulating proliferation, differentiation and migration of progenitors cells, observations essentially obtained *in vitro* (see Liu *et al.*, 2008), the role of purinergic receptors during development remains still largely unexplored. Nevertheless, very recently, our group contributed to show that A2ARs are involved in hippocampal interneurons migration (Silva *et al.*, in press). Moreover, there is also some *in vitro* evidence indicating that purinergic receptors can also influence neuronal development, in particular neurite outgrowth. In PC12 cells it was observed that A1R inhibits neurite outgrowth (Thevananther *et al.*, 2001)

More recently, it was reported a bidirectional modulation of axonal outgrowth in developing hippocampal neurons by P2Rs. While P2Y1Rs promote axonal outgrowth, P2X7Rs inhibit axonal elongation (del Puerto *et al.,* 2012*)*. Interestingly, it was shown that this effect is most likely mediated by signalling pathways converging in the modulation of GSK3β activity, an intracellular enzyme that upon activation induces growth cone collapse and arrests axonal elongation through the phosphorylation of CRMP2 (Yoshimura *et al.*, 2005), as mentioned above.

4 - Purinergic Signaling and Epileptogenesis

The ubiquitous relevance of purinergic signaling in the normal adult brain suggests that any impairment or change on this signaling system may give rise to pathological consequences. In fact, over the years, various studies have reported the involvement of purinergic receptors in a wide spectrum of pathological conditions (Burnstock *et al.*, 2011). Moreover, there is now increasing evidence that support ATP and adenosine as a dangerous signal, *i.e.*, upon noxious insults such as trauma, hypoxia or ischemia there is an increase in the extracellular levels of ATP and adenosine, which, in turn, brings purinergic receptors into play during these pathological conditions. Indeed, several reports have been showing the involvement of purinergic receptors in these pathological conditions, either as a protective mechanism or its role in aggravating the

deleterious conditions (reviewed in Burnstock *et al.*, 2011). The involvement of purinergic receptors have been also implicated in neurodegenerative diseases.

For instance, in Alzheimer's disease (AD) A1Rs density is significantly reduced in the hippocampus (Jansen *et al.*, 1990), alongside with an increase in A2ARs density (Albasanz *et al.*, 2008), which seems to be relevant in the etiology of the disease, as the blockade of A2ARs was shown to be neuroprotective and able to prevent memory loss in an animal model of Aβ-toxicity (Canas *et al.*, 2009). In addition, it has been shown that ATP increases the vulnerability of neurons to Aβ peptide-induced cell death (Haughney and Matson, 2003) and P2X7Rs seem to be also relevant in AD, since their up regulation in a mouse model of AD (Tg2576) (Parvathenani *et al.*, 2003) seems to exacerbate the inflammatory responses in AD brain associated to synaptotoxicity (Sanz *et al.*, 2009; Lee *et al.*, 2011; but see Delarasse *et al.*, 2011).

Purinergic signaling seems also to be relevant in epileptic phenomena. As reviewed in Dale and Frenguelli, 2009, epileptic seizures are apparently accompanied by a release of adenosine. In fact, high levels of adenosine and its breakdown products were observed in the rat brain after electroshock seizures (Lewin and Bleck, 1981). This report was followed by the observation that, after theophylline (P1Rs antagonist) exposure, it was possible to observe generalized convulsions (Persson and Erjefalt, 1982). These reports gave the first clues that purinergic signaling should be involved in epileptic phenomena.

Wieraszko *et al.*, 1989 reported an increase in the extracellular ATP levels in the hippocampus associated to a seizure-prone state. This is in agreement with the fact that glutamate is accompanied by ATP preferentially upon high-frequency stimulation in the hippocampus (Cunha *et al.*, 1996), the brain region mostly affected in temporal lobe epilepsy. Moreover it was observed that the injection of ATP analogs caused a generalized motor seizure (Knutsen *et al.*, 1997). These evidences indicate that extracellular ATP and/or adenosine are not only present during seizures, but may be also contributing to seizures. Accordingly, it was found an upregulation of P2X7Rs in the hippocampus of pilocarpine-induced epileptic rats (Vianna *et al.*, 2002) and our group has also observed an increase in the density of P2Y1R in the hippocampus upon SE-induction with KA (Oses *et al.*, 2006), an increase also recently reported to be observed in the hippocampus of patients with temporal lobe epilepsy (Padrao *et al.*, 2011). Furthermore, several evidences obtained previously in our group showed that the

pharmacological blockade or genetic deletion of the P2Y1R attenuates/prevents SE-induced hippocampal neuronal death, astrogliosis, microgliosis and synaptic loss (unpublished results).

Altogether, these evidences indicate that purinergic signaling and in particular the P2Y1Rs are operational during epileptogenesis. Moreover, as mentioned above, it was recently reported that P2Y1R bolsters axonal growth in developing hippocampal neurons and, more importantly, there is a mechanistic convergence between P2Y1R and KAR in the modulation of axonal development, *i.e.* through the modulation of GSK3β. This evidence strongly indicates that P2Y1R should be contributing to hippocampal mossy fiber sprouting, fulfilling the criteria previously defined to be considered a potential target to arrest mossy fiber sprouting: 1) P2Y1R is operational during epileptogenesis and 2) capable of modulating axonal outgrowth most likely through the modulation of CRMP2 function.

Aim

- Characterization of the role of P2Y1Rs in the establishment of neuronal polarity (axon specification) and axonal growth in mice developing hippocampal neurons: mechanistically relevance of CRMP2 protein.

- Role of P2Y1Rs-CRMP2 in the hippocampal MF sprouting in the KA-animal model of TLE.

Materials and Methods

1. Animals used

The primary cultures of hippocampal neurons were prepared from 18-19 day-old Wistar rat embryos handled according to European guidelines for use of experimental animals. The progenitors were anesthetized under halothane atmosphere and subsequently sacrificed by decapitation. For the animal model of temporal lobe epilepsy male Wistar rats (8-10 weeks old and weighting between 250-300 g) were used, handled according to European guidelines.

2. Rat hippocampal neurons

As previously described, the primary cultures of hippocampal neurons were obtained from 18-19 day-old Wistar rat embryos. The hippocampus were dissected and placed in Hank's Balanced Salt Solution (HBSS) (0.8% NaCl, 0.035% $NaHCO_3$, 0.006% KH_2PO_4, 0.04% KCl, 1% glucose, 0.014% $CaCl_2$, 0.0048% Na_2HPO_4, 0.0098% $MgSO_4$, Fenol Red 2% and at pH=7). After the complete removal of the meninges, the tissue was washed and incubated in HBSS containing 10% trypsin and 1% DNAse for 15 min. at 37ºC. The enzyme activity was stopped using 5 mL of DMEM medium (empresa)with 10% foetal bovineserum (FBS) (empresa) and 1% penicilin/streptomycin (P/S) (empresa) The hippocampi was then transferred to 2 mL of DMEM medium and the cells were mechanically dissociated through a 1 mL pipette and centrifuged at 10000rpm for 5min. The pellet was ressuspended in DMEM (31996) with 10% FBS, 1%P/S and were plated in poly-lysine 0.1mg/mL (Sigma) and laminine 0.01mg/mL (Sigma) coated 12 mm diameter coverslips or 6 wells dishes at different densities: 10,000 cells per coverslip for immunocytochemistry assays and $5x10^5$ cells per well for Western Blot analysis. Two hours after plating, cells were incubated with the drugs and cultured in Neurobasal medium (GIBCO, BRL, Life Technologies, Scotland, UK), supplemented with 0.25% glutamine, 0.25% glutamate, 2% B27 supplement and 1% P/S, at 37ºC in a humidified atmosphere of 5% CO_2 and 95% O_2.

At 3 DIV, cells were fixed with 4% warmed paraformaldehyde for immunocytochemical assays and morphological analysis. At 6 DIV, protein extracts were collected by scraping the cells into 100 µL of cell lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 1% TritonX-100, 0.1% SDS and protease inhibitors [Roche Diagnostics]; pH 7.4). The protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology).

3. Animal model of temporal lobe epilepsy

One of the best characterized animal model of mesial temporal lobe epilepsy (TLE) is the systemic or local administration of kainic acid, an AMPAR/KAR agonist, which induces acute seizure episodes and subsequent development of recurrent spontaneous seizures, the hallmark of epilepsy, and similar hippocampus sclerosis and mossy fiber sprouting to those observed in humans (Nadler, 2003). Seizures induced by kindling have been classified into five stages based on clinical signs and this scale was later adopted to describe seizures in other animal models of epilepsy (Racine, 1972). According to the Racine's scale to monitor rat behavior immediately after chemoconvulsant-induced seizures, there are five stages to be considered: stage I- facial automatisms; stage II- head nodding; stage III- forelimb clonus and lordotic posture; stage IV- forelimb clonus as the animal rears; stage V- forelimb clonus and rearing with falling over or loss of the righting reflex. Stages III, IV and V, together with "wet-dog" shake movements, are considered motor or convulsive seizures and should happen until two hours after KA injection to guarantee hippocampal neuronal degeneration.

3.1. Experimental procedure in the animal model of temporal lobe epilepsy

This model was performed using adult maleWistar rats (weighting 250-300 g). Animals were separated into 6 different groups: the saline group, which received artificial cerebrospinal fluid (aCSF, 124 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 10 mM glucose, 26 mM NaHCO3, 1 mM MgCl2 and 2 mM CaCl2) *icv* and saline (SAL, 0.9 % NaCl) *ip*; the kainate group, which received aCSF *icv* and 10 mg/kg kainate (KA) *ip*; the PPADS + KA group, that received 1 nmol/4 µl PPADS *icv* and 10 mg/kg kainate *ip*; the MRS2500 + KA group, that received 1 nmol/4 µl MRS2500 *icv* and 10 mg/kg kainate *ip* and, finally, the PPADS and MRS2500 groups that received 1 nmol/4 µl PPADS or MRS2500 *icv* and SAL *ip*. Three days before the *ip* administration of kainate or saline, the animals were anaesthetized with sodium thiopental 50 mg/kg i*p* and placed in a stereotaxic apparatus. The skull was opened and a guide cannula (CMA11 guide cannula) was introduced into the lateral ventricle and fixed to the skull with the help of two drills and cement at the following coordinates relative to Bregma: -0,8 mm antero-posterior; -1,5 mm lateral and -3,5 mm dorso-ventral. On the *icv* injection day, animals were

taken into a needle (4.2 mm length, 26-guage) connected to a PE 50 polyethylene tube. After unscrewing the cap, the needle was gently inserted through the guide cannula, 1 mm bellow, into the ventricle and drugs or saline were slowly manually infused. Administration was done at the rate of 0.5 µl/ min. At the end of the injection, the needle remained in place for 3 min before being slowly removed from the cannula, to avoid reflux. The convulsive behavior was monitorized for 3h after KA injection and classified according to the Racine's scale. Only the animals reaching stage4-5 were used. Twenty-four hours later, hippocampi were removed for total protein extract preparation for Western blot analyses.

4. Total protein extracts

. Animals were sacrificed and hippocampi isolated as previously described. The tissue was homogenised in a 0.32 M sucrose solution containing 1 mM EDTA, 10 mM HEPES and 1 mg/ml BSA, pH7.4 at 4 ºC. Then, the homogenates were centrifuged at 3000g for 10 min, at 4 ºC. The pellet (P1) was discarded and the supernatant (S1) was further centrifuged at 100,000 x g for 30 min at 4 ºC. The supernatants (S2) were discarded and the pellet (P2), correspondent essentially to total cytoplasmic membranes, was resuspended in a 5% Sodium Dodecyl Sulphate (SDS) solution with 0.1 mM PMSF. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce Biotechnology).

5. Western Blot analysis

Protein extracts either from hippocampal cultured neurons or total hippocampal protein extracts were diluted in 6x SDS-PAGE sample buffer (0.35 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue, pH 6.8), boiled at 95 ºC for 10 min and then separated by 12% SDS-polyacrilamide gel electrophoresis (12% resolving gel with a 4% stacking gel). The phosphorylated formof CRMP2 protein at Thr514was detected using the rabbit antibody against p-CRMP2^{T514} (Cell Signaling; 1:500) in TBS-T with 5% BSA . The phosphorylated form of GSK3β at Ser9 was detected using the rabbit antibody against p-GSK3β^{S9} (Abcam; 1:1000) in TBS-T with 5% PhosphoBLOCKER Blocking Reagent (Cell Biolabs). Total CRMP2 protein was detected using a rabbit antibody against CRMP2 (Abcam; 1:4000) in TBS-T with 5% milk. Total GSK3β was detected using a rabbit antibody against GSK3β (Abcam; 1:500) in TBS-T with 5% milk. A rabbit antibody against GAPDH (Abcam; 1:1000) was used as a loading control. The

membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (Pierce; 1:5000), followed by incubation with SuperSignal West Pico Chemiluminescent Substrate (Pierce) in the case of total CRMP2and GAPDH or with Luminata Forte Western HRP Substrate (Millipore) in the case of p-CRMP2^{T555}, p-CRMP2^{T514,} GSK3β, p-GSK3β^{S9} and visualized using the VersaDoc 3000 system by chemiluminescense and analyzed using Quantity One software (BioRad, Portugal).

6. Immunocytochemistry in rat hippocampal neurons

Cultured hippocampal neurons fixed at 3 DIV were washed 3x with phosphate buffered saline medium (PBS, in mM: 140 NaCl, KCl, 26 NaH2PO4, 15 KH2PO4, pH 7.4) and permeabilized with PBS 0.2% Triton X-100 for 10 min. After washing twice with PBS, cells were blocked with PBS 3% BSA for 30 minutes to avoid non-specific binding and then incubated with primary antibodies in blocking solution for 1 h at room temperature (RT)Cells were incubated with mouse anti-Smi-31 R-100 (1:1000, Covance)as an axonal marker, rabbit anti- β-III-tubulin (1:1000, Empresa) as a neuronal marker. After washing 3x with PBS, cells were incubated with the Alexa-488 conjugated donkey anti-mouse and Alexa-555 donkey anti-rabbit antibodies (1:400; Invitrogen) for 1 h at RT. Coverslips were mounted into slides on DAPI-supplemented mounting medium (Vectashield, Vector Laboratories) and visualized using Zeiss Axiovert 200 fluorescence microscope equipped with AxioCam HRc. Images were acquired using Axiovision software 4.6 (PG-Hitec, Portugal) and the morphological analysis was performed using Neurolucida software (MBF Bioscience).

7. Data presentation and statistical analyses

All the presented data are mean ± S.E.M. of *n* experiments. Axonal length was expressed as relative values in relation to respective control conditions as fold change quantified from a minimum of 100 cells per condition per culture, taken as single measurements. Modifications in the phosphorylation levels observed by Western blot were also presented as fold change in relation to respective control conditions. The effects presented as relative data were analyzed by one-sample *t*-test comparing with hypothetical value of 1 (control condition). Group differences were analyzed using unpaired *t*-test to compare two independent groups and one-way ANOVA to compare several independent groups as in the number of axons per neuron, also quantified

from a minimum of 100 cells per condition per culture. When the ANOVA provided significant general group effect, Dunnet's *post-hoc* test was used to compare different groups with the control group. The distribution of the neurons with different number of axons was represented as percentages, quantified from a minimum of 100 cells per condition per culture, taken as single measurements. The impact of the conditions tested on the percentage of neurons with different number of axons was performed using two-way ANOVA. Whenever an interaction was found significant, *post-hoc* comparisons were performed with Bonferroni`s *post-hoc* test. In all the analysis performed, a family-wise 95% confidence levels ($p<0.05$) was applied. All data processing and analysis were performed with GraphPad Prism 5.0 software (Graphpad).

Results

1 – Role of purinergic signaling in the establishment of neuronal polarity (axon specification and axonal outgrowth in rat hippocampal neurons

1.1 - P2Y1Rs promote while P2X7Rs inhibt axonal growth in rat hippocampal neurons

The first aim of this study was to evaluate if the previously shown modulation of axonal growth in developing hippocampal neurons by P2Rs through the modulation of GSK3β activity (del-Puerto *et al.*, 2012) also involved the downstream regulation of CRMP2 protein. For that purpose, we first attempted to confirm that P2Y1R promotes, while P2X7R inhibits axonal growth in rat hippocampal neurons as previously described (del-Puerto *et al.*, 2012). As shown in Figure 2, it was observed an increase in the axon length in neurons cultured in the presence of ADPβS (5 µM), an agonist of P2Y1R, P2Y12R and P2Y13R. The observation of a prevention of this increase in the presence of the selective antagonist of P2Y1Rs, MRS2179 (10 µM), shows that this increase in axonal length is induced by P2Y1R activation (Figure 2). Moreover, neurons cultured in the presence of MRS2179 (10 µM) presented a significant lower axonal length

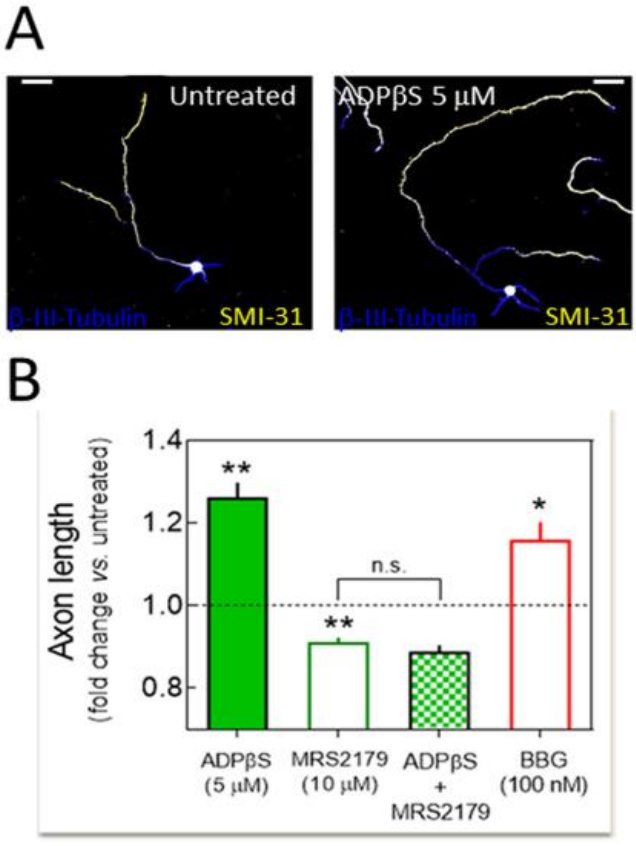

Figure 2. P2Y1R promotes, while P2X7R inhibits axonal growth in rat hippocampal neurons. **(A)** Representative images of rat hippocampal neurons (3 DIV) in untreated conditions and cultured in the presence of ADPβS (5 µM), immunolabeled with an anti-SMI-31 antibody (yellow; axonal marker) and anti-β-III-tubulin (blue; neuronal marker). Co-localization appears as white. Scale bars are 50 µm. **(B)** Histogram summarizing the axonal length quantification. The selective agonist of P2Y1R, P2Y12R and P2Y13R, ADPβS (5 µM), increased the axonal length. This effect was prevented in the presence of the selective antagonist of P2Y1R, MRS2179 (10 µM) (p>0.05, unpaired t-test ADPβS + MRS2179 *vs.* MRS2179), which reduced axonal length by itself. The blockade of P2X7R with the selective antagonist brilliant blue G (BBG; 100 nM) increased axonal length. The data are expressed as the fold change (mean ± s.e.m.) relative to untreated cells quantified from 4different cultures, analyzing a minimum of 100 cells per condition and culture. *p<0.05, **p<0.01 and ***p<0.001, one-sample t-test.

showing for the existence of a tonic action of P2Y1R promoting axonal growth. On the other hand, the presence of a selective antagonist of P2X7R, brilliant blue G (BBG; 100 nM), induced an increase in the axonal length showing a tonic inhibitory action of P2X7R in axonal outgrowth (Figure 2).

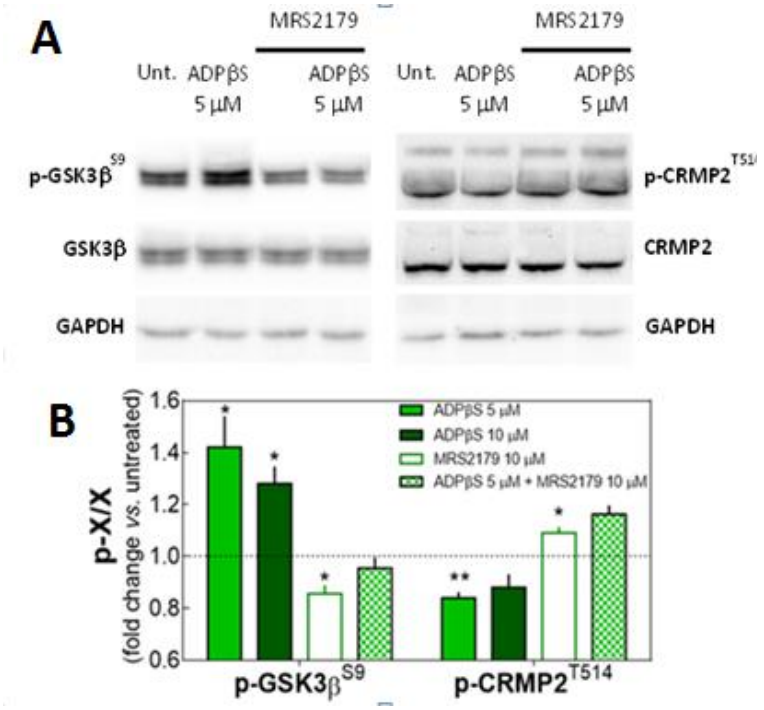

Figure 3. Activation of P2Y1Rs or blockade of P2X7Rs diferentially regulates the phosphorilation pattern of GSK3β and CRMP2 in rat hippocampal neurons. **(A)** Representative Western Blot obtained from cultured neurons exposed to ADPβS (5 μM), MRS2179(5 μM) MRS2179 (10 μM) + ADPβS (5 μM). Bands showing GSK3β, phosphorylated GSK3β at S9, CRMP2, phosphorylated CRMP2 at T514 all loaded with GPDAH. **(B)** Histogram summarizing the ratios of phosphorylation (p-proteint:total protein) of either GSK3b at Ser9 (p-GSK3bS9) or CRMP2 at Thr514 (p-CRMP2T514) ADPβS, a selective agonist of P2Y1R, P2Y12R and P2Y13R, induced an increase in the phosphorylation of GSK3β at S9 (at 5 μM and 10 μM) a decrease in the phosphorylation of CRMP2 at T514 (at 5 μM but not at 10 μM), effects prevented in the presence of the selective antagonist of P2Y1R, MRS2179 ($p>0.2$ ADPbS+MRS2179 vs. MRS2179 for both proteins, unpaired t-test, n=4). Neurons cultured in the presence of MRS2179 (10 μM) alone presented a decrease in the phosphorylation of GSK3β at S9 and a corresponding increase in the phosphorylation of CRMP2 at T514. The data are expressed as the fold change (mean ± s.e.m.) relative to untreated cells quantified from 4 different cultures. *$p<0.05$ and **$p<0.01$.

Altogether, these results are concordant with the results previously reported (del-Puerto *et al.*, 2012), showing a bidirectional receptor-dependent modulation of axonal growth, promoting through P2Y1R and inhibiting through P2X7R.

1.2 – P2Y1R decreases the phosphorylation levels of CRMP2 at Thr514

It was previously shown that either the promotion of axonal growth by P2Y1R involved the inhibition of GSK3β activity by inducing an increase in the phosphorylation levels at the inhibitory phosphorylation site Ser9 (del-Puerto *et al.*, 2012). By Western Blot analysis, we could also observe that ADPβS (5 μM and 10 μM) increased the levels of p-GSK3β^{S9}, an effect prevented by the selective antagonist of P2Y1R, MRS2179 (10 μM) (Figure 3- left bars). Moreover, MRS2179 alone induced a reduction in the levels of p-GSK3β^{S9} (Figure 3), showing

once again a tonic action of P2Y1R, as observed regarding axonal length (see Figure 2).

As mentioned before, GSK3β modulates axonal growth through the downstream regulation of CRMP2 protein (Yoshimura *et al.*, 2005). CRMP2 is a cytosolic phosphoprotein crucial in neuronal development, being essential to the establishment of neuronal polarity (axon specification), axonal growth and guidance. Two different and independent phosphorylation pathways of CRMP2 protein have been described: one operated by Cdk5 at Ser522 (priming kinase) and GSK3β at Thr514 (Uchida *et al.*, 2005) and the other one by Rho kinase or directly by PKC at Thr555 (Arimura *et al.*, 2000), both inhibiting the ability of CRMP2 to promote axonal growth by disrupting CRMP2-tubulin binding, and hence microtubule assembly, resulting in growth cone collapse (Fukata *et al.* 2002; Arimura *et al.*, 2005; Yoshimura *et al.*, 2005). This prompt the hypothesis that the promotion of axonal elongation by P2Y1R involves the desinhibition of CRMP2 through the dephosphorylation at Thr514, reflecting the inhibition of GSK3β. Indeed, we could observe that ADPβS (5 μM and 10 μM) that increased the levels of p-GSK3β^{S9}, reduced concomitantly the phosphorylation levels of CRMP2^{T514}, once again an effect no longer observed in the presence of MRS2179 (10 μM). Similarly, we could once again observe a tonic action of P2Y1R, since MRS2179 alone induced an increase in the levels of p-CRMP2^{T514}. These data suggest that P2Y1R-induced inhibition of GSK3β promotes axonal growth through the downstream desinhibition of CRMP2.

1.3 – Purinergic signaling affects neuronal polarity

To further test the effects of endogenous ATP, neurons were cultured in the presence of apyrase (3.6.1.5.), an enzyme that catabolizes ATP and ADP into AMP (as reviewed in Clark and Roux, 2011). Surprisingly, we observed a significant increase in the number of axons per neuron (Figure 4B) and in the percentage of neurons with more than one axon (Fig.4C), which shows for the first time that purinergic signaling is involved in the establishment of neuronal polarity in rat hippocampal neurons.

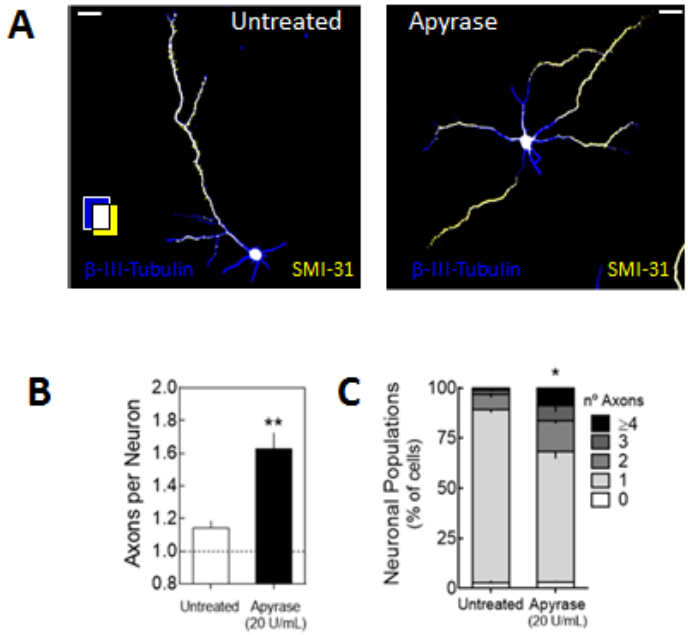

Figure 4. Apyrase induces the formation of secondary axons in rat hippocampal neurons. **(A)** Representative images of rat hippocampal neuron (3 DIV) in untreated conditions and cultured in the presence of apyrase, immunolabeled with and anti-SMI-31 antibody (yellow, axonal marker) and anti-β-III-tubulin (blue, neuronal marker). Co-localization appears as white. Scale bars are 50µm. **(B)** Histogram summarizing the axons per neuron in untreated cultures and in cultures exposed to apyrase. Apyrase induced an increase in the number of axons per neuron and **(C)** an increase in the percentage of cells with more than one axon. The data are expressed as mean ± s.e.m. of the number of axons in **(B)** and of the percentage of neurons with different number of axons in **(C)** quantified from 4 different cultures, analyzing a minimum of 100 cells per condition and culture, taken as single measurements. ***p<0.01 one sample t-test in **(B)** and *p<0.05 two-way ANOVA with Bonferroni's ***post-hoc*** test in **(C).**

1.3.1. A2AR activation induces the formation of secondary axons in rat hippocampal neurons.

The formation of secondary axons induced by apyrase could be due or to a reduced/lack of activation of P2Rs (lower levels of ATP and ADP) or to an increase in the activation of P1Rs, either A1R or A2AR (higher levels of adenosine). The first hypothesis would be feasible since it was shown that GSK3β-CRMP2 pathway is also involved in neuronal polarization/axon specification (Yoshimura *et al.*, 2005). However, neither the selective antagonist of P2Y1R, MRS2179 (10 µM), nor the selective antagonist of P2X7R (BBG 100 nM), nor the generic antagonist of P2R (PPADS 10 µM), mimicked the effect of apyrase (Figure 5). The second hypothesis was that this increase in the number of secondary axons was due to an increased activation of adenosine P1Rs due to the expected higher levels of extracellular adenosine. Indeed, we found that the activation of A2AR with the selective agonist CGS21680 (30 nM), but not of A1R with the selective agonist CPA (100 nM), mimicked the effect of apyrase, increasing also significantly the number of axons per neuron (Figure 6A) and the percentage of neurons with more than one axon (Figure 6B).

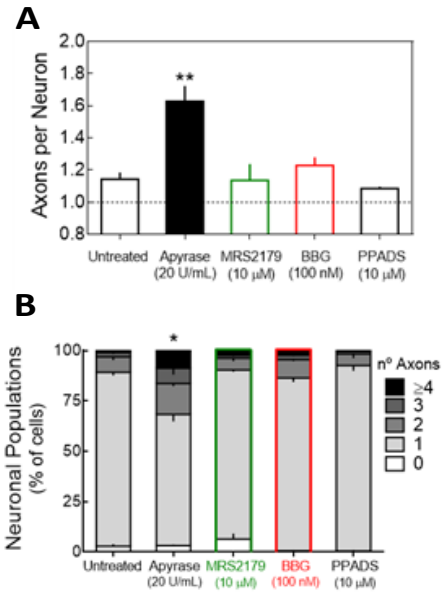

Figure 5. Blockade of P2Rs did not induce the formation of secondary axons. **(A)** Neither the selective antagonist of P2Y1R, MRS2179 (10 μM), nor the selective antagonist of BBG (100 nM), nor a generic antagonist of P2R, PPADS (10μM mimicked neither the increase in the number of axons per neuron **(B)** nor the increase in the percentage of neurons with more than one axon induced by apyrase (20 U/mL). The data are expressed (mean ± s.e.m.) of the number of axons per neurons in (**A**) and the percentage of neurons with different number of axons in (**B**) from 4 different cultures, analyzing a minimum of 100 cells per condition and culture. $^{**}p<0.01$ one-sample *t*-test in (**A**) and $^{*}p<0.05$ two-way ANOVA with Bonferroni's *post-hoc* test

1.3.2 - P1Rs differentially regulate the phosphorylation of CRMP2^{T514}

The observation of a mechanistic convergence in the bidirectional receptor-dependent modulation of axonal growth by P2Rs, involving a differential regulation of GSK3β activity and most likely of CRMP2 phosphorylation, together with the well know role of GSK3β-CRMP2 pathway in axon formation (Yoshimura *et al*., 2005), we decided to test if adenosine P1Rs also modulate the phosphorylation levels of GSK3β^{S9} and CRMP2^{T514}Western Blot analysis showed that the GSK3β-CRMP2 pathway is also targeted by adenosine P1Rs, being differentially regulated by A2ARs and A1R. While A2ARs activation (CGS21680 30 nM)induced an increase in the phosphorylation of GSK3β^{S9} and a reduction in the phosphorylation of CRMP2^{T514}, A1R activation (CPA 100 nM) induced a decrease in the phosphorylation of GSK3β^{S9} and an increase in CRMP2^{T514} (Figure 7). These data show that purinergic signaling is not only capable to modulate axonal outgrowth through the activation of P2Rs (and eventually through P1Rs; see discussion), but is also involved in neuronal polarization by the regulation of axon specification through A2ARs.

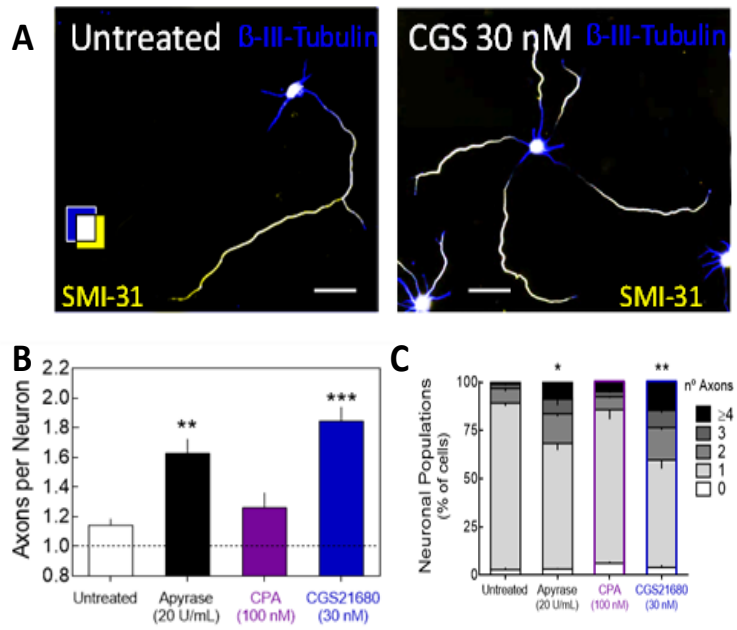

Figure 6. Pharmacological activation of A2ARs induces the formation of secondary axons **(A)** Representative images of rat hippocampal neurons (3 DIV) cultured in the presence of CGS, immunolabeled with an anti-SMI-31 antibody (yellow; axonal marker) and anti-β-III-tubulin (blue; neuronal marker). The selective A2ARs agonist CGS21680 (30 nM), but not the selective agonist of A1R CPA (100 nM), mimicked the effect of apyrase increasing significantly the number of axons per neuron (**B**) and (**C**) the percentage of neurons with more than one axon. The data are expressed as mean ± s.e.m.of the number of axons per neuron in (**B**) and the percentage of neurons with different number of axons in (**C**) quantified from 4 different cultures, analyzing a minimum of 100 cells per condition and culture. $^{**}p<0.01$ and $^{***}p<0.001$ one-sample *t*-test in (**A**) and $^{*}p<0.05$ and $^{**}p<0.01$ two-way ANOVA with Bonferroni's *post-hoc* test

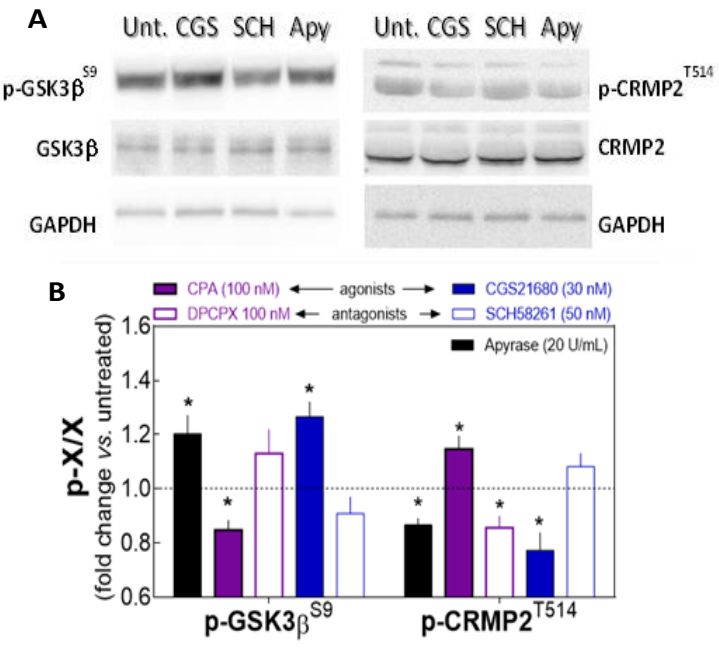

Figure 7. Activation or blockade of A1Rs and A2ARs diferentially regulates the phosphorilation pattern of GSK3β and CRMP2 in rat hippocampal neurons. **(A)** Representative Western Blot obtained from cultured neurons exposed to apyrase (20U/mL), the selective A2AR agonist CGS21680 (30nM) and the selective A2AR antagonist SCH58261 (50nM). Bands showing GSK3β, phosphorylated GSK3β at S9, CRMP2, phosphorylated CRMP2 at T514 all loaded with GPDAH. **(B)** Histogram summarizing the ratios of phosphorylation (p-proteint:total protein) of either GSK3β at Ser9 (p-GSK3bS9) or CRMP2 at Thr514 (p-CRMP2T514). CGS21680, a selective agonist of A2ARs, induced an increase in the phosphorylation of GSK3β at S9 and a decrease in the phosphorylation of CRMP2 at T514. The selective A2AR antagonist, SCH58261, presented no significant effects. The specific A1R agonist significantly decreased the phosphorylation of GSK3β and increased the phosphorylation of CRMP2. The selective A1R antagonist only presented a significant decrease in the phosphorylation of CRMP2. The data are expressed as the fold change (mean ± s.e.m.) relative to untreated cells quantified from 4 different cultures. $^{*}p<0.05$

2 . P2Y1R-CRMP2 signaling module in KA-animal model of temporal lobe epilepsy

It was recently reported an increase in the phosphorylated form of GSK3β^{S9} and an increase of CRMP2 density in the hippocampus of animals injected with pilocarpine (Lee *et al.*, 2012), which can be involved in mossy fiber sprouting (see Wilson *et al*., 2012). We now observed in developing hippocampal neurons that in addition to the increase in the phosphorylation of GSK3β^{S9}, P2Y1R induces a dephosphorylation of CRMP2^{T514}. In addition, as mentioned before, our group could gather several evidence showing that the pharmacological blockade or genetic deletion of P2Y1R prevents neuronal death, astrogliosis and neuroinflammation caused by SE-induction by systemic administration of KA in rats (unpublished results), indicating that P2Y1R is operational during epileptogenesis. Altogether these evidences prompt the hypothesis that P2Y1R-GSK3b-CRMP2 found in developing hippocampal neurons may be reactivated during epileptogenesis and may be inducing/contributing to the abnormal mossy fiber sprouting. To test this hypothesis we first aimed to evaluate if the SE-induced modifications in GSK3β-CRMP2 pathway involved P2Y1R activation. For that purpose, we evaluated if the modifications in the phosphorylation state of either GSK3β^{S9} or CRMP2^{T514} in the hippocampus induced by SE could be prevented with the blockade of P2Y1R. We used the systemic administration of KA to induce SE because we had previously observed a P2Y1R activity in this animal model of TLE. However, Western Blot analysis revealed an unexpected increase in the truncated form of CRMP2 of 55 kDa (Figure 8) and concomitant reduction in CRMP2A (70 kDa) and CRMP2B (62 kDa) levels, known to be a consequence of excitotoxicity (Bretin *et al*. 2006), in the hippocampus 24 h after KA injection, in contrary to the reported in the pilocarpine-model (Lee *et al.*, 2012). This observation impaired the use of the KA-animal model to evaluate the involvement of P2Y1R-GSK3β-CRMP2 in hippocampal mossy fiber sprouting, being the pilocarpine-injection a better model to explore that hypothesis. Nevertheless, we could observe that the *icv* injection of the selective antagonist of P2Y1R, MRS2500 (1 nmol), prior to KA administration prevented the KA-induced cleavage of CRMP2 (Figure 8) showing that P2Y1R is required in excitotoxicity-induced cleavage of CRMP2. This prevention was not mimicked by the injection of the generic P2Rs antagonist, PPADS (1 nmol *icv*), probably due to the action of other P2Rs with opposing effects regarding P2Y1R.

As mentioned before, our group could gather several phenomenological evidences showing a neuroprotective action of the blockade of P2Y1R in KA-induced excitotoxicity (unpublished data). This observation not only constitutes an additional evidence for a crucial role of P2Y1R in excitotoxicity, but also provides a first hint regarding the mechanism underlying it, because it was reported that CRMP2 cleavage is mediated by calpain activity (Bretin *et al*., 2006),which is responsible for SE-induced neuronal death (Araujo *et al*., 2008).

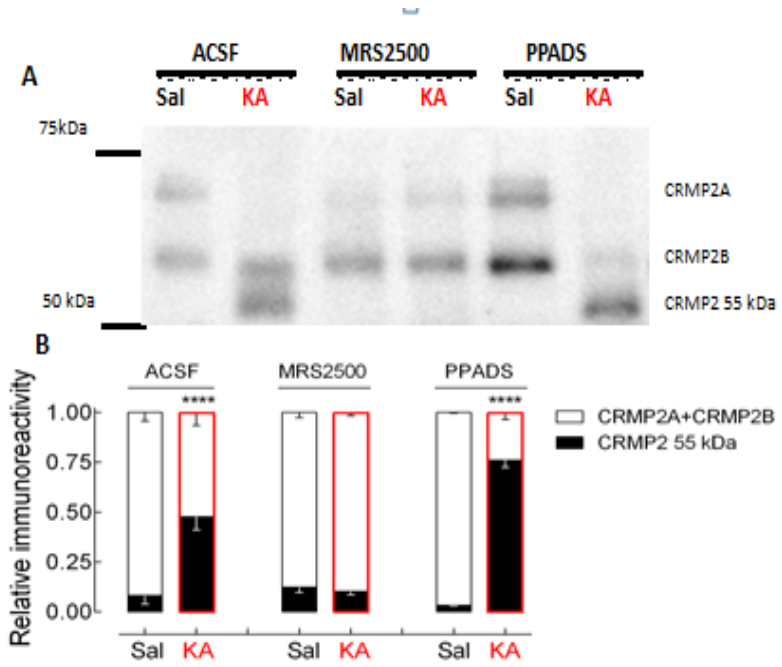

Figure 8. Cleavage of CRMP2 in the rat hippocampus 24 h upon KA-injection requires P2Y1R activation. **(A)** Representative Western Blots of CRMP2 protein in total protein extracts of the hippocampus 24 h upon the *ip* injection of either Saline (0.9% NaCl) or KA (10 mg/kg) together with the *icv* injection either with ACSF, MRS2500 (1 nmol) or PPADS (1 nmol), 15 min before. **(B)** Systemic administration of KA induced the cleavage of CRMP2A and CRMP2B into the truncated form CRMP2 55 kDa, increasing the relative distribution of CRMP2 55 kDa with the concomitant reduction of CRMP2A and CRMP2B. The selective antagonist of P2Y1R, MRS2500, but not the generic P2Rs antagonist PPADS, prevented the cleavage of CRMP2. Data are presented as mean ± s.e.m. of the relative distribution of truncated CRMP2 55 kDa form and CRMP2A+CRMP2B. ****$p<0.0001$, n=4, two-way ANOVA with Bonferroni's *post-hoc* test.

To confirm that P2Y1R is involved in the SE-induced calpain activity, we evaluated if the blockade of P2Y1R was also able to attenuate/prevent the cleavage of spectrin, a well-known calpain substrate (Araujo *et al*. 2005). As previously reported (Araujo *et al*., 2005 and 2008), using an antibody against full-length spectrin that also recognizes spectrin breakdown products (SBDP) (Nath *et al*. 1996), we observed a significant increase in the calpain-dependent 145 kDa SBDP in the hippocampus 24 h after the systemic administration of KA (Figure 9). The pharmacological blockade of P2Y1R attenuated the KA-induced formation of SBDP, similarly to the observed for CRMP2 (see Figure 9C). These results together with the previous data obtained by our group demonstrate that SE-induced neuronal death through calpain activity requires P2Y1R.

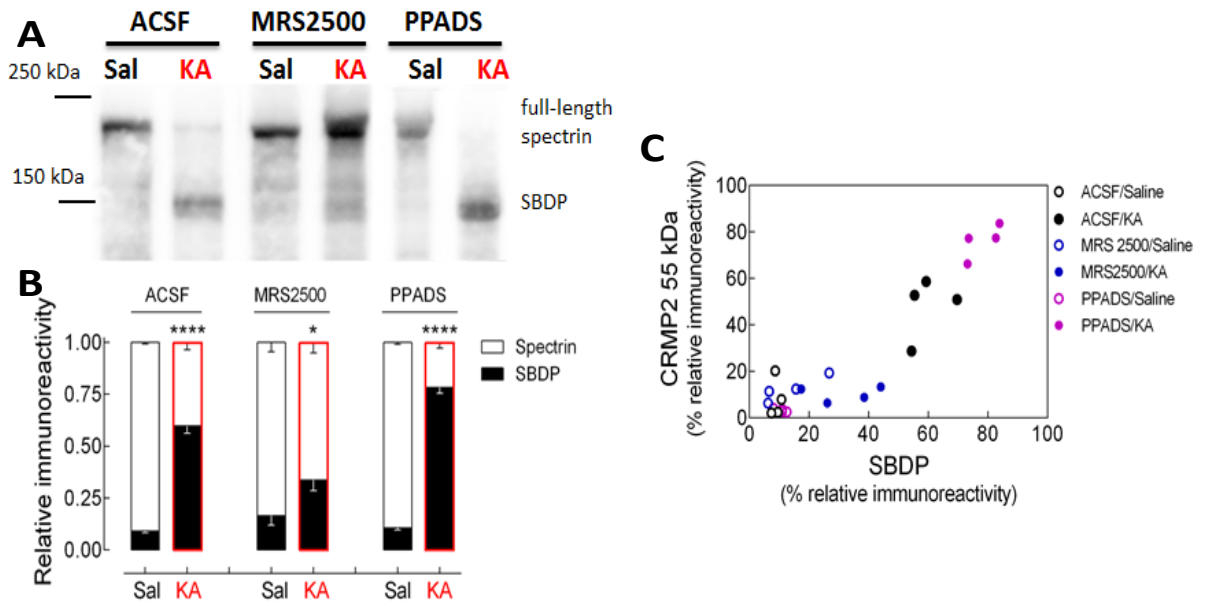

Figure 9. Cleavage of the calpain substrate spectrin involves P2Y1R activation. **(A)** Representative Western blots using an antibody against full-length spectrin also labelling spectrin breakdown products (SBDP) of total protein extracts of the hippocampus 24 h upon the *ip* injection of either Saline (0.9% NaCl) or KA (10 mg/kg) together with the *icv* injection either with ACSF, MRS2500 (1 nmol) or PPADS (1 nmol), 15 min before. **(B)** Systemic administration of KA increased significantly the ratio SBDP/Spectrin, an effect attenuated by the selective antagonist of P2Y1R, MRS2500, but not by the generic P2Rs antagonist PPADS (C) similarly to the observed for CRMP2. .Data are presented as mean ± s.e.m. of the relative distribution of SBDP and full-length spectrin in (**B**). **p*<0.05, *****p*<0.0001 *n*=4, two-way ANOVA with Bonferroni's *post-hoc* test. In (**C**) it is plotted the individual relative distribution obtained in each individual rat of CRMP2 55 kDa *vs.* SBDP.

Discussion

The present study further highlights the mechanisms involved in the P2Y1R-driven promotion of axonal outgrowth in developing hippocampal neurons, also unveiling a new role of purinergic signaling in neuronal development in the regulation of neuronal polarity. Furthermore, it also re-enforces the involvement of P2Y1R in excitotoxicity, particularly showing that P2Y1R is required for SE-induced calpain activity

The bidirectional receptor-dependent modulation of axonal growth in developing hippocampal neurons through facilitatory P2Y1R and inhibitory P2X7R previously reported (del Puerto *et al*. 2012) was reproduced successfully. It was also reported that this modulation involves a differential regulation of GSK3β through the modulation of the phosphorylation at S9. We now provide evidence that P2Y1R not only increases the phosphorylation of $GSK3\beta^{S9}$, but also induces the dephosphorylation of CRMP2, previously shown to be the GSK3β target in the modulation of axonal growth (Yoshimura *et al*., 2005). Hence, these data support the following working hypothesis: P2Y1 receptor activation should promote axonal outgrowth through the dephosphorylation and hence desinhibiton of CRMP2, reflecting the upstream inhibition of GSK3β. Further experiments should be now done to get a functional causal demonstration for this working hypothesis.

In this study we could also find that besides the modulation of axonal outgrowth, purinergic signaling is also capable to modulate neuronal polarity. We observe that the catabolism of ATP and ADP into adenosine by apyrase induces the formation of secondary aberrant axons. This should be due to increased levels of extracellular adenosine since this effect was mimicked by pharmacological activation of A2AR. This data demonstrates for the first time that purinergic signaling has a broader modulatory role in neuronal development, not only modulating axonal growth, being also involved in axon specification, in receptor-dependent manner. Interestingly, this A2AR-induced formation of secondary axons should also converge mechanistically in the modulation of GSK3β-CRMP2, previously shown to be also involved in axon formation (Yoshimura *et al*., 2005), since we observed that pharmacological activation of A2ARs induces similar modifications in the phosphorylation pattern of GSK3β and CRMP2 as observed upon P2Y1R activation. However, P2Y1R activation did not induce the formation of secondary axons. A possible explanation for this apparent discrepancy should rely on a differential subcellular and time-dependent expression of both receptors. While A2ARs should be expressed at the neurites before differentiation, P2Y1R should be only expressed upon the

specification/differentiation of the neurites into axons. It was previously shown that P2Y1R is localized at the axonal tip. We are now attempting to address if there is a differential time- and maturation-dependent expression of both A2AR and P2Y1R.

As mentioned before, the evidences showing that (1)P2Y1R is operational during epileptogenesis, (2) GSK3β-CRMP2 pathway is most likely involved in aberrant axonal sprouting in epileptic phenomena (Wilson *et al*., 2012; Lee *et al.*, 2012) and (3) P2Y1R promotes axonal outgrowth through the inhibition of GSK3β, prompt the hypothesis that P2Y1R may be contributing to hippocampal mossy fiber sprouting through GSK3β-CRMP2 pathway. Indeed we now found that in developing hippocampal neurons that P2Y1R also regulates CRMP2 phosphorylation. To address this hypothesis we first attempted to evaluate if the modifications in the phosphorylation pattern of GSK3β and CRMP2 induced by SE, recently reported using the pilocarpine-animal model (Lee *et al*, 2012), involved P2Y1R. However, the use of KA-animal model revealed not appropriate for that purpose since it induces the cleavage of CRMP2, in contrary to the observed in the pilocarpine-induced SE model. Nevertheless, we could observe that P2Y1R is required in the SE-induction of cleavage of CRMP2, an effect mediated by calpain activity, which further re-enforces the group's previous findings that the blockade of P2Y1R is neuroprotective in excitotoxicity conditions by preventing/attenuating calpain activity.

Regarding the role of P2Y1R-CRMP2 signaling module in mossy fiber sprouting, we are now using the pilocarpine-animal model of TLE. Moreover, it should be noticed that our group could also gather evidences that also the blockade of A2ARs is neuroprotective in SE-induced hippocampal neuronal death, which is also indicative that besides P2Y1Rs, A2ARs are also active during epileptogenesis. Hence, the observation that A2ARs induces the formation of aberrant secondary axons in developing hippocampal neurons, most likely through CRMP2 regulation as well, adds A2ARs as an additional potential target to be involved in mossy fiber sprouting through the modulation of mossy fiber sprouting.

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