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PURINERGIC MODULATION OF NEUROTRANSMITTER TRANSPORTERS IN HUMAN MESIAL TEMPORAL LOBE EPILEPSY (MTLE)

Tese de candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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"*Valeu a pena? Tudo vale a pena/ Se a alma não é pequena*."
(Fernando Pessoa, *In* Mar Português)

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

A-438079, 3-[[5-(2,3-dichlorophenyl)-1*H*-tetrazol-1-yl]methyl]pyridine hydrochloride

AC, Adenylate cyclase

ADA, Adenosine deaminase

ADP, Adenosine diphosphate

AEDs, Antiepileptic drugs

AK, Adenosine kinase

AMP, Adenosine monophosphate

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA, analysis of variance

AOAA, Aminooxyacetic acid

ATP, Adenosine triphosphate

BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)

BCA, Bicinchoninic acid

BDNF, Brain derived neurotrophic factor

BGT1, Betaine-GABA transporter

BSA, Bovine serum albumin

BzATP, 2'(3')-O-(4-Benzoylbenzoyl)ATP

CA, Ammon's horn or *Cornu Ammonis*

Ca^{2+}, Calcium

cAMP, Cyclic AMP

CD11b, Alpha M integrin

CD73, Ecto-5'-nucleotidase

CHP-HGSA, Centro Hospitalar do Porto – Hospital Geral de Santo António

Cl^-, Chloride

CNS, Central nervous system

DAG, Diacylglycerol

DAPI, 4',6-diamidino-2-phenylindole

DG, Dentate gyrus

DIC, Differential interference contrast

DL-TBOA, DL-*threo*-β-benzyloxyaspartic acid

EAATs, Excitatory amino acid transporter

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

E-NPPs, Ectonucleotide pyrophosphatase and/or phosphodiesterases

E-NTPDases, Ectonucleoside triphosphate diphosphohydrolases

EPSC, Excitatory postsynaptic current

GABA, γ-aminobutyric acid

GABAT, GABA transaminase

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

GATs, GABA transporters

GFAP, Glial fibrillary acidic protein

GPCR, G-protein coupled receptors

HEPES, 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid

HGNC, HUGO Gene Nomenclature Committee

IP_3, Inositol 1,4,5-trisphosphate

K_{ir}, Inwardly rectifying potassium channels

KCC2, K^+-Cl^- cotransporter

K_m, Michaelis constant

LDH, Lactate dehydrogenase

LTD, Long-term depression

LTP, Long term potentiation

MTLE, Mesial temporal lobe epilepsy

Na^+, Sodium

NAD^+, Nicotinamide adenine dinucleotide

NKCC1, Na^+-K^+-Cl^- cotransporter

NF200, Neurofilament 200

NMDA, N-methyl-D-aspartate

NMDG, *N*-methyl-D-glucamine

PBS, phosphate-buffered saline

PKC, Protein kinase C

PLC, Phospholipase C

PSD95, postsynaptic density-95

PVDF, polyvinylidene difluoride

RIPA, Radio-Immunoprecipitation Assay Buffer

SE, *Status epilepticus*

SBFI-AM, Bis(acetyloxymethyl)4-[6-[13-[2-[2,4-bis(acetyloxymethoxycarbonyl) phenyl]-5-methoxy-1-benzofuran-6-yl]-1,4,10-trioxa-7,13-diazacyclopentadec-7-yl]-5-methoxy-1-benzofuran-2-yl]benzene-1,3-dicarboxylate

slc1, Solute carrier family

SDS, Sodium dodecyl sulphate

SKF89976A, 1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride)

TTX, Tetrodotoxin

UDP, Uridine diphosphate

UTP, Uridine triphosphate

VAMP-1, Synaptic vesicle-associated membrane protein 1 or synaptobrevin 1

VGLUT, Vesicular glutamate transporters

VIAAT, vesicular inhibitory amino acid transporter

V_{max}, Maximum velocity

ρ, Pearson's coefficient

RESUMO

Embora a epilepsia seja reconhecida há vários séculos como uma das doenças neurológicas mais prevalentes, a sua base celular e molecular não é ainda completamente conhecida. Contudo, acredita-se que a sua causa seja multifatorial e envolva um desequilíbrio entre a neurotransmissão GABAérgica e glutamatérgica. Cerca de 30% dos doentes epiléticos são refratários à terapia medicamentosa e a maioria destes doentes sofre de epilepsia do lobo mesial temporal (MTLE). Apenas uma fração dos doentes resistentes aos fármacos atualmente disponíveis pode ser submetida a cirurgia como tratamento de último recurso. Os restantes doentes permanecem com um limitado recurso ao tratamento médico. Isto realça a necessidade de encontrar novas ferramentas farmacológicas, capazes de controlar as convulsões e/ou o processo epileptogénico.

Os transportadores de elevada afinidade para o GABA e o glutamato, são proteínas chave no controlo da excitabilidade no sistema nervoso central. Considerando o seu papel crucial no controlo dos níveis extracelulares de neurotransmissores, torna-se evidente que a sua atividade deva ser fortemente regulada. O trifosfato de adenosina (ATP) e a adenosina surgem como potenciais candidatos capazes de modular o transporte dos neurotransmissores rápidos, uma vez que os níveis extracelulares de ambos aumentam rapidamente no cérebro durante um estímulo neuronal de elevada frequência ou em situações patológicas, permitindo a coabitação espacial e temporal destas duas purinas com os neurotransmissores, GABA e glutamato. Tendo em consideração trabalhos anteriores que sugerem que o recetor P2X7, sensível ao ATP, é um alvo potencial para o controlo das crises epiléticas e que a ativação deste recetor é capaz de modular os níveis extracelulares dos neurotransmissores aminoacídicos, este estudo focou-se no papel do recetor P2X7 na modulação sincronizada da captação de GABA e glutamato na epilepsia refratária ao tratamento medicamentoso.

Assim, os objetivos deste trabalho visaram avaliar: (1) o papel dos recetores P2X7 no transporte de elevada afinidade de GABA e glutamato em terminais nervosos (sinaptossomas) isolados do cérebro humano (controlos cadavéricos) e de ratazanas Wistar; (2) a via de sinalização subjacente à modulação do transporte de GABA e de glutamato mediada pela ativação do recetor P2X7; e (3) as

alterações causadas pela variação da densidade e localização do recetor P2X7 no transporte de elevada afinidade de GABA e glutamato em indivíduos epiléticos – quer em ratazanas com epilepsia induzida por injecção de pilocarpina, quer em doentes com epilepsia refratária ao tratamento medicamentoso (MTLE e não-MTLE). Além disso, avaliou-se se a densidade e a localização de outros recetores purinérgicos estaria alterada em amostras de cérebro humano de doentes MTLE, em comparação com dadores cadavéricos, a fim de inferir sobre o seu envolvimento na epilepsia. Uma vez que a MTLE afeta o hipocampo e o neocórtex adjacente, estas duas estruturas cerebrais foram incluídas sempre que possível nas análises realizadas neste trabalho.

A acumulação de GABA e de glutamato (marcados radioativamente) foi avaliada por espectrometria de cintilação líquida. A densidade e distribuição dos recetores purinérgicos no tecido cerebral foram avaliadas por *Western blotting* e por imunofluorescência acoplada à microscopia confocal, respetivamente.

Os resultados obtidos mostram que a ativação de recetores P2X7 modula negativamente a captação de GABA e de glutamato por terminais nervosos isolados do neocórtex humano e de ratazanas Wistar. O mecanismo subjacente à modulação negativa da captação desencadeada pela ativação do recetor P2X7 parece ser comum a ambos os neurotransmissores e envolve o colapso parcial do gradiente de Na^+ – a força motriz para a captação de aminoácidos – que é causado pelo influxo de Na^+ através do recetor P2X7. Esta conclusão foi alcançada, uma vez que: (1) o efeito inibitório do agonista do recetor P2X7, BzATP, na captação de aminoácidos foi mimetizado por dois ionóforos de Na^+; (2) o aumento da concentração intracelular de Na^+, bem como a diminuição da concentração extracelular de Na^+, diminuíram a captação de ambos os neurotransmissores, como previsto pelas alterações nos potenciais de reversão dos transportadores; (3) o efeito inibitório do BzATP foi atenuado quando o gradiente transmembranar de Na^+ foi diminuído; (4) a ativação do recetor P2X7 aumentou significativamente os níveis de intracelulares de Na^+; e (5) o bloqueio seletivo da entrada de Na^+ através do recetor P2X7 impediu o efeito inibitório de BzATP na captação de glutamato e de GABA. Além disso, os resultados obtidos em sinaptossomas de córtex de ratazana mostram que, paralelamente à diminuição da captação de GABA e de glutamato quando o Ca^{2+} extracelular se encontra diminuído, a ativação do recetor P2X7 também promove a libertação de ambos os neurotransmissores, embora a

magnitude e o mecanismo subjacente à libertação de GABA e de glutamato sejam significativamente diferentes: a ativação do recetor P2X7 promove a libertação de pequenas quantidades de GABA através da reversão do transportador GAT1, enquanto o glutamato é libertado em maiores quantidades através do próprio recetor P2X7.

Contrariamente às nossas expectativas, não foram encontradas diferenças na densidade nem na função do recetor P2X7 em terminais nervosos isolados do córtex de ratazanas epiléticas comparativamente com os animais controlo. Estes resultados contrastam com os resultados obtidos em terminais nervosos isolados do neocórtex cerebral de doentes epiléticos refratários à medicação, nos quais o recetor P2X7 se encontra aumentado, estando este aumento correlacionado com uma maior potência do BzATP (agonista dos recetores P2X7) para inibir a captação de GABA, mas não a captação de glutamato.

Dado que a atividade epilética também origina grandes quantidades de adenosina e que os recetores A_{2A} da adenosina têm sido implicados tanto no controlo dos níveis sinápticos de GABA e glutamato como na epilepsia, foi avaliado, pela primeira vez, a densidade e localização destes recetores em amostras de cérebro humano de doentes epiléticos refratários à medicação. Contrariamente à localização pré-sináptica dos receptores P2X7, os resultados obtidos mostraram que os recetores A_{2A} são principalmente expressos em astrócitos, estando a sua densidade aumentada de forma significativa em amostras do cérebro de doentes MTLE. É muito provável que a modulação negativa da captação de glutamato promovida pelo colapso do gradiente de Na^+ transmembranar após a ativação do recetor A_{2A} astrocítico, tal como descrito na literatura, seja bastante semelhante à descrita neste estudo para a modulação da captação de glutamato e GABA devida à ativação do recetor P2X7, e que ambas possam estar aumentadas em doentes epiléticos.

No seu conjunto, os resultados deste trabalho sugerem que a ativação transitória de recetores P2X7 e A_{2A} – motivada pelo aumento da quantidade extracelular de ATP libertado aquando de um estímulo nervoso de elevada frequência e o subsequente catabolismo extracelular deste nucleótido em adenosina – poderá facilitar não só a neurotransmissão glutamatérgica, mas também, a neurotransmissão GABAérgica cuja atividade promove a neuro-inibição tónica após estímulo intenso. Embora esta modulação fina pareça ser

fisiologicamente relevante para a formação da memória e a aprendizagem através da potenciação da neurotransmissão, a sobre expressão dos recetores P2X7 e A_{2A} no cérebro epilético (respetivamente nos terminais nervosos e astrócitos) poderá ser perigosa e fomentar as crises epiléticas. Isto pode ser explicado pelo facto do aumento da densidade dos recetores P2X7 e A_{2A} promover, para além da sustentação da sinalização glutamatérgica excitatória, a persistência da neurotransmissão GABAérgica, devido à diminuição da captação deste neurotransmissor, que poderá culminar no *rundown* GABAérgico, um processo através do qual o efeito inibitório do GABA é convertido num efeito pró-convulsivo promovendo assim a hiperexcitabilidade neuronal, em vez da neuro-inibição.

Em suma, este estudo vem preencher uma lacuna na compreensão do cérebro epilético humano, em relação ao papel pro-epileptogénico do ATP e da adenosina em condições em que os níveis sinápticos destas purinas aumentam rapidamente, como por exemplo durante estímulos de elevada frequência. Assim sendo, o uso de antagonistas seletivos dos recetores P2X7 neuronais e dos recetores A_{2A} nos astrócitos poderá constituir uma alternativa terapêutica nova e valiosa para controlar a epilepsia refratária. Para além do contexto da epilepsia, existe um aumento significativo de resultados que indiciam o envolvimento de ambos os recetores, P2X7 e A_{2A}, na fisiopatologia de vários distúrbios neurológicos, nos quais o antagonismo destes dois subtipos de recetores purinérgicos poderá também apresentar benefícios clínicos através do controlo dos "sinais perigosos" provenientes da sua disfunção.

ABSTRACT

Although epilepsy is recognized for centuries as one of the most prevalent neurological disorders, its cellular and molecular basis is still largely unknown. It is, however, believed that the cause is multifactorial and involves an unbalance between GABAergic and glutamatergic neurotransmission. About 30% of epilepsy patients remain drug-refractory and the majority of them suffer from Mesial Temporal Lobe Epilepsy (MTLE). Only a fraction of these patients can be submitted to surgery as a last resource treatment, leaving remaining patients with an unmet medical need. This highlights the need to search for new pharmacological tools controlling seizures and/or epileptogenesis.

High-affinity transporters of fast neurotransmitters, like γ-aminobutyric acid (GABA) and glutamate, are key proteins in the control of excitability in the central nervous system (CNS). Given their major role in the control of neurotransmitters levels in the extracellular space, it became clear that these transporters must be highly regulated. Adenosine triphosphate (ATP) and adenosine emerge as potential candidates to modulate neurotransmitters transport since their extracellular levels rapidly increase in the brain during high-frequency neuronal firing or under pathological conditions allowing the spatial and temporal cohabitation of these two purines with GABA and glutamate neurotransmitters. Taking into consideration previous findings suggesting that the ATP-sensitive P2X7 receptor, may be a potential target to control seizures and that this receptor is able to modulate the extracellular levels of amino acid neurotransmitters, we focus our attention on the study of the role of the P2X7 receptor on synchronized modulation of GABA and glutamate uptake in drug-resistant epilepsy.

Therefore, the goals of this work were to evaluate: (1) the role of the ATP-sensitive P2X7 receptor on high-affinity transport of GABA and glutamate into nerve terminals (synaptosomes) isolated from brain samples from Wistar rats and human cadaveric controls; (2) the signaling pathway underlying the P2X7 receptor-mediated modulation of GABA and glutamate transport; (3) the changes in the expression and cellular localization of the P2X7 receptor, as well as the P2X7 receptor-mediated modulation of high-affinity transport of amino acid, in epileptic individuals – both rats injected with pilocarpine and human drug-refractory epileptic

patients (MTLE and non-MTLE). In addition, we also evaluated if the expression and the localization of other purinoceptors were altered in human brain samples from MTLE patients as compared with cadaveric controls in order to infer about their involvement in epilepsy. Since the MTLE disorder affects the hippocampus and adjacent neocortex, these two regions were included whenever possible in the analysis performed in this study.

Radiolabeled GABA and glutamate accumulation was evaluated by liquid scintillation spectrometry. Brain tissue density and distribution of purinoceptors were assessed by Western blot analysis and immunofluorescence confocal microscopy, respectively.

Results clearly show that activation of the pre-synaptic P2X7 receptor downregulates [^{3}H]GABA and [^{14}C]glutamate uptake by nerve terminals of the neocortex of human and Wistar rats. The mechanism underlying downmodulation of the uptake of amino acids triggered by activation of the P2X7 receptor seems to be common for both neurotransmitters and it involves the partial collapse of the Na^+ driving-force for neurotransmitters uptake caused by the influx of Na^+ through the P2X7 receptor channel. This conclusion was achieved, since: 1) the inhibitory effect of the P2X7 receptor agonist, 2'(3')-O-(4-Benzoylbenzoyl)ATP (BzATP), on amino acids uptake was mimicked by two Na^+ ionophores; (2) the increase in intracellular Na^+ concentration, as well as the decrease in extracellular Na^+ concentration downmodulated the uptake of both neurotransmitters, as predicted by the changes in transporter reversal potentials; (3) the inhibitory effect of BzATP was attenuated when transmembrane Na^+ gradient was decreased; (4) the activation of the P2X7 receptor significantly increased intrasynaptosomal Na^+ levels; and (5) the selective blockade of Na^+ entry through the P2X7 receptor prevented the inhibitory effect of BzATP on GABA and glutamate uptake. Additionally, obtained data using rat cortical synaptosomes show that besides decreasing GABA and glutamate uptake under low Ca^{2+} conditions, activation of the P2X7 receptor also favors the release of both neurotransmitters, although the magnitude and the mechanisms underlying GABA and glutamate release differ significantly: P2X7 receptor activation causes the outflow of small amounts of GABA through reversal of GAT1 transporter, while glutamate is released in higher quantities through the P2X7 receptor pore.

Contrary to our expectations, we found no differences in the P2X7 receptor expression and function on rat cortical nerve terminals of pilocarpine-induced

epileptic rats, compared to their control littermates. These findings contrast with our data using nerve terminals isolated from human cerebral neocortices obtained from drug-resistant epileptic patients, where the P2X7 receptor was upregulated and the increase in the P2X7 receptor density correlates with a higher potency of the P2X7 receptor agonist, BzATP, concerning inhibition of GABA, but not glutamate uptake by nerve terminals of the neocortex of MTLE patients.

Since epileptic activity also produce large amounts of adenosine and that adenosine A_{2A} receptors have been implicated both in controlling synaptic levels of GABA and glutamate and in epilepsy, we assessed, for the first time, the density and localization of A_{2A} receptors in human brain samples of drug-resistant epileptic patients. In contrast to the pre-synaptic localization of the P2X7 receptor, data from this study show that A_{2A} receptors are mainly expressed in astrocytes being its density significantly increased in brain samples from MTLE patients. It is highly possible that the downmodulation of glutamate uptake promoted by the partial collapse of the transmembrane Na^+ gradient described in the literature is owed to a surplus in astrocytic A_{2A} receptor activation and may be enhanced in epileptic patients. This is quite similar to what we have found in the modulation of GABA and glutamate uptake by the activation of the P2X7 receptor.

All together, results from this study suggest that transient activation of P2X7 and A_{2A} receptors by massive quantities of ATP released under high-frequency nerve firing and its subsequent extracellular catabolism into adenosine, may facilitate the glutamatergic neurotransmission while promoting the endurance of GABAergic neurotransmission ensuring, therefore, tonic neuro-inhibition following an intense period of stimulation. While this fine-tuning modulation may be physiologically relevant under conditions of memory formation and learning by potentiating neurotransmission, the overexpression of P2X7 and A_{2A} purinoceptors (respectively in nerve terminals and astrocytes) in the epileptic brain may be dangerous and foment epileptic discharges. This may be explained by the fact that the increase in the levels of P2X7 and A_{2A} receptors promotes, besides the endurance of excitatory glutamatergic signaling, the persistence of GABA-mediated neurotransmission, due to a decrease in GABA uptake, that may result in GABAergic rundown, a process by which GABAergic inhibition is converted into a pro-convulsive action by promoting neuronal hyperexcitability, instead of inhibition.

In summary, this study fills a gap in the understanding of the human epileptic brain, regarding the pro-epileptic role of both ATP and adenosine in the extracellular milieu, in conditions where the synaptic levels of these purines rapidly increase, such as during intense neuronal discharges. Taking this into account, pharmacological targeting of neuronal P2X7 and astrocytic A_{2A} receptors with selective antagonists may constitute a novel and valuable therapeutic alternative to control drug-resistant epilepsy in humans. Outside the epilepsy context, there is an explosion of data indicating that both P2X7 and A_{2A} receptors are involved in the pathophysiology of several neurological syndromes, where the blockage of both purinoceptors may also present clinical benefits to curb “dangerous signals” emanating from an underlying dysfunction of these two purinoceptors.

CHAPTER 1: INTRODUCTION

Epilepsy: an old disorder still unsolved

Epilepsy was first described by Hippocrates in the 5th century BC. It is estimated that over 50 million people around the world suffer from epilepsy nowadays. This makes this disease one of the world's oldest recognized disorders, the second most common neurological disease after stroke, and a major burden for public health systems (Madsen et al., 2010; Pitkänen and Lukasiuk, 2011).

Epilepsy is a chronic and heterogeneous neurological disorder that is characterized by the propensity of the brain to generate spontaneous recurrent seizures having severe impact on patients' quality of life due to devastating behavioral, social and occupational consequences. Epileptic seizures arise from an excessively synchronous and sustained discharge of a group of neurons and reflects periods of enhanced excitability (Engelborghs et al., 2000). Its clinical presentation depends on the brain region in which the seizures start and spread, and can vary from light and nearly undetectable symptoms to loss of consciousness and severe convulsions culminating in *status epilepticus* (SE), a state of continuous seizures that is considered the most extreme form of seizure (Trinka et al., 2015).

Regarding its etiology, epilepsy can be classified into three groups: structural/metabolic, genetic and unknown (Berg and Scheffer, 2011). In the structural/metabolic epilepsy, there is a distinct structural or metabolic condition of disease that has been demonstrated to be associated with a substantially increased risk of developing epilepsy. When of genetic origin, there is a separate disorder interposed between the gene defect and the epilepsy. The genetic epilepsy is, as best as understood, the direct result of a known or presumed genetic defect(s) in which seizures are the core symptom of the disorder. In the unknown epilepsy, as the name suggests, the nature of the underlying cause is unidentified. It is also called idiopathic epilepsy.

Although epilepsy has been (and continue to be) one of the most studied neurological disorders, its cellular and molecular basis are still largely unknown (O'Dell et al., 2012; Pavlov et al., 2013) and there is still no cure for this disorder. Nevertheless, it is believed that the cause of epilepsy is multifactorial and involves several mechanisms, namely an imbalance between glutamatergic and GABAergic neurotransmission, changes in function and/or composition of the ionotropic receptors – which promote alterations of ionic gradients of Na^+, Ca^{2+}, Cl^- or K^+ – or

deficiency in endogenous neurotransmitters and neuromodulators (Engelborghs et al., 2000; Miles et al., 2012; Lerche et al., 2013; Pavlov et al., 2013; Waszkielewicz et al., 2013). The enormous complexity of this neurological disorder makes it impossible to fully understand the mechanisms underlying epileptogenesis and seizure generation only through clinical evaluation of human patients. Therefore the researchers developed rodent animal models in which the induced pathology resembles some aspects of the human pathology. There are several animal models of epilepsy currently being used, which are induced by neurochemical agents (e.g. pilocarpine and kainic acid), electrical stimulation protocols (e.g. electroshock-induced seizures, afterdischarges and kindling), hyperthermal or hypoxic insults, traumatic injuries, optogenetics and rodent strains with idiopathic or audiogenic-induced seizures (Curia et al., 2008; Pitkänen et al., 2011; O'Dell et al., 2012; Kandratavicius et al., 2014). The diversity of animal models to study epilepsy reflects the existence of not only one type of epilepsy, but the existence of different forms of epilepsy or epileptic syndromes. Therefore, the use of an appropriate animal model is essential when studying a particular form of epilepsy. One must, however, bear in mind that resemblance of any animal model with human pathology is associated with many limitations (Kandratavicius et al., 2014), namely, by (1) the anatomical and functional differences between species, (2) the chronicity of the disorder – the evolution of epilepsy in humans occurs in several years whereas in animal models occurs in only a few weeks – and (3) the origin and neuronal network adaptation to the initial cause of the disease.

Most of the antiepileptic drugs (AEDs) currently used as primary therapy for epilepsy were discovered by screening, without a rationale regarding to the mechanism of action (Engelborghs et al., 2000). The posterior finding of the mechanism of action of such drugs clarified that most of AEDs exert their antiepileptic properties through only a few neurochemical mechanisms. Basically, most AEDs decrease membrane excitability through the interaction with ionic channel conductance – mainly Na^+ (*e.g.* phenytoin and carbamazepin), K^+ (*e.g.* retigabine) and Ca^{2+} (*e.g.* ethosuximide) channels – or with neurotransmitters receptors – essentially by the promotion of inhibitory neurotransmission (e.g. barbiturates and benzodiazepines) or by the inhibition of excitatory neurotransmission (e.g. lamotrigine and felbamate) (Engelborghs et al., 2000; Beck and Yaari, 2012; O'Dell et al., 2012). Some of these AEDs, such as valproate, exert

their action by acting on two or more different targets. In addition to the antiepileptic properties, a few novel AEDs, such as levetiracetam – which may affect exocytosis upon binding selectively to synaptic vesicle protein SV2A (Lynch et al., 2004; Yan et al., 2005; Russo et al., 2010) –, seem to have also some effects in the prevention or modification of epileptogenesis (Yan et al., 2005; Russo et al., 2010; Löscher, 2012). Recently, some authors have emphasized neuronal cell transplantation as an alternative therapy for restraining spontaneous recurrent seizures in drug-resistant epileptic individuals due to their ability for giving rise to neurons synthesizing the inhibitory neurotransmitter GABA, thereby recovering the neuronal loss and the adverse effects resulting therefrom (Shetty, 2012).

Despite the intense research of the last decades, few new AEDs were approved to clinical practice and only 50% of the patients are adequately treated for their symptoms with the currently available AEDs (Madsen et al., 2010). Of the remaining patients, 20% are treated at expense of severe side effects, while about 30% of patients are refractory to medication continuing to have spontaneous seizures, even when treated with three or more different drugs (Beck and Yaari, 2012). Currently, in these refractory epilepsies the most promising treatment is the complete surgical removal of ‘‘the minimum amount of tissue that must be resected to produce seizure freedom’’, also known as epileptogenic zone, or the complete disconnection of this zone (Rosenow and Lüders, 2001). To define the location and boundaries of the epileptogenic zone, epileptologists use various diagnostic tools, such as electrophysiological recordings, analysis of seizure semiology, functional testing and neuroimaging techniques. However, only a subset of patients, in which the seizures are generated over a well-localized area that can be removed safely, are able to profit from this type of treatment, leaving the remaining patients with an unmet medical need. In this context, the research of new potential pharmacological targets able to control seizures is an urgent need. In addition to controlling seizures, the identification of a therapy that could prevent or modify epileptogenesis would represent a major advance over the majority of current treatment options.

MTLE as an unmet medical need

MTLE is the most common and devastating form of human intractable epilepsy. This chronic disease is characterized by a dynamic process that progressively alters neuronal excitability, establishes critical interconnections and

develops structural and biochemical changes (Henshall et al., 2013) that affect the hippocampus (O'Dell et al., 2012) – being MTLE frequently associated with hippocampal sclerosis characterized by neuronal loss of the Ammon's horn or *Cornu Ammonis* (CA; hence the subdivisions CA1 through CA4) and concomitant astrogliosis (Cendes et al., 2014; Thom, 2014) – as well as the affection of several neocortical regions (Doherty et al., 2003; Bartolomei et al., 2005; Scanlon et al., 2011; Alhusaini et al., 2012; Biagini et al., 2013; Di Maio, 2014; Kandratavicius et al., 2014). These pathological features often acquired over decades of disease limit the possibility of successful therapeutic approaches, often rendering the disease refractory and increasing the incapacitating nature of this specific epileptic disorder (Di Maio, 2014).

The disease is normally initiated by a traumatic event, also defined as primary insult – including febrile seizures, SE, trauma or infection – after which the disease remains invisible during 5–15 years. This period is characterized by freedom of symptoms or complications and it is called latency period (Sharma et al., 2007; O'Dell et al., 2012; Di Maio, 2014). After this period, the patient begins to suffer from spontaneous seizures, which at the onset are controllable with medication (silent period; O'Dell et al., 2012). As the disease progresses, patients commonly develop intractable symptoms that cannot be managed with the available AEDs. At this time and when the epileptogenic zone is conveniently detected, amygdalo-hypocampectomy surgery appears as the only available treatment of last resource.

The irreversible structural and biochemical changes are thought to occur during the latent period. However, several authors propose that these changes continue to accumulate with each new insult over the course of the disease (Yang et al., 2010). These changes can include neurodegeneration, neurogenesis, astroglial scar and astrogliosis, aberrant sprouting of mossy fiber or granule cell axon, dendritic plasticity, blood-brain barrier damage, recruitment of inflammatory cells into brain tissue, reorganization of the extracellular matrix, and reorganization of the molecular architecture of individual brain cells (de Lanerolle et al., 2003; Sharma et al., 2007; Bae et al., 2010; Yang et al., 2010; Pitkänen and Lukasiuk, 2011; O'Dell et al., 2012).

GABA and glutamate neurotransmission in CNS

Glutamatergic neurotransmission: an overview

Although glutamate effects in mammalian CNS were known for more than 75 years, it was only in 1984 that this non-essential amino acid was really acknowledged as fulfilling the criteria of a neurotransmitter (reviewed by Niciu et al., 2012). Currently it is known that glutamate is the main excitatory neurotransmitter in the CNS, playing an important role in many essential brain functions including cognition, memory and learning. Although the brain contains huge amounts of glutamate, normally only a small fraction of this neurotransmitter is present in the extracellular space (3-10 μM) (Danbolt, 2001). This high concentration gradient of glutamate across the plasma membrane is due to the fact that elevated concentrations of extracellular glutamate are toxic to neurons and may promote neuronal death in a process now referred as “excitotoxicity” (Niciu et al., 2012; Willard and Koochekpour, 2013).

Glutamate cannot permeate the blood-brain barrier and, therefore, this amino acid must be synthesized from local precursors, such as glucose or glutamine. Neurons cannot perform the synthesis of glutamate from glucose, since these cells lack both the glutamine synthesizing and the pyruvate carboxylase enzymes (Rowley et al., 2012). Astrocytes, on the other hand, express both of these enzymes and are therefore essential for maintaining a supply of glutamine to neurons (Kersanté et al., 2013). Glutamine produced in glial cells is then transported to nerve terminals, where it is metabolized to glutamate, a process known as the glutamate-glutamine cycle (Danbolt, 2001).

Once synthesized in nerve terminals, glutamate is actively transported into synaptic vesicles by vesicular glutamate transporters (VGLUT) and then it is released by exocytosis of synaptic vesicles, in a Ca^{2+}-dependent manner. Although most of the focus has been on synaptic release of glutamate from nerve terminals by exocytosis, this is not the only mechanism responsible to supply the extracellular fluid with glutamate (Danbolt, 2001; Zhou and Danbolt, 2014). In fact, there are several non-vesicular and non-exocytotic mechanisms through which glutamate can be released to extracellular space, namely through: glutamate-cystine antiporter (xCT; slc7a11), anion channels, reversal of high-affinity glutamate transporters, gap junctions (connexins and pannexins) and ATP-sensitive P2X7 receptors (Danbolt,

2001; Marcoli et al., 2008; Niciu et al., 2012; Zhou and Danbolt, 2014; Orellana et al., 2015). In addition, growing evidences show that brain astrocytes also contribute to the supply of extracellular glutamate, being the mechanisms of release similar to those found in neurons (Malarkey and Parpura, 2008; Orellana et al., 2011; Zhou and Danbolt, 2014).

The released glutamate exerts its signaling by acting on ionotropic and metabotropic receptors which are located on the surface of cells expressing them (Willard and Koochekpour, 2013; Martinez-Lozada and Ortega, 2015). It is interesting to note that most cells in the CNS express at least one type of glutamate receptor. Ionotropic glutamate receptors can be subdivided into three groups of receptors, according to their selective agonist: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate. All of these receptors are ligand-gated non-selective cation channel that induce a rapid excitatory postsynaptic current (EPSC), being NMDA family also gated by voltage. In contrast to ionotropic receptors, the responses mediated by metabotropic glutamate receptors are slower, since these receptors are G-protein coupled receptors (GPCR). Based on sequence homology, G-protein coupled and pharmacology, metabotropic glutamate receptors can be categorized in three groups. The receptors of group I (mGluR1 and mGluR5) are generally expressed on postsynaptic cells and are coupled to G_q protein promoting neuronal excitation through the activation of phospholipase C (PLC) and consequent release of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The receptors of group II (mGluR 2 and mGluR 3) and group III (mGluR 4 and mGluR 6-8) are mostly expressed on presynaptic cells and are coupled to a G_i protein promoting neuronal inhibition through the inhibition of adenylate cyclase (AC) and consequent decrease of cyclic AMP (cAMP) levels (Willard and Koochekpour, 2013). In general, it appears that both families of glutamate receptors – ionotropic and metabotropic – play crucial roles in the synaptic plasticity, a term used to describe changes in synaptic strength and efficacy either due to physiological alterations in neuronal activity (as in memory and learning) as well as due to pathological disturbances (Niciu et al., 2012): a long-lasting increase in synaptic strength is known as long-term potentiation (LTP), while its counterpart is termed long-term depression (LTD). Several glutamate receptors have been largely implicated in several neurological disorders, such as Schizophrenia, Alzheimer's disease, Parkinson's disease, epilepsia and anxiety

(Willard and Koochekpour, 2013; Pitsikas, 2014; Crépel and Mulle, 2015; Nicoletti et al., 2015).

After dissociation from the receptor, glutamate is transported back into the presynaptic nerve terminal or into surrounding astrocytes via high-affinity glutamate transporters (see section Neurotransmitter transporters) thereby terminating glutamate's excitatory action. Other mechanisms such as glutamate diffusion and binding to transporters also contributes to the control the extracellular concentration of glutamate (Danbolt, 2001; Fontana, 2015).

GABAergic neurotransmission: an overview

GABA is the main inhibitory neurotransmitter in the mammalian CNS, playing a fundamental role in the control of neuronal excitability, neuronal plasticity and network synchronization in tandem with glutamate (see previous section). The synthesis of GABA occurs by decarboxylation of glutamate through the enzyme glutamate decarboxylase, which is almost exclusively expressed in GABAergic neurons (Schousboe et al., 2014). The fact that GABA synthesis is closely connected with glutamate synthesis makes it very difficult to affect the synthesis of one neurotransmitter without affecting the synthesis of the other.

After synthesis, GABA is transported into synaptic vesicles via a vesicular inhibitory amino acid transporter (VIAAT) and it is released in response to a specific stimulus through a Ca^{2+}-dependent mechanism or by other Ca^{2+}-independent pathways, namely by the reversal of the Na^{+}-coupled high-affinity GABA transporters or through ATP-sensitive P2X7 receptor (Wang et al., 2002; Richerson and Wu, 2003; Papp et al., 2004; Wu et al., 2007; Lee et al., 2011).

Released GABA exerts its function through the activation of widely distributed ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors. $GABA_B$ receptors are coupled to $G_{i/0}$ protein and mediate slow and prolonged inhibitory transmission. Presynaptic $GABA_B$ receptors inhibit the release of GABA ($GABA_B$ auto-receptors) and other neurotransmitters ($GABA_B$ hetero-receptors), such as glutamate and noradrenaline, via blockage of Ca^{2+} channels and second messenger-mediated effects downstream of Ca^{2+} entry, while postsynaptic $GABA_B$ receptors diminish excitability by the activation of inwardly rectifying potassium (K_{ir}) channels (Kumar et al., 2013; Wu and Sun, 2015). Ionotropic GABA receptors are

ligand-gated receptors that mediate the hyperpolarization of the cell through Cl^- influx (Madsen et al., 2010).

The most important inhibitory receptor in the CNS is the ionotropic $GABA_A$ receptor (Sigel and Steinmann, 2012). This receptor is permeable to Cl^- and HCO_3^- in a proportion of 5:1 and it is localized at the synaptic level, as well as, outside the synapse, being in this case referred to as perisynaptic or as extrasynaptic (Kaila et al., 1993; Miles et al., 2012; Sigel and Steinmann, 2012; Braat and Kooy, 2015; Schipper et al., 2015). Synaptically located $GABA_A$ receptors have low affinity for GABA and mediate fast inhibitory transmission (also referred as phasic or transient inhibition) in response to high concentrations (low micromolar) of intermittently released GABA, whereas extrasynaptic $GABA_A$ receptors display high-affinity for GABA, sensing low ambient GABA concentrations in the extracellular space in order to generate a form of tonic (persistent) inhibition (Brickley and Mody, 2012; Sigel and Steinmann, 2012; Braat and Kooy, 2015). Extrasynaptic $GABA_A$ receptors show no, or very little, desensitization in contrast to their synaptic counterparts (Madsen et al., 2010). Thus, tonic inhibitory currents are due to GABA that is not captured synaptically and produce a slow temporal inhibition by persistent activation of extrasynaptic $GABA_A$ receptors. Regarding its structure, $GABA_A$ receptor consists of heteromeric pentamers made up of various combinations of different subunits – α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ and ρ_{1-3} – resulting in a complex heterogeneity of receptors with distinct physiological and pharmacological profiles (Braat and Kooy, 2015). It is believed that specific subunits are characteristic of extrasynaptic $GABA_A$ receptors, while other subunits are expressed only in synaptic $GABA_A$ receptors. For instance, synaptic $GABA_A$ receptors contain an α_{1-3}, β_n and γ_2 subunits – being γ_2 critical for the maintenance of receptors at the synapse – and extrasynaptic $GABA_A$ receptors contain α_{4-6} and δ subunit (Semyanov et al., 2004; Palma et al., 2007; Braat and Kooy, 2015).

After dissociation from the receptor, and similarly to glutamate, GABA is transported back into presynaptic nerve endings or to astrocytes via Na^+-coupled high-affinity GABA transporters (see section Neurotransmitter transporters) or it is diffused, thus terminating GABA's action. Most of the GABA taken up into nerve terminals is reutilized as a neurotransmitter, after its incorporation into synaptic vesicles. A small amount of neuronal GABA, as well GABA taken up by astrocytes is metabolized into succinate, through the concerted action of GABA transaminase

(GABAT) and succinic semialdehyde dehydrogenase (Rowley et al., 2012; Schousboe et al., 2014).

As previously said, GABA is the main inhibitory neurotransmitter. However, this only seems to be true in mature tissue (adult), since different studies have shown that, during the early development, GABA acting on $GABA_A$ receptors is depolarizing and often excitatory (Köhling, 2002; Ben-Ari et al., 2012; Ben-Ari, 2014; Wu and Sun, 2015). The "polarity" of GABA action (Figure 1) seems to be dependent on the intracellular Cl^- concentration (Ben-Ari et al., 2012) and has been associated with two major cation-chloride transporters that regulates the intracellular Cl^- concentration: the inwardly directed mediated by Na^+-K^+-Cl^- cotransporter (NKCC1) – largely responsible for the high intracellular Cl^- concentration – and the Cl^- extruding K^+-Cl^- cotransporter (KCC2). The expression of NKCC1 is high in the developing and postnatal brain, while the expression of KCC2 is low, thus promoting high intracellular Cl^- concentration at this period (Blaesse et al., 2009; Ben-Ari, 2014). Under these circumstances, the activation of $GABA_A$ receptors promotes Cl^- efflux (Figure 1A) causing membrane depolarization and the excitatory GABA effect (Sigel and Steinmann, 2012; Ben-Ari, 2014; Braat and Kooy, 2015; Wu and Sun, 2015). The "GABA effect shift", *i.e.*, the change in the GABA effect from depolarizing in developing brain to hyperpolarizing in the adult brain (Figure 1) is due to a decline in intracellular Cl^- concentration mediated by increased expression of KCC2 in a developmentally time-dependent manner (Wu and Sun, 2015). In these conditions of low intracellular Cl^- concentration, the activation o $GABA_A$ receptors promotes Cl^- influx (Figure 1B) triggering membrane hyperpolarization and the conventional inhibitory GABA effect (Ben-Ari, 2014; Braat and Kooy, 2015; Wu and Sun, 2015). Moreover, some authors have proposed that the "GABA shift" may even occur in adult tissue, being involved in several neurological disorders characterized by neuronal excitability, such as epilepsy (Cohen et al., 2002; Köhling, 2002; Huberfeld et al., 2007; Blaesse et al., 2009; Lagostena et al., 2010; Viitanen et al., 2010; Miles et al., 2012; Pavlov et al., 2013). In this case, the "GABA shift" has been largely associated with a decrease in the expression of KCC2 and with an increase in the expression of NKCC1 (Miles et al., 2012; Ben-Ari et al., 2014),(Miles et al., 2012) leading to an effect of GABA that may easily switch from hyperpolarizing into depolarizing.

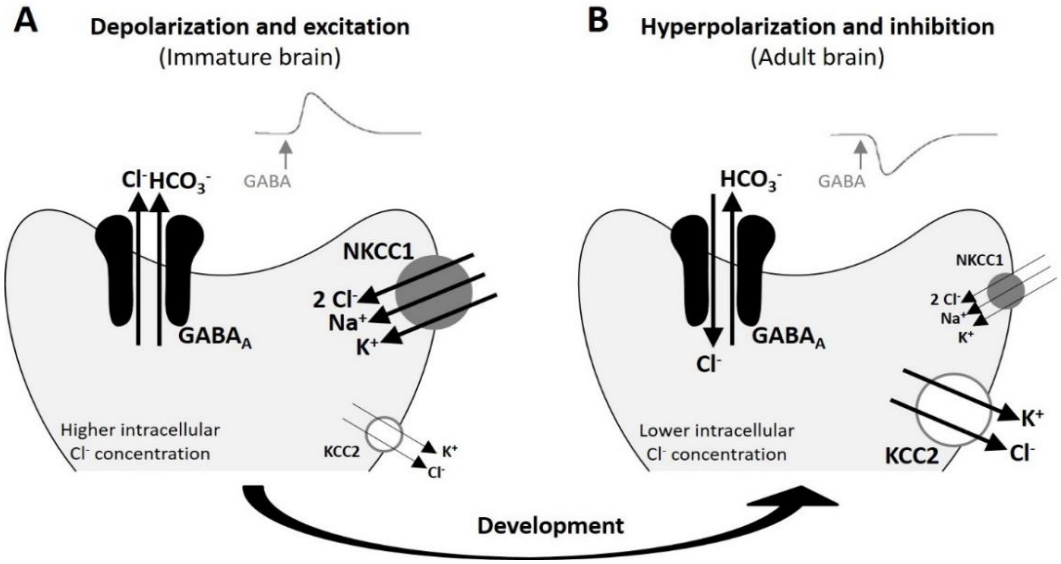

Figure 1 – Schematic diagram of "GABA shift" along the development and its relation with changes in intracellular Cl^- concentration. Panel A shows the high intracellular Cl^- concentration in immature brain due to the high expression of NKCC1, which determines the efflux of Cl^- upon $GABA_A$ receptor activation and the consequent membrane depolarization and promotion of neuronal excitability. In panel B it is shown the low intracellular Cl^- concentration in adult brain due to the predominant expression of KCC2, causing the influx of Cl^- into the cell upon $GABA_A$ receptor activation, membrane hyperpolarization and neuronal inhibition. Adapted from Ben-Ari (2014).

Neurotransmitter transporters: key proteins in the regulation of neuronal excitability

The actions of GABA and glutamate in the brain highlight the need to maintain the balance between the extracellular levels of these neurotransmitters at synapses. The maintenance of low levels of glutamate is crucial to prevent overexcitation of brain neurons, while the regulation of extracellular GABA levels controls the excitability of cells. If neurotransmitters are not rapidly removed from the synaptic cleft (in a millisecond time scale), receptors will be in a desensitized or persistently activated status and brain function compromised (Niciu et al., 2012).

There are no extracellular enzymes that can metabolize glutamate or GABA with significance. Hence, when these neurotransmitters are released they must be rapidly and efficiently cleared from the extracellular space through high-affinity amino acid transporters (Danbolt, 2001; Conti et al., 2004; Zhou and Danbolt, 2013; Scimemi, 2014). Both GABA and glutamate transporters belong to a large family of sodium symporters. These transporters are widely expressed throughout the brain and they are mainly located in plasma membranes of neurons and glia, essentially in close apposition to the synapse (Allen et al., 2004; Kanner, 2006; Scimemi, 2014) to more effectively shape synaptic transmission. The transport of amino acid

neurotransmitters across the plasma membrane is electrogenic and it is performed against very high concentration gradients of GABA or glutamate using the power from ion gradients maintained by Na^+/K^+-ATPase, *i.e.*, high extracellular Na^+ concentration and high intracellular K^+ concentration (secondary active transport) (Kanner, 2006; Scimemi, 2014). Ionic requirement for the transport depends on the amino acid being taken up into the cell and it is schematically represented in Figure 2.

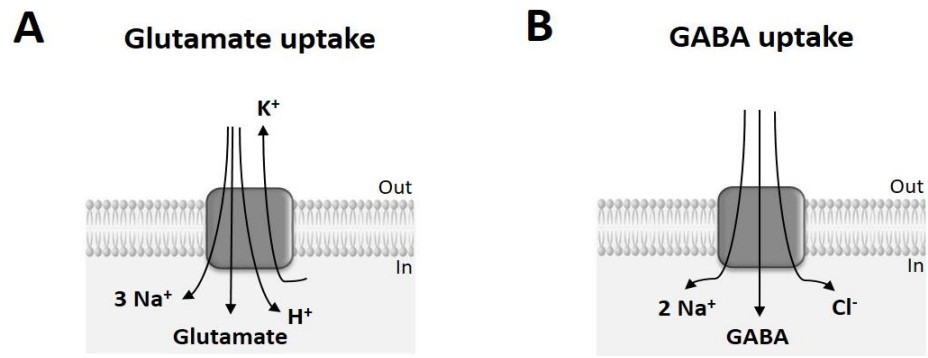

Figure 2 – Schematic representation of ionic fluxes coupled to glutamate (panel A) and GABA (panel B) uptake. Adapted from Kanner (2006).

To date, five distinct subtypes of glutamate transporters have been identified: excitatory amino acid transporters (EAAT) 1-5. These transporters belong to a solute carrier 1 (slc1) family – gene name approved by HUGO Gene Nomenclature Committee (HGNC) (Hediger et al., 2013) – and mediate co-transport of glutamate, Na^+ and H^+ with counter-transport of K^+ (Figure 2A) in the proportion of $1Glutamate^-:3Na^+:1H^+:1K^+$ (Kanner, 2006; Zhou and Danbolt, 2014), thus allowing the maintenance of high glutamate concentration gradient across the membrane. EAAT are widely distributed throughout the brain and their cellular and subcellular localization is summarized in Table 1. EAAT2 (also known as GLT-1) is the most important glutamate transporter, since it accounts for more than 80% of the total glutamate transport activity (Danbolt, 2001; Fontana, 2015; Takahashi et al., 2015). Although it was thought that EAAT2 was exclusively expressed in astrocytes, a number of recent studies have suggested that this glutamate transporter is also expressed in nerve terminals of some neurons (Beart and O'Shea, 2007; Bjornsen et al., 2007; Holmseth et al., 2012; Fontana, 2015). Only 6% of the EAAT2-immunoreactivity was detected in the plasma membrane of nerve terminals, however the EAAT2 in nerve terminals account for more than half of the uptake of

exogenous glutamate by hippocampal slice preparation (Schmitt et al., 2002; Furness et al., 2008), suggesting that terminal EAAT2 is more active than astroglial EAAT2. In turn, EAAT1 (also known as GLAST) seems to be exclusively expressed in brain astrocytes, while EAAT3 (also known as EAAC1) and EAAT4 are mostly expressed in neurons (Kim et al., 2011; Holmseth et al., 2012; Zhou and Danbolt, 2013). The expression of EAAT5 in the brain is very low, being these transporters preferentially expressed in the Müller cells of retina (Héja et al., 2006).

Contrarily to other EAAT's that are mainly present at the plasma membrane, EAAT3 is mainly intracellular and its targeting to the cytoplasmic membrane seems to be rapidly regulated by several mechanisms, such as protein kinase C (PKC) activation (Beart and O'Shea, 2007; Bianchi et al., 2013). It is believed that the function of EAAT3 is related to the subtle regulation of glutamatergic transmission through the prevention of the spillover of glutamate at excitatory synapses and it is important for the production of intracellular glutathione and subsequent protection from oxidative stress, since these transporters also can transport cysteine (Bianchi et al., 2013).

Table 1 – Nomenclature and localization of the different subtypes of glutamate transporters.

Subtypes	Other names	Gene symbol *	Cellular localization	Sub-cellular localization
EAAT1	GLAST	slc1a3	Astrocytes	Astrocytic plasma membrane
EAAT2	GLT-1	slc1a2	Mainly astrocytes, some neurons	Astrocytic plasma membrane, Axon terminals
EAAT3	EAAC1	slc1a1	Mainly neurons, some oligodendrocytes	Soma, dendrites
EAAT4	_	slc1a6	Mainly neurons, some astrocytes	Soma, Dendrites, Spines, Astrocytic plasma membrane
EAAT5	_	slc1a7	Müller cells	Plasma membrane endfeet

** An abbreviated form of the gene name approved by HGNC. Adapted from Héja et al. (2006), Kim et al. (2011), Bianchi et al. (2013), Zhou and Danbolt (2013) and Šerý et al. (2015) .*

Until now, four subtypes of GABA transporters (GATs) are known: GAT1, GAT2, GAT3 and betaine-GABA transporter (BGT1). The nomenclature of GABA transporters between mice, rat and human is different and somewhat confusing (see the summary in Table 2) and for simplicity, it will be used the same terminology regardless of the species being investigated. GABA transporters belong to a solute carrier 6 family (slc6; gene name approved by HGNC) and they catalyze the co-transport of GABA, Na^+ and Cl^- (Figure 2B) with the stoichiometry of

1GABA:2Na^+:1Cl^- (Richerson and Wu, 2003; Kanner, 2006; Hediger et al., 2013; Scimemi, 2014). GAT1 and GAT3 are expressed exclusively in the brain, whereas GAT2 and BGT1 are expressed in multiple other organs (Madsen et al., 2010). The cellular and subcellular localization of GABA transporters is summarized in Table 2. GAT1 was the first molecularly identified GABA transporter and it is the most important transporter for the removal of synaptically released GABA, given its main localization at nerve terminals. Additionally, GAT1 is also found in astrocytes (Zhou and Danbolt, 2013; Melone et al., 2015), as well as, in neuronal cell bodies for a short time during development (Zhou and Danbolt, 2013). In contrast to GAT1, GAT3 is predominantly expressed in astrocytes throughout the CNS, although it is also expressed in nerve terminals (Melone et al., 2015). The expression of GAT2 and BGT1 in brain is scarce, being these transporters more expressed in hepatocytes in the liver and kidney. Within the brain, the expression of GAT2 is higher in the leptomeninges and some large blood vessels and the expression of BGT1 is higher in the leptomeninges (Zhou and Danbolt, 2013; Scimemi, 2014). Additionally, BGT1 has been found also in the cortex and hippocampus where it is located in an extrasynaptic region involving, to a large extent, astrocytes (Madsen et al., 2010).

Table 2 – Nomenclature and localization of the different subtypes of plasma membrane GABA transporters.

Subtypes	Other names	Gene symbol *	Cellular localization	Sub-cellular localization
GAT1	–	slc6a1	Mainly neurons, some astrocytes	Axon terminals, Astrocytic plasma membranes
GAT2	Rat GAT2, human GAT2, mouse GAT3	slc6a13	Hepatocytes, leptomeningeal cells and some blood vessel cells	Plasma membranes
GAT3	Rat GAT3, human GAT3, mouse GAT4,GAT-B	slc6a11	Mainly astrocytes, some neurons	Distal astrocytic processes, nerve terminals
BGT1	Rat BGT1, rat NTBE, rat GAT-4, human GAT-4, mouse GAT-2	scl6a12	Hepatocytes, leptomeningeal cells and some astrocytes	Plasma membranes

** An abbreviated form of the gene name approved by the HGNC. Adapted from Héja et al. (2006), Madsen et al. (2010), Melone et al. (2015) and Zhou and Danbolt (2013).*

It has long been recognized that the action of high-affinity transporters for GABA and glutamate can be highly modified via short- and longer-term mechanisms. In fact, several studies have demonstrated that amino acid transporters are regulated, not only by kinases/phosphatases and scaffolding proteins, but also by diverse influences that seem to affect trafficking of the transporters: platelet-derived growth factor, thyroid hormone, trophic factors – e.g. brain derived neurotrophic factor (BDNF) – among others (Casado et al., 1993; Corey et al., 1994; Gonçalves et al., 1997; Quick et al., 1997; Beckman et al., 1998; Cordeiro et al., 2000; Beart and O'Shea, 2007; Hu and Quick, 2008; Cristóvão-Ferreira et al., 2009; Vaz et al., 2011; Melo et al., 2013; Yu et al., 2015). Short-term mechanisms include: (1) changes in ionic concentrations and membrane potential responsible for the transport, (2) changes in the trafficking of transporters through dynamic redistribution of these proteins between intracellular space and the plasma membrane, (3) changes in transporters lateral mobility via interaction with other synaptic proteins, and (4) post-translational modifications, such as phosphorylation. Longer-term mechanisms are due to altered expression and abundance of GABA and glutamate transporters. However, it remains unclear how these changes in the expression, mobility and activity of GABA and glutamate transporters affect the time course and spatial spread of GABAergic and glutamatergic signals in the brain.

As previously seen, the transport of neurotransmitters depends on the electrochemical gradients across the plasma membrane. The implication is that, as already mentioned, an alteration of the ionic gradients can promote an alteration of the transporter function. Interestingly, disruption of the electrochemical gradients can, on its own, be sufficient to reverse the transporter function and promote the translocation of GABA and glutamate from the cytoplasm back into the extracellular space, instead of the uptake into the cell (Richerson and Wu, 2003; Allen et al., 2004; Héja et al., 2006; Wu et al., 2006; Scimemi, 2014). In the last few years, the reverse mode function of the transporters has been described under depolarizing conditions, where electrochemical gradients are sufficiently weakened, *i.e.*, when there is a decrease of the ratio between extracellular Na^+ concentration and intracellular K^+ concentration and/or when there is an increase in the ratio between intracellular Na^+ and extracellular K^+ concentrations (Fontana, 2015). Such loss of energy supply due to ionic gradient disruption may occur under acute pathological states – such as during epileptic seizures or ischemia. The reverse mode of amino

acid transporters function and the consequent augmentation of extracellular GABA and glutamate levels have been described in these pathologies (Allen et al., 2004). Some studies reach the conclusion that reversion of neurotransmitter transporters mode may be critical for neurotransmitters release under such conditions, as this can be the main pathway for the translocation of amino acids outwards (Héja et al., 2006; Zhou and Danbolt, 2013). Furthermore, it has been shown the reversal of some transporters in response to physiologically relevant stimuli. This is the case of GABA transporters, for which a small increase in the extracellular K^+ concentration to 12 mM, which may be achieved during high-frequency neuronal firing, is sufficient to revert the transport. GABA transporters revert easier than glutamate transporters (Richerson and Wu, 2003; Allen et al., 2004; Wu et al., 2007), since they are powered by co-transport of only 2 Na^+ ions, while glutamate transporters co-transport 3 Na^+ ions. This is in keeping with theoretical calculations showing a close relation between the reversal potential of GABA transporters and resting membrane potential of neurons (Richerson and Wu, 2003; Allen et al., 2004; Wu et al., 2006). The emerging view of reverse function of neurotransmitter transporters adds complexity to the traditional and simplistic view of transporters as “tiny vacuum cleaners” (Richerson and Wu, 2003).

The field of transport biology of GABA and glutamate has tremendously grown over the past years and is nowadays recognized as playing an important role in the manifestation and even in the treatment of several neurological disorders, including cerebral ischemia, traumatic brain injury, Parkinson’s disease, Alzheimer’s disease, Huntington’s disease and epilepsy (O'Shea, 2002; Sheldon and Robinson, 2007; Madsen et al., 2010; Vizi et al., 2010; Hediger et al., 2013).

Dysfunction of GABAergic and glutamatergic neurotransmission in epilepsy: focusing on high-affinity transporters

The imbalance between GABAergic and glutamatergic neurotransmission that leads to aberrant neuronal excitability has long been considered to be the hallmark of epileptic conditions. The crucial role of high-affinity transporters in the regulation of the extracellular levels of GABA and glutamate and consequent prevention of neuronal damage has attracted much attention in the last years highlighting these transporters as hopeful therapeutic targets to control seizures

and/or epileptogenesis (Sheldon and Robinson, 2007; Madsen et al., 2010; Tian et al., 2010; Rowley et al., 2012; Schousboe et al., 2014; Fontana, 2015).

Increased levels of extracellular glutamate have been extensively found in epilepsy conditions (Janjua et al., 1992; During and Spencer, 1993; Soukupova et al., 2015) and have been associated with a dysfunction of the EAAT (Danbolt, 2001; Beart and O'Shea, 2007). However we are facing “the chicken or egg” dilemma, since we do not know whether the changes in the expression/function of these transporters contribute to the pathogenesis of epilepsy or they result from epilepsy.

The glutamate transporter that has been more extensively studied is the EAAT2 (Takahashi et al., 2015), given its dominant role in glutamate uptake. Different studies have shown that the expression of EAAT2 was decreased in rodent models of epilepsy (Sakurai et al., 2015), as well as in patients with intractable temporal lobe epilepsy (Proper et al., 2002; Sarac et al., 2009). Additionally, the development of lethal spontaneous seizures that exacerbates brain injury was observed in mice lacking EAAT2 (Tanaka et al., 1997; Petr et al., 2015), EAAT2 downregulation was correlated with thalamic neuronal death following kainic acid-induced SE (Sakurai et al., 2015) and a neuroprotection phenotype was conferred by the increased EAAT2 expression (Kong et al., 2012; Kong et al., 2014). These observations lead to the hypothesis that overexpression or simple activation of EAAT2 could be a promising therapeutic approach through the inhibition of excitotoxicity (Takahashi et al., 2015). However some authors have called attention to the fact that effects obtained with the promotion of EAAT2 activity may be contrary to the desired effect due to the reversion of transporter during pathological excitotoxic conditions (Danbolt, 2001; Richerson and Wu, 2003; Allen et al., 2004; Sheldon and Robinson, 2007; Fontana, 2015). So, the clarification of whether the EAAT2 activation under excitotoxic conditions will facilitate glutamate clearance or, instead, intensify reverse transport is a question still unanswered.

Similarly to EAAT2, EAAT1 is also decreased in patients with intractable temporal lobe epilepsy (Proper et al., 2002; Sarac et al., 2009), the generalized seizure duration of amygdala-kindled seizures in mice lacking EAAT1 was significantly prolonged in these animals (Watanabe et al., 1999) and EAAT1 knockout mice showed more severe stages of pentylenetetrazol-induced seizures than wild type mice (Watanabe et al., 1999).

EAAT3 involvement in epilepsy is controversial. While some studies show that, similarly to EAAT2 and EAAT1, the expression of EAAT3 is decreased in human neocortical epileptic loci (Rakhade and Loeb, 2008), as well as in epileptic models induced by kainate (Simantov et al., 1996) and in brain malformations (Harrington et al., 2007), other studies show that this transporter is increased in intractable temporal lobe epilepsy (Crino et al., 2002; Proper et al., 2002) as well as in acute seizures induced by 4-aminopyridine (Medina-Ceja et al., 2012), pilocarpine or kainate (Ross et al., 2011). Furthermore genetic deletion of the EAAT3 decreases seizure-induced neuronal death after pilocarpine-induced SE (Lane et al., 2014).

There are growing evidences that GABAergic signaling is altered in refractory temporal lobe epilepsy (Loup et al., 2000; Cohen et al., 2002; D'Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Loup et al., 2009; Mazzuferi et al., 2010; Miles et al., 2012; Pallud et al., 2014). Since GATs are responsible for termination of GABA action, it is possible that these transporters are altered in epilepsy and, therefore, its targeting may constitute a valuable therapeutic approach.

Among GABA transporters the most studied is GAT1. However, studies relating its alteration in epilepsy are not consistent. While in a rat model of seizures induced with corticotropin-releasing hormone GAT1 seems to be overexpressed, the same is not true for a rat model of seizures induced by hyperthermia (Orozco-Suarez et al., 2000). In a model of acute seizures induced by 4-aminopyridine, the expression of GAT1 increases 60 min after the induction and it decreases 120 min later (Medina-Ceja et al., 2012). Studies performed in human epileptic brain tissue resected from drug-resistant patients have shown a reduction of GAT1, in both neocortex and hippocampus (Lee et al., 2006; Aronica et al., 2007).

In the last years, several studies have showed that the promotion of GABAergic signaling by inhibition of GABA transporters may be beneficial to decrease neuronal excitability. In this way, pharmacological approaches were designed to inhibit GABA transport and consequently increase extracellular levels of this inhibitory neurotransmitter. Tiagabine is one inhibitor of GABA transporters, inhibiting preferentially GAT1 rather than GAT2 or GAT3, which has demonstrated clinical anticonvulsant efficacy and has been approved for clinical use in the treatment of epilepsy (Nielsen et al., 1991; Engelborghs et al., 2000; Madsen et al., 2010). However, tiagabine is associated with severe adverse effects and it is not recommended in some seizure types where the effect obtained is opposite to the

desired neuro-inhibition (Madsen et al., 2010). Some authors suggest that unwanted effects obtained with the blockage of GABA transporters may be explained by less GABA being released via reversal of GABA transporters when neurons depolarize (Richerson and Wu, 2003; Allen et al., 2004). On the contrary, others authors have suggested that the blockage of GABA transporters and the consequent augmentation of extracellular GABA concentration by decreasing the uptake is not always the best strategy since “friendly” inhibitory GABA may become “foe” due to $GABA_A$ rundown (Köhling, 2002).

Actually, several authors have proposed that the “GABAergic rundown” is responsible for the promotion of synchronous neuronal discharges underlying focal seizures in refractory temporal lobe epilepsy (Cohen et al., 2002; D’Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012; Pallud et al., 2014). This phenomenon could be (1) due to desensitization of $GABA_A$ receptor upon repetitive stimulation producing a reduction of the evoked GABA current in an use-dependent manner (Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010), or even (2) due to depolarization and excitatory actions stemming from changes in intracellular concentration of Cl^- promoted essentially by the downregulation of KCC2 – responsible for Cl^- extrusion, a situation that has been observed in epileptic tissue (D’Antuono et al., 2004; Huberfeld et al., 2007; Blaesse et al., 2009; Ben-Ari et al., 2012; Miles et al., 2012). This implies that instead of GABAergic signaling counterbalancing the depolarizing effects of glutamate, it may also contribute to the excitatory tone. The doubts over the anti- or pro-epileptic roles of GABAergic signaling in epilepsy suggest that treatments targeting the cell firing control and the consequent reduction of intrinsic neuronal excitability should be carefully examined, since these treatments may beneficially restrain sudden surges in network activity according to whether GABA is a “friend” or “foe” at this time (Köhling, 2002; Pavlov et al., 2013).

Purinergic signaling

Research during the last decades has shown that ATP is not only a component of nucleic acids and the principal fuel inside the cell, but also has a great importance in cellular communication. The concept of purinergic neurotransmission in which extracellular purines (namely ATP and adenosine) and pyrimidines act as

signaling molecules was introduced in 1972 by Burnstock (reviewed by Abbrachio et al., 2009; Burnstock et al., 2011a; Burnstock et al., 2011b). Subsequently, ATP was identified as a co-transmitter in sympathetic and parasympathetic nerves, and it is now recognized that ATP acts either as sole transmitter or a co-transmitter in most nerves in both peripheral and central nervous systems (Abbracchio et al., 2009; Burnstock et al., 2011a). Growing evidences have shown that purinergic signaling appears to play important roles in neurodegeneration, neuroprotection and neuroregeneration (Burnstock, 2015).

In basal physiological conditions, intracellular concentration of ATP is high (5-8 millimolar) while the extracellular concentration of ATP is low (nanomolar to micromolar range). However under physiological high-frequency stimulation conditions and under pathological conditions, such as epilepsy and cellular injury, the extracellular concentration of ATP increases reaching millimolar levels (Cunha et al., 1996b; Frenguelli et al., 2007; Dale and Frenguelli, 2009; Heinrich et al., 2012; Sáez-Orellana et al., 2015). Under these conditions, ATP is released, or co-released with other classical neurotransmitters (such as glutamate and GABA) by several mechanisms (Figure 3A), that involve constitutive and regulated exocytosis (Ca^{2+}-dependent), ATP-binding cassette proteins, pannexins, connexins, ATP-sensitive P2X7 receptor and osmolytic transporters linked to anion channels (Lazarowski, 2012; Sáez-Orellana et al., 2015). Additionally, it has been suggested that a leak in events of cell membrane damage could contribute to hugely increase extracellular ATP levels, conditioning in this way the synaptic activity (Sáez-Orellana et al., 2015).

After ATP being released, it is rapidly metabolized by a cascade of plasma membrane-bound enzymes, known as ectonucleotidases (Figure 3B). This process is physiologically relevant since it controls the lifetime of ATP, produce ATP metabolites that also act as signaling molecules and protect some purinergic receptors from desensitization (Cardoso et al., 2015). According to substrate specificity and products formation, ectonucleotidases are divided into four major families, which are all expressed in the brain (Abbracchio et al., 2009; Sáez-Orellana et al., 2015): ectonucleoside triphosphate diphosphohydrolases (E-NTPDases), ectonucleotide pyrophosphatase and/or phosphodiesterases (E-NPPs), alkaline phosphatases, and ecto-5'-nucleotidase (CD73). E-NTPDases and E-NPPs hydrolyze ATP and adenosine diphosphate (ADP) to adenosine monophosphate (AMP), which is then hydrolyzed to adenosine by CD73. Alkaline phosphatases

equally hydrolyze nucleoside tri, di and monophosphates. Dinucleoside polyphosphates, nicotinamide adenine dinucleotide (NAD^+) and uridine diphosphate (UDP) sugars, are substrates exclusively for E-NPPs (Abbracchio et al., 2009).

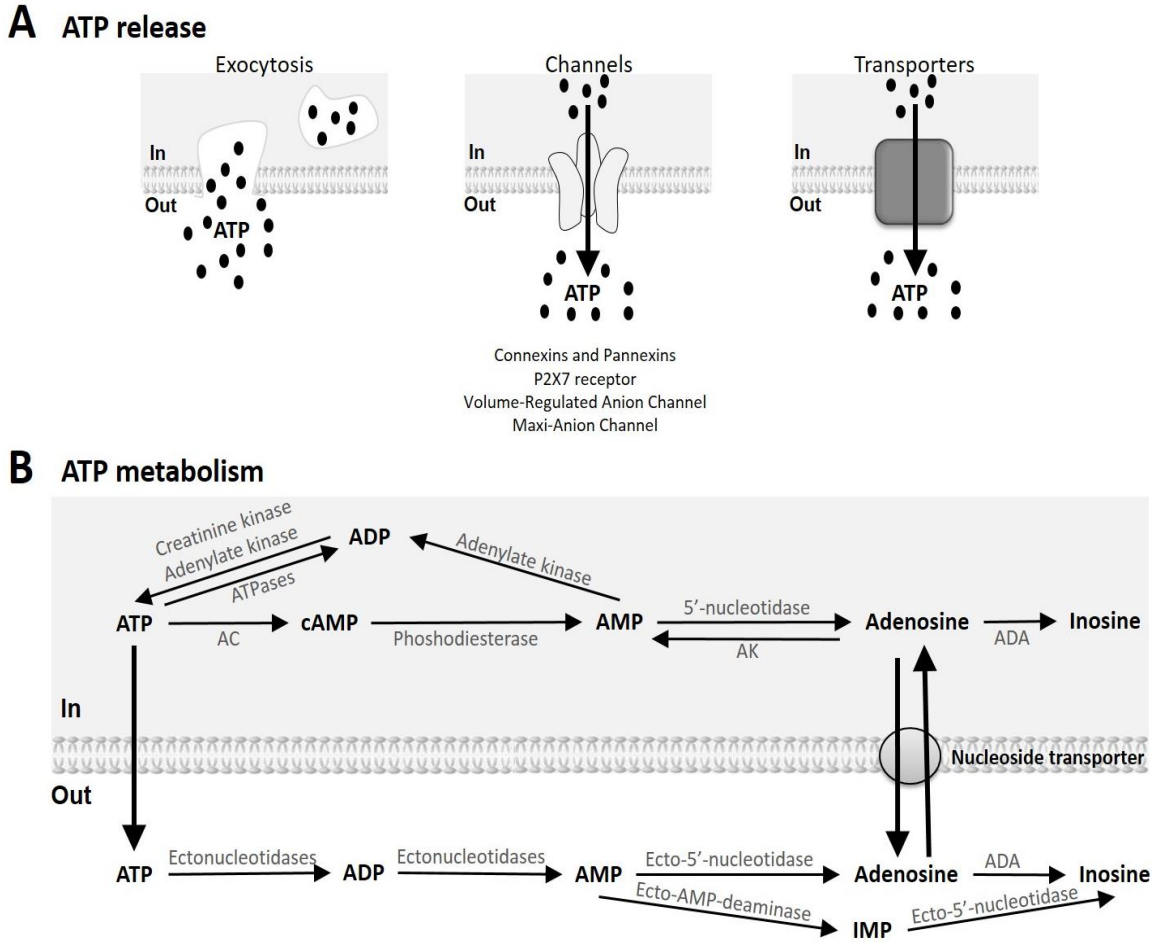

Figure 3 – Schematic representation of ATP release and its metabolism. Panel A illustrates the different mechanisms of ATP release. Panel B represents the metabolism of ATP, inside and outside of the cell.

Although adenosine is produced from the extracellular catabolism of ATP (Figure 3B), it can also be released as such into the extracellular medium by equilibrative nucleoside transporters (Bonan, 2012; Heinrich et al., 2012; Wall and Dale, 2013; Sims and Dale, 2014). In addition, there is increasing evidence for other mechanisms of adenosine release, including exocytotic release and retrograde signaling (Dale and Frenguelli, 2009). When in the extracellular milieu, adenosine is metabolized to inosine by adenosine deaminase (ADA) or taken up by cells via nucleoside transporters (Correia-de-Sá and Ribeiro, 1996). The driving motor for adenosine uptake by cells via equilibrative nucleoside transporters is the

phosphorylation of the nucleoside into AMP by high-affinity intracellular adenosine kinase (AK).

As discussed above, the metabolism of ATP originates several extracellular ubiquitous nucleotides and nucleosides, which exert signaling effects through the activation of purinoceptors (see next section).

Purinoceptors

Purinergic receptors are a family of membrane receptors expressed in a wide variety of tissues, including the brain. These receptors were defined for the first time in 1976 and were subdivided 2 years later into two types of purinoceptors, classified as adenosine-sensitive P1 and nucleotide-activated P2 (Figure 4; reviewed by Burnstock et al., 2007) . P2 receptors were then subdivided into ionotropic P2X and metabotropic P2Y receptors (Figure 4) based on their mechanism of action, pharmacology and molecular cloning (Ralevic and Burnstock, 1998; Abbracchio et al., 2009).

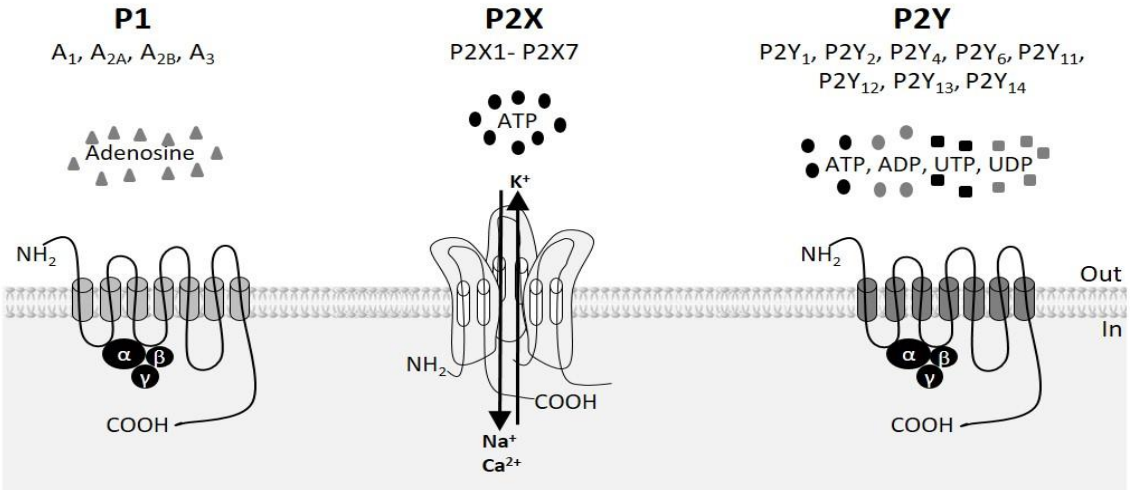

Figure 4 – Schematic representation of the three different families of purinergic receptors: metabotropic P1 receptors, ionotropic P2X receptors and metabotropic P2Y receptors.

P1 receptors

P1 receptors comprise four types of GPCR, A_1, A_{2A}, A_{2B} and A_3, displaying distinct pharmacological and functional properties. In common with other GPCR, P1 receptors contain seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus (Figure 4). Activation of A_1 and A_3 receptors favors (1) the inhibition of AC, (2) the activation of several types of K^+ channels, (3) the

inactivation of Ca^{2+} channels, and (4) the activation of PLC-β (Burnstock et al., 2011a). In contrast, activation of A_{2A} and A_{2B} receptors stimulates AC resulting in the production of cAMP. Despite of this dominant pathway, A_{2A} and A_{2B} receptors may also be associated with intracellular Ca^{2+} mobilization (Burnstock et al., 2011a).

The A_1 and A_{2A} receptors are the most relevant receptors in the brain (Burnstock, 2007; Boison, 2008). The A_1 receptor is the most expressed adenosine receptor under physiological conditions. The latter receptor is highly expressed in the hippocampus (Burnstock et al., 2011a), while the A_{2A} receptor is expressed at low levels in this structure (Schiffmann et al., 1991; Dixon et al., 1996), but this balance can change with ageing (Cunha et al., 1995; Rebola et al., 2003).

The key agonists and antagonists of the P1 adenosine receptor subtypes are summarized in Table 3.

Table 3 – Characteristics of P1 receptors

Receptor	Transduction pathway	Main agonists	Antagonists
A_1	G_i/G_0: ↓ cAMP	CCPA; R-PIA	DPCPX, N-040, MRS1754
A_{2A}	G_s: ↑ cAMP	NECA; CGS21680	KF17837, SCH58261, ZM241385
A_{2B}	G_s: ↑ cAMP	NECA	Enprofylline, MRE2029-F20, MRS17541
A_3	G_i/G_0, G_q/G_{11}: ↓ cAMP, PLC-β activation	IB-MECA, NECA, 2-Cl-IB-MECA	MRS1220, L-268605, MRS1191, MRS1523

Adapted from IUPHAR and Burnstock (2007)

In the last couple of years, the A_{2A} receptor has been implicated in neuronal excitability and synaptic plasticity (Kanno and Nishizaki, 2012; Matos et al., 2012; Valadas et al., 2012; Matos et al. 2013), as well as in diverse pathological conditions of the CNS, such as Parkinson's disease (Uchida et al., 2015), Alzheimer's disease (Orr et al., 2015) and epilepsy (see section Purinergic signaling and epilepsy). Initially it was believed that the A_{2A} receptor was mostly located in neurons, both pre- and post-synaptically (Rebola et al., 2005; Rodrigues et al., 2015). However, growing evidences have shown that this receptor is also expressed in glial cells (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013; Orr et al., 2015), where the A_{2A} receptor is critical for glutamate transport modulation (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013) and memory formation (Orr et al., 2015).

P2X receptors

P2X receptors are ligand-gated ion channels that promote fast cell depolarization through the influx of Na^+ and Ca^{2+} ions (Figure 4). These receptors are composed by three individual subunits and are designated as P2X1 to P2X7 according to the historical order of cloning. P2X receptors share a common topology (see Figure 4) with two transmembrane domains, a glycosylated extracellular ligand binding loop with several conserved cysteines connecting the two transmembrane domains, and intracellular N- and C-termini (North, 2002; Abbracchio et al., 2009; Baroja-Mazo et al., 2013). Trimeric P2X receptors are assembled in the endoplasmic reticulum, and then trafficked to the cell membrane (Sáez-Orellana et al., 2015). With the exception of P2X6 subunit, all subunits undergo homotrimerization (Abbracchio et al., 2009; Sáez-Orellana et al., 2015). The key agonists and antagonists of the homomeric P2X receptors are summarized in Table 4.

Table 4 – Characteristics of P2X receptors

Receptor	Transduction pathway	Main agonists	Antagonists
P2X1	Ion channel (Ca^{2+} and Na^+)	ATP, 2-MeSATP	TNP-ATP, IP5I, NF023, NF449
P2X2	Ion channel (mainly Ca^{2+})	ATP, ATPγS	Suramin, isoPPADS, RB2, NF770, NF279
P2X3	Ion channel	2-MeSATP, ATP	TNP-ATP, PPADS, A317491, NF110, Ip5I,
P2X4	Ion channel (mainly Ca^{2+})	ATP, Ivermectin potentiation	5-BDBD, TNP-ATP, BBG
P2X5	Ion channel	ATP, 2-MeSATP, ATPγS	Suramin, PPADS, BBG
P2X6	Ion channel	_	_
P2X7	Ion channel and large pore with prolonged activation	BzATP, ATP	A-438079, O-ATP, A-740003

Adapted from IUPHAR and Burnstock (2007).

One atypical member of the P2X family is the homomeric P2X7 receptor, since this receptor displays unusually large ionic conductance, high EC_{50} for ATP (>100 μM) and slow desensitization (Khakh and North, 2006; Jarvis and Khakh, 2009; Pankratov et al., 2009). In contrast to other P2X receptors, the P2X7 receptor has a long intracellular C-terminus (239 amino acids) and upon repeated or prolonged activation it opens a non-selective pore that allows the permeation of organic cations with large molecular weight (600-800 Da) (North, 2002; Sperlágh

and Illes, 2014). Another peculiar characteristic of P2X7 receptor is the increase of its activity by reducing the concentration of divalent ions, like Ca^{2+} and Mg^{2+} (Virginio et al., 1997; North, 2002; Jiang, 2009; Yan et al., 2011). Growing evidences implicate P2X7 receptor in crucial physiological phenomena, such as LTP (Chu et al., 2010), as well as in the pathophysiology of several neurological syndromes, namely neurotrauma (Kimbler et al., 2012), multiple sclerosis (Gu et al., 2015), amyotrophic lateral sclerosis (Yiangou et al., 2006), Alzheimer's disease (McLarnon et al., 2006; Miras-Portugal et al., 2015; Sáez-Orellana et al., 2015), Huntington's disease (Díaz-Hernández et al., 2009), psychiatric mood disorders (Kongsui et al., 2014) and epilepsy (see section Purinergic signaling and epilepsy).

Recent studies have shown that the P2X subunits can form hetero-trimers due to the assembly of at least two different individual subunits leading to the existence of a large number of P2X receptor phenotypes (North, 2002; Burnstock and Kennedy, 2011; Baroja-Mazo et al., 2013). To date, seven functional hetero-trimers have been reported: P2X2/3, P2X4/6, P2X1/5, P2X2/6, P2X1/4, P2X1/2 and P2X4/7 (Burnstock and Kennedy, 2011). The most physiologically relevant of these heteromeric receptors are the P2X1/2, P2X2/3, P2X1/4 and P2X1/5 receptors (Baroja-Mazo et al., 2013).

P2Y receptors

P2Y receptors belong to GPCR family and share the common topology of seven transmembrane domains, an extracellular N-terminus and an intracellular C-terminus (Figure 4). The first P2Y receptor (P2Y1) was cloned in 1993 (Baroja-Mazo et al., 2013) and so far, eight subtypes of metabotropic P2Y are described: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$.

P2Y receptors can be activated by adenine and uridine nucleotides – ATP, ADP, UTP and UDP (Figure 4, Table 5) – and therefore they are frequently grouped according to their preferential endogenous agonist. $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ receptors are only activated by adenine nucleotides: $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors preferentially bind ADP and $P2Y_{11}$ has a high-affinity for ATP (Baroja-Mazo et al., 2013). The $P2Y_2$ receptor and rodent $P2Y_4$ receptor can be activated by either adenine or uridine nucleotides, while the human $P2Y_4$ and $P2Y_6$ receptors are selective for uridine nucleotides. In contrast to $P2Y_2$ and $P2Y_4$ receptors that

use mainly triphosphate nucleotides, $P2Y_6$ receptor binds preferentially to UDP (Baroja-Mazo et al., 2013). The $P2Y_{14}$ receptor subtype is activated by UDP sugars: UDP-glucose and UDP-galactose (Weisman et al., 2012; Baroja-Mazo et al., 2013). The key agonists and antagonists of the P2Y receptors are summarized in Table 5.

Table 5 – Characteristics of P2Y receptors

Receptor	Transduction pathway	Main agonists	Antagonists
P2Y1	G_q/G_{11}, G_i/G_0: PLC-β activation, ion channel (K^+ and $Ca2^+$)	MRS2365, 2-MeSADP, ADPβS	MRS2179, MRS2500
P2Y2	G_q/G_{11} and possibly G_i/G_0: PLC-β activation	2-thio-UTP, UTP, ATP	Suramin, RB-2, AR-C126313
P2Y4	G_q/G_{11} and possibly G_i: PLC-β activation, PLA_2 stimulation	UTP,ATP	RB2, Suramin
P2Y6	G_q/G_{11}: PLC-β activation,	3-Phenacyl-UDP, UDPβS, UDP	MRS2578
P2Y11	G_q/G_{11} and G_s: PLC-β activation, ↑ cAMP	AR-C67085MX, BzATP, ATPγS	Suramin, RB-2, NF157
P2Y12	$G_{i\alpha}$: ↓ cAMP	2-MeSATP, 2-MeSADP, ADP	CT50547, INS49266, AZD6140, PSB0413, ARL66096
P2Y13	G_i/G_0: ↓ cAMP	ADP, 2-MeSADP	MRS2211, 2-MeSAMP
P2Y14	G_i/G_0: ↓ cAMP, PLC stimulation	UDP-glucose, UDP-galactose	–

Adapted from IUPHAR and Burnstock (2007)

Moreover, P2Y receptors can also be grouped according to the phylogenetic similarity and coupling to specific G proteins. (1) G_q protein-coupled receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) activate PLC-β; (2) G_i protein-coupled receptors ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) inhibit AC and regulate ion channels; and (3) the G_s protein-coupled receptor ($P2Y_{11}$) activates AC and regulates ion fluxes. Note that $P2Y_{11}$ receptor is coupled to different G proteins, G_q and G_s, evidencing an agonist-specific signaling pathway (White et al., 2003).

P2Y receptors can homodimerize or heterodimerize with other P2Y receptors, as well as with other transmitter receptors, including the adenosine A_1 receptor (Abbracchio et al., 2009).

Purinergic signaling and epilepsy

Several lines of evidence led to the conclusion that ATP exerts a pro-epileptic role by activating P2 purinoceptors, leading to the hypothesis that purinergic signaling could be a target for the treatment of drug-refractory epilepsy. It has been

evidenced that (1) microinjection of ATP analogues into the pre-piriform cortex can induce generalized motor seizures (Knutsen and Murray, 1997); (2) adenine nucleotides modulate epileptiform activity in hippocampal slices (Ross et al., 1998); (3) ATP participates in the pathophysiology of pilocarpine-induced temporal lobe epilepsy (Vianna et al., 2002), and (4) extracellular levels of ATP and adenosine rapidly increase during high-frequency neuronal firing, as well as upon noxious brain conditions such as epilepsy-associated seizures (Dale and Frenguelli, 2009; Heinrich et al., 2012; Wall and Dale, 2013; Sims and Dale, 2014) Different P2 receptors, mostly excitatory ionotropic P2X receptors activated by ATP, were identified as putative targets to control seizures, (Burnstock and Kennedy, 2011; Henshall et al., 2013; North and Jarvis, 2013; Sáez-Orellana et al., 2015).

The P2X7 receptor has been considered the P2 receptor more committed to epilepsy, although controversy still exists in the literature regarding its pro- or anticonvulsant effects in distinct animal models. For instance, pilocarpine-induced seizures were increased in mice lacking the P2X7 receptor, although no such effect was observed in the kainate epilepsy model (Kim and Kang, 2011). *In vitro* studies also argue that the P2X7 receptor activation can be inhibitory. These include a report showing that presynaptic P2X7 receptor reduce neurotransmitter release in hippocampal slices (Armstrong et al., 2002). On the other hand, it has been shown that the P2X7 receptor is upregulated in both hippocampus and cerebral cortex of rodents with temporal lobe epilepsy (either induced with pilocarpine or kainate); these findings were observed both in acute and chronic phases of the disorder (Avignone et al., 2008; Doná et al., 2009; Jimenez-Pacheco et al., 2013). Despite this, the expression of P2X7 receptor may transiently decrease to control levels in the latent phase of the disease (Doná et al., 2009) and this finding may complicate interpretation of the results obtained by various research groups. An increase in the density of the P2X7 receptor was also observed in the neocortex from patients with drug-resistant temporal lobe epilepsy (Jimenez-Pacheco et al., 2013). Manipulation of the P2X7 receptor tone with the selective antagonist, A-438079, exerts an anticonvulsant effect in rodent models of epilepsy (Engel et al., 2012a; Engel et al., 2012b; Henshall et al., 2013; Jimenez-Pacheco et al., 2013). The apparently contradictory findings regarding the role of P2X7 receptor in epilepsy suggest that the contribution of this receptor may change along disease stages and the underlying epileptogenic process.

Despite P2X4 and P2X7 subunits may form functional heterotrimers the involvement of P2X4 subunits in epilepsy is less studied, but the results obtained are also controversial. Doná et al. (2009) found no changes in the P2X4 receptor expression and function in the epileptic hippocampus of rats injected with pilocarpine. Conversely, Ulmann et al. (2013) using a model of kainate-induced SE detected increased levels of the P2X4 receptor in activated glial cells of the hippocampus.

The ATP metabolite, adenosine, has long been considered an endogenous antiepileptic substance acting via inhibitory A_1 receptors. This is why some authors propose the augmentation of extracellular adenosine as a solution to resolve drug-refractory epilepsies (Boison, 2007, 2012; Boison and Stewart, 2009). Nevertheless, one must not forget that adenosine may also activate excitatory A_{2A} receptors in certain brain regions (e.g. hippocampus) besides the predominant inhibitory A_1 receptor (Boison, 2008). In this regard, it should be noticed that excitatory A_{2A} receptors become more abundant in epileptic animals (Chen et al., 2007; Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013; Orr et al., 2015). Experimental results in rodents demonstrate that adenosine A_{2A} receptor antagonists may offer protection against diverse epileptic syndromes, including temporal lobe epilepsy, leading several authors to propose that the adenosine A_{2A} receptor may be an attractive pharmacological target for treatment of epilepsy (Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013; Orr et al., 2015).

Interplay between GABA, glutamate and purines in epilepsy: clinical relevance of this study

As discussed previously, epilepsy is largely characterized by an imbalance between GABAergic and glutamatergic neurotransmission and nowadays only 50% of the epileptic patients are adequately treated for their symptoms with currently available AEDs. High-affinity transporters have a key role in the maintenance of synaptic transmission and growing evidences indicates the pharmacological modulation of GABA and glutamate transporters as relevant therapeutic targets

(Madsen et al., 2010; Rowley et al., 2012; Schousboe et al., 2014; Zhou and Danbolt, 2014). As a matter of fact, several efforts have been made to increase brain network inhibitory tone or decrease excitation by targeting GABA and glutamate transporters. Additionally, the approved clinical use of the GAT1 inhibitor, tiagabine, for the therapeutic management of epilepsy (Nielsen et al., 1991; Madsen et al., 2010) makes us to believe that the "fine-tuning" modulation of amino acid transporters by fast endogenous regulators, namely ATP, may be a promising strategy through promotion of appropriate concentrations of GABA and glutamate in the right places at the right time. ATP emerges as a potential candidate, since it is co-released with neurotransmitters GABA and glutamate and its extracellular levels increase significantly during high-frequency neuronal firing (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012). Furthermore, ATP co-inhabits with GABA and glutamate at the synapse, being this nucleotide in the right place to rapidly modulate the extracellular levels of GABA and glutamate.

Besides the ATP and given that this nucleotide is extracellularly metabolized by a cascade of ectonucleotidases into adenosine resulting in high extracellular concentrations of adenosine, it is probable that adenosine is also able to modulate the extracellular levels of GABA and glutamate. In fact, several works have shown that adenosine, through the activation of A_{2A} receptors, can modulate the extracellular levels of GABA and glutamate, altering both the uptake and the release of these amino acid neurotransmitters (Gonzalez et al., 2006; Cristóvão-Ferreira et al., 2009; Vaz et al., 2011; Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013; Vaz et al., 2015). Although it is already known that the A_{2A} receptor is increased in rodent models of epilepsy (Chen et al., 2007; Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013; Orr et al., 2015), no attempts have been made to investigate alterations in the A_{2A} receptor density in human brain samples from drug-resistant epileptic patients. Likewise, there are no studies, to our knowledge where the modulation of extracellular levels of GABA and glutamate by adenosine A_{2A} receptor activation has been tested in epileptic conditions.

So, in this work, we aim at contributing to find new drug therapies to control seizures through the finding of "fine-tuning" modulatory mechanisms that rapidly regulate the extracellular levels of GABA and glutamate in accordance with the needs of a balanced synapse. Besides its value in controlling seizure activity in

epileptic patients, it is possible that these drugs acting selectively on GABA and/or on glutamate transporters, by modulating its activity, may constitute a potential therapeutic candidate for the treatment of other neurological and psychiatric diseases associated with the GABAergic/glutamatergic balance, such as anxiety, sleep disorders, among many others.

CHAPTER 2: GOALS

Given that, as discussed above (see section Purinergic signaling and epilepsy), (1) the excitatory P2X7 receptor has been proposed as a target to control seizures, (2) the opening of the P2X7 receptor channel by ATP increases Na^+-influx that is characteristic of increased brain activity (Lo et al., 2008; Yu et al., 2010), and (3) GABA and glutamate transporters are crucially dependent on the transmembrane Na^+ gradient, we decided to investigate the modulatory role of ATP acting via the P2X7 receptor on the uptake of GABA and glutamate by isolated nerve terminals measured in parallel. Our hypothesis is that full opening of the P2X7 receptor channel leads to extensive Na^+-influx, which might dissipate the Na^+ gradient across the plasma membrane and, thus, decrease the transport driving force for GABA and glutamate. Since energy modulation by changing the transmembrane Na^+ gradient may affect both GABA and glutamate transport systems and given the overall effects of these two neurotransmitters on neuronal networking, it seems necessary to study the uptake of the two amino acids in parallel, *i.e.* under the same modulatory conditions, in order to appreciate putative differential effects caused by activation of the P2X7 receptor in control and epileptic brain tissue. Our hypothesis that the activation of the P2X7 receptor may alter neurotransmitters uptake was borne out by studies conducted in the RBA-2 type-2-like astrocytic cell line showing that the P2X7 receptor stimulation increases intracellular Na^+ concentration and, thereby, causes downmodulation of the glutamate transport (Lo et al., 2008). In keeping with hypothesis, other authors showed (1) that the P2X7 receptor activation may modulate the uptake of glutamate in microglial cells via the activation of a cascade of extracellular signal-regulated kinases and the production of oxidants (Morioka et al., 2008), and (2) that P2X receptors are associated with increases in the amount of GABA in the extracellular milieu due to a reduction of its uptake in the rat retina (Neal et al., 1998). In this context, the specific aims of this study were to investigate: (1) the role of the ATP-sensitive P2X7 receptor on high-affinity GABA and glutamate transport in brain tissues from *Wistar* rats and Human controls (cadaveric organ donors), (2) the molecular pathway underlying modulation of GABA and glutamate transport operated by the P2X7 receptor activation, and (3) whether the expression and cellular localization of P2X7 receptors and the P2X7 receptor-mediated modulation of amino acid transporters change in epileptic individuals, both rats injected with pilocarpine and human MTLE and non-MTLE patients that underwent neurosurgical

ablation of the epileptic focus at Centro Hospitalar do Porto – Hospital Geral de Santo António. The use of the animal model allowed us to test extensively our hypothesis that ATP acting via the P2X7 receptor could modulate GABA and glutamate uptake by changing the transmembrane Na^+ gradient before going to brain samples from Human controls and patients with drug-refractory epilepsy, which for obvious reasons are more difficult to obtain. Given the similarity of the results obtained with nerve terminals isolated from brain samples of both species, we are now in conditions to say that the pilocarpine-injected epileptic rat model is suitable for testing the role of purines in the context of amino acid uptake modulation, which was a secondary goal of the present study.

Changes in the P2X7 receptor-mediated downmodulation of GABA and glutamate uptake were mostly assessed in the absence of extracellular Ca^{2+} in order to mimic conditions that occur under high-frequency neuronal firing and/or pathologic brain activity, such as prolonged or repeated seizures (Engel et al., 2012a). Under these conditions the extracellular Ca^{2+} concentration falls up to 90% (Heinemann et al., 1977; Rusakov and Fine, 2003; Engel et al., 2012a; Torres et al., 2012; Jimenez-Pacheco et al., 2013) and Na^+-conductance through the P2X7 receptor is largely enhanced, as this receptor is highly sensitive to changes in the extracellular concentration of divalent cations (Virginio et al., 1997; Yan et al., 2011). Performing experiments in low extracellular Ca^{2+} conditions also excludes the involvement of Ca^{2+}-regulation of amino acid transporters (directly or via plasmalemmal and mitochondrial Na^+/Ca^{2+} exchangers), as well as the interference of Ca^{2+}-activated neurotransmitter release (Cordeiro et al., 2000; Cordeiro et al., 2003; Ashpole et al., 2013; Romei et al., 2015).

Additionally, we also evaluated if the expression of other purinergic receptors was altered in Human brain samples of MTLE patients as compared with cadaveric organ donors. Besides the P2X7 receptor (see above), the excitatory adenosine A_{2A} receptor that is expressed in epileptic region targets, both cerebral cortex and hippocampus, called our attention by the additional following reasons: (1) the A_{2A} receptor is a metabotropic receptor whose function has been associated with the ability to modulate GABA and glutamate transport (Nishizaki et al., 2002; Vaz et al., 2011; Kanno and Nishizaki, 2012; Matos et al., 2012), (2) several studies demonstrate that the A_{2A} receptor expression may be altered in rodent models of epilepsy (Chen et al., 2007; Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009;

Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013; Orr et al., 2015) and 3) it has been shown that A_{2A} receptor antagonists can offer protection against diverse epileptic syndromes induced in rodents (Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013).

It is worth noting that, since the MTLE disorder affects both brain regions hippocampus and neocortex (Doherty et al., 2003; Bartolomei et al., 2005; Scanlon et al., 2011; Alhusaini et al., 2012; O'Dell et al., 2012; Biagini et al., 2013; Di Maio, 2014; Kandratavicius et al., 2014), this study was performed whenever possible using to the two cerebral regions according to the availability of human tissue.

The ultimate goal of this study is to contribute to unravel the “mysteries” of drug-resistant epilepsies, such as MTLE, in order to prompt for novel and more accurate drugs targeting the purinergic signaling cascade, which might be deeply involved both in epileptogenesis and in neuronal damage secondary to prolonged or repetitive seizures.

CHAPTER 3: ORIGINAL RESEARCH PAPERS

The results obtained in this thesis were published or prepared for publication as original research papers, as follows:

Paper 1: **Barros-Barbosa AR,** Lobo MG, Ferreirinha F, Correia-de-Sá P, Cordeiro JM. P2X7 receptor activation downmodulates Na^+-dependent high-affinity GABA and glutamate transport into rat brain cortex synaptosomes. *Neuroscience. 2015. 306:74-90. DOI: 10.1016/j.neuroscience.2015.08.026; PMID: 26299340.*

Paper 2: **Barros-Barbosa AR,** Lukoyanov N, Soares J, Lobo MG, Ferreirinha F, Correia-de-Sá P, Cordeiro JM. Inhibition of GABA and glutamate uptake by cortical nerve terminals produced by P2X7 receptors activation is kept unaltered in epileptic rats. *In Preparation.*

Paper 3: **Barros-Barbosa AR,** Fonseca AL, Guerra-Gomes S, Ferreirinha F, Santos A, Rangel R, Lobo MG, Correia-de-Sá P, Cordeiro JM. Up regulation of P2X7 receptor-mediated inhibition of GABA uptake by nerve terminals of the human epileptic neocortex. *Epilepsia. 2015. In press. DOI: 10.1111/epi.13263.*

Paper 4: **Barros-Barbosa AR,** Ferreirinha F, Mendes M, Lobo MG, Santos A, Rangel R, Cordeiro JM, Correia-de-Sá P. The A_{2A} receptor is upregulated in hippocampal astrocytes of human patients with mesial temporal lobe epilepsy (MTLE). *In Preparation.*

PAPER 1

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P2X7 receptor activation downmodulates Na^+-dependent high-affinity GABA and glutamate transport into rat brain cortex synaptosomes

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Abstract

Sodium-dependent high-affinity amino acid transporters play crucial roles in terminating synaptic transmission in the CNS. However, there is a lack of information about the mechanisms underlying the regulation of amino acid transport by fast-acting neuromodulators, like ATP. Here, we investigated whether activation of the ATP-sensitive P2X7 receptor modulates Na^+-dependent GABA and glutamate uptake into nerve terminals (synaptosomes) of the rat cerebral cortex. Radiolabeled neurotransmitter accumulation was evaluated by liquid scintillation spectrometry. The cell-permeant sodium selective fluorescent indicator, SBFI-AM, was used to estimate Na^+ influx across plasma membrane. BzATP, 3-300µM, a prototypic P2X7 receptor agonist, concentration-dependently decreased [^{3}H]GABA (14%) and [^{14}C]glutamate (24%) uptake; BzATP decreased transport V_{max} without affecting the K_m values. The selective P2X7 receptor antagonist, A-438079 (3µM), prevented inhibition of [^{3}H]GABA and [^{14}C]glutamate uptake by BzATP (100µM). The inhibitory effect of BzATP coincided with its ability to increase intracellular Na^+ and was mimicked by Na^+ ionophores, like gramicidin and monensin. Increases in intracellular Na^+ (with veratridine or ouabain) or substitution of extracellular Na^+ by $NMDG^+$ all decreased [^{3}H]GABA and [^{14}C]glutamate uptake and attenuated BzATP effects. Uptake inhibition by BzATP (100 µM) was also attenuated by calmidazolium, which selectively inhibits Na^+ currents through the P2X7 receptor pore. In conclusion, disruption of the Na^+ gradient by P2X7 receptor activation downmodulates high-affinity GABA and glutamate uptake into rat cortical synaptosomes. Interference with amino acid transport efficacy may constitute a novel target for therapeutic management of cortical excitability.

Introduction

GABA and glutamate are key neurotransmitters in CNS where they play a fundamental role in controlling neuronal excitability, information processing and neuronal plasticity. Therefore, maintenance of the balance between GABAergic inhibition and glutamatergic excitation in the brain is crucial under normal and pathological conditions. Under physiological conditions, Na^+-dependent high-affinity transporters rapidly clear amino acid neurotransmitters from the extracellular space. These transporters are located in the plasma membrane of both neurons and glia exhibiting high density at the synaptic regions (Kanner, 2006). In order to adapt transport function to the synaptic environment, transporters should be rapidly modulated. However, little is known about the signaling molecules responsible for fast regulation of GABA and glutamate transport.

ATP is a likely candidate, as the nucleotide is co-released with neurotransmitters from both neurons and glia under physiologic and pathologic conditions, such as during epileptic seizures (Khakh and North, 2006; Pankratov et al., 2009; Burnstock et al., 2011b). ATP controls cerebral functions through the activation of ionotropic P2X and metabotropic P2Y receptors. The slowly-desensitizing homomeric P2X7 receptor channel is a unique member of the ATP-gated P2X receptor family with a characteristic long intracellular C-terminus (239 amino acids), which displays unusually large ionic conductance and high EC_{50} for ATP (>100 μM) (Jarvis and Khakh, 2009; Jiang, 2009; Pankratov et al., 2009). P2X7 receptors are widely expressed in the CNS in many cell types including neurons and glial cells (Khakh and North, 2006; Henshall et al., 2013). These purinoceptors have been implicated in LTP phenomena (Chu et al., 2010) and in many pathological conditions, including epilepsy (Engel et al., 2012a; Henshall et al., 2013; Jimenez-Pacheco et al., 2013; Sperlágh and Illes, 2014).

The driving-force for high-affinity GABA and glutamate transport is crucially dependent on Na^+-gradient across the plasma membrane (Wonnemann et al., 2000; Richerson and Wu, 2003; Allen et al., 2004; Kanner, 2006). Brain activity is accompanied by significant Na^+-influx from the extracellular space, through a variety of paths including permeation via ligand-gated cation channels, like P2X purinoceptors (Lo et al., 2008; Yu et al., 2010). Activation of the P2X7 receptor results in intense Na^+-influx that may alter the Na^+ equilibrium and lead to GABA

and glutamate uptake downmodulation by lowering the transport driving-force. This rationale was borne out from studies conducted in the RBA-2 type-2-like astrocytic cell line showing that P2X7 receptor stimulation increase intracellular Na^+ concentration and induce the downregulation of glutamate transport (Lo et al., 2008).

Although P2X7 receptors display low affinity for ATP (Jarvis and Khakh, 2009), high levels of ATP released during high-frequency stimuli (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) are able to activate this receptor *in vivo* under physiological conditions, thus promoting spatio-temporal coincidence of ATP with GABA and glutamate. Under conditions of high-frequency neuronal firing, there is also a decline in extracellular Ca^{2+} (Heinemann et al., 1977; Borst and Sakmann, 1999; Engelborghs et al., 2000; Stanley, 2000; Massimi and Amzica, 2001; Rusakov and Fine, 2003; Engel et al., 2012a; Torres et al., 2012; Jimenez-Pacheco et al., 2013) that can both trigger ATP release from astrocytes (Torres et al., 2012) and increase the activity of P2X7 receptors, since divalent ions (like Ca^{2+}) are negative modulators of this purinoceptor subtype (Virginio et al., 1997; Jiang, 2009; Yan et al., 2011) rendering P2X7 receptor activation an intense but short-lived modulatory capacity over Na^+-coupled high-affinity transport energy. Such low-affinity high-capacity mechanism may precede other forms of modulation involving activation of adenosine receptors following extracellular ATP breakdown (Nishizaki et al., 2002; Cristóvão-Ferreira et al., 2009) as well as low intracellular Ca^{2+} concentration (micromolar levels) modulation of both GABA and glutamate high-affinity transporters through enzymatic signaling cascades (Casado et al., 1993; Corey et al., 1994; Gonçalves et al., 1997; Beckman et al., 1998; Cordeiro et al., 2000; Cordeiro et al., 2003; Ashpole et al., 2013). It is, therefore, tempting to focus our research efforts on the energetic component of Na^+-coupled transport by performing most of the experiments in the absence of extracellular Ca^{2+}.

Since Na^+-coupled transport energy modulation may affect both GABA and glutamate transporters and given the antagonistic effects of GABA and glutamate on neuronal networks, added to evidences suggesting these neurotransmitter transporters may act concertedly to regulate extracellular levels of neurotransmitters, it seems necessary to study these in parallel, under the same modulatory conditions. Hence, the purpose of this study was to investigate whether

the stimulation of ATP-sensitive P2X7 receptors, in the absence of extracellular Ca^{2+} ions, modulates Na^{+}-dependent high-affinity GABA and glutamate uptake into synaptosomes of the rat cerebral cortex.

Experimental procedures

Drugs and solutions

HEPES and triton X-100 were from Merck Millipore (Darmstadt, Germany). GABA, SDS (sodium dodecyl sulphate), 2-mercaptoethanol, sodium deoxycholate, Tris (trizma-base), BSA (bovine serum albumin), bromophenol blue, carbenoxolone, glycerol, tween 20, BzATP, ouabain**,** EGTA (ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid), monensin sodium salt, NMDG (*N*-methyl-D-glucamine), aminooxyacetic acid (AOAA) and BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)) were obtained from Sigma-Aldrich (St. Louis, MO). L-Glutamic acid, A-438079, DL-TBOA (DL-*threo*-β-benzyloxyaspartic acid), H1152 ((S)-(+)-2-Methyl-1-[(4-methyl-5-iso-quinolinyl)sulfonyl]-hexahydro-1H-1,4-diazepine dihydrochloride), veratridine and TTX (tetrodotoxin) were obtained from Tocris Bioscience (Bristol, UK); SBFI-AM (1,3-benzenedicarboxylic acid, 4,4'-[1,4,10-trioxa-7,13-diazacyclopentadecane-7,13-diylbis (5-methoxy-6,12- benzofurandiyl)]bis-, tetrakis[(acetyloxy)methyl] ester) and Pluronic F-127 were from Invitrogen (Carlsbad, CA); gramicidin was from Life Technologies (Carlsbad, CA); SKF89976A hydrochloride (1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride) and calmidazolium (3-[bis(4-chlorophenyl)methyl]-1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy) ethyl] -1H-imidazolium chloride) were from Abcam (Cambridge, UK); [^{14}C]Glutamate and [^{3}H]GABA were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All stock solutions were stored as frozen aliquots. Dilutions of stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used, were observed.

Animals

Animal care and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and followed the European

Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for Care and Use of Laboratory animals (NIH Publications No. 80-23) revised 1996. All studies involving animals are reported in accordance with ARRIVE guidelines for reporting experiments involving animals (McGrath et al*.,* 2010). All efforts were made to minimize animal suffering and to reduce the number of animals used. Wistar rats (150-200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21 ºC) and a regular light (06.30–19.30 h)–dark (19.30–06.30 h) cycle, with food and water *ad libitum*.

Preparation of synaptosomes from the rat cerebral cortex

Synaptosomes were isolated as previously described by Helme-Guizon et al. (1998) and then modified by Bancila et al. (2009). Briefly, the cerebral cortex was dissected out and gently homogenized in cold oxygenated (95% O_2 and 5% CO_2) Krebs solution (in mM: glucose 5.5, NaCl 136, KCl 3, $MgCl_2$ 1.2, Na_2HPO_4 1.2, $NaHCO_3$ 16.2, $CaCl_2$ 0.5, pH 7.40). Homogenates were filtered through a nylon filter (mesh size 100 µm). The filtrate was left to sit during 30–45 min until formation of a pellet, which was re-suspended into Krebs solution and left at room temperature. Protein concentration determined by the BCA method (bicinchoninic acid; Pierce™, Thermoscientific, Rockford USA) was adjusted to 6.25 mg protein mL^{-1} (uptake and release experiments) or to 6 mg protein mL^{-1} (Na^+-influx measurement experiments).

[^{3}H]GABA and [^{14}C]glutamate uptake experiments

[^{3}H]GABA uptake by synaptosomes was measured as described elsewhere (Cordeiro et al., 2003). [^{3}H]GABA (0.25 µCi mL^{-1}; 70 Ci $mmol^{-1}$) uptake reactions were initiated by adding [^{3}H]GABA (0.5 µM, except otherwise specified) to media containing synaptosomes (final concentration 0.25 mg protein mL^{-1}), at 30 ºC. Unless otherwise indicated, uptake reactions were performed in media containing (in mM) NaCl 128, $MgCl_2$ 1.2, KCl 3, glucose 10, HEPES–Na 0.01 (pH 7.4), EGTA 0.1 and AOAA 0.01 (used to prevent GABA metabolism by GABAT). Experiments were performed in the absence of extracellular Ca^{2+} (except otherwise specified), in order to focus research on the energetics of Na^+-coupled transport. The reactions were stopped by rapid filtration through glass fibre prefilters (Merck Millipore, Cork, IRL), prewashed with cold sucrose 320 mM, Tris–HCl 10 mM (pH 7.4) and EGTA

0.1 mM. The filters were then washed with the same medium and plunged into vials containing scintillation cocktail (Insta-Gel Plus, Perkin Elmer, Boston, USA) for radioactivity measurement by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA). The values for [^{3}H]GABA taken up by synaptosomes were expressed as pmol mg protein^{-1} after subtraction of blank values obtained by filtering reaction medium aliquots. [^{14}C]Glutamate uptake (0.25 μCi mL^{-1}; 0.270 Ci mmol^{-1}) by synaptosomes was measured as described above for [^{3}H]GABA uptake but using [^{14}C]glutamate (10 μM) without adding AOAA (10 μM) to the medium. Unless stated otherwise, uptake assays were carried out during 90 seconds since this time is in the linear phase of [^{3}H]GABA and [^{14}C]Glutamate accumulation (see e.g. Figure 6).

All modifier drugs tested were allowed to equilibrate with the synaptosomes at least for 10 min before adding the test drug, which was applied 10 min before addition of the radioactive neurotransmitter. Control samples were incubated for the same amount of time in the absence of drugs. To assess synaptosomal integrity before and after incubation with the P2X7 receptor agonist (BzATP) we evaluated the activity of the intracellular enzyme, lactate dehydrogenase (LDH, EC 1.1.1.27), in the incubation medium by the method of Stolzenbach and Kaplan (1976).

[^{3}H]GABA and [^{14}C]glutamate release experiments

[^{3}H]GABA release by synaptosomes was measured after loading the synaptosomes with [^{3}H]GABA (0.25 μCi mL^{-1}; 70 Ci mmol^{-1}; 0.5 μM) during 10 min, at 30 ºC. Aliquots of a synaptosomal suspension containing 0.5 mg protein mL^{-1} were layered onto glass fiber filters (Merck Millipore, Cork, IRL), which were mounted in 365 μL chambers of a semi-automated 12-sample superfusion system (SF-12 Suprafusion 1000, Brandel, Gaithersburg, MD, USA). Filters containing the synaptosomes were superfused (flow rate of 0.5 mL min^{-1}) with a Ca^{2+}-free physiological solution (in mM: NaCl 128, $MgCl_2$ 1.2, KCl 3, glucose 10, HEPES–Na 0.01 (pH 7.4), EGTA 0.1 and AOAA 0.01), at 30 ºC. After a 26–min equilibration period, 2-min fractions of the superfusate were automatically collected using the SF-12 suprafusion system; this procedure was prolonged for 34 min. Ten-min after beginning fraction collection, synaptosomes were challenged with BzATP (100 or 300 μM) or veratridine (10 μM) during 2 min by changing the inlet tube from one flask to another containing the test drug. The P2X7 receptor antagonist, A-438079

(3 µM), was added to the superfusion solution 10 min before BzATP. The radioactive content of collected fractions and that remaining in the filters at the end of the experimental protocol was measured by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA). [^{14}C]Glutamate release (0.25 µCi mL^{-1}; 0.270 Ci mmol^{-1}; 10 µM) by synaptosomes was measured as described for [^{3}H]GABA without adding AOAA (10 µM) to the superfusion solution.

Experiments to measure Na^+-influx by synaptosomes

Measurements of Na^+ influx into synaptosomes was performed as described previously (Tretter et al., 1998), with minor modifications. Synaptosomes (6 mg protein mL^{-1}) were incubated in Na^+-free medium (in mM: NMDG 128, $MgCl_2$ 1.2, $CaCl_2$ 0.1, KCl 3, glucose 10 and HEPES–Na 0.01; pH 7.4) with the cell-permeant sodium selective fluorescent indicator, SBFI-AM (15 µM), for 60 min at 37 °C. After sedimentation and washing with Na^+-free medium, the pellet was re-suspended in the same medium, and 20 µL aliquots were used in wells of a 96-well plate containing 180 µL of reaction medium (in mM: NaCl 128, $MgCl_2$ 1.2, $CaCl_2$ 0.1, KCl 3, glucose 10 and HEPES–Na 0.01; pH 7.4) in a final concentration of 6 mg mL^{-1}. SBFI-AM fluorescence trapped within synaptosomes was measured using a multi detection microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA) according to manufacturer's instructions**.** After a 5 min equilibrium period, synaptosomes were stimulated with medium (control) or BzATP and SBFI fluorescence was allowed to reach a new steady-state. Thereafter, the Na^+ ionophore, gramicidin (10 µM), was applied to provide maximum SBFI signal. When the influence of a modifier drug over the BzATP was assayed, modifier drug was allowed to equilibrate with the synaptosomes for 10 min before the addition of BzATP. Control samples were incubated for the same amount of time in the absence of drugs.

Immunofluorescence staining and confocal microscopy

Brain slices

Brain samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; in mM: NaCl 137, KCl 2.6, Na_2HPO_4 4.3, KH_2PO_4 1.5; pH=7.4) for about 48 h (4 ºC), cryopreserved in 30% sucrose in PBS and stored in a tissue freezing medium at -80 ºC.

Free floating 30 µm brain slices were incubated for 1 h, at room temperature, with blocking buffer I (foetal bovine serum 10%, BSA 1%, triton X-100 0.5%, NaN_3 0.05%) and subsequently incubated overnight, at 4 ºC, with the following primary antibodies; rabbit anti-P2X7 receptor (1:50, #APR004, Alomone, Jerusalem, Israel), goat anti-synaptic vesicle-associated membrane protein 1 (VAMP-1 or synaptobrevin 1; 1:20, R&D Systems, Minneapolis, MN), mouse anti-glial fibrillary acidic protein (GFAP; 1:350, Chemicon, Temecula, CA), mouse anti-S-100 clone 15E2E2 (1:200; Chemicon, Temecula, CA) and mouse anti-CD11b (1:50, Santa Cruz Biotechnology, Dallas, TX) diluted in blocking buffer II (foetal bovine serum 5%, BSA 0.5%, triton X-100 0.5%, NaN3 0.05% in PBS). Sections were rinsed in PBS supplemented with triton X-100 0.5% (3 cycles of 10 min) and incubated for 120 min with species specific secondary antibodies conjugated with fluorescent dyes (donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 568; donkey anti-goat Alexa 633) diluted in blocking buffer II, at room temperature. After rinsing in PBS, slices were mounted on optical-quality glass slides using VectaShield (Vector Labs, Peterborough, UK) as mounting media. Observations were performed with a laser scanning confocal microscopy (Olympus FV1000, Tokyo, Japan). Controls were performed by following the same procedure but replacing the primary antibodies with the same volume of blocking buffer II. Images were analyzed using the Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, Japan). Co-localization was assessed by calculating the staining overlap and the Pearson's coefficient (ρ) for each confocal micrograph stained with two fluorescent dyes. Overlap between two stainings gives a value between +1 and 0 inclusive, where 1 is total overlap and 0 is no overlap. ρ is a measure of the linear correlation between two variables (stainings), giving a value between +1 and −1 inclusive, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation.

Cortical synaptosomes

The immunocytochemical analysis in cortical synaptosomes was performed as previously described (Miras-Portugal et al., 2003; Rodrigues et al., 2005; Marcoli et al., 2008). Briefly, synaptosomes were placed onto chamber slides coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO), fixed with 4% paraformaldehyde in

PBS for 15 min, and washed twice with PBS. Synaptosomes were permeabilized in PBS with 0.2% triton X-100 for 10 min and then blocked for 1h in PBS with 3% BSA and 5% foetal bovine serum. The synaptosomes were then washed twice with PBS and incubated overnight, at 4 ºC, with the following primary antibodies diluted in 3% BSA in PBS: rabbit anti-P2X7 receptor (1:50, #APR004, Alomone, Jerusalem, Israel) and goat anti-VAMP-1 (1:20, R&D Systems, Minneapolis, MN). Then, synaptosomes were rinsed with 3% BSA in PBS (3 cycles of 10 min) and incubated 1h at room temperature with species specific secondary antibodies conjugated with fluorescent dyes: donkey anti-rabbit IgG Alexa Fluor 488 and donkey anti-goat IgG Alexa Fluor 568. After rinsing in PBS and mounting on slices using VectaShield (Vector Labs, Peterborough, UK) as mounting media, the synaptosomes were observed with a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan). Controls were performed by following the same procedure but replacing the primary antibodies by the same volume of 3% BSA in PBS. Images were also analyzed using Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, Japan). Co-localization was assessed by calculating the staining overlap and the ρ for each confocal micrograph stained with two fluorescent dyes.

SDS-PAGE and Western blot analysis

Total membrane lysates and synaptosomes of the rat cerebral cortex were homogenized in Radio-Immunoprecipitation Assay (RIPA) buffer containing: Tris-HCl (pH 7.6) 25 mM, NaCl 150 mM, sodium deoxycholate 1%, triton-X-100 1%, SDS 0.1%, EDTA 5 mM and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein content of the samples was evaluated using the BCA method. Samples were solubilized at 70 °C in SDS reducing buffer (Tris-HCl (pH 6.8) 125 mM, SDS 4%, bromophenol blue 0.005%, glycerol 20%, and 2-mercaptoethanol 5%) for 10 min, subjected to electrophoresis in 12.5% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Merck MilliPore, Temecula, CA). Membranes were blocked for 1 h in Tris-buffered saline (TBS; in mM: Tris-HCl 10 (pH 7.6), NaCl 150) containing Tween 20 0.05% and BSA 5% and, subsequently, incubated overnight, at 4 ºC, with primary antibodies: rabbit anti-P2X7 receptor (1:300, #APR004, Alomone, Jerusalem, Israel), rabbit anti-β-actin antibody (1:2500, Abcam, Cambridge, UK), mouse anti-synaptophysin (1:1000, Chemicon, Temecula, CA) and mouse anti-GFAP (1:500, Chemicon,

Temecula, CA). Membranes were washed three times for 10 min in 0.05% Tween 20 in TBS and then incubated with horseradish anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies for 120 min, at room temperature. The antigen-antibody complexes were visualized by chemiluminescence with the Immun-Star WesternC Kit (Biorad Laboratories, Hercules, CA) using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA). To test for specificity of the bands corresponding to P2X7 the anti-P2X7 receptor antibody was pre-adsorbed with a control peptide antigen corresponding to the amino acid residues 576-595 of the intracellular C-terminus of the rat P2X7 receptor, before incubation with the membrane. Gel band image densities were quantified with Image J (National Institute of Health, USA).

Data presentation and statistical analysis

The uptake of [^{3}H]GABA, [^{14}C]glutamate and Na^{+} by synaptosomes of the rat cerebral cortex was expressed as a percentage of control values obtained in the same synaptosomal batch without adding any drug. The release of [^{3}H]GABA and [^{14}C]glutamate by synaptosomes was expressed as the ratio of the peak area obtained with test drug and the peak area obtained with a reference compound, veratridine. Results are expressed as mean ± SEM, with *n* (showed in graphs) indicating the number of individual experiments performed in a given situation. Because of limited inter-individual variation, randomly chosen groups of at least three animals of the same strain and weight (Wistar rats of 150-200 g) were considered sufficient to replicate each experimental protocol; individual experiments were performed in triplicate (uptake experiments) or in duplicate (release experiments and Na^{+} measurement experiments). Statistical analysis of data was carried out using GraphPad Prism 6.04 software (La Jolla, CA, USA). Unpaired Student's *t*-test with Welch correction was used for statistical analysis when parametric data was considered. For multiple comparisons, one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test was used. $P < 0.05$ (two tailed) values were considered to show significant differences between means.

Results

Synaptosomes of the rat cerebral cortex are enriched in synaptophysin-positive nerve terminals

In order to confirm that the synaptosomal fraction of the rat cerebral cortex (see Materials and Methods) is enriched in nerve terminals, we analyzed the relative protein density of synaptophysin and GFAP, which are well-recognized markers respectively of synaptic nerve terminals and astrocytic glial cells, in total lysates and synaptosomal fractions by Western blot. Figure 5 shows that cortical synaptosomal fractions are highly (~60-fold) enriched in synaptophysin compared to total lysates, where the protein density difference between synaptophysin and GFAP is less evident. This indicates that, under the present experimental conditions, the functional results obtained with synaptosomes of the rat cerebral cortex are most likely to be attributed to synaptic nerve terminal enrichment, with a residual (if at all) participation of glial subcellular particles (gliosomes, see Figure 5B; Milanese et al., 2009; Carney et al., 2014).

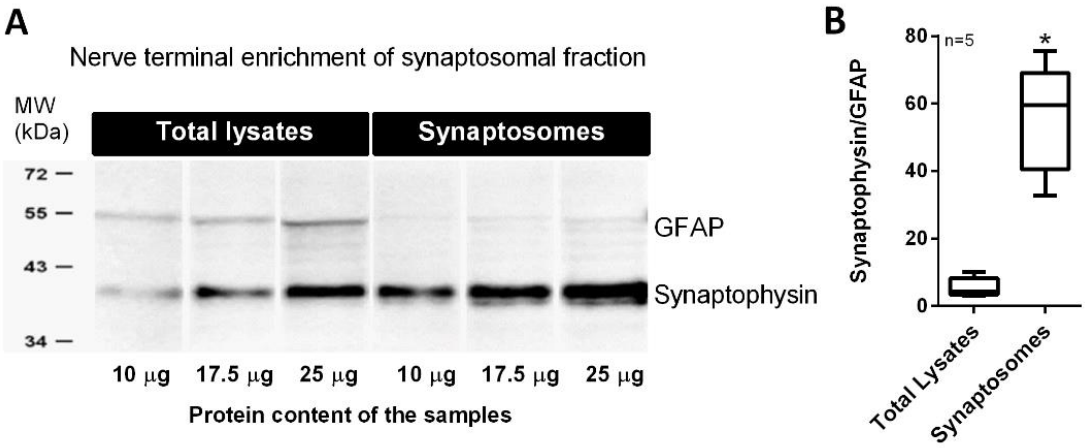


Figure 5 – Western blot analysis of synaptophysin and GFAP contents in synaptosomal fractions and total lysates of the rat cerebral cortex. Panel (A), shows representative immunoblots of five distinct animals loaded with three different protein amounts (10, 17.5 and 25 µg). In Panel (B), shown are "Box and Whiskers" plots representing the ratios between synaptophysin and GFAP protein densities obtained in total lysates and synaptosomal fractions of five distinct animals; in each box represented is the minimum, the 25th percentile, the median, the 75th percentile, and the maximum. **P*<0.05 (unpaired Student's *t*-test with Welch correction) represents significant differences as compared to total lysates. Please note that under the present experimental conditions the synaptosomal fraction is highly enriched in synaptophysin-immunoreactive synaptic nerve terminals, whereas the negative GFAP immunodetection denotes very little (if at all) contamination of this fraction by glial subcellular particles (gliosomes).

GABA and glutamate uptake is mediated by Na^+-dependent high-affinity transporters in synaptosomes of the rat cerebral cortex

Before evaluating the influence of any drug on the accumulation of GABA and glutamate by synaptosomes of the rat cerebral cortex, experiments were designed to assess the accumulation of both neurotransmitters over time and to determine the most adequate time of incubation with [^{3}H]GABA and [^{14}C]glutamate for the subsequent experiments. Under the present experimental conditions, the time course of [^{3}H]GABA and [^{14}C]glutamate uptake by synaptosomes of the rat cerebral cortex (Figure 6A and 6B) was similar to that previously described (Rauen et al., 1992; Cordeiro et al., 2003). All subsequent experiments were performed with a 90s incubation time so that [^{3}H]GABA and [^{14}C]glutamate uptake were measured in the linear accumulation phase.

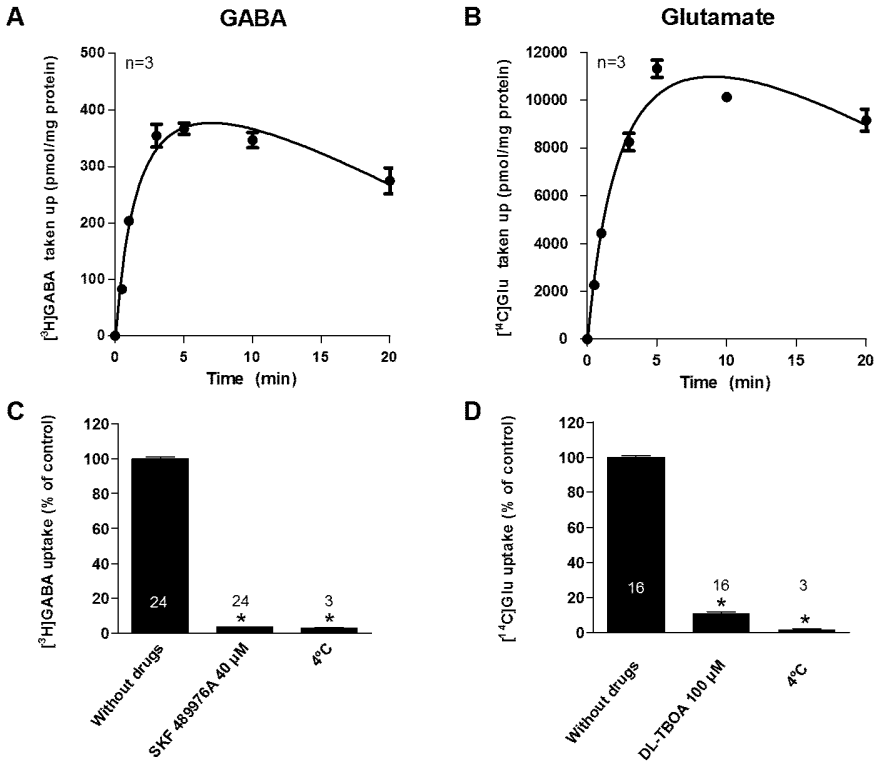


Figure 6 – Rat cortical synaptosomes take up GABA and glutamate through Na^+-dependent high-affinity transporters. Illustrated is the accumulation of [^{3}H]GABA (A) and [^{14}C]glutamate (B) into rat cortical synaptosomes over time (0-20 min). Panels (C) and (D) show that the accumulation of [^{3}H]GABA and [^{14}C]glutamate (t=90 s) occurs through Na^+-dependent high-affinity transporters since the incubation of synaptosomes with inhibitors of transporters, SKF489976A (40 µM; GAT1 selective inhibitor) and DL-TBOA (100 µM; non-specific EAAT inhibitor), abolished neurotransmitter uptake, respectively. The results are expressed as mean ± SEM; the vertical bars represent SEM and are shown when they exceed the symbols in size; the *n* number of individual experiments is shown in the graphs. **P*<0.05 (unpaired Student's *t*-test with Welch correction) represents significant differences as compared to the control situation. SKF 489976A and DL-TBOA were incubated 20 min before addition of [^{3}H]GABA or [^{14}C]Glutamate, respectively.

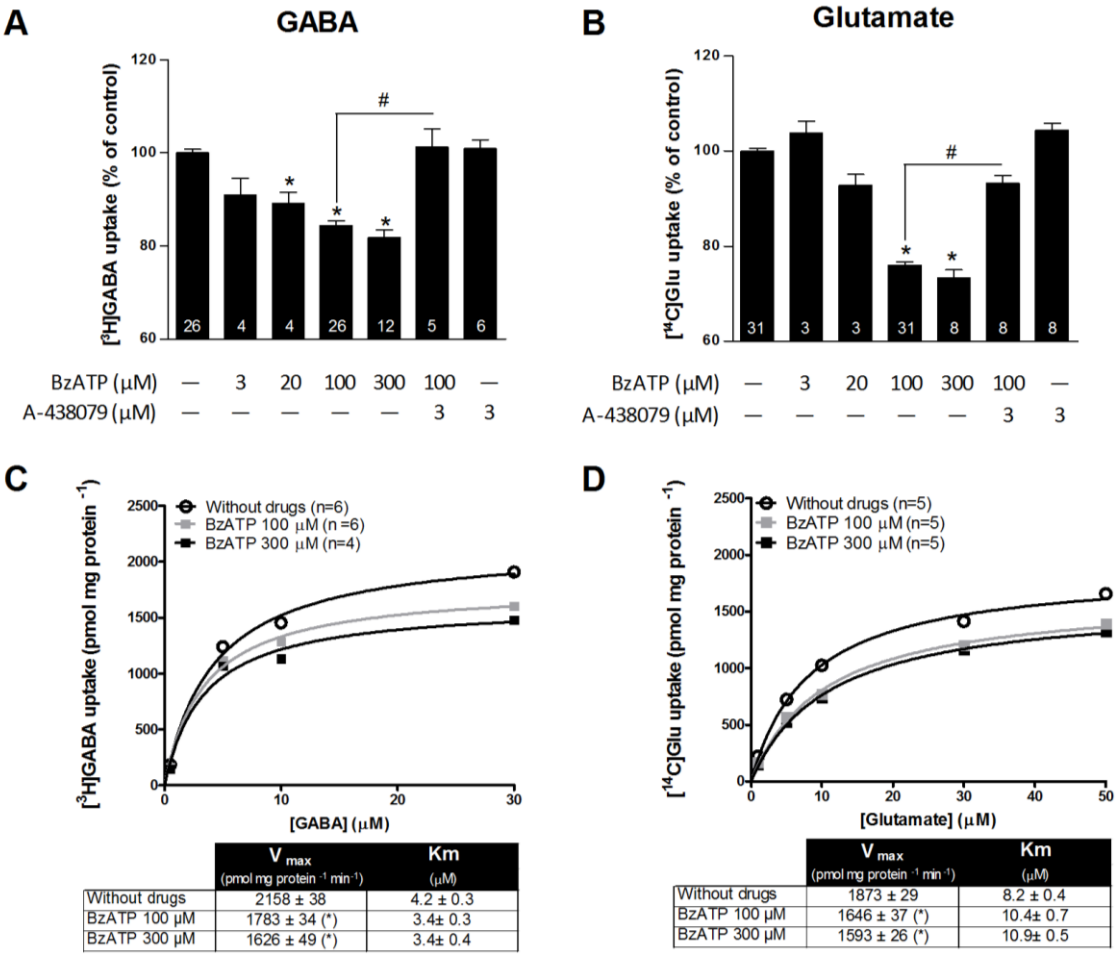
We observed that [^{3}H]GABA accumulation was significantly inhibited (96.4±0.2%) in the presence of a potent inhibitor of GABA transporter 1 (GAT1) SKF89976A (40 µM) (Figure 6C). Likewise, [^{14}C]glutamate uptake was significantly inhibited (89.3±1.1%) in the presence of DL-TBOA (100 µM) (Figure 6D); here, we used a non-selective inhibitor because subtype-specific glutamate transport inhibitors are still missing. Data suggest that the uptake of both neurotransmitters by synaptosomes occurs almost exclusively through high-affinity transporters, making unlikely the participation of other membrane translocation mechanisms for GABA and glutamate, like connexin hemichannels, that are expected to open in the absence of extracellular Ca^{2+}; GAT1 seems to be the dominant GABA transporter in synaptosomes of the rat cerebral cortex.

P2X7 receptor activation downmodulates high-affinity [^{3}H]GABA and [^{14}C]glutamate uptake

To evaluate the role of the P2X7 purinoceptor in the modulation of GABA and glutamate uptake, cortical synaptosomes were incubated with the prototypic P2X7 receptor agonist, BzATP (3-300 µM), during 10 minutes before the addition of [^{3}H]GABA and [^{14}C]glutamate. BzATP concentration-dependently decreased the uptake of [^{3}H]GABA (Figure 7A) and [^{14}C]glutamate (Figure 7B) by synaptosomes of the rat cerebral cortex. The inhibitory effects produced by 100 µM BzATP (15.6±1.0% and 24.0±0.8%, respectively for GABA and glutamate) were significantly ($P<0.05$) attenuated by a selective P2X7 receptor antagonist, A-438079 (3 µM; Figure 7A and 7B); on its own A-438079 (3 µM) did not affect the uptake of neurotransmitters. These results suggest that activation of the P2X7 purinoceptor negatively modulates the uptake of GABA and glutamate by cortical synaptosomes. The amplitude of glutamate uptake downmodulation by P2X7 was greater than that of GABA.

We, then, assessed the influence of the P2X7 receptor activation on the kinetics of amino acid transporters by evaluating changes in the Michaelis-Menten constant (K_m) and in the maximum uptake velocity (V_{max}). As can be concluded from the saturation curves depicted in Figure 7, BzATP (100-300 µM) decreased the V_{max} of [^{3}H]GABA (Figure 7C) and [^{14}C]glutamate (Figure 7D) uptake in a concentration-dependent manner, with minor changes in K_m values, contributing to the decrease in transport efficiency; 100 and 300 µM BzATP decreased ($P<0.05$) V_{max} values for

the uptake of [^{3}H]GABA from 2158±38 to 1783±34 and to 1626±49 pmol mg protein^{-1} min^{-1} and for the uptake of [^{14}C]glutamate from 1873±29 to 1646±37 and to 1593±26 pmol mg protein^{-1} min^{-1}, respectively.



	V_{max} (pmol mg protein $^{-1}$ min^{-1})	Km (μM)
Without drugs	2158 ± 38	4.2 ± 0.3
BzATP 100 μM	1783 ± 34 (*)	3.4± 0.3
BzATP 300 μM	1626 ± 49 (*)	3.4± 0.4

	V_{max} (pmol mg protein $^{-1}$ min^{-1})	Km (μM)
Without drugs	1873 ± 29	8.2 ± 0.4
BzATP 100 μM	1646 ± 37 (*)	10.4± 0.7
BzATP 300 μM	1593 ± 26 (*)	10.9± 0.5

Figure 7 - Activation of the P2X7 receptor downmodulates Na^+-dependent high-affinity GABA and glutamate transport into synaptosomes of the rat cerebral cortex. Illustrated is the inhibition of [^{3}H]GABA (A) and [^{14}C]glutamate (B) uptake triggered by activation of the P2X7 receptor with BzATP (3-300 μM), a prototypical P2X7 receptor agonist. The inhibitory effect of 100 μM BzATP was prevented by the P2X7 receptor antagonist, A-438079 (3 μM). On its own, A-438079 (3 μM) did not influence GABA nor glutamate uptake. Panels (C) and (D) show the saturation curves depicting the amount of [^{3}H]GABA and [^{14}C]glutamate, respectively, taken up as a function of the concentration of neurotransmitter. The tables below each panel show the K_m (μM) and V_{max} (pmol.mg protein^{-1}.min^{-1}) values determined from the saturation curves obtained in the absence and in the presence of BzATP (100-300 μM),using the GraphPad Prism 6.04 software (La Jolla, CA, USA). Data are expressed as mean ± SEM; the vertical bars represent SEM and are shown when they exceed the symbols in size; the *n* number of individual experiments is shown in the graphs. *P<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation where no drugs were added; #P<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of BzATP 100 μM applied alone. BzATP was incubated for 10 min before addition of [^{3}H]GABA or [^{14}C]Glutamate and A-438079 was added 10 min before the P2X7 receptor agonist.

BzATP (100 µM) did not significantly (*P*>0.05) change the activity of LDH in the incubation fluid of rat cortical synaptosomes (0.27±0.02 mU mL^{-1}, n=3) compared to the control situation in the absence of the ATP analogue (0.29±0.06 mU mL^{-1}, n=3). Data indicate that rat cortical synaptosomes keep their integrity after being exposed to BzATP, a situation that is in clear contrast with the findings obtained after disrupting synaptosomal membranes with 1% of triton X-100, which leads to extrusion of intrasynaptosomal content increasing LDH activity in the extracellular media to 3.27±0.10 mU mL^{-1} (*P*<0.05 , n=3).

The P2X7 receptor is expressed predominantly on nerve terminals of the rat cerebral cortex

The presence of functional P2X7 receptors on cortical nerve terminals (synaptosomes) of the rat has been demonstrated by measuring fluorescent intracellular Ca^{2+} signals and by immunocytochemistry staining (Miras-Portugal et al., 2003; Alloisio et al., 2008; Marcoli et al., 2008; Marín-García et al., 2008). Here, we used slices of the rat cerebral cortex stained with an antibody that specifically reacts with the P2X7 receptor in the brain (Messemer et al., 2013) to evaluate the distribution of this receptor among distinct neuronal cells by immunofluorescence confocal microscopy (Figure 8A). To this end, we labelled the cells with antibodies directed towards: (1) a synaptic nerve terminal marker, the VAMP-1; (2) two astrocytic markers, the Ca^{2+} binding protein A1/B1 (S-100, clone 15E2E2) and GFAP; and (3) a microglial cell marker, the alpha M integrin (CD11b). Figure 8A shows that the highly specific antibody directed against the C-terminal of the rat P2X7 receptor (#APR-004, green) co-localize extensively (staining overlap of 0.73±0.04; ρ=0.59±0.04) with the synaptic nerve terminal marker, VAMP-1 (red), giving the final yellow labelling when merging the two fluorescence channels. The P2X7 receptor labelling was also observed in some CD11b positive (red) microglial cells of the rat cerebral cortex (staining overlap of 0.46±0.04; ρ=0.32±0.06), but no color merge was detected with GFAP (staining overlap of 0.07±0.01; ρ=0.01±0.01) nor with S-100 (staining overlap of 0.16±0.02; ρ=-0.01±0.01) astrocytic cells (Figure 8A). Likewise, co-localization of P2X7 (green) and VAMP-1 (red) staining was also observed in synaptosomes isolated from the rat cerebral cortex (staining overlap of 0.62±0.04; ρ=0.61±0.04) (Figure 8B), which is in agreement with findings from electron microscopy and immunofluorescence labelling of P2X7 receptors found in

the literature (Deuchars et al., 2001.; Sperlágh et al., 2002; Miras-Portugal et al., 2003; Cavaliere et al., 2004).

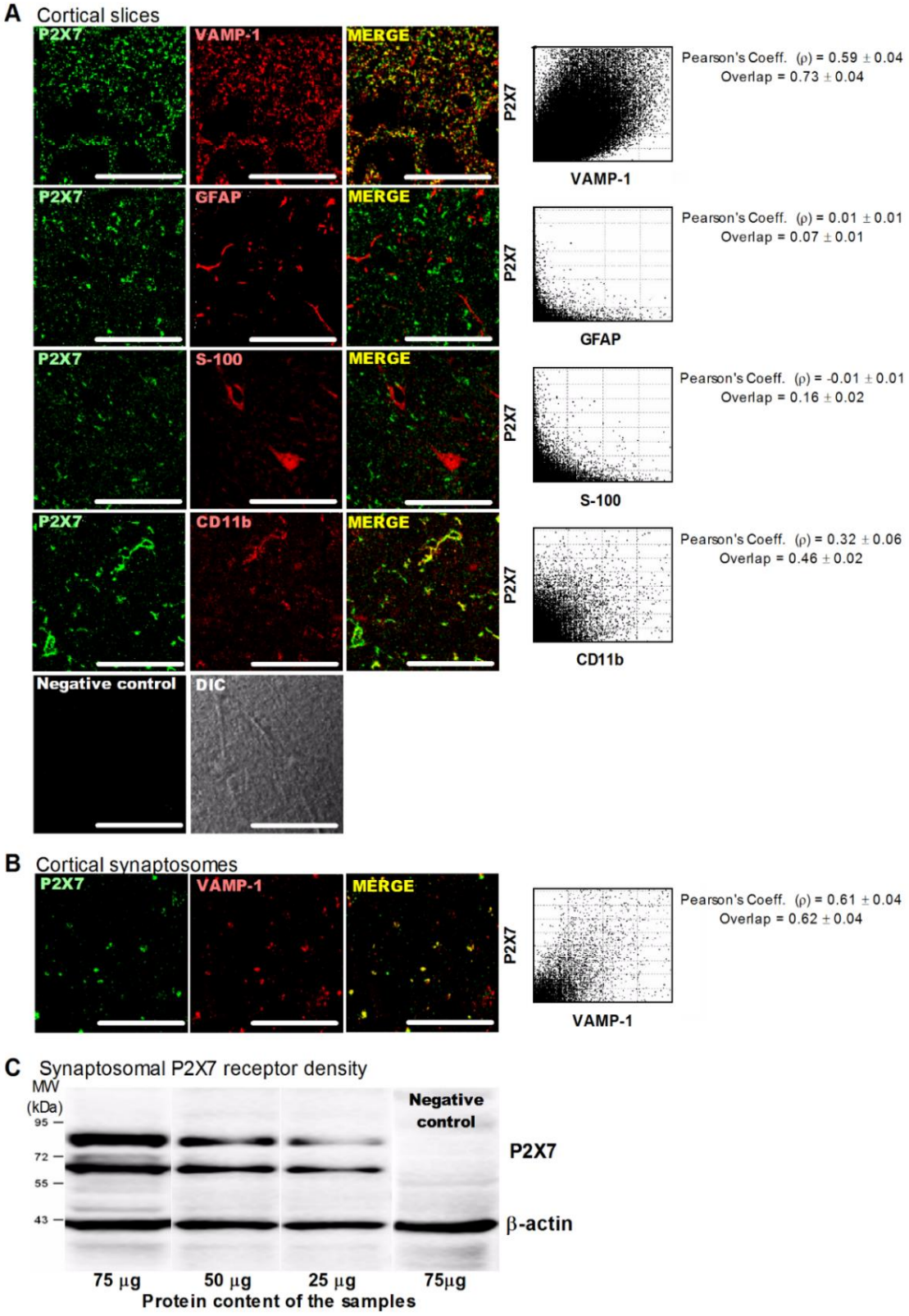


Figure 8 - The P2X7 receptor is present predominantly on nerve terminals of the rat cerebral cortex. Panel (A) shows the immunolocalization of the P2X7 receptor in confocal micrographs of rat cortical

slices. Synaptic nerve terminals are stained with VAMP-1; astrocytes are labelled with the Ca^{2+} binding protein A1/B1 (S-100, clone 15E2E2) and GFAP, whereas CD11b was used to stain microglial cells. A negative control resulting from the incubation with the anti-rabbit secondary antibody without previous addition of the rabbit anti-P2X7 receptor primary antibody (#APR004) is also shown; differential interference contrast (DIC) image is shown for comparison in the latter situation. Confocal micrographs shown in Panel (B), show that VAMP-1-positive synaptosomes (red) of the rat cerebral cortex are endowed with the P2X7 receptor (green). Yellow staining denotes co-localization of P2X7 receptors (green) and type-specific cell markers (red). Fluorescence intensity scatter plots shown on the right-hand side of the figure were used to estimate co-localization by calculating the staining overlap and the Pearson´s Coefficient (ρ). Scale bars = 50 μm. In Panel (C), shown are representative immunoblots of the P2X7 receptor in cortical synaptosomes from eight rats; gels were loaded with 25, 50 and 75 μg of protein. Please note that rat cortical synaptosomes express the P2X7 receptor as a double band at ~ 72 kDa; both bands are specific as they disappeared after pre-adsorption of antibody with the control peptide antigen corresponding to the amino acid residues 576-595 of the intracellular C-terminus of the rat P2X7 receptor (negative control; lane 4). β-actin was used as a reference protein.

In addition, Western blot analysis confirmed that rat cortical synaptosomes express the P2X7 receptor at the protein level (Figure 8C). Please note that rat cortical synaptosomes expressed the P2X7 receptor as a double band at ~72 kDa (Künzli et al., 2007; Jimenez-Pacheco et al., 2013; Yu et al., 2013), which densities increase with the amount of protein (25-75 μg) loaded to the gels. Both bands disappeared after pre-adsorption of synaptosomal membranes with the control peptide antigen corresponding to the amino acid residues 576-595 of the intracellular C-terminus of the rat P2X7 receptor (Figure 8C). Taken together, these results support our pharmacological data implicating the P2X7 receptor in the negative control of [^{3}H]GABA (Figure 7A) and [^{14}C]glutamate (Figure 7B) uptake by nerve terminals (synaptosomes) of the rat cerebral cortex.

Downmodulation of [^{3}H]GABA and [^{14}C]glutamate transport by P2X7 receptor activation correlates with drug-induced shifts in the synaptosomal Na^+ concentration

Experiments were designed to unravel the mechanism(s) underlying downmodulation of GABA and glutamate uptake by activation of the P2X7 purinoceptor.

Knowing that P2X7 receptor activation promotes the influx of Na^+ and Ca^{2+} from the extracellular milieu and taking into account that extracellular Ca^{2+} was removed from the reaction medium (see Materials and Methods), it was hypothesized that Na^+-entry through the P2X7 receptor channel and subsequent dissipation of the Na^+-gradient may be responsible for the negative modulation of

high-affinity GABA and glutamate transport, which is crucially dependent on the Na^+-gradient.

To test the validity of this hypothesis, the effects of two Na^+ ionophores, monensin and gramicidin, were evaluated. Monensin, is a polyether antibiotic isolated from *Streptomyces cinnamonensis*; ionophore properties of monensin are related to the preference of crown ethers to form complexes with monovalent cations, such as Li^+, Na^+, K^+, Rb^+, Ag^+, and Ti^+, thus conferring these ions the possibility to cross lipid cell membranes in both electrogenic and electroneutral (*i.e.* non-depolarizing) conditions (Huczyński et al., 2012). Na^+ influx caused by monensin may favor Na^+/Ca^{2+} exchange and Ca^{2+}-induced activation of PLC (Wang et al., 1999), yet this mechanism is negligible under extracellular null Ca^{2+} conditions, as in this study. Gramicidin is a heterogeneous mixture of three linear pentadecapeptide antibiotic compounds, gramicidins A, B and C, obtained from the soil bacterial species *Bacillus brevis*. Gramicidin chains assemble inside of the hydrophobic interior of the cellular lipid bilayer to form a β-helix, which then dimerizes to form the elongated channel that spans the whole membrane. Its mode of action results from increasing cell membrane permeability selectively to inorganic monovalent cations, like Na^+, which travel unrestricted through the channel in a single file coordinated with the same number of water molecules thereby destroying the ion gradient between the cytoplasm and the extracellular environment (Burkhart et al., 1999). Interestingly, divalent cations, like Ca^{2+}, block the channel by binding near its mouth, so gramicidin channel is essentially impermeable to divalent cations; it also excludes anions, like Cl^-, because its hydration shell is thermodynamically stronger than that of most monovalent cations. Figure 9 shows that the inhibitory effect of BzATP (3-300 μM) was mimicked by monensin (0.1-1μM) and gramicidin (0.1-1μM), although the ATP analogue exhibited a lesser potency. At 1 μM concentration, the two Na^+ ionophores, monensin and gramicidin, decreased [^{3}H]GABA uptake by 36.7±2.0% and 56.7±4.3% (Figure 9A) and [^{14}C]glutamate uptake by 35.3±2.3% and 31.7±4.6% (Figure 9B), respectively.

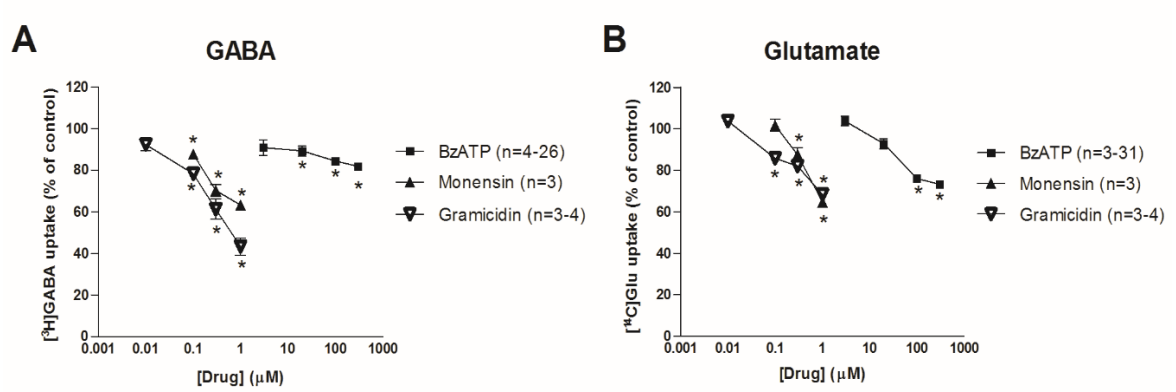


Figure 9 - Intracellular Na^+-accumulation decreases GABA and glutamate uptake. Illustrated is the role of two Na^+ ionophores, monensin (0.1-1μM) and gramicidin (0.1-1μM), on [^{3}H]GABA (A) and [^{14}C]glutamate (B) uptake into rat cortical synaptosomes carried out during 90 seconds. The effect of BzATP (3-300 μM) is also shown for comparison. The results are expressed as mean ± SEM; the vertical bars represent SEM and they are expressed when they exceed the symbols in size; the *n* number of individual experiments is shown in the graphs. **P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation; BzATP, monensin and gramicidin were incubated 10 min before addition of [^{3}H]GABA or [^{14}C]Glutamate.

We, then evaluated whether manipulation of intracellular Na^+-levels could modulate GABA and glutamate uptake, using for this purpose two drugs with distinct mechanisms of action: ouabain (an inhibitor of Na^+/K^+-ATPase) and veratridine (an activator of voltage-sensitive Na^+ channels). Activation of voltage-gated Na^+ channels with veratridine (1-10 μM) concentration-dependently decreased the uptake of [^{3}H]GABA (Figure 10A) and [^{14}C]glutamate (Figure 10B). The inhibitory effects produced by 10 μM veratridine (77.1±1.3% and 43.7±1.5%, for GABA and glutamate respectively) were prevented by blocking voltage-gated Na^+ channels with TTX (1 μM; Figures 10A and 10B), while the inhibitory effects produced by 100 μM BzATP on the uptake of [^{3}H]GABA and [^{14}C]glutamate were not significantly altered by TTX (1 μM; data not shown). On its own TTX (1 μM) did not influence the uptake of both neurotransmitters. The inhibitory effects of 100 μM BzATP on [^{3}H]GABA and [^{14}C]glutamate uptake (37.7±2.3% and 36.3±2.0%, respectively) were still visible when the P2X7 agonist was applied on top of 1 μM veratridine, which on its own decreased the uptake of [^{3}H]GABA by 26.5±1.0% and [^{14}C]glutamate by 21.5±2.3%. The uptake inhibition of both neurotransmitters by 100 μM BzATP becomes less apparent upon increasing the concentration of veratridine to 3 or 10 μM applied together with the P2X7 receptor agonist (Figures 10A and 10B).

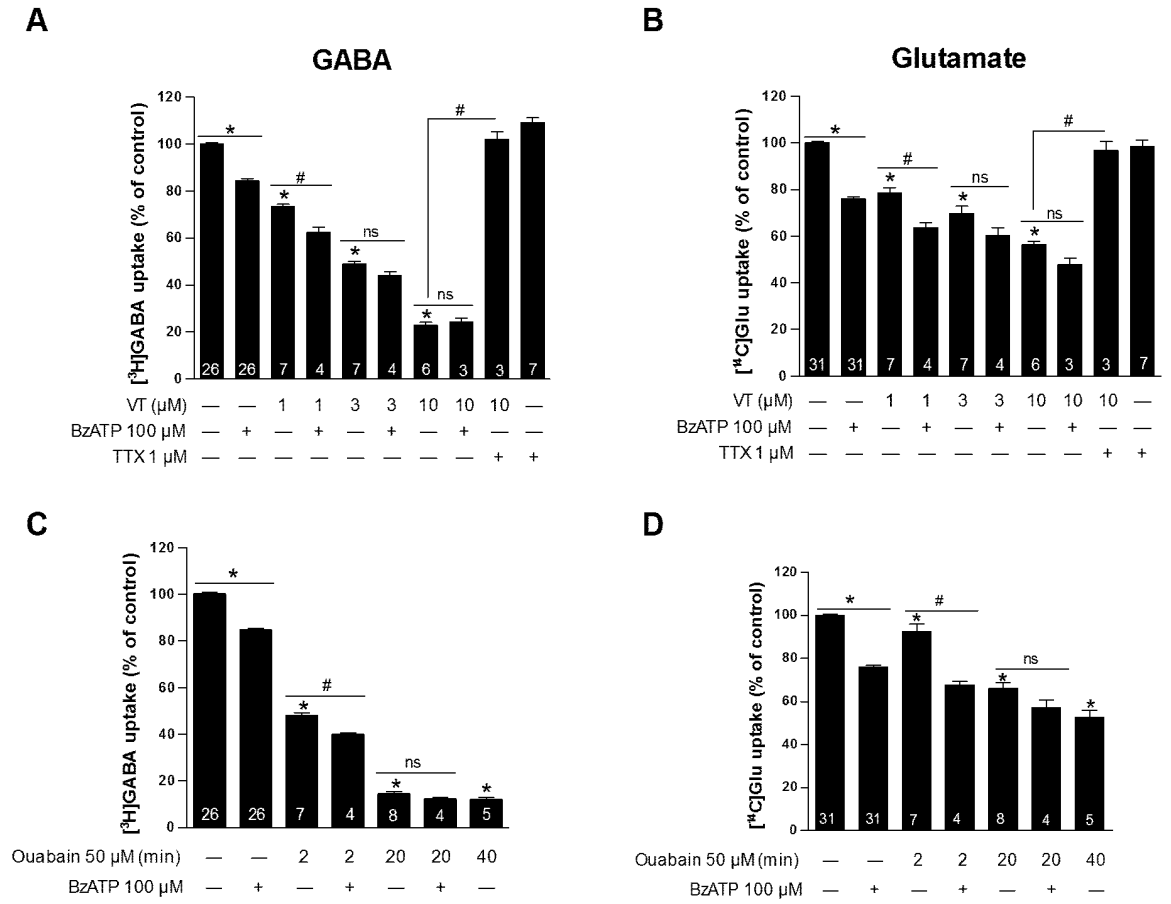


Figure 10 - Increase in intracellular Na^+-levels attenuates the inhibitory effect of BzATP on GABA and glutamate uptake. Top panels show the inhibitory effect of veratridine (1-10 μM), an activator of voltage-gated Na^+-channels, on [^{3}H]GABA (A) and [^{14}C]glutamate (B) uptake into rat cortical synaptosomes evaluated in the absence and in the presence of BzATP (100 μM). The effect of veratridine 10 μM was inhibited by the voltage-gated Na^+-channel blocker, TTX (1 μM). On its own, TTX (1 μM) did not influence GABA nor glutamate uptake. Bottom panels show the inhibitory effect of the Na^+/K^+-ATPase inhibitor, ouabain (50 μM), on [^{3}H]GABA (C) and [^{14}C]glutamate (D) uptake into rat cortical synaptosomes carried out during 90 seconds as a function of the time of incubation (2-40 min) measured in the absence and in the presence of BzATP (100 μM). The results are expressed as mean ± SEM; the vertical bars represent SEM; the *n* number of individual experiments is shown in the graphs. **P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation; #*P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of veratridine (top panels) or ouabain (bottom panels) alone; ns, non-significant. BzATP and veratridine were incubated 10 min before addition of [^{3}H]GABA or [^{14}C]Glutamate and ouabain was incubated for 2, 20 and 40 min before addition of [^{3}H]GABA or [^{14}C]Glutamate. TTX was incubated 10 min before veratridine application.

Inhibition of Na^+/K^+ ATPase with 50 μM ouabain progressively decreased the uptake of [^{3}H]GABA (to a maximum of 88.2±1.0%; Figure 10C) and [^{14}C]glutamate (to a maximum of 47.4±3.2%; Figure 10D) depending on the time of incubation with this drug (2-40 min). Different incubation times with ouabain were used in order to promote a gradual increase of intracellular Na^+ levels until full collapse of the Na^+-

gradient. The inhibitory effect of 50 µM ouabain incubated for 2 minutes (52.2±1.5% and 7.5±3.5%, for GABA and glutamate respectively) was amplified by 100 µM BzATP (to 60.3±0.8% and 32.5±1.8%, respectively). The effect of 100 µM BzATP on [^{3}H]GABA and [^{14}C]glutamate uptake was significantly attenuated by prolonging to 20 min the exposure time of the synaptosomes to 50 µM ouabain (85.8±1.2% and 33.9±2.6%, for GABA and glutamate respectively; Figure 10C and 10D).

To exclude the participation of hemichannels on the inhibitory effect of BzATP on [^{3}H]GABA and [^{14}C]glutamate uptake by rat cortical synaptosomes, we performed interaction experiments with carbenoxolone (10 µM, a non-selective inhibitor of Cx26, Cx30, Cx43 and Cx46, which also blocks pannexin-1 hemichannels) and H1152 (3 µM, a Rho kinase inhibitor that affects hemichannels pore permeability) (see *e.g.* Pinheiro et al., 2013; Timóteo et al., 2014), which were added to the incubation solution 10 minutes before the P2X7 agonist. Neither of these compounds affected (*P*>0.05) the BzATP (100 µM)-induced downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake by synaptosomes of the rat cerebral cortex (Figure 11).

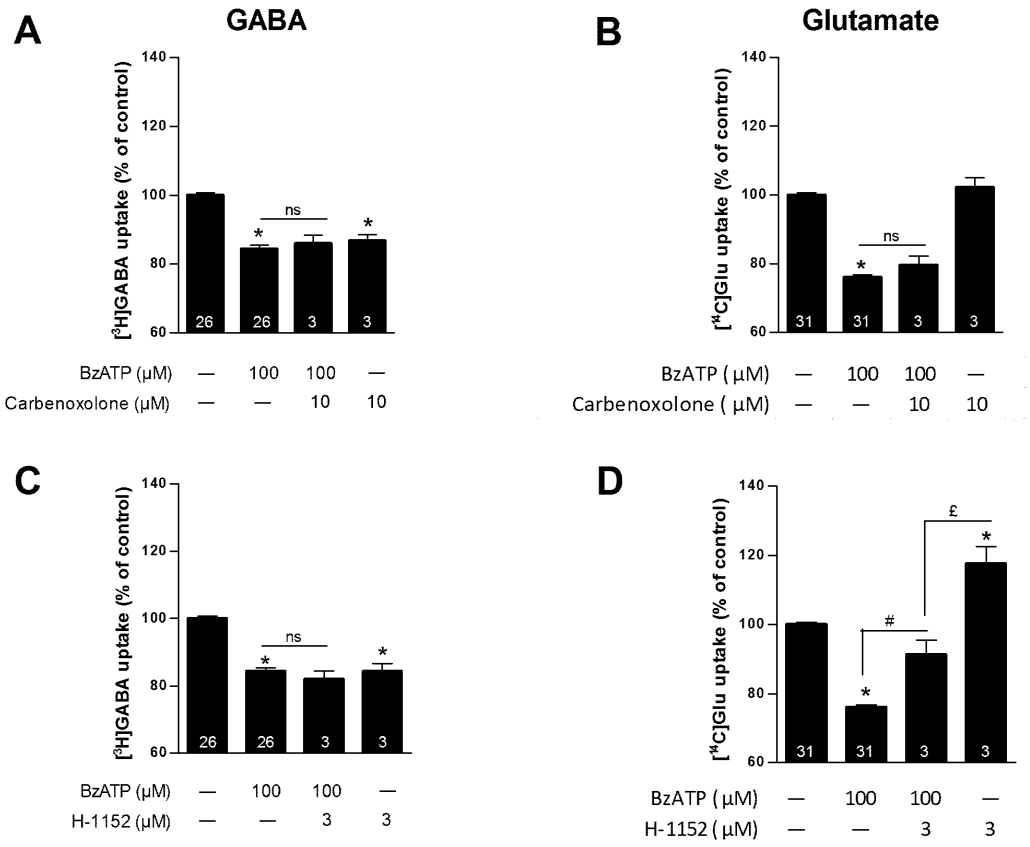


Figure 11 – Hemichannels do not participate in the inhibitory effect of BzATP. Top panels show that the inhibitory effect of BzATP (100 µM) on [^{3}H]GABA (A) and [^{14}C]glutamate (B) uptake into rat

cortical synaptosomes is not altered by carbenoxolone (10 μM), a non-selective inhibitor of Cx26, Cx30, Cx43 and Cx46, which can also block pannexin-1. On its own, carbenoxolone (10 μM) decreases GABA, but not glutamate uptake. Bottom panels show that the inhibitory effect of BzATP (100 μM) on [^{3}H]GABA (C) and [^{14}C]glutamate (D) uptake into rat cortical synaptosomes is not altered by H-1152 (3 μM), a Rho kinase inhibitor that affects hemichannels pore permeability. On its own, H-1152 (3 μM) decreases GABA uptake and increases glutamate uptake. The results are expressed as mean ± SEM; the vertical bars represent SEM; the *n* number of individual experiments is shown in the graphs. **P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation; #*P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of BzATP alone; £ *P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of H-1152 alone; ns, non-significant. BzATP was incubated 10 min before addition of [^{3}H]GABA or [^{14}C]Glutamate. Carbenoxolone and H-1152 were incubated 10 min before BzATP application.

All together these results suggest that increasing intracellular Na^+ concentration, *i.e.* by partial collapsation of the Na^+-gradient, downmodulates GABA and glutamate uptake similarly to that occurring with the activation of the P2X7 receptor. Furthermore, P2X7 downmodulation of amino acids transport closely accompany the thermodynamic changes operated by increasing intracellular Na^+ concentration.

Blockage of Na^+-influx through P2X7 receptors prevents the inhibitory effect of BzATP on the uptake of [^{3}H]GABA and [^{14}C]glutamate

Next, we evaluated whether manipulation of the extracellular Na^+ concentration could modulate GABA and glutamate uptake. Substitution of extracellular Na^+ by $NMDG^+$, so that the concentration of Na^+ in the reaction chamber decreased from 129 mM to 101, 69 and 9 mM, also diminished the uptake of [^{3}H]GABA (Figure 12A) and [^{14}C]glutamate (Figure 12B) by synaptosomes of the rat cerebral cortex; the collapse of the Na^+-gradient (9 mM plus 120 mM $NMDG^+$) reduced [^{3}H]GABA and [^{14}C]glutamate uptake by 92.6±0.7% and 73.3±2.6%, respectively. Interestingly, the inhibitory effect of the P2X7 receptor agonist, BzATP (100 μM), on the uptake of [^{3}H]GABA and [^{14}C]glutamate was significantly attenuated upon decreasing the amount of Na^+ in the reaction fluid from 129 mM to 101 and 69 mM (Figure 12A and 12B), reinforcing the idea that P2X7 downmodulation of GABA and glutamate transport closely accompanies the dynamics of the Na^+ transport.

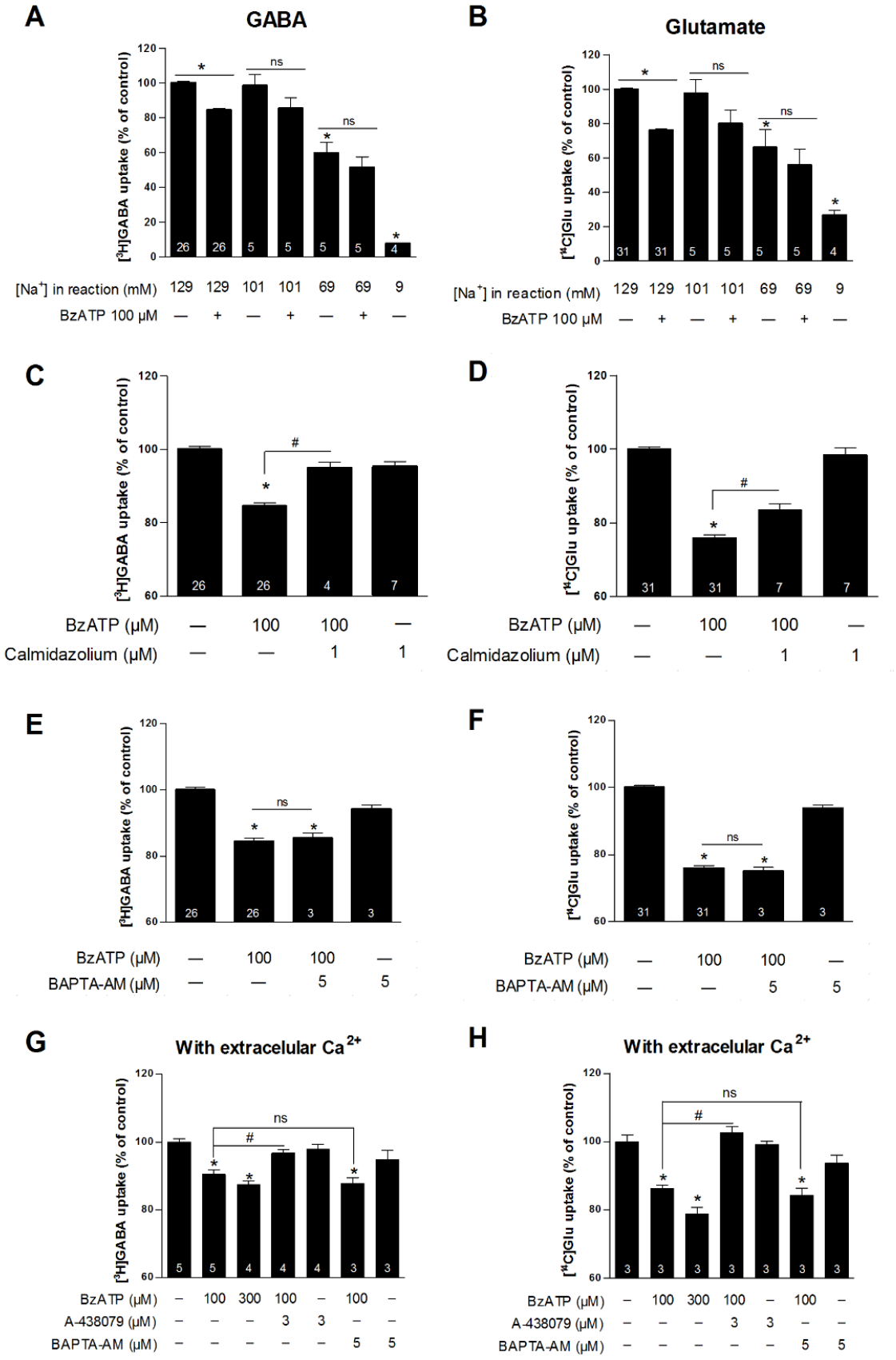


Figure 12 - Blockage of Na^+-entry through the P2X7 receptor pore prevents the inhibitory effect of BzATP on GABA and glutamate uptake. Shown is the inhibitory effect of BzATP (100 µM) on the uptake of [^{3}H]GABA (A) and [^{14}C]glutamate (B) carried out during 90 seconds upon decreasing the extracellular concentration of Na^+ by its replacement by $NMDG^+$. Inhibition of [^{3}H]GABA and

[^{14}C]glutamate uptake by the P2X7 receptor agonist, BzATP (100 µM), was significantly attenuated upon decreasing the amount of Na^+ in the reaction fluid from 129 mM to 101 and 69 mM. Panels (C) and (D) illustrate the blockage of the inhibitory effect of BzATP (100 µM) on the uptake of [^{3}H]GABA (C) and [^{14}C]glutamate (D) in the absence and in the presence of calmidazolium (1 µM), which inhibits small cation currents through the P2X7 receptor pore but not the permeation of the channel by organic molecules. On its own, calmidazolium (1 µM) did not influence GABA nor glutamate uptake. In panels (E) and (F), shown is that the effect of BzATP (100 µM) is not significantly changed by the cell-permeant fast Ca^{2+}-chelator, BAPTA-AM (5 µM). Experiments shown in panels (A-F) were performed in Ca^{2+}-free conditions. Bottom panels show that downmodulation of [^{3}H]GABA (G) and [^{14}C]glutamate (H) uptake caused by P2X7 receptors activation with BzATP (100-300 µM) is also verified in the presence of external $CaCl_2$ (2.2 mM). Under these physiologically relevant conditions, the selective P2X7 receptor antagonist, A-438079 (3 µM), prevented the effect of BzATP (100 µM), which was still not modified by BAPTA-AM (5 µM). On their own, A-438079 (3 µM) and BAPTA-AM (5 µM) were devoid of effect on GABA and glutamate uptake. The results are expressed as mean ± SEM; the vertical bars represent SEM; the *n* number of individual experiments is shown in the graphs. **P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation; #*P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of BzATP 100 µM alone; ns, non-significant. BzATP was incubated 10 min before addition of [^{3}H]GABA or [^{14}C]Glutamate; calmidazolium and A-438079 were incubated 10 min before BzATP application. BAPTA-AM was incubated for 33.5 min before addition of [^{3}H]GABA or [^{14}C]Glutamate.

To confirm that the inhibitory effect of BzATP on the transport of GABA and glutamate is mediated by Na^+-influx, calmidazolium was tested. This drug inhibits small cation currents through the P2X7 receptor pore, without affecting permeation of hydrophilic organic molecules with a mass up to 900 Da (Virginio et al., 1997). The results show that the inhibitory effects produced by 100 µM BzATP (14.1±1.1% and 24.0±0.8%, for GABA and glutamate respectively) were significantly attenuated by calmidazolium (1 µM), which was incubated with synaptosomes 10 minutes before the addition of BzATP (Figure 12C and 12D). In the absence of BzATP, calmidazolium (1 µM) did not influence the uptake of neurotransmitters. The fact that blocking Na^+-influx through the P2X7 receptor attenuates the inhibitory action of BzATP on the GABA and glutamate uptake suggests that the effect of the P2X7 receptor activation is mediated by Na^+-permeation through the receptor pore.

Knowing that calmidazolium also inhibits calmodulin-regulated enzymes, and to prove that observed BzATP effects on the high-affinity transport of GABA and glutamate are directly mediated by Na^+-influx and not by possible recruitment of intracellular Ca^{2+}, BAPTA-AM (5 µM; a cell-permeant fast Ca^{2+}-chelator) was tested. The results show that the inhibitory effects produced by 100 µM BzATP were not significantly ($P>0.05$) modified by BAPTA-AM, despite this drug has been pre-incubated with synaptosomes for as long as 33.5 min to ensure high intracellular free BAPTA guaranteeing strong intracellular Ca^{2+}-chelation (Figure 12E and 12F).

On its own BAPTA-AM did not influence the uptake of both neurotransmitters. These experiments demonstrate that Ca^{2+}-signaling was not involved in calmidazolium blockade of BzATP effect on neurotransmitter uptake. Moreover, the fact that under our experimental conditions BzATP effect was unchanged in presence of BAPTA-AM also rules-out the involvement of intracellular Ca^{2+}-triggered cytosolic GABA or glutamate release proposed recently by Romei et al. (2015).

Taking into consideration our findings showing that Na^+-dependent downmodulation of [^{3}H]GABA and [^{14}C]glutamate transport was observed in Ca^{2+}-free media, we questioned ourselves whether it could also occur under physiologically-compatible conditions where extracellular Ca^{2+} was present. In the presence of 2.2 mM external $CaCl_2$, the inhibitory effects of BzATP (300 μM) on [^{3}H]GABA (12.6±1.3%) (Figure 12G) and [^{14}C]glutamate (21.2±1.9%) (Figure 12H) uptake were about the same magnitude to those obtained in the Ca^{2+}-free conditions (Figure 7A and 7B, respectively). Likewise, downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake in the presence of 2.2 mM external $CaCl_2$ was fully prevented by the selective P2X7 antagonist, A-438079 (3 μM). Please note that the fast intracellular Ca^{2+} chelator, BAPTA-AM (5 μM) was also devoid of effect on BzATP-induced downmodulation of amino acid uptake in the presence of external Ca^{2+} (Figures 12G and 12H).

Activation of the P2X7 receptor triggers Na^+ influx into cortical synaptosomes

To ultimately prove that activation of the P2X7 receptor promotes an elevation of intrasynaptosomal Na^+-levels, we used the cell-permeant sodium selective fluorescent indicator, SBFI-AM, to estimate plasma membrane Na^+-gradients. Results show that BzATP (100-300 μM) causes a concentration-dependent increase in intrasynaptosomal Na^+-levels (Figure 13), being the effect of 100 μM BzATP (25.7±1.7%) fully prevented by the selective P2X7 receptor antagonist, A-438079 (3 μM; Figure 13). On its own A-438079 (3 μM) did not influence intrasynaptosomal Na^+-levels. These results confirm the hypothesis that Na^+-influx through the P2X7 receptor pore decreases the transport energetic for the uptake of GABA and glutamate by decreasing the Na^+ gradient across the synaptosomal membrane.

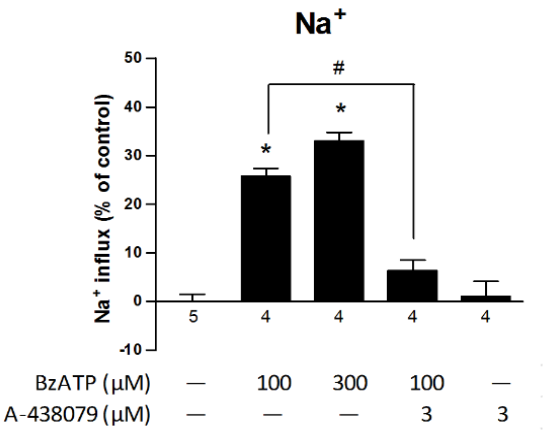


Figure 13 - Activation of the P2X7 receptor increases the intrasynaptosomal Na^+-concentration. Illustrated is the effect of BzATP (100 and 300 μM) on Na^+ influx into synaptosomes of the rat cerebral cortex loaded with the cell-permeant sodium selective fluorescent indicator, SBFI-AM, either in the absence or in the presence of the P2X7 receptor antagonist, A-438079 (3 μM). The results are expressed as mean ± SEM; the vertical bars represent SEM; the *n* number of individual experiments is shown in the graphs. **P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation; #*P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of BzATP 100 μM applied alone. BzATP was applied 10 min before addition of [^{3}H]GABA or [^{14}C]Glutamate; A-438079 was incubated for 10 min before BzATP application. On its own A-438079 (3 μM) did not influence intrasynaptosomal Na^+ levels.

The higher inhibitory effect of BzATP on [^{14}C]glutamate uptake may stem from a concomitant leakage of the neurotransmitter directly through the P2X7 receptor pore

As mentioned above, BzATP inhibition was preferential on [^{14}C]glutamate uptake, whereas changes in the transmembrane Na^+ gradient (caused by veratridine and ouabain) were more effective in decreasing the uptake of [^{3}H]GABA (Figure 7A and 7B). One possibility to explain these findings may be that repeated or prolonged activation of the P2X7 receptor opens a non-selective pore allowing the permeation of large molecular weight hydrophilic organic cations up to 900 Da (Virginio et al., 1997) providing a route for the release of glutamate (Duan et al., 2003; Marcoli et al., 2008; Cervetto et al., 2013; Fu et al., 2013; Sperlágh and Illes, 2014). In keeping with this hypothesis, we found that rat cortical synaptosomes challenged with BzATP (100-300 μM) in Ca^{2+}-free media release five-fold more [^{14}C]glutamate than [^{3}H]GABA when neurotransmitter release amounts were normalized by the stimulatory effect of veratridine (10 μM) under the same experimental conditions (Figure 14). It is worth noting that [^{14}C]glutamate release produced by BzATP (100 μM) was prevented in the presence of the P2X7 receptor antagonist, A-438079 (3 μM) and it was not altered by the glutamate transport inhibitor, DL-TBOA (100 μM; Figure 14B). These results suggest that the higher potency of the P2X7 receptor-induced decline in [^{14}C]glutamate uptake may be owe

to concurrent non-vesicular [^{14}C]glutamate leakage from rat cortical synaptosomes through the P2X7 receptor pore. This hypothesis may also explain why blockage of Na^+ currents, but not permeation of organic molecules, through activated P2X7 receptors with calmidazolium was unable to fully prevent the inhibitory effect of BzATP on [^{14}C]glutamate uptake (Figure 12D), in contrast to that observed with the uptake of [^{3}H]GABA under the same experimental conditions (Figure 12C).

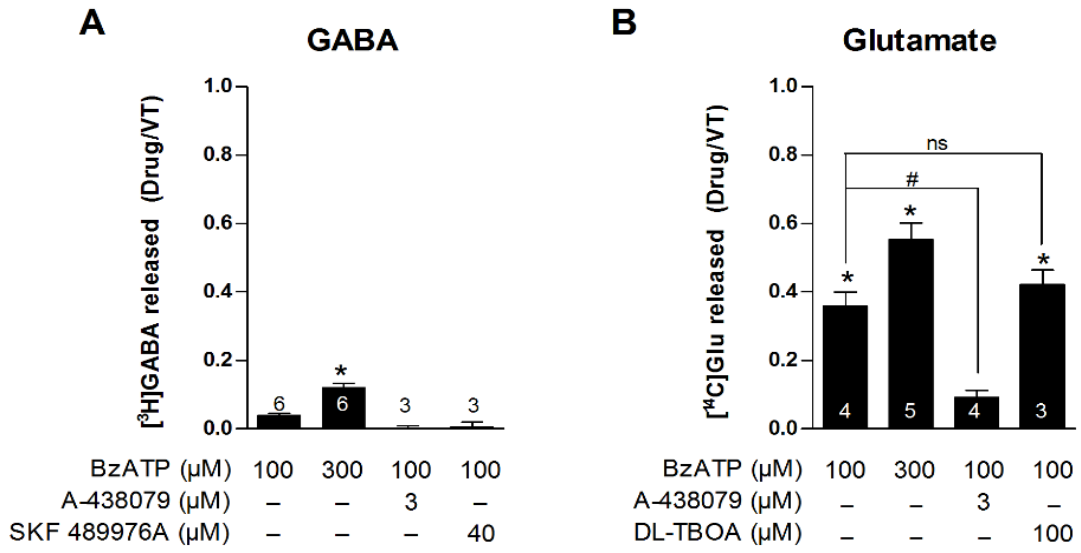


Figure 14 – Activation of the P2X7 receptor with BzATP exerts a predominant facilitory role on [^{14}C]glutamate compared to [^{3}H]GABA release from synaptosomes of the rat cerebral cortex. The radioactivity of superfusates was measured by liquid scintillation spectrometry in 2-min fractions collected automatically during 34 min. Ten min after initiation of fraction collection, synaptosomes retained in fibre glass filters were challenged during 2 min with BzATP (100 or 300 μM) or veratridine (10 μM) in Ca^{2+}-free media by changing the inlet tube from one flask to another containing the test drug. The P2X7 receptor antagonist, A-438079 (3 μM) and inhibitors of the high-affinity GABA and glutamate transport, SKF 489976A (40 μM) and DL-TBOA (100 μM), were added to the superfusion solution from the beginning of the release period, *i.e.* 10 min before BzATP. For comparison purposes, [^{3}H]GABA and [^{14}C]glutamate release produced by BzATP was normalized by the stimulatory effect of veratridine (10 μM) under the same experimental conditions (Drug/VT). Data are expressed as mean ± SEM; the vertical bars represent SEM. The *n* number of individual experiments is shown in the graphs. **P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the control situation; #*P*<0.05 (one way ANOVA followed by Bonferroni's multiple comparison test) represents significant differences as compared to the effect of BzATP 100 μM applied alone; ns, non-significant.

Discussion and conclusions

This study was performed to evaluate, in parallel and under the same experimental conditions, the role of P2X7 receptor activation on [^{3}H]GABA and [^{14}C]glutamate uptake by synaptosomes of the rat cerebral cortex, which are highly enriched in nerve terminals expressing P2X7 receptors as we demonstrated by

Western blot analysis and immunofluorescence confocal microscopy (Miras-Portugal et al., 2003; Alloisio et al., 2008; Marín-García et al., 2008). Results indicate that, either in the presence or in the absence of extracellular Ca^{2+} ions, stimulation of the P2X7 receptor downmodulates Na^{+}-dependent high-affinity GABA and glutamate transport into rat cortical synaptosomes, being this effect more evident for the glutamate uptake. This suggests that transient P2X7 receptor activation following ATP release by high-frequency stimulus (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) may constitute a mechanism facilitating local glutamatergic neurotransmission while promoting, albeit to a lesser extent, the endurance of GABAergic neurotransmission ensuring tonic and more diffuse neuro-inhibition following intense stimulus. This mechanism may be particularly relevant in processes such as memory and learning where transient glutamate endurance in the synaptic cleft may facilitate the induction of LTP while preventing excytotoxicity by the concomitant promotion of diffuse GABAergic inhibition.

Stimulation of the P2X7 receptor decreases the maximum transport capability (V_{max}) of rat cortical synaptosomes to take up GABA and glutamate, keeping virtually unaltered the affinity for the substrates (K_m). A similar result was observed regarding the uptake of glutamate by primary cultures of the rat spinal microglia (Morioka et al., 2008), yet in this case a metabotropic pathway seems to involve activation of the extracellular signal-regulated kinase cascade and production of anti-oxidants via a mechanism independent on extracellular Na^{+} and Ca^{2+} ions. It is, however, uncertain whether the same occurs in CD11b positive microglial cells of the rat cerebral cortex expressing the P2X7 receptor (see Figure 7). Taking into account that (1) the slowly-desensitizing P2X7 receptor works as a non-selective cationic channel promoting the influx of Na^{+} under low Ca^{2+} conditions (Jarvis and Khakh, 2009), (2) GABA and glutamate uptake are crucially dependent on plasma membrane Na^{+}-gradient (Kanner, 2006), and (3) extracellular Ca^{2+} was withdrawn from the reaction medium in the majority of the experiments, it is probable that the transportation decline (V_{max}) caused by the P2X7 agonist may be owed to an impairment of the transport energetics, *i.e.* to the partial disruption of the Na^{+}-gradient via the activation of a low-affinity high-capacity ATP signal. Moreover, the fact that transport inhibition by BzATP was observed in the absence of intra and extracellular Ca^{2+} excludes any effects requiring Ca^{2+} activation, like: (1) exo- and

endocytosis (amenable to alter transporter membrane density), (2) Ca^{2+}-dependent neurotransmitter release, or (3) the activation of Ca^{2+}-dependent enzymatic cascades. In agreement with this hypothesis, we showed that (1) the inhibitory effect of BzATP on neurotransmitter transport was mimicked by two Na^+ ionophores, gramicidin and monensin; (2) the increase in intracellular Na^+ concentration via voltage-sensitive Na^+ channels activation with veratridine or through inhibition of Na^+/K^+ ATPase with ouabain downmodulates the transport of both neurotransmitters; (3) the decrease in extracellular Na^+ concentration by substitution with $NMDG^+$ also downmodulates the transport of both neurotransmitters as predicted by the changes in transporter reversal potentials (Richerson and Wu, 2003; Allen et al., 2004); (4) the inhibitory effect of BzATP was attenuated when Na^+-gradient was disrupted in accordance with changes in Na^+-equilibrium; and, finally, (5) the activation of P2X7 receptors significantly increased intrasynaptosomal Na^+-levels. Moreover, when Na^+-entry through the P2X7 receptor pore was selectively blocked with calmidazolium the effect of BzATP on GABA and glutamate uptake was prevented, thus giving a strong indication that BzATP effects were mainly (if not exclusively) mediated by lowering the Na^+-coupled transporter driving-force. Hence, our results point towards a novel mechanism implicated in the decrease of GABA and glutamate uptake by synaptic nerve terminals, which might depend on the decline of the Na^+ driving-force necessary for amino acid transportation across the plasma membrane operated by P2X7 receptor activation (Wonnemann et al., 2000; Richerson and Wu, 2003; Kanner, 2006; Lo et al., 2008; Yu et al., 2010). A similar dependence on transmembrane Na^+-gradient was also observed for the glutamate uptake by the RBA-2 astrocytic cell line (Lo et al., 2008). Contrarily to what happens in cell cultures, it seems that the P2X7 protein density found in astrocytes *in situ* is below immunofluorescence detection while both nerve terminals and microglia exhibit clear P2X7 immunoreactivity (see Figure 7) (Deuchars et al., 2001.; Sperlágh et al., 2002; Miras-Portugal et al., 2003; Cavaliere et al., 2004).

As previously mentioned, activation of P2X7 receptors requires high ATP concentrations, since this receptor has low affinity for its endogenous ligand (Jarvis and Khakh, 2009). Thus, under basal conditions, P2X7 receptors are probably dormant, but they may become unmasked after high-frequency stimuli (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) or

under pathologic brain activity, such as during prolonged or repeated seizures (Engel et al., 2012a; Henshall et al., 2013). Different studies have demonstrated that Ca^{2+} concentration in the extracellular milieu drops progressively to as much as 90% under strong, yet physiological, stimulus as well as in pathological conditions, such as epilepsy (Heinemann et al., 1977; Borst and Sakmann, 1999; Engelborghs et al., 2000; Stanley, 2000; Massimi and Amzica, 2001; Rusakov and Fine, 2003; Engel et al., 2012a; Poornima et al., 2012; Torres et al., 2012; Zhou et al., 2012; Jimenez-Pacheco et al., 2013). Under such low extracellular Ca^{2+} conditions, Ca^{2+}-dependent neurotransmitter release is significantly diminished while Na^{+} conductance via P2X7 receptors is largely enhanced (Virginio et al., 1997; Jiang, 2009; Yan et al., 2011) and synaptic ATP is highest. So, it seems plausible to admit that the mechanism unraveled in this work might play a role in physiological conditions by potentiating neurotransmission under both normal (high extracellular Ca^{2+}) and low quantal content (low extracellular Ca^{2+}) conditions. Such a typically short temporal-spanning amplification mechanism might gain a different meaning under excitotoxic conditions like during epileptic seizures, since in the latter situation the extracellular Ca^{2+} concentration falls during prolonged periods of time in synchrony with ATP endurance and the expression of P2X7 receptors is increased resulting in a predominantly pro-epileptic action (Burnstock et al., 2011b; Engel et al., 2012a; Jimenez-Pacheco et al., 2013).

Besides epileptic seizures, there is an explosion of data indicating that P2X7 receptors are involved in the pathophysiology of several neurological syndromes (*e.g.* stroke, neurotrauma, neuropathic pain, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease) and psychiatric mood disorders (reviewed in Sperlágh and Illes, 2014). This may be due to the release of large quantities of ATP following any kind of cell injury and its widespread involvement as a key regulator of the inflammasome complex. It is possible that the decrease in the driving-force for neurotransmitters uptake caused by Na^{+}-influx via P2X7 receptors occurs synchronously to other Na^{+}-triggered events, like the activation of Na^{+}/Ca^{2+}-exchangers and subsequent activation of Ca^{2+}-dependent neurotransmitter exocytosis following a significant increase in intracellular Na^{+} concentration that is a characteristic event associated with tissue injury (Yu et al., 2010; Romei et al., 2015).

In summary, data from this study suggest that Na^+-influx through activated P2X7 receptors on synaptic nerve terminals may disrupt plasma membrane Na^+-gradient, which is the driving-force for the uptake of GABA and glutamate by brain cells. Hence, downmodulation of Na^+-dependent high-affinity uptake of amino acids may constitute a novel mechanism by which ATP might influence neuronal excitation and, thus, synaptic transmission in the CNS, in particular when the extracellular concentration of the nucleotide increases to levels high enough to stimulate low affinity ionotropic P2X7 receptors, such as during high-frequency nerve firing or under pathological conditions leading to increased nerve activity and/or neuroinflammation.

PAPER 2

MANUSCRIPT IN PREPARATION

Inhibition of GABA and glutamate uptake by cortical nerve terminals produced by P2X7 receptors activation is kept unaltered in epileptic rats

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Abstract

Recently, we demonstrated that nerve terminals from the rat cerebral cortex express ATP-sensitive P2X7 receptors, whose activation downmodulates high-affinity GABA and glutamate uptake by decreasing the transmembrane Na^+ gradient. Increasing evidences made in several animal models point towards a significant role of P2X7 receptor in the pathophysiology of epilepsy. Increased expression of the P2X7 receptor was also observed by us in the hippocampus and neocortex of patients with drug-resistant MTLE. In this context, we aimed at understanding the pathological significance of the alterations of the P2X7 receptor-mediated downmodulation of GABA and glutamate uptake in the pilocarpine-induced epileptic rat model, which is most frequently used to compare with MTLE in humans. Results demonstrate that the P2X7 receptor-induced inhibition of GABA and glutamate uptake by synaptosomes of epileptic rats were not different from their control littermates. In contrast to that observed in human epileptic patients, we detected no significant changes in the density of P2X7 receptors in cortical slices of epileptic rats compared to control animals, thus indicating that the pilocarpine-induced animal model might not reproduce drug-resistant MTLE in humans at least under the present experimental conditions.

Introduction

Epilepsy is an old and prevalent disorder, still unresolved, with a great negative impact in the quality of life of patients, mainly for those patients in which seizures are not controlled by the currently available drugs. Despite intense research during the last decades, few new AEDs were approved for clinical practice (Madsen et al., 2010). Only a subset of drug-refractory patients meet the criteria for surgical ablation of damaged tissue as last resource treatment, leaving remaining patients with an unmet medical need. Therefore, it is urgent to find new pharmacological targets to control seizures and prevent epileptogenesis.

In a recent study, we showed that activation of the P2X7 receptor downmodulates GABA and glutamate uptake by nerve terminals (synaptosomes) of the rat cerebral cortex (Paper 1; Barros-Barbosa et al., 2015b). Despite the P2X7 receptor-mediated inhibition being preferential on glutamate uptake, which may stem from a concomitant leakage of the neurotransmitter directly through the P2X7 receptor pore, downmodulation of GABA uptake was by some means unexpected given the pro-convulsive actions of the P2X7 receptor in most literature reports (Vianna et al., 2002; Fernandes et al., 2009; Padrão et al., 2011; Engel et al., 2012a; Jimenez-Pacheco et al., 2013). At this point, it is worth mentioning that during prolonged and/or repetitive seizures increases in the extracellular levels of GABA might not have a protective role but, conversely, may exert a pro-epileptic one due to GABAergic “rundown” (Cohen et al., 2002; D’Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012). This can be defined as a decrease in GABAergic inhibition or even transient excitatory GABAergic actions, resultant from desensitization of $GABA_A$ receptor upon repetitive stimulation (Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010) or due to depolarization stemming from changes in Cl^- homeostasis (D’Antuono et al., 2004; Miles et al., 2012).

The present study was designed to understand the pathological significance of the alterations of the P2X7 receptor-mediated downmodulation of the uptake of GABA and glutamate in the pilocarpine-induced epileptic rat model, which is most frequently used to compare with drug-resistant MTLE in humans (Sharma et al., 2007; Curia et al., 2008; O'Dell et al., 2012). Pilocarpine is a muscarinic acetylcholine receptor agonist that reproduces many morphological and clinical

aspects of MTLE in rodents (Sharma et al., 2007). Using a suitable animal model will guide us in the work we plan to execute with samples obtained from the brain of drug-resistant MTLE and non-MTLE patients submitted to neurosurgery for ablation of the epileptic focus, as human tissue available for research is very scarce.

Experimental procedures

Drugs and solutions

HEPES and triton X-100 were from Merck Millipore (Darmstadt, Germany). GABA, SDS, 2-mercaptoethanol, sodium deoxycholate, Tris, BSA, bromophenol blue, glycerol, tween 20, BzATP, scopolamine methyl bromide, pilocarpine and AOAA were obtained from Sigma-Aldrich (St. Louis, MO). L-Glutamic acid, A-438079 and DL-TBOA were from Tocris Bioscience (Bristol, UK); SKF89976A hydrochloride was from Abcam (Cambridge, UK); [^{14}C]Glutamate and [^{3}H]GABA were from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All stock solutions were stored as frozen aliquots. Dilutions of stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used, were observed.

Animals

Animal care and experimental procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and followed the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the National Institutes of Health Guide for Care and Use of Laboratory animals (NIH Publications No. 80-23) revised 1996. This study was done in accordance with ARRIVE guidelines (McGrath et al*.,* 2010). All efforts were made to minimize animal suffering and to reduce the number of animals used. Wistar rats (150-200 g) of either sex (Charles River, Barcelona, Spain) were kept at a constant temperature (21 ºC) and a regular light (06.30–19.30 h)–dark (19.30–06.30 h) cycle, with food and water *ad libitum*.

Pilocarpine-induced epileptic rat model

The generation of the epilepsy model was made as previously described byCardoso et al. (2011). Ten-week old rats were pretreated with scopolamine methyl bromide (1 mg/kg, subcutaneous) to minimize peripheral cholinergic side effects of pilocarpine. Thirty minutes later, the rats received a single high-dose of pilocarpine (350 mg/kg, intraperitoneal). The onset of SE was defined as the appearance of behavioral symptoms corresponding to stage 4 or 5 seizures on the Racine scale (Racine, 1972), *i.e.* rearing, falling and generalized convulsions. SE onset was detected usually 30–60 min following the pilocarpine injection. It has been previously reported that pilocarpine-induced SE, if lasting several hours, can be associated with high mortality rates ranging between 15 and 50% depending on the dose of pilocarpine and other experimental conditions. Therefore, special efforts were made to improve the survival rate of the animals. To this end, the rats were injected with diazepam (2.5 mg/kg, intraperitoneal) 2 h after the beginning of SE in order to cease convulsions. Albeit considerably reduced in severity seizure activity was only abrogated after an additional dose of diazepam (2.5 mg/kg) given to the rats 30 min after the first injection. During the first 24 h of the recovery period, the animals were periodically hydrated with saline injected subcutaneously. Following the induction of SE by a single-injection of pilocarpine, the rats were observed daily for spontaneous behavioral seizures at random times between 08:00 h and 20:00 h. Animals exhibiting spontaneous seizures were used for the experimental work, which was performed 2 months after pilocarpine injection.

Preparation of synaptosomes from the rat cerebral cortex

Synaptosomes were isolated as previously described by Helme-Guizon et al. (1998) and then modified by Bancila et al. (2009). Briefly, the cerebral cortex was dissected out and gently homogenized in cold oxygenated (95% O_2 and 5% CO_2) Krebs solution (in mM: glucose 5.5, NaCl 136, KCl 3, $MgCl_2$ 1.2, Na_2HPO_4 1.2, $NaHCO_3$ 16.2, $CaCl_2$ 0.5, pH 7.40). Homogenates were filtered through a nylon filter (mesh size 100 µm). The filtrate was left to sit during 30–45 min until formation of a pellet, which was re-suspended into Krebs solution and left at room temperature. Protein concentration determined by the BCA method (Pierce™, Thermoscientific, Rockford USA) was adjusted to 6.25 mg protein mL^{-1}.

[^{3}H]GABA and [^{14}C]glutamate uptake experiments

[^{3}H]GABA uptake by synaptosomes was measured as described elsewhere (Cordeiro et al., 2003; Barros-Barbosa et al., 2015a; Barros-Barbosa et al., 2015b). [^{3}H]GABA (0.25 μCi mL^{-1}; 70 Ci mmol^{-1}) uptake reactions were initiated by adding [^{3}H]GABA (0.5 μM) to media containing synaptosomes (0.25 mg protein mL^{-1}), at 30 ºC. Uptake reactions were performed in media containing (in mM) NaCl 128, $MgCl_2$ 1.2, KCl 3, glucose 10, HEPES–Na 0.01 (pH 7.4), EGTA 0.1 and AOAA 0.01 (used to prevent GABA metabolism by GABAT). Experiments were performed in the absence of extracellular Ca^{2+}, in order to focus research on the energetics of Na^+-coupled transport. The reactions were stopped by rapid filtration through glass fibre prefilters (Merck Millipore, Cork, IRL), prewashed with cold sucrose 320 mM, Tris–HCl 10 mM (pH 7.4) and EGTA 0.1 mM. The filters were then washed with the same medium and plunged into vials containing scintillation cocktail (Insta-Gel Plus, Perkin Elmer, Boston, USA) for radioactivity measurement by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA). The values for [^{3}H]GABA taken up by synaptosomes were expressed as pmol mg protein^{-1} after subtraction of blank values obtained by filtering reaction medium aliquots. [^{14}C]Glutamate uptake (0.25 μCi mL^{-1}; 0.270 Ci mmol^{-1}) by synaptosomes was measured as described above for [^{3}H]GABA uptake but using [^{14}C]glutamate (10 μM) without adding AOAA (10 μM) to the medium. Unless stated otherwise, uptake assays were carried out during 90 seconds since this time is in the linear phase of [^{3}H]GABA and [^{14}C]Glutamate accumulation.

All modifier drugs tested were allowed to equilibrate with the synaptosomes at least for 10 min before adding the test drug, which was applied 10 min before addition of the radioactive neurotransmitter. Control samples were incubated for the same amount of time in the absence of drugs.

Immunofluorescence staining and confocal microscopy

Brain samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; in mM: NaCl 137, KCl 2.6, Na_2HPO_4 4.3, KH_2PO_4 1.5; pH=7.4) for about 48 h (4 ºC), cryopreserved in 30% sucrose in PBS and stored in a tissue freezing medium at -80 ºC.

Free floating 30 μm brain slices were incubated for 1 h, at room temperature, with blocking buffer I (foetal bovine serum 10%, BSA 1%, triton X-100 0.5%, NaN_3

0.05%) and subsequently incubated overnight, at 4 ºC, with the primary antibodies diluted in blocking buffer II (foetal bovine serum 5%, BSA 0.5%, triton X-100 0.5%, NaN3 0.05% in PBS): rabbit anti-P2X7 receptor (1:50, #APR004, Alomone, Jerusalem, Israel), goat anti-VAMP-1 (1:20, R&D Systems, Minneapolis, MN), mouse anti-GFAP (1:350, Chemicon, Temecula, CA). Sections were rinsed in PBS supplemented with triton X-100 0.5% (3 cycles of 10 min) and incubated for 120 min with species specific secondary antibodies conjugated with fluorescent dyes (donkey anti-rabbit IgG Alexa Fluor 488, donkey anti-mouse IgG Alexa Fluor 568; donkey anti-goat Alexa 633) diluted in blocking buffer II, at room temperature. After rinsing in PBS, slices were mounted on optical-quality glass slides using VectaShield (Vector Labs, Peterborough, UK) as mounting media. Observations were performed with a laser scanning confocal microscopy (Olympus FV1000, Tokyo, Japan). Controls were performed by following the same procedure but replacing the primary antibodies with the same volume of blocking buffer II. Images were analyzed using the Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, Japan). Co-localization was assessed by calculating the staining overlap and the ρ for each confocal micrograph stained with two fluorescent dyes. Both parameters were automatically calculated per image with the Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, Japan). Overlap between two stainings gives a value between +1 and 0 inclusive, where 1 is total overlap and 0 is no overlap. ρ is a measure of the linear correlation between two variables (stainings), giving a value between +1 and −1 inclusive, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation.

SDS-PAGE and Western blot analysis

Synaptosomes of the rat cerebral cortex were homogenized in RIPA buffer containing: Tris-HCl (pH 7.6) 25 mM, NaCl 150 mM, sodium deoxycholate 1%, triton-X-100 1%, SDS 0.1%, EDTA 5 mM and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein content of the samples was evaluated using the BCA method. Samples were solubilized at 70 °C in SDS reducing buffer (Tris-HCl (pH 6.8) 125 mM, SDS 4%, bromophenol blue 0.005%, glycerol 20%, and 2-mercaptoethanol 5%) for 10 min, subjected to electrophoresis in 12.5% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (Merck MilliPore, Temecula, CA). Membranes were blocked for 1 h in Tris-buffered saline

(TBS; in mM: Tris-HCl 10 (pH 7.6), NaCl 150) containing Tween 20 0.05% and BSA 5% and, subsequently, incubated overnight, at 4 ºC, with primary antibodies: rabbit anti-P2X7 receptor (1:300, #APR004, Alomone, Jerusalem, Israel) and rabbit anti-β-actin antibody (1:2500, Abcam, Cambridge, UK). Membranes were washed three times for 10 min in 0.05% Tween 20 in TBS and then incubated with horseradish anti-rabbit or anti-mouse peroxidase-conjugated secondary antibodies for 120 min, at room temperature. The antigen-antibody complexes were visualized by chemiluminescence with the Immun-Star WesternC Kit (Biorad Laboratories, Hercules, CA) using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA). To test for specificity of the bands corresponding to P2X7 receptor, the anti-P2X7 receptor antibody was pre-adsorbed with a control peptide antigen corresponding to the amino acid residues 576-595 of the intracellular C-terminus of the rat P2X7 receptor, before incubation with the membrane. Gel band image densities were quantified with Image J (National Institute of Health, USA).

Data presentation and statistical analysis

The uptake of [^{3}H]GABA and [^{14}C]glutamate by synaptosomes of the rat cerebral cortex was expressed as a percentage of control values obtained in the same synaptosomal batch without adding any drug. Results are expressed as mean ± SEM, with *n* (showed in graphs) indicating the number of individual experiments performed in a given situation. Because of limited inter-individual variation, randomly chosen groups of at least three animals of the same strain and weight (Wistar rats of 150-200 g) were considered sufficient to replicate each experimental protocol; individual uptake experiments were performed in triplicate. Statistical analysis of data was carried out using GraphPad Prism 6.04 software (La Jolla, CA, USA). Unpaired Student's *t*-test with Welch correction was used for statistical analysis when parametric data was considered. For multiple comparisons, one-way ANOVA followed by Bonferroni's Multiple Comparison Test was used. For multiple comparisons between multiple groups, two-way ANOVA followed by Bonferroni´s Multiple Comparison Test was used. $P < 0.05$ values were considered to show significant differences between means.

Results

[^{3}H]GABA and [^{14}C]glutamate uptake by cortical nerve terminals isolated from both control and epileptic animals is mediated by high-affinity transporters

First, we evaluated if the uptake of both neurotransmitters, [^{3}H]GABA and [^{14}C]glutamate, into isolated nerve terminals from the cerebral cortex of control and epileptic rats occurs through Na^+-dependent high affinity transporters. Figure 15 shows that selective inhibition of GAT1 with SKF89976A (40 μM) decreased ($P<0.05$) [^{3}H]GABA uptake by cortical nerve terminals of control and epileptic rats by 96.4±0.2% and 96.8±0.3%, respectively (Figure 15A). Likewise, the non-selective glutamate transporter inhibitor, DL-TBOA (100 μM), decreased [^{14}C]glutamate uptake by cortical nerve terminals from control and epileptic rats by 89.3±1.1% and 95.3±0.8%, respectively (Figure 15B). These results suggest that the uptake of [^{3}H]GABA and [^{14}C]glutamate by rat cortical nerve terminals occurs predominantly via high-affinity amino acid transporters.

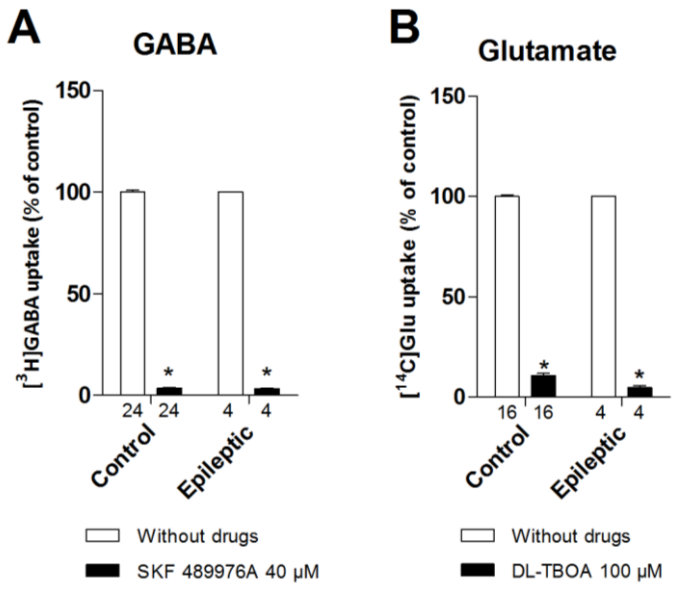

Figure 15 – Nerve terminals isolated from the cerebral cortex of control and epileptic rats take up [^{3}H]GABA and [^{14}C]glutamate through Na^+-dependent high-affinity transporters. Shown is the inhibition of Na^+-coupled high-affinity transport of [^{3}H]GABA (panel A) and [^{14}C]glutamate (panel B) with SKF489976A (40 μM; GAT1 selective inhibitor) and DL-TBOA (100 μM; non-specific inhibitor of the EAAT), respectively, into nerve terminals of control and pilocarpine-induced epileptic rats. SKF 489976A and DL-TBOA contacted with nerve terminals for 20 min before application of [^{3}H]GABA and [^{14}C]glutamate into the reaction chamber. Data are expressed as mean ± SEM; the *n* number of experiments is shown below each bar. *P < 0.05 (Student's *t*-test with Welch correction) represents significant differences as compared to the situation where no drugs were added.

Downmodulation of GABA and glutamate uptake produced by activation of the P2X7 receptor is kept unchanged in epileptic rats

Like that observed in isolated nerve terminals of the cerebral cortex of control rats (Paper1; Barros-Barbosa et al., 2015b), the prototypic P2X7 receptor agonist,

BzATP (1-300 µM), downmodulates [^{3}H]GABA (Figure 16A) and [^{14}C]glutamate uptake by cortical synaptosomes of epileptic animals (Figure 16B). The magnitude of the inhibitory effect of BzATP was dependent on the concentration; downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake was more evident at concentrations within the range (20-300 µM), indicating that the nucleotide activates preferentially low-affinity P2X7 receptors (North, 2002; Jarvis and Khakh, 2009); the negative modulation of [^{3}H]GABA and [^{14}C]glutamate uptake by BzATP was roughly similar in control and epileptic animals (Figure 16A and 16B).

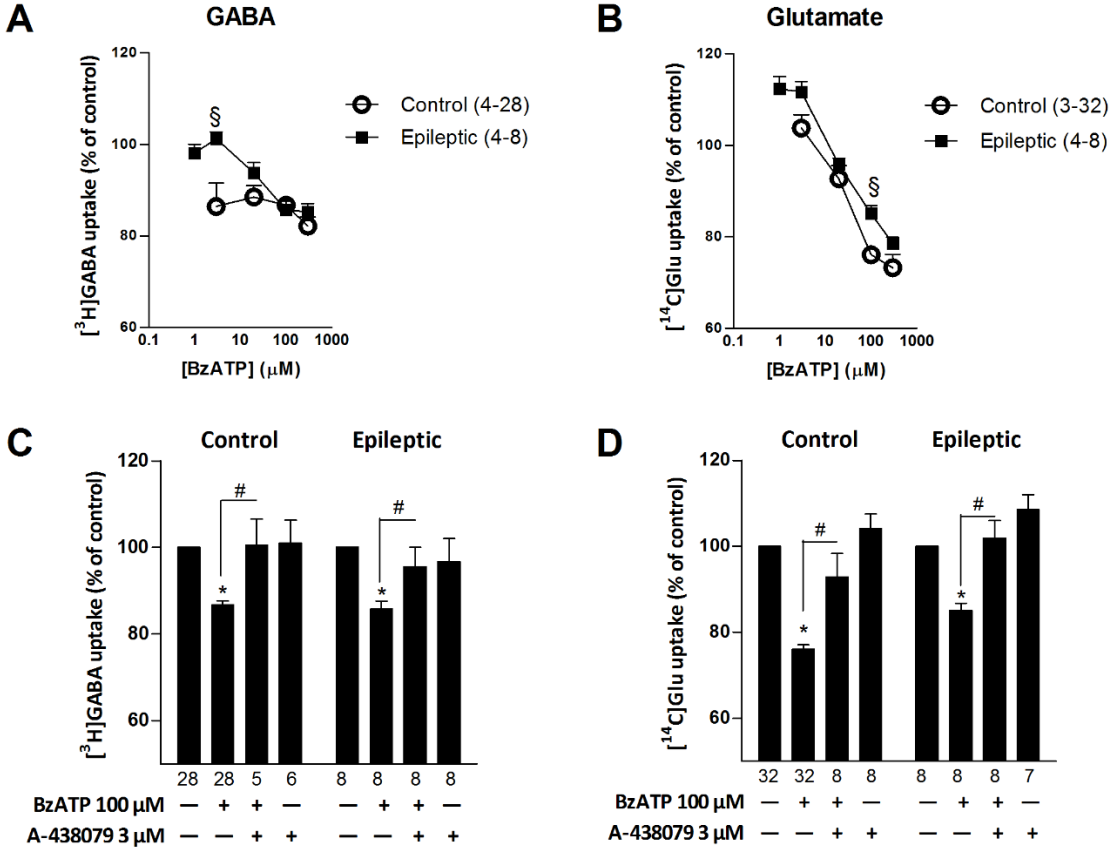

Figure 16 – Downmodulation of GABA and glutamate uptake caused by the P2X7 receptor agonist, BzATP, has about the same magnitude in cortical synaptosomes of control and pilocarpine-induced epileptic rats. Panels A and B illustrate the effect of BzATP (3-300 µM) on [^{3}H]GABA and [^{14}C]glutamate uptake by nerve terminals isolated from the cerebral cortex of control and epileptic rats. The selective P2X7 receptor antagonist, A-438079 (3 µM), prevented BzATP (100 µM)-induced inhibition of [^{3}H]GABA (C) and [^{14}C]glutamate (D) uptake by cortical synaptosomes of control and epileptic rats. BzATP contacted with nerve terminals for 10 min before application of [^{3}H]GABA and [^{14}C]glutamate into reaction chamber; A-438079 was added 10 min before BzATP. Data are expressed as mean ± SD; the *n* number of animals per experimental condition is shown below each bar. §P < 0.05 (two way ANOVA followed by Bonferroni´s Multiple Comparison Test) represents significant differences as compared to control group. *P < 0.05 and #P< 0.05 (one-way ANOVA followed by Bonferroni´s Multiple Comparison Test) represents significant differences as compared to the situation where no drugs were added and to the effect of BzATP (100 µM) alone, respectively.

When used in the low micromolar range, BzATP increased (12%) rather than decreased the uptake of [^{14}C]glutamate into isolated cortical nerve terminals of epileptic rats but not in control animals (Figure 16B), an effect that may be mediated by a distinct P2 purinoceptor (North, 2002; Jarvis and Khakh, 2009). Conversely, the nucleotide failed to inhibit the uptake of [^{3}H]GABA into cortical synaptosomes of epileptic rats when used in a 3-µM concentration (Figure 16A), suggesting either that (1) another purinoceptor activated by BzATP may be counteracting the P2X7 receptor-mediated inhibition, or that (2) the P2X7 receptor agonist lost its potency in epileptic rats.

The selective P2X7 receptor antagonist, A-438079 (3 µM), prevented ($P<0.05$) the inhibitory effects of BzATP (100 µM) on the uptake of [^{3}H]GABA (Figure 15C) and [^{14}C]glutamate (Figure 16D) into synaptosomes of the cerebral cortex of control and epileptic rats. These results strengthen our assumption that the P2X7 receptor is equally involved in the inhibition of high-affinity GABA and glutamate uptake by cortical nerve terminals produced by BzATP in both control and epileptic rats.

No changes were observed in the cellular localization and expression density of P2X7 receptors in the cerebral cortex of control and epileptic rats

Using immunofluorescence confocal microscopy and Western blot assays, we showed that the P2X7 receptor is predominantly localized in cortical nerve terminals of healthy rats (Paper1; Barros-Barbosa et al., 2015b). Our results are in keeping with reports from other authors suggesting the presence of functional P2X7 receptors on nerve terminals of the rat cerebral cortex (Miras-Portugal et al., 2003; Alloisio et al., 2008; Marcoli et al., 2008; Marín-García et al., 2008). Here, we evaluated if the same occurs in the cerebral cortex of pilocarpine-injected epileptic rats and whether one could detect changes in the density of expression of the P2X7 receptor in these animals.

The analysis of confocal micrographs shown in Figure 17 indicates that the antibody directed against the P2X7 receptor (green) co-localizes with the synaptic nerve terminal marker, VAMP-1 (red, Figure 17A), whereas no significant co-localization was detected with the astrocytic cell marker, GFAP (red, Figure 17B), within the same brain region. Co-localization was assessed by evaluating the Pearson´s coefficient (ρ) and staining overlap obtained by merging the two

fluorescent channels (yellow staining). Data suggest that no changes were observed in the cellular localization of the P2X7 receptor in slices of the cerebral cortex of both control and epileptic rats; the dominant localization of the P2X7 receptor in cortical nerve terminals was kept unaltered in epileptic animals assayed after two months of pilocarpine injection.

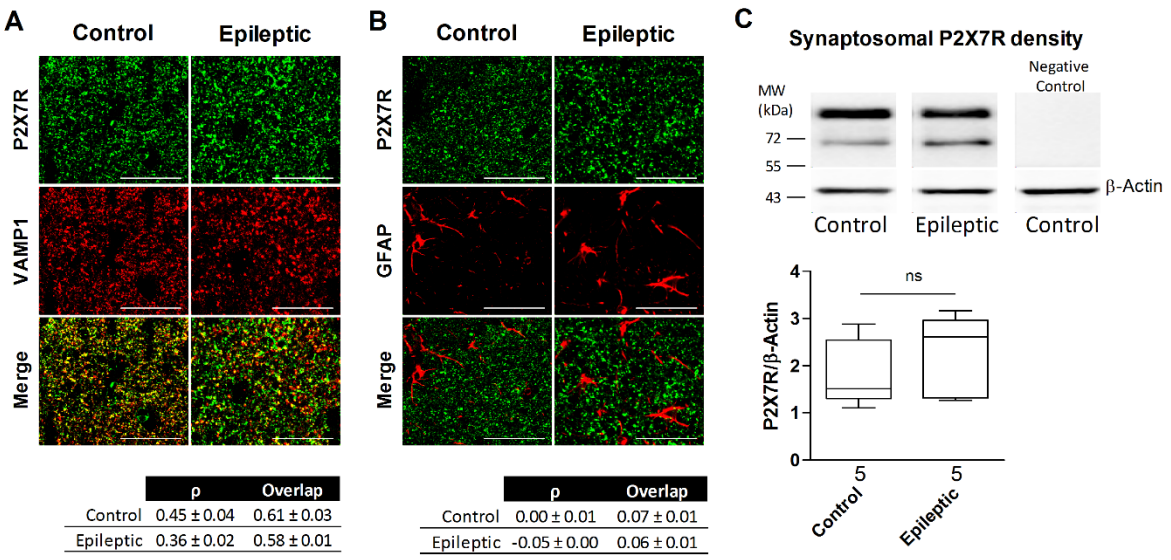

Figure 17– Immunoreactivity against the P2X7 receptor in nerve terminals isolated from the cerebral cortex of epileptic rats is similar to that found in control rats. Panels A and B illustrate confocal micrographs of rat cortical slices from control and epileptic rats stained against the P2X7 receptor. Synaptic nerve terminals were identified with VAMP-1 antibody, whereas astrocytes were stained with an antibody against the GFAP. Note that VAMP-1-positive nerve terminals (red) are endowed with the P2X7 receptor (green) (panel A), but no significant co-localization was observed between P2X7 receptor (green) and GFAP (red) (panel B). Data below confocal micrographs correspond to staining overlap and Pearson´s Coefficient (ρ) and were used to estimate co-localization of P2X7 receptor and type-specific cell markers (yellow staining). Panel C illustrates representative blots of the P2X7 receptor immunoreactivity in synaptosomes of the cerebral cortex of control and epileptic rats; gels were loaded with 75 µg of protein. Please note that both bands are specific as they disappeared after pre-adsorption of antibody with the control peptide antigen (negative control; lane 3). β-actin was used as a reference protein. Data are expressed as mean ± SEM and the number of animals per group is shown below each bar. ns (Student's *t*-test with Welch correction) represents non-significant differences as compared to control rats. Scale bars = 50 µm.

Regarding the expression density of the P2X7 receptor in the cerebral cortex of control and pilocarpine-injected epileptic rats, Western blot analysis shows that the P2X7 receptor expression normalized by β-actin was kept fairly constant in the two animal groups (Figure 17C). Like that observed in cortical synaptosomes of control rats, two bands corresponding to the P2X7 receptor appear at ~72 kDa (Künzli et al., 2007; Jimenez-Pacheco et al., 2013; Yu et al., 2013) in the samples

isolated from epileptic animals. Please note that both bands disappeared after pre-adsorption of the antibody against P2X7 receptor with excess of the control peptide antigen corresponding to amino acid residues 576-595 of the intracellular C-terminus of the rat P2X7 receptor, confirming the specificity of the antibody used in this study (Figure 17C, lane 3, Negative Control).

Discussion and conclusions

Despite the fact that P2X7 receptor has been considered the P2 receptor more committed to epilepsy, controversy still exists regarding its pro- or anticonvulsant effects in distinct animal models. Pilocarpine-induced seizures were increased in mice lacking the P2X7 receptor, although no such effect was observed in the kainate epilepsy model (Kim and Kang, 2011). *In vitro* studies also argue that the P2X7 receptor activation can be inhibitory. These include a report showing that presynaptic P2X7 receptor reduce neurotransmitter release in hippocampal slices (Armstrong et al., 2002). Conversely, increasing evidences suggest that the P2X7 receptor may be upregulated in the hippocampus and cerebral cortex of rodents with temporal lobe epilepsy induced either with pilocarpine or kainate; these findings were observed both in acute and chronic phases of the disorder (Avignone et al., 2008; Doná et al., 2009; Jimenez-Pacheco et al., 2013). Nevertheless, it might happen that the P2X7 receptor expression may transiently decrease to control levels in the latent phase of the disease (Doná et al., 2009) and this finding may complicate interpretation of the results obtained by various research groups. An increase in the density of the P2X7 receptor was also observed in the neocortex from patients with drug-resistant temporal lobe epilepsy (Jimenez-Pacheco et al., 2013), as well as in the hippocampus of patients with temporal lobe epilepsy associated with hippocampal sclerosis (abstracts published by Fernandes et al., 2009; Padrão et al., 2011). Manipulation of the P2X7 receptor tone with the selective antagonist, A-438079, exerts an anticonvulsant effect in rodent models of epilepsy (Engel et al., 2012a; Engel et al., 2012b; Henshall et al., 2013; Jimenez-Pacheco et al., 2013).

In this study, we show for the first time that activation of the P2X7 receptor in isolated nerve terminals of the rat cerebral cortex downregulates high-affinity GABA and glutamate uptake by a similar extent in epileptic rats and healthy control littermates when the former where tested 2 months after induction of the SE by

injecting pilocarpine. Immunofluorescence confocal microscopy data and Western blot analysis show that the P2X7 receptor is highly enriched in nerve terminals of the rat cerebral cortex labeled with VAMP1, with no immunoreactivity being observed in GFAP-positive astrocytes. In contrast to previous findings (Avignone et al., 2008; Doná et al., 2009; Jimenez-Pacheco et al., 2013), we observed no significant differences in the P2X7 receptor expression density normalized by β-actin in synaptosomes isolated from healthy controls and epileptic rats. This may explain why no changes were detected in the magnitude of the inhibitory effect of BzATP on GABA and glutamate uptake by synaptosomes of the cerebral cortex of the two groups of animals, when the nucleotide was tested in the high micromolar concentration range. Whether these contradictory findings regarding the role of the P2X7 receptor in different animal models of epilepsy are attributed to changes in the receptor expression and function along the disease progression (Doná et al., 2009) and/or to the underlying epileptogenic process remains to be elucidated.

In fact, the use of animal models for studying epilepsy has been associated with many limitations (Kandratavicius et al., 2014), which are mainly due to (1) anatomical and functional differences between species, (2) the chronicity of the disease stage, and/or (3) the origin and neuronal network adaptation to the initial cause of the disease. It is, therefore, plausible that under the present experimental conditions, epileptic animals used 2 months after induction of the SE with pilocarpine require extra time to develop measurable changes in cortical P2X7 receptor expression and function comparable to those found in the neocortex of more severe epileptic animal models and, eventually, of MTLE patients, as for the latter the epileptogenic process lasts several years (see original data in the following two papers).

PAPER 3

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Up regulation of P2X7 receptor-mediated inhibition of GABA uptake by nerve terminals of the human epileptic neocortex

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Abstract

Thirty percent of epileptic patients are refractory to medication. The majority of these patients have MTLE. This prompts for new pharmacological targets, like ATP-mediated signaling pathways, since the extracellular levels of the nucleotide dramatically increase during *in vitro* epileptic seizures. In this study, we investigated whether sodium-dependent GABA and glutamate uptake by isolated nerve terminals of the human neocortex could be modulated by ATP acting via slow-desensitizing P2X7 receptor.

Modulation of [^{3}H]GABA and [^{14}C]glutamate uptake by ATP, through activation of the P2X7 receptor, was investigated in isolated nerve terminals of the neocortex of cadaveric controls and drug-resistant epileptic (non-MTLE and MTLE) patients submitted to surgery. Tissue density and distribution of the P2X7 receptor in the human neocortex was assessed by western blot analysis and immunofluorescence confocal microscopy.

The P2X7 receptor agonist, BzATP (3-100 μM) decreased [^{3}H]GABA and [^{14}C]glutamate uptake by nerve terminals of the neocortex of control and epileptic patients. The inhibitory effect of BzATP (100 μM) was prevented by the selective P2X7 receptor antagonist, A-438079 (3 μM). Downmodulation of [^{14}C]glutamate uptake by BzATP (100 μM) was roughly similar in control and epileptic patients, but the P2X7 receptor agonist inhibited more effectively [^{3}H]GABA uptake in the epileptic tissue. Neocortical nerve terminals of epileptic patients express higher amounts of the P2X7 receptor protein than control samples.

High-frequency cortical activity during epileptic seizures releases huge amounts of ATP, which by acting on low-affinity slowly-desensitizing ionotropic P2X7 receptor, leads to downmodulation of neuronal GABA and glutamate uptake. Increased P2X7 receptor expression in neocortical nerve terminals of epileptic patients may, under high-frequency firing, endure GABA signaling and increase GABAergic rundown, unbalancing glutamatergic neuroexcitation. This study highlights the relevance of the ATP-sensitive P2X7 receptor as an important negative modulator of GABA and glutamate transport and prompts for novel anti-epileptic therapeutic targets.

Introduction

Epilepsy is the second most common neurological disorder, affecting ~50 million people worldwide (Pitkänen and Lukasiuk, 2011). Nearly 30% of epileptic patients are drug refractory and most of them exhibit MTLE with unknown etiology (Pitkänen and Lukasiuk, 2011). Amygdalohyppocampectomy surgery is the last resource treatment for some, leaving remaining patients with an unmet medical need. This prompts for new pharmacological tools to control seizures and/or epileptogenesis (Pitkänen and Lukasiuk, 2011).

Sodium-coupled high-affinity GABA and glutamate transporters located in the plasma membrane of both neurons and glial cells emerge as potential targets, since they are crucial in terminating amino acid neurotransmission and synaptic excitability by removing neurotransmitters from the synaptic cleft. In order to adapt the uptake of amino acids to the synaptic environment, transporters should be rapidly modulated. However, little is known about the signaling molecules responsible for fast regulation of GABA and glutamate transport in the epileptic human brain.

ATP is a likely candidate, since *in vitro* studies have shown that the extracellular levels of this nucleotide dramatically increase during high-frequency neuronal firing and/or pathologic brain activity, such as prolonged or repeated seizures (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012). ATP exerts its action through the activation of P2X and P2Y receptors. Increasing evidences have implicated the participation of ionotropic P2X7 receptor in epilepsy (Vianna et al., 2002; Fernandes et al., 2009; Padrão et al., 2011; Engel et al., 2012a; Jimenez-Pacheco et al., 2013). It has been shown that P2X7 receptor levels increase after *SE* in hippocampus and cortex of mice (Engel et al., 2012a; Jimenez-Pacheco et al., 2013) and other animal models (Vianna et al., 2002). Increased neocortical expression of the P2X7 receptor has also been demonstrated in human patients with pharmaco-resistant temporal lobe epilepsy (Jimenez-Pacheco et al., 2013), as well as in the hippocampus of patients with temporal lobe epilepsy associated with hippocampal sclerosis (published in abstract form) (Fernandes et al., 2009; Padrão et al., 2011). Additionally, an anticonvulsant effect of P2X7 receptor antagonists has been observed in animal models of epilepsy (Engel et al., 2012a; Jimenez-Pacheco et al., 2013).

Although P2X7 receptor shows low affinity (0.1–1 mM) for its agonist (Jarvis and Khakh, 2009), high levels of ATP released during intense and/or high-frequency stimuli (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) activate this receptor leading to spatial-temporal coincidence of high concentrations of ATP, glutamate and GABA at the synapse. Additionally, under conditions of high-frequency nerve firing extracellular Ca^{2+} drops (Heinemann et al., 1977; Rusakov and Fine, 2003; Engel et al., 2012a; Torres et al., 2012; Jimenez-Pacheco et al., 2013) allowing for full receptor activation, since Ca^{2+} is a negative modulator of these P2X7 receptor (Virginio et al., 1997; Yan et al., 2011). Full opening of the P2X7 receptor pore leads to extensive Na^{+}-influx, which might dissipate the Na^{+}-gradient across the plasma membrane and, thus, the transport driving force for GABA and glutamate (Lo et al., 2008; Yu et al., 2010). Astrocytic and neuronal P2X7 receptor activation may also contribute to neuronal damage by promoting the release of ATP, glutamate and GABA, unbalancing excitability (Tian et al., 2005) and/or causing a direct neurotoxic effect (Volonté et al., 2003).

Given our previous study showing that the P2X7 receptor activation downmodulates Na^{+}-dependent high-affinity GABA and glutamate transport into rat brain cortical synaptosomes (Paper 1; Barros-Barbosa et al., 2015b) , here we investigated if this modulation also occurs in the neocortex of cadaveric controls and epileptic (non-MTLE and MTLE) human patients. Taking into consideration the overall antagonistic effects of GABA and glutamate on synaptic networking, the uptake of the two amino acids was evaluated in parallel, *i.e.* under the same modulatory conditions, in order to appreciate putative differential effects caused by activation of the P2X7 receptor in control and epileptic tissue.

Experimental procedures

Drugs and solutions

HEPES and triton X-100 were from Merck Millipore (Darmstadt, Germany); GABA, SDS, 2-mercaptoethanol, sodium deoxycholate, Tris, BSA, bromophenol blue, glycerol, tween 20, BzATP, ouabain, EGTA, NMDG, protease inhibitors and AOAA were from Sigma-Aldrich (St. Louis, MO, USA); L-Glutamic acid, A-438079 and DL-TBOA were from Tocris (Bristol, UK); SKF89976A was from Abcam

(Cambridge, UK); [^{14}C]Glutamate and [^{3}H]GABA were from American Radiolabeled chemicals (St. Louis, MO, USA).

No statistical differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used, were observed.

Subjects

Human epileptic brain samples were obtained from patients undergoing surgery at the Department of Neurosurgery of the Centro Hospitalar do Porto – Hospital Geral de Santo António (CHP-HGSA). This study and all its procedures were approved by the Ethics Committees of CHP-HGSA and Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto. All epileptic patients signed an informed consent for using the biological material. The amount of tissue removed did not differ from the strict amount necessary for successful surgery practice. Resected tissue was kept in cold artificial cerebral-spinal fluid (in mM: glucose 10, NaCl 124, KCl 3, $MgCl_2$ 1, NaH_2PO_4 1.2, $NaHCO_3$ 26, $CaCl_2$ 2, pH 7.40) and used within 2-6 hours after collection. Surgical specimens were divided into two groups: MTLE and non-MTLE (composed by patients that have epilepsy other than MTLE).

Control brain samples were obtained from four human cadavers, with no previous history of neurological disease, submitted to forensic autopsy performed within 4-7 hours *post-mortem*, which corresponds to the tissue viability window for functional assays. Previous experiments from our group demonstrated that Ca^{2+} influx into nerve terminals of the human neocortex depolarized by veratridine (10 µM) decreases drastically (P<0.05) when brain samples are collected more than 7 hours after death, reaching zero activity 8h *post-mortem* (manuscript in preparation); due to legal constrains, it is virtually impossible to obtain forensic brain samples within the first 4 hours *post-mortem*. Brain samples were made available by the Instituto Nacional de Medicina Legal e Ciências Forenses – Delegação do Norte (INMLCF-DN), according to Decree-Law 274/99, of 22 July, published in Diário da República - 1st SERIE A, No. 169, of 22-07-1999, Page 4522, regarding the regulation on the ethical use of human cadaveric tissue for research. After collection, brain tissue was kept in cold artificial cerebral-spinal fluid until usage.

Clinical information on the composition of the control and epileptic groups is provided in Table 6. The investigation conforms to the principles outlined in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

Table 6 – Comparison of clinical variables of the three groups: control, non-MTLE and MTLE

	Control	Non-MTLE	MTLE
Age (years) ± SD	60.5 ± 16.3	29.0 ± 22.6	43.4 ± 6.0
Male : Female	3 : 1	6 : 3	4 : 6
Epilepsy onset (years) ± SD	NA	10.1 ± 15.5	11.05 ± 9.2
Duration (years) ± SD	NA	19.0 ± 19.4	32.35 ± 11.6
pmi (h) ± SD	5.1 ± 1.3	NA	NA
Pathology	Acute myocardial infarction (4)	Dysplasia (6), Dystrophy (1), Ganglioma (1), Cavernous angiome (1)	MTLE

NA= not applicable; pmi= *post mortem* interval; SD=standard deviation

Isolation of nerve terminals from human neocortex

Nerve terminals (synaptosomes) from the human neocortex were isolated as previously described (Bancila et al., 2009). Briefly, the neocortex was gently homogenized in cold oxygenated (95% O_2 /5% CO_2) Krebs solution (in mM: glucose 5.5, NaCl 136, KCl 3, $MgCl_2$ 1.2, Na_2HPO_4 1.2, $NaHCO_3$ 16.2, $CaCl_2$ 0.5, pH 7.40). Homogenates were filtered through a nylon filter (mesh size 100 µm). The filtrate was left to sit until pellet formation, which was re-suspended into Krebs solution and left at room temperature. Protein concentration determined by the BCA method (Pierce™, Thermoscientific, Rockford USA) was adjusted to 6.25 mg protein mL^{-1}. In order to take full advantage of scarce cadaveric human brain tissue meeting the criteria for inclusion in this study (Table 6), we used re-sealed nerve terminal membranes in some control experiments required to implement the technique for using human neocortex in our lab (Cordeiro et al., 2000; Cordeiro et al., 2003). Compared to synaptosomes, isolation of re-sealed nerve terminal membranes of the human neocortex allowed processing larger amounts of tissue while taking up [^{3}H]GABA and [^{14}C]glutamate via Na^+-dependent high-affinity transporters with a similar kinetics.

[^{3}H]GABA and [^{14}C]glutamate uptake experiments

[^{3}H]GABA uptake was measured as described elsewhere (Cordeiro et al., 2000; Cordeiro et al., 2003; Barros-Barbosa et al., 2015b). [^{3}H]GABA (0.25 µCi ml^{-1}; 0.5 µM) uptake reactions, carried out at 30 ºC, were initiated by adding either synaptosomes or re-sealed nerve terminal membranes to the reaction media containing AOAA, which prevents GABA metabolism by GABAT. Reactions were stopped by rapid filtration through glass-fiber prefilters (Merck Millipore, Cork, IRL), prewashed with cold saline. The filters were washed with the same medium and the radioactivity was measured by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA). [^{3}H]GABA uptake values were expressed as pmol mg protein^{-1} after subtraction of blank values. Assays were performed in triplicates. All experiments were performed in the absence of extracellular Ca^{2+} (Barros-Barbosa et al., 2015b).

[^{14}C]glutamate uptake (0.25 µCi mL^{-1}) was measured as described above for [^{3}H]GABA uptake, but using [^{14}C]glutamate at 10 µM and without AOAA.

Ninety seconds was set as the incubation time in all experiments, so that [^{3}H]GABA and [^{14}C]glutamate uptake were measured in the linear accumulation phase. When the influence of a modifier drug was assayed, it equilibrated with the synaptosomes for 10 min before the addition of the test drug, which was added 10 min before the beginning of reaction. Control samples were incubated for the same amount of time in the absence of drugs.

Immunofluorescence confocal microscopy

Brain samples were fixed in 4% paraformaldehyde in PBS, cryopreserved in 30% sucrose and stored in a tissue freezing medium at -80ºC. Free floating 30 µm brain sections were incubated for 1 h, with blocking buffer (foetal bovine serum 10%, BSA 1%, triton X-100 0.5%, NaN_3 0.05%) and incubated overnight with the primary antibodies: rabbit anti-P2X7 receptor (1:50, Alomone #APR-004, Jerusalem, Israel), goat anti-VAMP-1 (1:20, R&D Systems #AF4828, Minneapolis, MN, USA) and mouse anti-GFAP (1:200, Chemicon #MAB360, Temecula, CA, USA). Sections were rinsed and incubated 2 h with species specific secondary antibodies (donkey anti-rabbit Alexa Fluor 488, goat anti-mouse Alexa Fluor 633 and donkey anti-goat Alexa Fluor 633; Molecular Probes,

Eugene, OR). After mounting, sections were observed and analyzed with a laser scanning confocal microscope (Olympus FV1000, Tokyo, Japan).

Co-localization was assessed by calculating the staining overlap and the ρ for each confocal micrograph using the Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, Japan). Overlap between two colors gives values between +1 (total overlap) and 0 (no overlap); the ρ is a measure of the linear correlation between two variables (stainings), giving values between +1 and −1 inclusive, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation.

Western blot analysis

Total membrane lysates and synaptosomes of the human cerebral neocortex were homogenized in RIPA buffer (Tris-HCl 25 mM (pH 7.6), NaCl 150 mM, sodium deoxycholate 1%, Triton-X-100 1%, SDS 0.1%, EDTA 5 mM and protease inhibitors). Samples were solubilized in SDS buffer (Tris-HCl 125 mM (pH 6.8), SDS 4%, bromophenol blue 0.005%, glycerol 20%, and 2-mercaptoethanol 5%), subjected to electrophoresis in SDS-polyacrylamide gels and electrotransferred onto PVDF membranes (Merck Millipore, Darmstadt, Germany). Membranes were blocked in Tris-buffered saline (in mM: Tris-HCl 10 (pH 7.6), NaCl 150) containing Tween 20 0.05% and BSA 5% and incubated with the primary antibodies: mouse anti-GFAP (1:500, Chemicon, Temecula, CA), mouse anti-synaptophysin (1:750, Chemicon, Temecula, CA), mouse anti-postsynaptic density-95 (PSD95) (1:600, Chemicon, Temecula, CA) and rabbit anti-P2X7 receptor (1:200; Alomone #APR-004, Jerusalem, Israel). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody.

For normalization purpose, membranes were incubated with rabbit anti-β-actin antibody (1:1000; Abcam, Cambridge, UK), rabbit anti-β-tubulin antibody (1:2500; Abcam, Cambridge, UK) or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:200; Santa Cruz Biotechnology, Dallas, TX) following the procedures described above. The antigen-antibody complexes were visualized using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA). Gel band image densities were quantified with Image J (National Institute of Health, USA). To test for specificity of the bands corresponding to

P2X7 receptor, the anti-P2X7 receptor antibody was pre-adsorbed with a control peptide antigen corresponding to the amino acid residues 576-595 of the intracellular C-terminus of the P2X7 receptor.

Data presentation and statistical analysis

The uptake of [^{3}H]GABA and [^{14}C]glutamate was expressed in percentage of control values obtained in the same batch without adding any drug. P2X7 receptor density was expressed as fold change of control individuals. Results are expressed as mean ± SD. *n* shown in graphs represent the total number of replicas in a given situation. Statistical analysis was carried out using Graph Pad Prism 6.04 software (La Jolla, CA, USA). Unpaired Student's *t*-test with Welch correction was used for statistical analysis when parametric data was considered. For multiple comparisons, one-way ANOVA followed by Dunnett's Multiple Comparison Test was used. For multiple comparisons between multiple groups, two-way ANOVA followed by Bonferroni´s Multiple Comparison Test was used. $P < 0.05$ values were accepted as significant.

Results

Human neocortical synaptosomes are enriched in synaptic nerve terminals

Western blot analyses (Figure 18) indicate that synaptosomes isolated from the neocortex of human cadaveric controls (Figure 18B) and MTLE patients (Figure 18A and 18C) present lesser ($P<0.01$) amounts of the astrocytic marker, GFAP, compared to total lysates. Similar levels of the postsynaptic density marker, PSD95, were found in synaptosomes and total lysates of the neocortex of control individuals and MTLE patients. More importantly, synaptosomes isolated from the neocortex of control individuals (Figure 18B) and MTLE patients (Figure 18A and 18C) using our methodology are highly enriched ($P<0.001$) in synaptic nerve terminals specifically labelled with synaptophysin compared to total lysates of the same brain region.

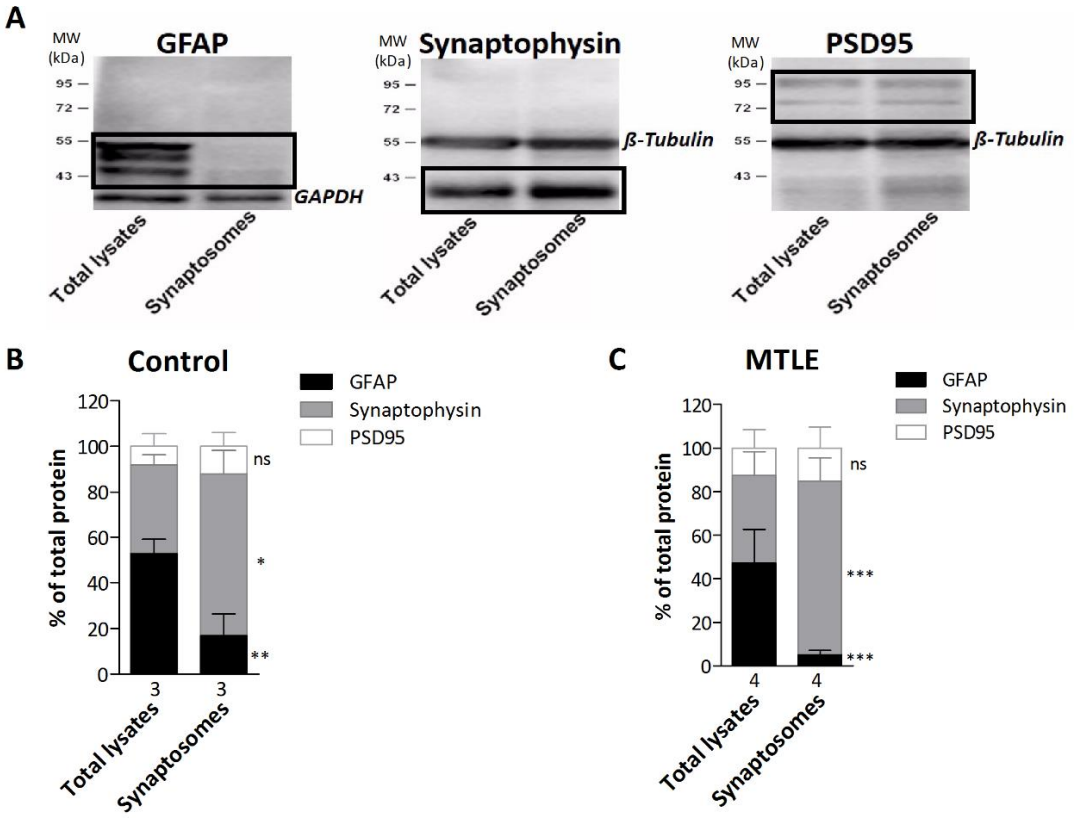

Figure 18 – Synaptosomes isolated from the neocortex of cadaveric controls and MTLE patients are enriched in synaptic nerve terminals. Panel A illustrates representative blots of GFAP, synaptophysin and PSD95 immunoreactivity (bands inside black boxes) in total lysates and synaptosomes of the neocortex of an MTLE patient. Bottom panels illustrate the average composition in GFAP, synaptophysin and PSD95 of total lysates and synaptosomes from three controls (B) and four MTLE (C) individuals. Data are expressed as mean ± SD. *P < 0.05, **P < 0.01 and ***P < 0.001 represents significant differences as compared to total lysates (two way ANOVA followed by Bonferroni´s Multiple Comparison Test); ns, non-significant.

[^{3}H]GABA and [^{14}C]glutamate uptake by isolated nerve terminals of the human neocortex is mediated by high-affinity transporters

Figure 19 (panels A and B) shows that isolated nerve terminals from the neocortex of cadaveric controls take up [^{3}H]GABA and [^{14}C]glutamate over time. Selective inhibition of the GABA transporter 1 (GAT1) with SKF89976A (40 µM) significantly decreased [^{3}H]GABA uptake by neocortical nerve terminals from cadaveric controls (85.0±6.1%), non-MTLE (94.4±1.7%) and MTLE (95.2±2.0%) patients (Figure 19C). Likewise, [^{14}C]glutamate uptake was significantly inhibited by DL-TBOA (100 µM) in neocortical nerve terminals from cadaveric controls (65.8±7.3%), non-MTLE (95.5±3.1%) and MTLE (90.4±4.2%) patients (Figure 19D); here, we used a non-selective inhibitor because subtype-specific glutamate transport inhibitors are still lacking. Data suggest that the uptake of [^{3}H]GABA

and [^{14}C]glutamate by human neocortical nerve terminals occurs mainly via high-affinity transporters, a situation that is even more obvious in epileptic patients where transport blockers reduced the uptake of both amino acids by more than 90%.

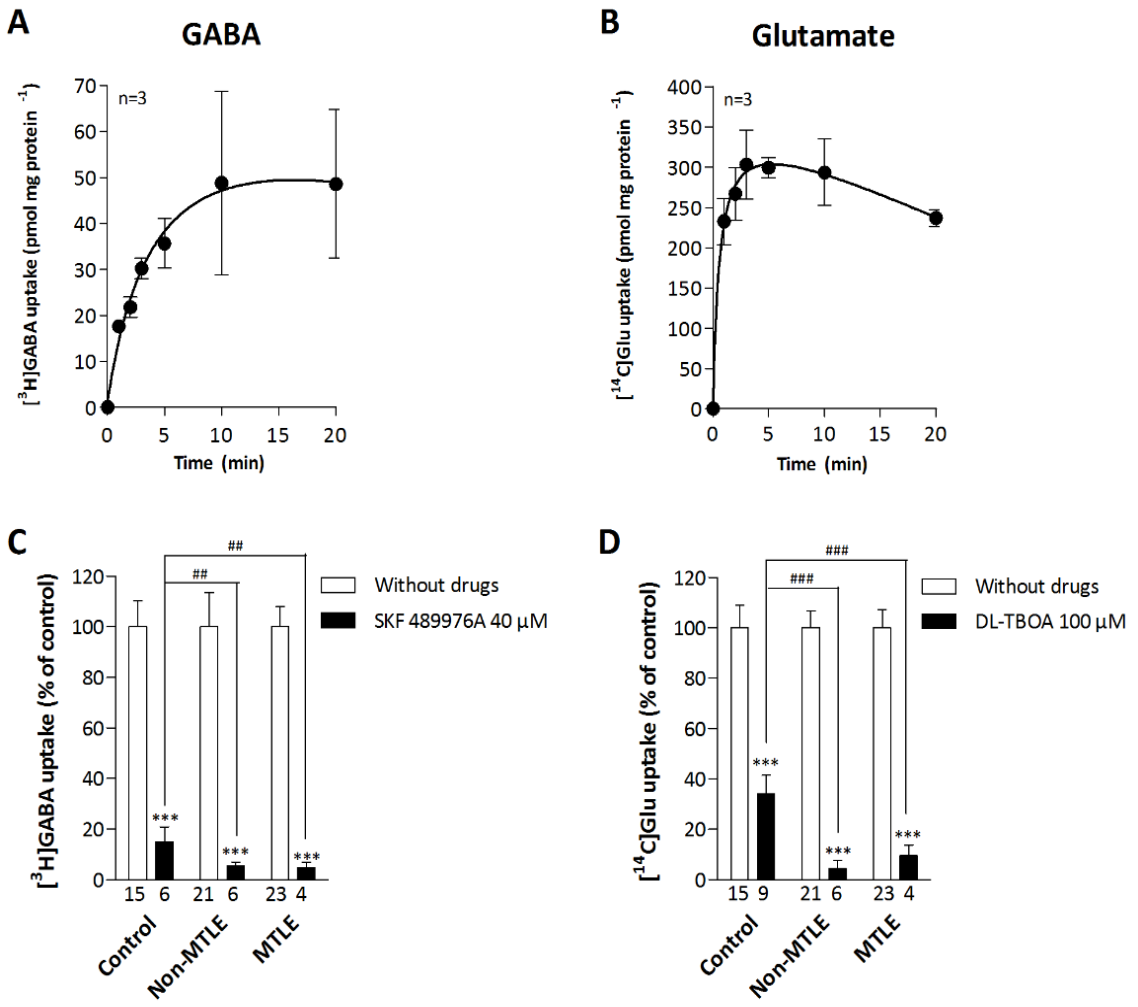

Figure 19 – Nerve terminals isolated from the human neocortex take up [^{3}H]GABA and [^{14}C]glutamate through Na^{+}-dependent high-affinity transporters. Illustrated is the accumulation of [^{3}H]GABA (A) and [^{14}C]glutamate (B) into nerve terminals from the neocortex of control individuals over time (0-20 min). Panels C and D show the inhibition of Na^{+}-coupled high-affinity transport of [^{3}H]GABA and [^{14}C]glutamate with SKF489976A (40 µM; GAT1 selective inhibitor) and DL-TBOA (100 µM; non-specific inhibitor of the EAAT), respectively, into nerve terminals of control individuals and epileptic (non-MTLE and MTLE) patients. SKF 489976A and DL-TBOA contacted with nerve terminals for 20 min before application of [^{3}H]GABA and [^{14}C]glutamate into the reaction chamber. Data are expressed as mean ± SD; the *n* number of replicas is shown below each bar. *P < 0.05 and ***P < 0.001 (Student's *t*-test with Welch correction) represents significant differences as compared to the situation where no drugs were added. ## P < 0.01 and ### P < 0.001 (one way ANOVA followed by Dunnett's Multiple Comparison Test) represents significant differences when compared to control individuals.

Neocortical nerve terminals of epileptic patients express higher levels of P2X7 receptor

The existence of functional P2X7 receptors on cortical nerve terminals of the rat has been previously demonstrated by microfluorimetric intracellular Ca^{2+}-concentration measurements and immunochemical staining (Miras-Portugal et al., 2003; Barros-Barbosa et al., 2015b). Here, we evaluated by immunofluorescence confocal microscopy if the P2X7 receptor is also present in nerve terminals of the human cerebral neocortex.

Figure 20A shows that the antibody directed against P2X7 receptor (green) co-localizes with the synaptic nerve terminal marker, VAMP-1 (red), in the three groups of individuals, controls, non-MTLE and MTLE, but no significant co-localization was detected with the astrocytic cell marker, GFAP; this was observed despite intense astrogliosis is documented in the epileptic brain (Figure 20B). Co-localization was assessed by evaluating the Pearson´s coefficient (ρ) and staining overlap obtained by merging the two fluorescent channels (yellow staining). These results are presented on the right-hand side of confocal micrographs (Figure 20A and 20B).

Co-localization of the P2X7 receptor with VAMP-1 was significantly higher in the neocortex of epileptic patients as compared to controls (Figure 20A), giving the impression that epileptic nerve terminals express more P2X7 receptor compared to control individuals. We confirmed this hypothesis by Western blot analysis. Figure 20D shows that the P2X7 receptor protein density increased five-fold in neocortical synaptosomes of MTLE patients compared to cadaveric controls, as previously described in the literature (Jimenez-Pacheco et al., 2013). Enrichment of the P2X7 receptor protein density in neocortical nerve terminals of non-MTLE tissue was intermediate between control and MTLE patients (Figure 20D). Disappearance of P2X7 receptor bands after pre-adsorption with excess of the corresponding antigen peptide sequence confirmed the specificity of the antibody used in this study (Figure 20D, lane 4).

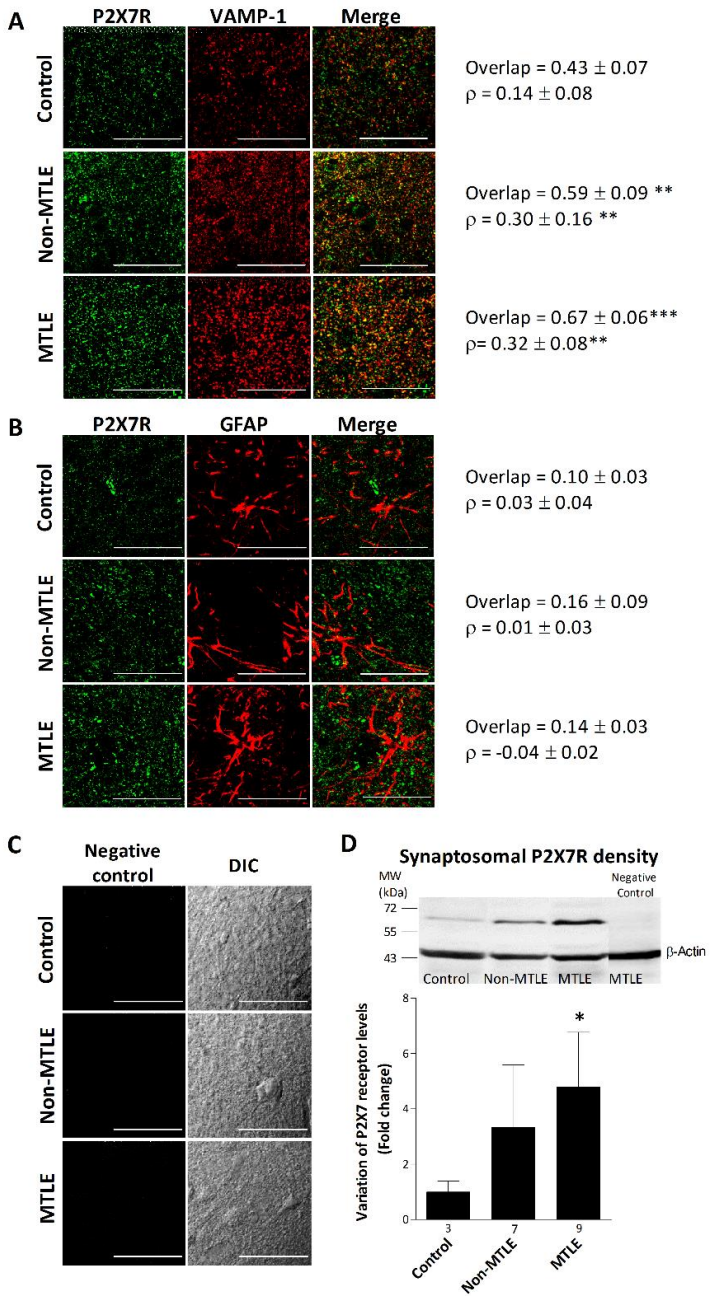

Figure 20 – Immunoreactivity against the P2X7 receptor is higher in nerve terminals isolated from the neocortex of epileptic patients than in control individuals. Panels A and B illustrate confocal micrographs of human neocortical slices from control, non-MTLE and MTLE individuals stained against the P2X7 receptor. Synaptic nerve terminals were identified with VAMP-1 antibody, whereas astrocytes were stained with an antibody against the GFAP. Note that VAMP-1-positive nerve terminals (red) are endowed with the P2X7 receptor (green) (panel A), but no significant co-localization was observed between P2X7 receptor (green) and GFAP (red) (panel B). Data on the right-hand side correspond to staining overlap and Pearson´s Coefficient (ρ) parameters calculated from 3-4 confocal micrographs per individual; at least three individuals from each group, control, non-MTLE and MTLE, were analyzed. These parameters were automatically calculated per image with the Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, Japan) and were used to estimate co-localization of P2X7 receptor and type-specific cell markers (yellow staining). Panel C shows negative controls resulting from the incubation of human neocortical slices from control, non-MTLE and MTLE individuals with the anti-rabbit secondary antibody without previous addition of the rabbit anti-P2X7 receptor primary antibody (#APR-004); DIC image is shown for comparison. Panel D illustrates representative blots of the P2X7 receptor immunoreactivity in synaptosomes of the human neocortex of control, non-MTLE and MTLE patients; gels were loaded with 100 µg of protein. Please note that the band corresponding to the molecular weight of native P2X7 receptor (~69 kDa) disappeared after pre-adsorption of the primary antibody (#APR-004) with a control peptide antigen equivalent to the amino acid residues 576-595 of the intracellular C-terminus of the P2X7 receptor (lane 4 - negative control); β-actin was used as a reference protein. Data are expressed as mean ± SD and the number of individuals per group is shown below each bar. *P < 0.05, **P < 0.01 and ***P < 0.001 (two way ANOVA followed by Bonferroni´s Multiple Comparison Test (panels A and B) and one way ANOVA followed by Dunnett's Multiple Comparison Test (panel D) represents significant differences as compared to control individuals. Scale bars = 50 µm.

Activation of the P2X7 receptor inhibits more potently the uptake of [^{3}H]GABA than of [^{14}C]glutamate by nerve terminals of the human epileptic neocortex

The prototypic P2X7 receptor agonist, BzATP (3-300 µM), concentration-dependently decreased [^{3}H]GABA (Figure 21A) and [^{14}C]glutamate (Figure 21B) uptake by neocortical nerve terminals of control individuals and epileptic patients. While downmodulation of [^{14}C]glutamate uptake by BzATP (100 µM) was roughly similar in control, non-MTLE and MTLE patients (Figure 21B), the P2X7 receptor agonist inhibited more effectively [^{3}H]GABA uptake in neocortical nerve terminals isolated from both non-MTLE and MTLE patients than from control individuals (Figure 21A). The selective P2X7 receptor antagonist, A-438079 (3 µM), significantly ($P<0.05$) attenuated the inhibitory effects of BzATP (100 µM) on [^{3}H]GABA (Figure 21C) and [^{14}C]glutamate (Figure 21D) uptake in epileptic patients, further confirming the involvement of the P2X7 receptor on BzATP-induced downmodulation of high-affinity GABA and glutamate uptake by human neocortical nerve terminals.

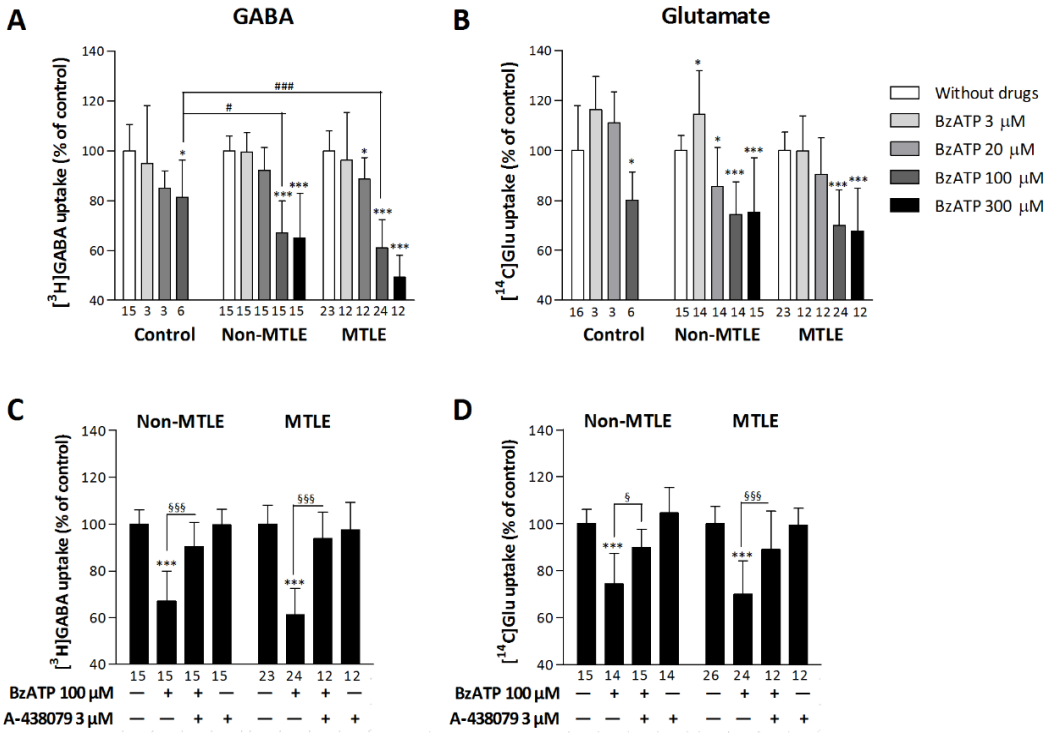

Figure 21 – Downmodulation of GABA, but not of glutamate, uptake by P2X7 receptor activation is upregulated in the neocortex of epileptic patients. Panels A and B illustrate the effect of BzATP (3-300 µM) on [^{3}H]GABA and [^{14}C]glutamate uptake by nerve terminals isolated from the neocortex of control, non-MTLE and MTLE patients. The selective P2X7 receptor antagonist, A-438079 (3 µM) prevented BzATP (100 µM)-induced inhibition of [^{3}H]GABA (C) and [^{14}C]glutamate

(D) uptake by neocortical synaptosomes of non-MTLE and MTLE patients. BzATP contacted with nerve terminals for 10 min before application of [^{3}H]GABA and [^{14}C]glutamate in to reaction chamber; A-438079 was added 10 min before BzATP. Data are expressed as mean ± SD; the *n* number of replicas is shown below each bar. *P < 0.05 and ***P < 0.001 (Student's *t*-test with Welch correction) represents significant differences as compared to the situation where no drugs were added; §P < 0.05 and §§§P < 0.001 (Student's *t*-test with Welch correction) represents significant differences as compared to BzATP (100 µM) alone; #P < 0.05 and ###P < 0.001 (two way ANOVA followed by Bonferroni´s Multiple Comparison Test) represents significant differences as compared to control individuals.

P2X7-induced downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake by human neocortical nerve terminals parallels the collapse of the transmembrane Na^+-gradient

Taking into consideration that (1) the P2X7 receptor works as a nonselective cationic channel promoting Na^+ entry under low Ca^{2+} conditions (Jarvis and Khakh, 2009), (2) GABA and glutamate uptake is critically dependent on transmembrane Na^+-gradient (Kanner, 2006), (3) extracellular Ca^{2+} was removed from the reaction medium (Barros-Barbosa et al., 2015b), and (4) Na^+ influx through P2X7 receptor and subsequent dissipation of the Na^+ gradient is responsible for the downmodulation of GABA and glutamate transport in rat cortex synaptosomes (Barros-Barbosa et al., 2015b), we evaluated if a similar mechanism could justify the P2X7-induced decrease of GABA and glutamate uptake in human neocortical nerve terminals.

Manipulation of intracellular Na^+ levels with ouabain (50 µM), an inhibitor of Na^+/K^+-ATPase, progressively decreased the uptake of [^{3}H]GABA (to a maximum of 97.1±2.0%; Figure 22) and [^{14}C]glutamate (to a maximum of 51.4±8.2%; Figure 22B) depending on the time of incubation with this drug (2-40 min). The inhibitory effects of ouabain (50 µM) incubated for 2 minutes (51.7±4.6% and 23.9±9.2% for GABA and glutamate, respectively) and for 20 min (93.9±2.1% and 42.2±6.0% for GABA and glutamate, respectively) were amplified in the presence of BzATP (100 µM). Magnification of ouabain (50 µM)-induced inhibition by BzATP (100 µM) decreased over time of incubation with the Na^+/K^+-ATPase inhibitor.

Furthermore, the substitution of extracellular Na^+ by $NMDG^+$, so that the Na^+ concentration in the reaction chamber decreased from 129 mM to 101, 69 and 9 mM, also diminished the uptake of [^{3}H]GABA (Figure 22C) and [^{14}C]glutamate (Figure 22D) by MTLE synaptosomes. Collapsing the Na^+ gradient

across the plasma membrane by reducing the extracellular Na^+ concentration to 9 mM (plus 120 mM $NMDG^+$) decreased the uptake of [^{3}H]GABA and [^{14}C]glutamate by 94.2±5.0% (Figure 22C) and 83.4±2.6% (Figure 22D), respectively. The inhibitory effect of BzATP (100 µM) on [^{3}H]GABA and [^{14}C]glutamate uptake diminished upon decreasing the amount of Na^+ in the reaction fluid from 129 mM to 101 mM and to a concentration (69 mM) below which no significant difference (P>0.05) from the control situation was observed, which in accordance with both Na^+-gradient and Na^+-coupled transport thermodynamics.

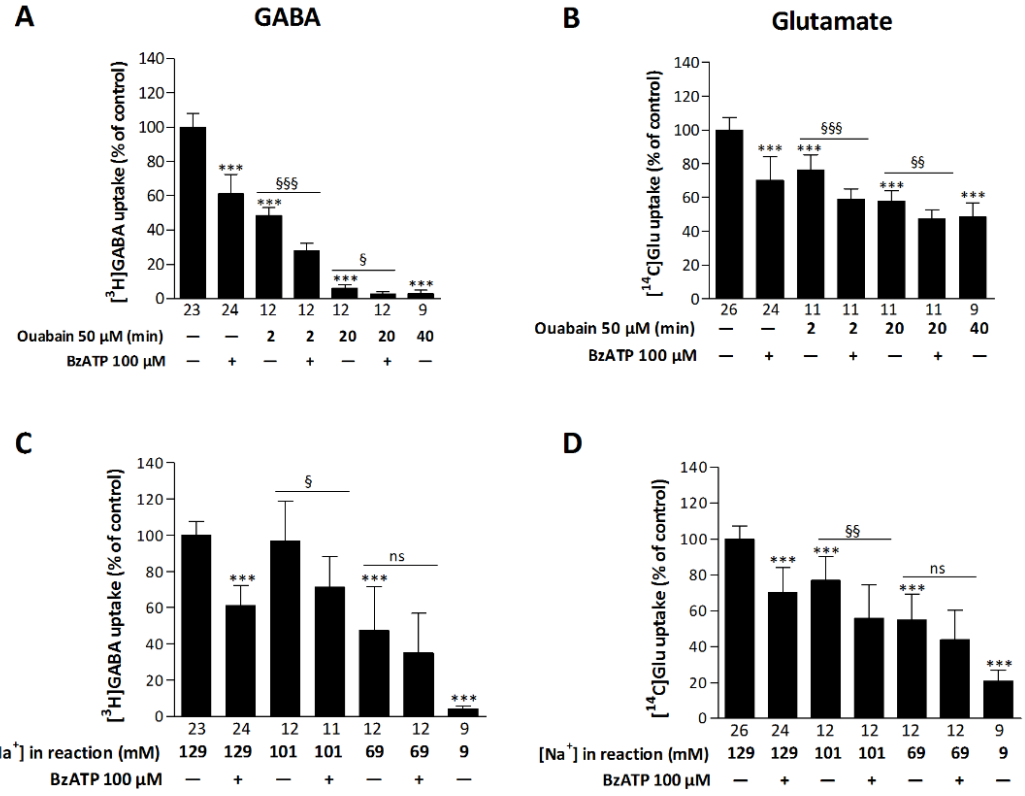

Figure 22 – P2X7 receptor-induced downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake by human neocortical nerve terminals parallels the collapse of transmembrane Na^+-gradient. Shown is the inhibitory effect of BzATP (100 µM) on [^{3}H]GABA (A and C) and [^{14}C]glutamate (B and D) uptake into neocortical synaptosomes of MTLE patients in the presence of the Na^+/K^+ ATPase inhibitor, ouabain (50 µM, applied for 2-40 min) (A and B) or after substituting extracellular Na^+ with $NMDG^+$, so that the Na^+-concentration in the reaction chamber decreased from 129 mM to 101, 69 and 9 mM, respectively (C and D). BzATP contacted with nerve terminals for 10 min before application of [^{3}H]GABA and [^{14}C]glutamate in to reaction chamber; ouabain was added 2, 20 and 40 min before BzATP. Data are expressed as mean ± SD; the *n* number of replicas is shown below each bar. *P < 0.05 and ***P < 0.001 (Student's *t*-test with Welch correction) represents significant differences as compared to the situation where no drugs were added; §P < 0.05 and §§§§P < 0.001 (Student's *t*-test with Welch correction) represents significant differences when comparing the effect of BzATP with the modifier alone; ns, non-significant differences.

All together these results suggest that increasing intracellular Na^+-concentration or decreasing extracellular Na^+-concentration, *i.e.* collapsing the Na^+-gradient, downmodulates [^{3}H]GABA and [^{14}C]glutamate uptake by nerve terminals of the human neocortex under low extracellular Ca^{2+} conditions, similarly to that occurring upon activation of the P2X7 receptor. Thus, P2X7 receptor-induced downmodulation of GABA and glutamate transport closely accompany the thermodynamic changes associated with Na^+-gradient across nerve terminal membranes.

Discussion and conclusions

This work demonstrates that neocortical nerve terminals isolated from drug-resistant epileptic human patients express higher-than-control amounts of P2X7 receptor, which negatively modulate Na^+-dependent high-affinity GABA and glutamate uptake. Additionally, our findings show for the first time that an increase in the density of P2X7 receptor in isolated nerve terminals of the epileptic neocortex correlates with a more potent P2X7 receptor-induced downmodulation of GABA uptake, but no significant changes were observed concerning downmodulation of the glutamate uptake. The minor difference in the P2X7 receptor protein density among MTLE and non-MTLE patients had no repercussion in the magnitude of the inhibitory effect of BzATP on [^{3}H]GABA and [^{14}C]glutamate uptake, suggesting that deregulation of the P2X7 receptor is characteristic of the epileptic neocortex independently of brain lesion characteristics.

Changes in the P2X7 receptor-mediated downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake were assessed in the absence of extracellular Ca^{2+} in order to mimic conditions that occur under high-frequency neuronal firing and/or pathologic brain activity, such as prolonged or repeated seizures (Engel et al., 2012a). Under these conditions the extracellular Ca^{2+} concentration falls up to 90% (Heinemann et al., 1977; Rusakov and Fine, 2003; Engel et al., 2012a; Torres et al., 2012; Jimenez-Pacheco et al., 2013) and Na^+ conductance through the P2X7 receptor is largely enhanced, as this receptor is highly sensitive to changes in the extracellular concentration of divalent cations (Virginio et al., 1997; Yan et al., 2011). Spatial-temporal coincidence of ATP, GABA and glutamate at

the synapse is possible, given that high levels of the nucleotide are released during intense and/or high-frequency neuronal activity (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) reaching high enough concentrations to activate low-affinity P2X7 receptor (Jarvis and Khakh, 2009). Performing experiments in low extracellular Ca^{2+} conditions also excludes the involvement of Ca^{2+} regulation of amino acid transporters (directly or via plasmalemmal and mitochondrial Na^+/Ca^{2+} exchangers), as well as the interference of Ca^{2+}-activated neurotransmitter release (Cordeiro et al., 2000; Cordeiro et al., 2003; Romei et al., 2015). It also allows focusing our research on alterations of the Na^+ driving-force for neurotransmitters uptake. Under these circumstances, downmodulation of [^{3}H]GABA and [^{14}C]glutamate uptake seems to occur almost exclusively through the decrease in the Na^+ driving-force available for transporters, as previously observed in the rat neocortex (Barros-Barbosa et al., 2015b). This is concluded because: 1) increases in intracellular Na^+ concentration or decreases in extracellular Na^+ concentration downmodulated the uptake of both neurotransmitters, and 2) the magnitude of the inhibitory effect of BzATP, was lowered upon reducing the Na^+ gradient as predicted by changes in transporters reversal potentials (Kanner, 2006; Lo et al., 2008; Yu et al., 2010). However, we cannot rule-out that minute amounts of P2X7-mediated GABA and glutamate release might also occur under our experimental conditions (Barros-Barbosa et al., 2015b). Furthermore, data using the Na^+/K^+-ATPase inhibitor, ouabain (Figure 22), indicate that [^{3}H]GABA uptake was more sensitive to variations in the intracellular Na^+-concentration, *i.e.* reduction of the transmembrane Na^+ gradient, compared to [^{14}C]glutamate. Our results may be of clinical interest because significant increases in intracellular Na^+ concentration constitute a characteristic event associated with tissue injury in epilepsy (Wang et al., 1996; Yu et al., 2010). Thus, we may speculate whether increases in Na^+ conductance leading to enhanced levels of the intracellular Na^+ concentration might be correlated with excessive ATP signals conveyed through up-regulated P2X7 receptor in nerve terminals of the epileptic neocortex.

The cellular localization of P2X7 receptor in the CNS of diverse animal species has been intensively investigated, but scarce information is available in the human brain (Narcisse et al., 2005; Fu et al., 2013; Rodrigues et al., 2015). Moreover, there is a gap in our knowledge concerning the presence of the P2X7

receptor in neurons of the human brain besides a couple of preliminary accounts suggesting that its levels increase in the hippocampus and neocortex of patients with TLE (Fernandes et al., 2009; Padrão et al., 2011; Jimenez-Pacheco et al., 2013). Here we show that the P2X7 receptor co-localizes with the synaptic nerve terminal marker, VAMP-1, in human neocortex, with only vestigial staining on GFAP-positive glial cells. Interestingly, the increase in staining overlap between P2X7 receptor and VAMP-1 did not occur with GFAP in the epileptic neocortex, despite the astrogliosis that usually accompanies epileptic brain lesions. This is apparently unexpected because astrocytes release ATP and P2X7 receptor have been reported to be present on astrocytes, yet no transcriptional activation of the P2X7 receptor was detected in astrocytes either after SE or in chronic epilepsy in mice (Engel et al., 2012a; Jimenez-Pacheco et al., 2013). Western blot data confirmed that isolated nerve terminals from epileptic human neocortices express increased amounts of P2X7 receptor, which is also compatible with previous findings (Fernandes et al., 2009; Padrão et al., 2011; Jimenez-Pacheco et al., 2013). While data from the present study do not exclude the putative participation of astrocytic and microglial P2X7 receptor towards unbalanced neuronal excitability and damage in epilepsy, gain of function of neuronal P2X7 receptor under the present experimental conditions deserve a leading role in deregulating GABA and glutamate uptake by nerve terminals of the epileptic neocortex.

It, thus, seems that under physiological conditions, transient P2X7 receptor activation following ATP release by high-frequency neuronal firing (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) may engage a mechanism that equally facilitates local glutamatergic neurotransmission while promoting the endurance of GABAergic signals, ensuring tonic and more diffuse neuroinhibition. This mechanism may be relevant in processes such as learning and memory, where transient glutamate endurance in the synaptic cleft may facilitate the induction of long term potentiation, while preventing excytotoxicity by concomitant promotion of diffuse GABAergic inhibition. In epileptic conditions, controversy still exists in the literature concerning the pro- or anticonvulsant effects of the P2X7 receptor in distinct animal models. Kang and colleagues reported that pilocarpine-induced seizures were increased in mice lacking the P2X7 receptor, although they found no influence of the receptor in the kainate model (Kim and Kang, 2011). Some *in*

vitro studies also argue that the P2X7 receptor activation can be inhibitory, including work showing that presynaptic P2X7 receptor on mossy fibres reduce neurotransmitter release in hippocampal slices (Armstrong et al., 2002). Conversely, Jimenez-Pacheco's group reported increased neocortical expression of the P2X7 receptor after *SE* induced by injecting kainic acid into the amygdala of mice and showed that the P2X7 receptor antagonist, A-438079, exerts an anticonvulsant effect (Engel et al., 2012a; Engel et al., 2012b; Jimenez-Pacheco et al., 2013). These apparently contradictory findings may suggest that the contribution of the P2X7 receptor may change along the disease stage and the underlying epileptogenic process.

In epileptic conditions, the increased downmodulation of GABA uptake might not have a protective role but, conversely, a pro-epileptic one due to GABAergic "rundown" (Cohen et al., 2002; D'Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012). This is a phenomenon defined as decreases in GABAergic inhibition or even transient GABAergic mediated excitation, resultant from desensitization of $GABA_A$ receptor upon repetitive stimulation (Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010) or even due to depolarization stemming from changes in Cl^- homeostasis (D'Antuono et al., 2004; Miles et al., 2012). There are evidences that GABAergic rundown promotes synchronous neuronal discharges underlying focal seizures in refractory TLE (Cohen et al., 2002; D'Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012). This implies that instead of GABAergic signaling counterbalancing the depolarizing effects of glutamate, it may also contribute to the excitatory tone. So, it is possible that the pro-epileptic role of the P2X7 receptor activation (Engel et al., 2012a; Engel et al., 2012b; Jimenez-Pacheco et al., 2013) may be explained by an increase in the downmodulation of GABA uptake contributing to GABAergic rundown in epileptic patients (Cohen et al., 2002; D'Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012). This hypothesis requires further investigations, which are being undertaken by our group. This study fills a gap regarding the pro-epileptic role of extracellular ATP in the human brain neocortex and led us to hypothesize, in line with previous suggestions from animal studies (Vianna et al., 2002; Engel et al., 2012a; Jimenez-Pacheco et al., 2013; Rodrigues et al., 2015), that targeting the

P2X7 receptor with selective brain-permeant antagonists, such as the recently characterized JNJ-42253432, which displays high affinity and selectivity for the P2X7 receptor (Lord et al., 2014), may constitute a valuable alternative to drug-resistant epilepsy treatment.

In summary, while the mechanism of the P2X7 receptor-mediated amino acid transporters downmodulation reported here in the human neocortex appears to have a plausible role under physiological conditions by potentiating neurotransmission, it might gain a different meaning during epileptic seizures. Under epileptic conditions, the extracellular Ca^{2+}-concentration falls in parallel to increases in the amount of released ATP (Engel et al., 2012b; Jimenez-Pacheco et al., 2013). Up-regulated P2X7 receptor bolsters ATP signals to a scenario that promotes GABA-mediated neurotransmission endurance, which combined with altered $GABA_A$ receptor subunit composition, might originate GABAergic rundown (Cohen et al., 2002; D'Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012). The parallel P2X7 receptor-induced glutamate transmission boost under epileptic conditions may ultimately lead to neuronal excytotoxicity. Data points towards Na^+-coupled high-affinity amino acid transport modulators, like the P2X7 receptor selective antagonists, as being putative new anti-epileptic drug targets.

PAPER 4

MANUSCRIPT IN PREPARATION

The A_{2A} receptor is upregulated in hippocampal astrocytes of human patients with mesial temporal lobe epilepsy (MTLE)

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Abstract

Most patients with MTLE are refractory to common AEDs or to the combinatorial usage of such medications. This prompts for the urgent need to find new and more accurate pharmacological targets to control seizures and/or the epileptogenic process. The adenosine A_{2A} receptor emerges as an interesting pharmacological target since the excitatory nature of this receptor partially counteracts the dominant antiepileptic role of endogenous adenosine acting via the most abundant inhibitory A_1 receptor in the brain. Gain of function of the excitatory A_{2A} receptor has been implicated in a significant number of CNS pathologies commonly characterized by neuronal excitotoxicity, such as Alzheimer's disease, Parkinson's disease, trauma, also including epilepsy. In this study, we investigated changes in the expression and cellular localization of the A_{2A} receptor in the hippocampus of control and MTLE human patients, in order to understand whether this receptor plays a role in human epilepsy.

The expression of the A_{2A} receptor was evaluated by Western blot analysis in total lysates and in isolated nerve terminals of the hippocampus of cadaveric controls and MTLE patients submitted to amygdalohippocampectomy for ablation of the epileptic focus. The distribution of the A_{2A} receptor in the human hippocampus was assessed by immunofluorescence confocal microscopy.

Results showed that hippocampi from MTLE patients exhibit higher amounts of the A_{2A} receptor protein than the control group. Immunoreactivity against the A_{2A} receptor colocalizes this receptor with the astrocytic cell marker, GFAP. No colocalization of the A_{2A} receptor was observed with the nerve terminal marker,

synaptotagmin 1/2, neither with axon neurofilament 200 (NF200). Moreover, results also show that astrogliosis that is characteristic of MTLE with sclerosis of the hippocampus is accompanied by an upregulation of the A_{2A} receptor density. Whether this feature contributes to an overall excitatory tone facilitating seizure occurrence in drug-resistant MTLE, remains to be elucidated by functional studies. In this context, our working hypothesis is that selective blockade of the adenosine A_{2A} receptor may be a useful therapeutic approach to control drug-refractory seizures in MTLE patients.

Introduction

MTLE is the most common and devastating form of human epilepsy (Pitkänen and Lukasiuk, 2011). This disorder is characterized by irreversible biochemical and structural changes in the hippocampus and several neocortical regions (Bartolomei et al., 2005; O'Dell et al., 2012; Biagini et al., 2013). Despite the extensive research of the last decades resulting in the discovery of new AEDs introduced into the clinical practice, most MTLE patients remain refractory to medication. The leading cause for this concern is our relative ignorance regarding the underlying mechanisms responsible for the transformation of brain networks into hyperexcitable connections more prone to intense and/or prolonged neuronal discharges. The last resource treatment for some drug-refractory patients is still the surgical ablation of defective tissue to curb epileptic crisis. Yet, a significant number of MTLE patients do not fill the requirements for this surgical procedure and are faced with an unmet medical need. This calls for the urgent need to find new and more accurate pharmacological targets to control seizures and/or the epileptogenic process (Pitkänen and Lukasiuk, 2011).

Increasing evidences show that the extracellular levels of ATP and adenosine dramatically increase during high-frequency neuronal firing and/or pathologic brain activity, such as prolonged or repeated seizures (Heinrich et al., 2012; Wall and Dale, 2013; Sims and Dale, 2014). Once in the extracellular space, adenosine, released directly from cells or produced from the extracellular catabolism of ATP (Heinrich et al., 2012; Wall and Dale, 2013; Sims and Dale, 2014), exerts its action through the activation of four types of adenosine receptors, A_1, A_{2A}, A_{2B} and A_3. The most relevant adenosine receptors in the brain are inhibitory A_1 and excitatory A_{2A} receptors (Boison, 2008). Recently, numerous studies have shown the contribution of A_{2A} receptor to control neuronal excitability and synaptic plasticity (Kanno and Nishizaki, 2012; Matos et al., 2012; Valadas et al., 2012; Matos et al., 2013). Involvement of this receptor subtype in diverse pathologies of the CNS, including epilepsy, has been demonstrated (Chen et al., 2007; Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013; Orr et al., 2015). Most studies implicating the adenosine A_{2A} receptor in epilepsy were performed in rodents, both in mice (El Yacoubi et al., 2008, 2009; Orr et al., 2015; Rombo et al., 2015) and rats (Hosseinmardi et al., 2007;

Fukuda et al., 2011; Rosim et al., 2011; Li et al., 2012; Huicong et al., 2013). Results from these investigations suggest that adenosine A_{2A} receptor antagonists might offer protection against diverse epileptic syndromes, such as temporal lobe epilepsy, highlighting the idea that the A_{2A} receptor may be an attractive pharmacological target for the treatment of epilepsy. To our knowledge, there are no studies investigating changes in the expression and function of the A_{2A} receptor in the brain of drug-resistant MTLE human patients, as we did in this work.

Though it was believed that the adenosine A_{2A} receptor was mostly located in neurons at the synaptic region (Rebola et al., 2005; Rodrigues et al., 2015), recent evidences showed that this receptor is also expressed in glial cells, both astrocytes and microglia (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013; Orr et al., 2015). The astrocytic A_{2A} receptor seems to be critical for the modulation of glutamate transport, either by decreasing the uptake (Matos et al., 2012; Matos et al., 2013) or by increasing the release (Kanno and Nishizaki, 2012) of glutamate. In view of this, astrocytic A_{2A} receptor have been implicated in memory formation (Orr et al., 2015). Furthermore, the same group demonstrated that astrocytic A_{2A} receptor levels are upregulated in humans with Alzheimer's disease, as well as in epileptic mice injected with kainate (Orr et al., 2015).

Given the excitatory (pro-convulsive) nature of the adenosine A_{2A} receptor activation in the brain and the need for clarification of its role in drug-resistant human epilepsy, we designed this study to investigate changes in the expression and cellular localization of the A_{2A} receptor in the hippocampus of control and MTLE human patients.

Experimental procedures

Subjects

Human MTLE brain samples were obtained from patients undergoing amygdalohippocampectomy at the Department of Neurosurgery of the CHP-HGSA. This study and all its procedures were approved by the Ethics Committees of CHP-HGSA and Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto. All MTLE patients signed an informed consent for using the biological material. The amount of tissue removed did not differ from the strict amount necessary for successful epileptic focus ablation surgery.

Control brain samples were obtained from four human cadavers submitted to forensic autopsy performed within 4-7 hours *post-mortem* (Barros-Barbosa et al., 2015a). Brain samples were made available by the Instituto Nacional de Medicina Legal e Ciências Forenses – Delegação do Norte (INMLCF-DN), according to Decree-Law 274/99, of 22 July, published in Diário da República - 1st SERIE A, No. 169, of 22-07-1999, Page 4522, regarding the regulation on the ethical use of human cadaveric tissue for research.

Clinical information on the composition of control and MTLE groups is provided in Table 1. The investigation conforms to the principles outlined in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

Table 7 – Comparison of clinical variables of control and MTLE patients.

	Control	MTLE
Age (years) ± SD	60.5 ± 16.3	38.9 ± 9.0
Male : Female	3 : 1	5 : 3
Epilepsy onset (years) ± SD	NA	9.9 ± 6.7
Duration (years) ± SD	NA	28.9 ± 12.8
pmi (h) ± SD	5.1 ± 1.3	NA
Cause of death	Acute myocardial infarction (4)	NA

NA= not applicable; pmi= *post mortem* interval; SD=standard deviation

Isolation of nerve terminals from human hippocampus

Nerve terminals (synaptosomes) from the human hippocampus were isolated as previously described (Bancila et al. 2009; Paper 1, Barros-Barbosa et al., 2015b; Paper 3; Barros-Barbosa et al., 2015a). Briefly, the hippocampus was gently homogenized in cold oxygenated (95% O_2 /5% CO_2) Krebs solution (in mM: glucose 5.5, NaCl 136, KCl 3, $MgCl_2$ 1.2, Na_2HPO_4 1.2, $NaHCO_3$ 16.2, $CaCl_2$ 0.5, pH 7.40). Homogenates were filtered through a nylon filter (mesh size 100 µm). The filtrate was left to sit during 30–45 min until pellet formation, which was re-suspended into Krebs solution and left at room temperature.

Western blot analysis

Western blot analysis was performed as previously described (Paper 1; Barros-Barbosa et al. 2015b). Total lysates and synaptosomes of the human cerebral hippocampus were homogenized in RIPA buffer. Samples were solubilized in SDS reducing buffer, subjected to electrophoresis in SDS-polyacrylamide gels

and electrotransferred onto PVDF membranes. Membranes were blocked in Tris-buffered saline containing Tween 20 0.05% and BSA 5% and incubated with the primary antibodies: mouse anti-GFAP (1:500, Chemicon, Temecula, CA), mouse anti-synaptophysin (1:750, Chemicon, Temecula, CA), mouse anti-PSD95 (1:600, Chemicon, Temecula, CA) and rabbit anti-A_{2A} receptor (1:250; Alpha Diagnostic, San Antonio, TX). Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. For normalization purpose, membranes were incubated with rabbit anti-β-tubulin antibody (1:2500; Abcam, Cambridge, UK) or mouse anti-GAPDH antibody (1:200; Santa Cruz Biotechnology, Dallas, TX) following the procedures described above. The antigen-antibody complexes were visualized using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA). Gel band image densities were quantified with Image J (National Institute of Health, USA). To test for specificity of the bands corresponding to A_{2A} receptor, the anti-A_{2A} receptor antibody was pre-adsorbed with a control/blocking peptide (# A2aR21-P, Alpha Diagnostic, San Antonio, TX).

Immunofluorescence confocal microscopy

Immunofluorescent staining and confocal microscopy analysis was performed as previously described (Paper 1; Barros-Barbosa et al. 2015b). Free floating 30 µm brain sections were incubated for 1 h, with blocking buffer and incubated overnight with the primary antibodies: mouse anti-A_{2A} receptor (1:300, Chemicon, Temecula, CA), rabbit anti-synaptotagmin 1/2 (1:500, Synaptic Systems GmbH, Goettingen, D), rabbit anti-GFAP (1:500, Dako, Glostrup, DK) and rabbit anti-NF200 (1/900, Abcam, Cambridge, UK). Sections were rinsed and incubated 2 h with species specific secondary antibodies: donkey anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 633 (Molecular Probes, Eugene, OR). After mounting, sections were observed and analyzed with a laser scanning confocal microscope (Olympus FV1000, Tokyo, JP). Co-localization was assessed by calculating the staining overlap and the Pearson´s Coefficient (ρ) for each confocal micrograph using the Olympus Fluoview 4.2 Software (Olympus FV1000, Tokyo, JP).

Data presentation and statistical analysis

Results are expressed as mean ± SD. *n* shown in graphs represent the number of individuals in a given situation. Statistical analysis was carried out using Graph Pad Prism 6.04 software (La Jolla, CA, USA). Unpaired Student's *t*-test with Welch correction was used for statistical analysis when parametric data was considered. For multiple comparisons, one-way ANOVA followed by Dunnett's Multiple Comparison Test was used. For multiple comparisons between multiple groups, two-way ANOVA followed by Bonferroni´s Multiple Comparison Test was used. $P < 0.05$ values were accepted as significant.

Results

Human hippocampal synaptosomes are enriched in synaptic nerve terminals

Figure 23 shows that synaptosomes isolated from the hippocampus of MTLE human patients present less amounts ($P<0.001$) of the astrocytic cell marker, GFAP, when compared to total lysates. Similar levels of the postsynaptic density marker, PSD95, were found in synaptosomes and total lysates of the hippocampus of MTLE patients. More importantly, synaptosomes isolated from the hippocampus of MTLE patients (Figure 23) using our methodology are highly enriched ($P<0.001$) in synaptic nerve terminals specifically labelled with synaptophysin compared to total lysates of the same brain region.

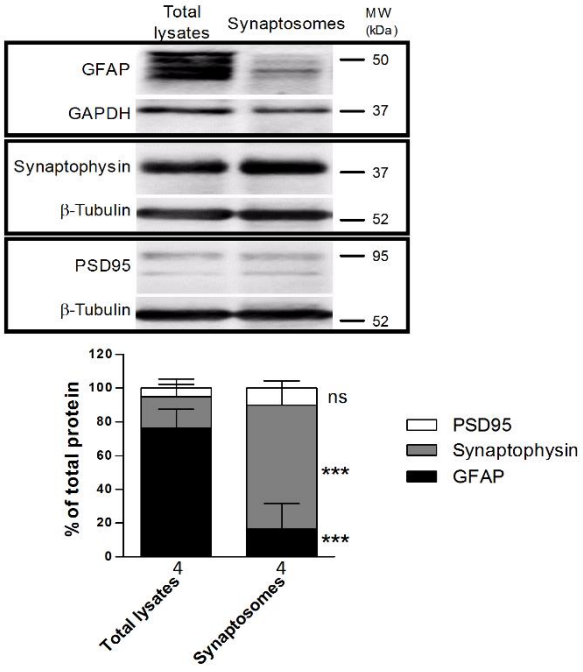


Figure 23 – Synaptosomes isolated from the hippocampus of MTLE human patients are enriched in synaptic nerve terminals. At the top of the figure are illustrated representative blots of GFAP, synaptophysin and PSD95 immuno-reactivity in total lysates and synaptosomes of the hippocampus of an MTLE patient. GAPDH and β-tubulin were used as reference proteins. At the bottom, it is illustrated the average composition in GFAP, synaptophysin and PSD95 of total lysates and synaptosomes from four MTLE patients. Data are expressed as mean ± SD. ***P < 0.001 represents significant differences as compared to total lysates (two way ANOVA followed by Bonferroni´s Multiple Comparison Test); ns, non-significant.

The A_{2A} receptor expression is upregulated in the hippocampus of MTLE human patients

Data from Western blot analysis show that the A_{2A} receptor protein (~46 kDa) levels are significantly ($P<0.001$) increased (by about three fold) in total lysates of the hippocampus of MTLE patients as compared to control individuals (Figure 24A). Please note that the bands corresponding to the A_{2A} receptor protein disappeared after pre-adsorption of the antibody with the control peptide (Figure 24A; negative control). It is worth noting that upregulation of the A_{2A} receptor protein in the hippocampus of MTLE patients compared to control individuals is more notorious ($P<0.01$) in total lysates than in isolated nerve terminal (synaptosomal) fractions, suggesting that the A_{2A} receptor is localized predominantly in non-neuronal cells, probably glial cells (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013; Orr et al., 2015).

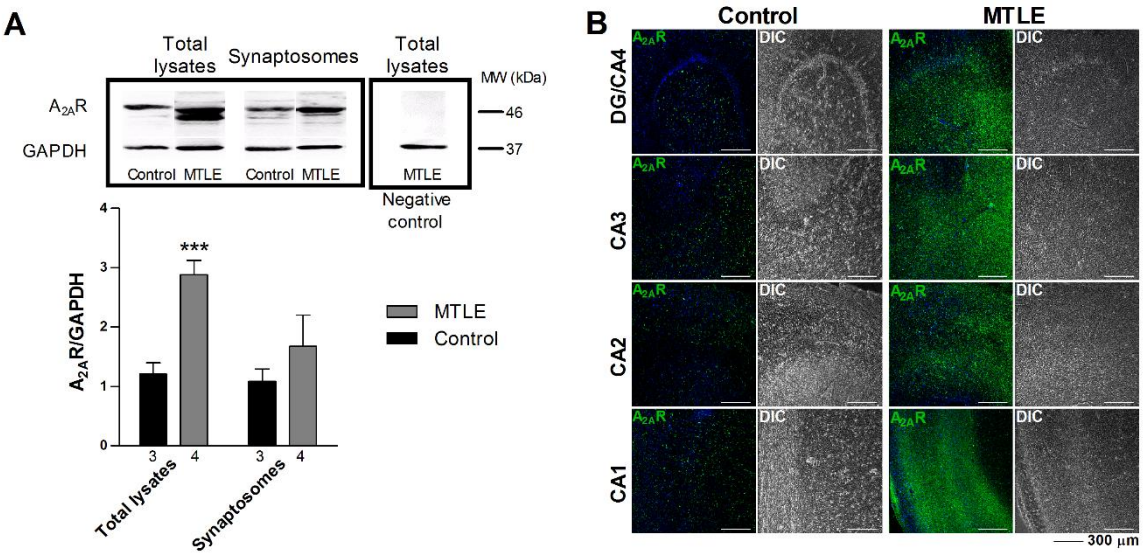


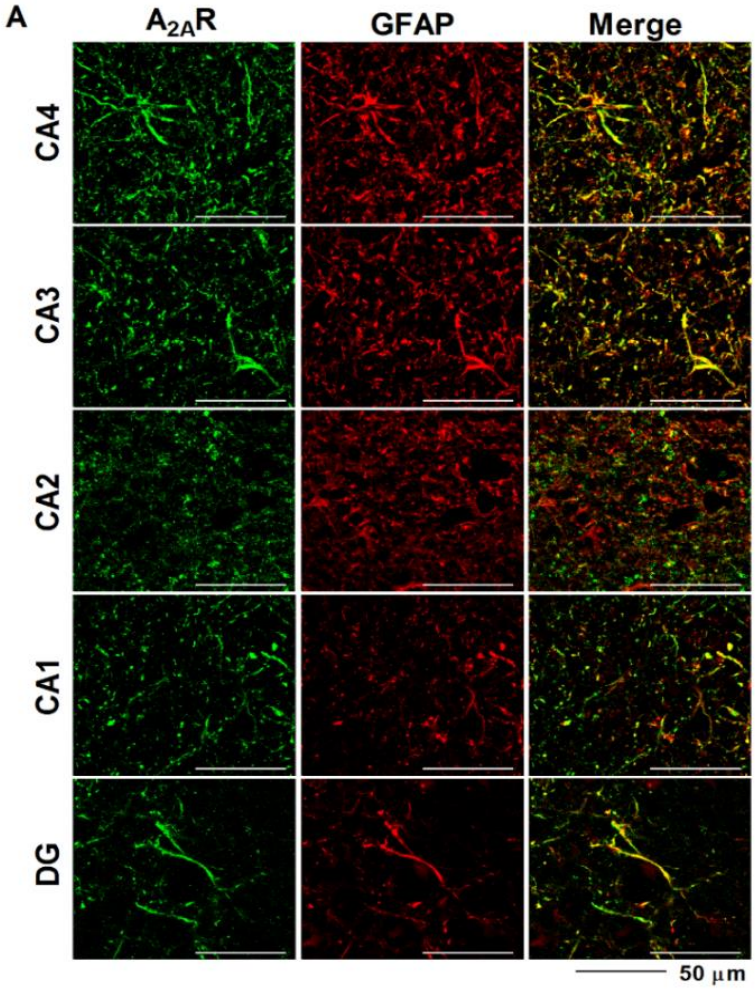
Figure 24 – The A_{2A} receptor protein density is greatly increased in the hippocampus of drug-refractory MTLE human patients. Panel A illustrates representative Western blots of the A_{2A} receptor immunoreactivity in total lysates and synaptosomes of the hippocampus of control and MTLE human patients; gels were loaded with 100 µg of protein. Please note that the bands corresponding to the molecular weight of native A_{2A} receptor (~46 kDa) disappeared after pre-adsorption of the primary antibody with a control peptide (lane 5; negative control); GAPDH was used as a reference protein. Data are expressed as mean ± SD and the number of individuals per group is shown below each bar. ***$P < 0.001$ (two way ANOVA followed by Bonferroni´s Multiple Comparison Test). Panel B shows representative confocal microscopy images from different regions of the human hippocampus demonstrating that immunoreactivity against the A_{2A} receptor (green) is more notorious in the hippocampus of MTLE patients than that from control individuals; nuclei are labeled with 4',6-diamidino-2-phenylindole (DAPI; blue); DIC images are shown for comparison; 2 confocal micrographs were obtained per individual; 3 individuals from each group (control and MTLE) were analyzed; scale bars = 300 µm.

Upregulation of the adenosine A_{2A} receptor expression in the hippocampus of MTLE patients compared to control individuals detected by Western blot analysis (Figure 24A) is supported by immunofluorescence confocal microscopy (Figure 24B). Confocal micrographs of the hippocampus of MTLE patients show increases in the A_{2A} receptor density (labelled in green) in all its regions, CA1, CA2, CA3 and DG/CA4, when compared to control individuals. Due to hippocampal sclerosis, the boundaries of each region are less evident in hippocampi of MTLE patients than of control individuals and this can be appreciated using the DIC images for comparison.

Altogether these results show that the adenosine A_{2A} receptor is upregulated (by about 3-fold) in the hippocampus of drug-refractory MTLE patients compared to control individuals.

Upregulated expression of adenosine A_{2A} receptors are mainly localized in astrocytes of the hippocampus of MTLE human patients

Results obtained by comparing A_{2A} receptor protein levels in total lysates and synaptosomal fractions of the human hippocampus (Figure 24A) led us to reach the conclusion that the A_{2A} receptor is localized predominantly in non-neuronal cells. Using immunofluorescence confocal microscopy, we show in Figure 25 that the adenosine A_{2A} receptor (green) colocalizes extensively with the astrocytic cell marker, GFAP (red), in all regions of the human hippocampus, including *Cornu Ammonis*, CA1, CA2, CA3, CA4, and dentate gyrus (DG). Co-localization between A_{2A} receptor and GFAP immunostainings detected by the yellow staining when merging the two fluorescence channels (Figure 25A, right-hand side of the image) was confirmed by the elevated scores obtained by calculating the staining overlap (overlap > 0.6) and the Pearson´s Coefficient ($\rho > 0.4$) in different areas of the human hippocampus (Figure 25B). Overlap between two colors gives values between +1 (total overlap) and 0 (no overlap) and the ρ value is a measure of the linear correlation between two variables (stainings), giving values between +1 and −1 inclusive, where 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation.



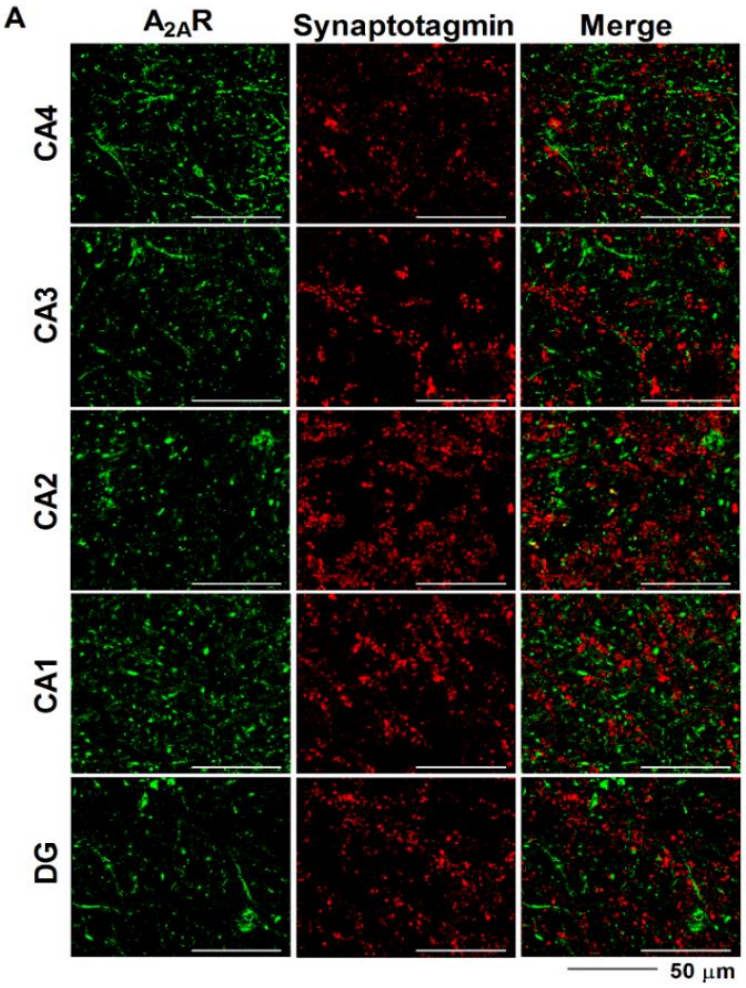
B

$A_{2A}R$ x GFAP

	Pearson´s Coefficient (ρ)	Overlap
CA4	0.59 ± 0.12	0.70 ± 0.11
CA3	0.56 ± 0.12	0.71 ± 0.06
CA2	0.50 ± 0.03	0.72 ± 0.09
CA1	0.40 ± 0.20	0.60 ± 0.08
DG	0.46 ± 0.10	0.65 ± 0.04

Figure 25 – The A_{2A} receptor is predominantly present in astrocytes. Panel A illustrates representative confocal micrographs of different regions of human hippocampus from MTLE patients stained against the A_{2A} receptor (green) and the astrocytic cell marker, GFAP (red). Co-localization is shown by the yellow labeling appearing when merging the two fluorescent channels (right-hand side of the image). Panel B shows the staining overlap and Pearson´s Coefficient (ρ) parameters (mean ± SD) calculated from 2-4 confocal micrographs per MTLE individual of 3 individuals. These parameters were automatically calculated per image and were used to quantify the co-localization of A_{2A} receptor and GFAP (yellow staining) in different hippocampal regions. Scale bars = 50 µm.

Conversely, we obtained reduced scores of colocalization when calculating the staining overlap (overlap < 0.25) and the Pearson´s Coefficient (ρ < 0.03) when we evaluated the colocalization of the A_{2A} receptor with the nerve terminal marker, synaptotagmin 1/2 (Figure 26), and the axonal marker, NF200 (Figure 27). Under these conditions, we could hardly see the yellow staining when merging the two fluorescent channels in any regions of the hippocampus of MTLE patients.



B

$A_{2A}R$ x Synaptotagmin

	Pearson´s Coefficient (ρ)	Overlap
CA4	–0.02 ± 0.02	0.22 ± 0.08
CA3	–0.02 ± 0.02	0.25 ± 0.09
CA2	–0.02 ± 0.02	0.25 ± 0.09
CA1	–0.04 ± 0.03	0.21 ± 0.07
DG	–0.03 ± 0.01	0.09 ± 0.02

Figure 26 – The A_{2A} receptor does not co-localize with the synaptic nerve terminal marker, synaptotagmin 1/2. Panel A shows representative confocal micrographs of different regions of the hippocampus of MTLE human patients labeled with antibodies against the A_{2A} receptor (green) and synaptotagmin 1/2 (red). The absence of colocalization is shown by the non-appearance of the yellow staining when merging the two fluorescent channels (right-hand side of the image). Panel B shows the staining overlap and the Pearson´s Coefficient (ρ) parameters (mean ± SD) calculated from 2-4 confocal micrographs per MTLE individual of 3 individuals. These parameters were automatically calculated per image and were used to quantify the co-localization of A_{2A} receptor and synaptotagmin 1/2 in different hippocampal regions. Scale bars = 50 µm.

These results suggest that the adenosine A_{2A} receptor is present predominantly in astrocytes of the hippocampus from MTLE patients, thus confirming our suspicion due to differences in the density of Western blot bands obtained with total lysates and synaptosomal fractions of the human hippocampus (Figure 24A). Our results are also in keeping with those obtained in the hippocampus of Alzheimer patients (Orr et al., 2015).

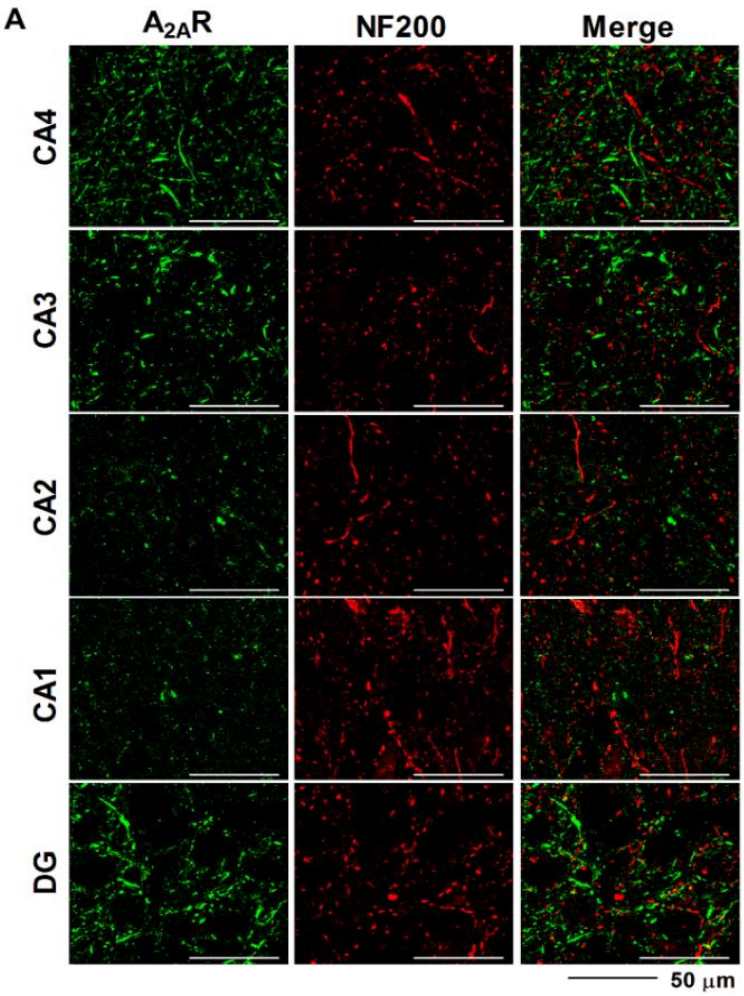


Figure 27 – The A_{2A} receptor does not co-localize with the axonal marker, NF200. Panel A shows representative confocal micrographs of different regions of the hippocampus of MTLE human patients labeled with antibodies against the A_{2A} receptor (green) and NF200 (red). The absence of colocalization is shown by the non-appearance of the yellow staining when merging the two fluorescent channels (right-hand side of the image). Panel B illustrates the staining overlap and the Pearson´s Coefficient (ρ) parameters (mean ± SD) calculated from 2-3 confocal micrographs per MTLE individual of 2 individuals. These parameters were automatically calculated per image and were used to quantify the colocalization of A_{2A} receptor and NF200 in different hippocampal region. Scale bars = 50 µm.

B

A_{2A}R x NF200

	Pearson´s Coefficient (ρ)	Overlap
CA4	0.00 ± 0.03	0.14 ± 0.05
CA3	0.02 ± 0.03	0.15 ± 0.07
CA2	0.03 ± 0.10	0.21 ± 0.09
CA1	–0.01 ± 0.03	0.23 ± 0.09
DG	0.03 ± 0.03	0.17 ± 0.09

Discussion and conclusions

The present study shows, for the first time, that the adenosine A_{2A} receptor is upregulated (by about 3-fold) in the hippocampus of drug-resistant MTLE human patients compared to control individuals. This feature is in agreement with previous studies showing that the A_{2A} receptor exists at relative low levels in the hippocampus (Schiffmann et al., 1991; Dixon et al., 1996), but its amount may increase in the hippocampus of epileptic animal models (Huicong et al., 2013; Orr et al., 2015). As

a matter of fact, increasing evidences show that activation of the adenosine A_{2A} receptor favors seizure activity in different epileptic syndromes (Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Huicong et al., 2013). Activation of the A_{2A} receptor has a pro-convulsive effect on piriform cortex kindled seizures in the rat (Hosseinmardi et al., 2007) and it decreases the seizure threshold of hyperthermia-induced convulsions in young rats (Fukuda et al., 2011). Reduction of seizures occurrence has been verified in rat models of temporal lobe epilepsy (induced by pilocarpine or by kindling) either by selective inhibition of the A_{2A} receptor with SCH58261 or ZM241385 (Rosim et al., 2011; Li et al., 2012) or by genetic deletion (knockdown) of the A_{2A} receptor (El Yacoubi et al., 2008, 2009). Curiously, deletion of the A_{2A} receptor does not protect against maximal electroshock-induced seizures that are originated from brainstem structures (El Yacoubi et al., 2008, 2009).

It is worth noting that increases in the extracellular levels of ATP and, consequently, augmentation of the levels of its metabolite, adenosine, that has been reported during high-frequency neuronal firing and/or during prolonged or repeated epileptic seizures (Heinrich et al., 2012; Wall and Dale, 2013; Sims and Dale, 2014), may be critical for the activation of the A_{2A} receptor. This is because adenosine resulting from the extracellular catabolism of released ATP, via CD73, preferentially activates the excitatory A_{2A} receptor in the hippocampus (Cunha et al., 1996a). Additionally, the CD73 enzyme seems to be increased in rat models of temporal lobe epilepsy induced by kainate or pilocarpine (Bonan et al., 2000).

Despite adenosine has been considered the main endogenous antiepileptic molecule, via the activation of inhibitory A_1 receptors, and some studies had proposed that augmentation of adenosine levels may be a solution to resolve drug-refractory epilepsies, this must be taken carefully since increased extracellular levels of adenosine may not only activate the inhibitory A_1 receptor, but can also trigger A_{2A} receptor-mediated excitation (Boison, 2008). Activation of the excitatory A_{2A} receptor is more likely to occur in epileptic patients, as this receptor is upregulated in the human epileptic hippocampus (this study, see e.g. Figure 24). According to the results described in this study, it is therefore advisable when proposing adenosine augmenting strategies for treating epilepsy to consider simultaneous inhibition of the A_{2A} receptor to prevent adenosine-induced excitability.

Another important finding from this study concerns the demonstration that the A_{2A} receptor is upregulated predominantly in GFAP-positive astrocytes of the hippocampus of drug-resistant MTLE patients. A recent study reached the same conclusion using epileptic rats injected with kainate, as well as hippocampi from patients with Alzheimer's disease (Orr et al., 2015). Data from our study revealed a low density score for colocalization of the A_{2A} receptor with immunoreactive neuronal markers, like synaptotagmin 1/2 and NF200, in the hippocampus of MTLE human patients. Whether upregulation of the A_{2A} receptor accompanies astrogliosis that is characteristic of hippocampal sclerosis observed in MTLE patients in detriment of neuronal loss and/or mossy fibers sprouting deserves further elucidation. Nevertheless, the astrocytic localization of the A_{2A} receptor highlights a role of these cells in epilepsy, which relevance may be associated with the proposed modulation of glutamate transport and release via the activation of astrocytic A_{2A} receptors (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013) and, subsequent, increase in the extracellular levels of this excitatory neurotransmitter resulting in neuronal excitation and/or excitotoxicity. The mechanism underlying the A_{2A} receptor-mediated control of glutamate release by astrocytes seems to involve the activation of PKA and intracellular Ca^{2+} mobilizations (Kanno and Nishizaki, 2012), while the mechanism underlying the A_{2A} receptor-mediated downmodulation of glutamate uptake seems to involve the decrease of the Na^+/K^+-ATPase activity, and, thereby, the disruption of transmembrane Na^+ gradient (Matos et al., 2013). Although the intracellular transduction pathway implicated in the A_{2A} receptor-mediated inhibition of glutamate uptake in astrocytes may differ, the final result (i.e. collapse of the Na^+-driving force to take up glutamate) is quite similar to what we found to be involved in the P2X7 receptor-mediated downmodulation of glutamate uptake in cortical nerve terminals (Barros-Barbosa et al., 2015a; Barros-Barbosa et al., 2015b).

In conclusion, our results suggest that inhibition of upregulated A_{2A} receptors localized in hippocampal astrocytes of drug-resistant MTLE human patients using selective antagonists may prevent neuronal excitation and confer neuroprotection, as previously demonstrated in epileptic animal models (Rosim et al., 2011; Li et al., 2012; Valadas et al., 2012; Rombo et al., 2015). Therefore, our hypothesis is that the hippocampal A_{2A} receptor may be an attractive pharmacological target for the treatment of drug-resistant MTLE. The mechanism by which the A_{2A} receptor

promotes excitability in epileptic patients remains unknown. However, it is possible that modulation of the glutamate transport operated by activation of astrocytic A_{2A} receptors (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013) might be involved, as it results in the augmentation of extracellular glutamate levels and, thus, promote neuronal excitability/excitotoxicity.

CHAPTER 4: DISCUSSION AND CONCLUSIONS

The main aim of this work was to contribute to the discovery of novel pharmacological targets to control drug-resistant epileptic seizures and/or epileptogenesis. Given that imbalance between GABAergic and glutamatergic neurotransmission leading to over synchronized neuronal firing has long been considered the hallmark of epilepsy, we investigated endogenous regulators that could fine-tuning modulate the extracellular levels of GABA and glutamate. Our focus was on ATP and adenosine whose extracellular accumulation rapidly increase in the brain during high-frequency neuronal firing or under pathological conditions (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013) allowing the spatial and temporal cohabitation of these two purines with GABA and glutamate neurotransmitters. Limitations in the use of animal models of epilepsy (Kandratavicius et al., 2014) encouraged us to address the changes in purinergic receptor-mediated modulation of Na^+-coupled neurotransmitter transporters directly on human tissue obtained from drug-resistant epileptic patients and from cadaveric controls. Despite the use of human brain samples, another major advantage of our approach compared with previous studies in the literature was the possibility of measuring simultaneously the transport of [^{3}H]GABA and [^{14}C]glutamate into isolated nerve terminals. However the scarcity of the human brain tissue and the difficulty in its achievement due to obvious ethical reasons and inherent complexity in coordinating clinical work and laboratory timings, lead us to first expand our evaluation about the mode of action of the purines on GABA and glutamate uptake in isolated nerve terminals (synaptosomes) of the cerebral cortex of control and epileptic Wistar rats injected with pilocarpine. This animal model was chosen because its clinical features and pathological characteristics most frequently compare with drug-resistant MTLE in humans (Sharma et al., 2007; Curia et al., 2008; O'Dell et al., 2012).

Taking into consideration previous findings suggesting that the ionotropic P2X7 receptor may be a potential target to control seizures and that it is able to modulate the extracellular levels of amino acid neurotransmitters (Neal et al., 1998; Lo et al., 2008; Morioka et al., 2008), we drew our attention to the study of the role of ATP acting via the P2X7 receptor on synchronized modulation of GABA and glutamate uptake into isolated cortical nerve terminals. Results clearly showed that activation of the ATP-sensitive P2X7 receptor downregulates Na^+-coupled high-affinity GABA and glutamate uptake by nerve terminals (synaptosomes) isolated

from the cerebral cortex of control rats and human patients. The mechanism underlying downregulation of amino acid transporters seems to be common for the two neurotransmitters and, in accordance with our initial hypothesis, it involves the partial collapse of the Na^+ driving-force for neurotransmitters uptake caused by the influx of Na^+ through the P2X7 receptor channel. It is worth to note that most of the experiments presented in this work were done in low extracellular Ca^{2+} conditions in order to mimic the synaptic ambience under strong physiological nerve firing or during epileptic seizures (Heinemann et al., 1977; Borst and Sakmann, 1999; Engelborghs et al., 2000; Stanley, 2000; Massimi and Amzica, 2001; Rusakov and Fine, 2003; Engel et al., 2012a; Poornima et al., 2012; Torres et al., 2012; Zhou et al., 2012; Jimenez-Pacheco et al., 2013). That is, under pathological epileptic conditions, the Ca^{2+} concentration in the extracellular milieu declines to a maximum of 90% and synaptic ATP is high enough to activate low-affinity P2X7 receptors (Jarvis and Khakh, 2009) in a situation where GABA and/or glutamate levels are maximal. This strategy allowed us to focus our research on alterations of the Na^+ driving-force for amino acids transportation, while excluding (1) the involvement of Ca^{2+} in the regulation of amino acid transporters either directly or via plasmalemmal and/or mitochondrial Na^+/Ca^{2+} exchangers, and (2) the interference of Ca^{2+}-activated neurotransmitter release (Cordeiro et al., 2000; Cordeiro et al., 2003; Romei et al., 2015).

Data using rat cortical synaptosomes show that besides decreasing GABA and glutamate uptake under low Ca^{2+} conditions, activation of the P2X7 receptor also favors the release of both neurotransmitters. However, the magnitude and the mechanism underlying GABA and glutamate release triggered by the P2X7 receptor activation differ significantly. While activation of the P2X7 receptor causes the outflow of small amounts of GABA through reversal of the GAT1, glutamate is released in higher quantities through the P2X7 receptor pore (see also Marcoli et al., 2008; Cervetto et al., 2013). These differences may contribute to explain why the P2X7 receptor agonist, BzATP, was more potent in decreasing the uptake of glutamate by nerve terminals of the rat cerebral cortex, as compared to GABA uptake as the concomitant leakage of glutamate directly through the P2X7 receptor pore may downsize [^{14}C]glutamate incorporation inside cortical synaptosomes.

Contrary to our expectations, we found no differences in the P2X7 receptor expression and function on nerve terminals from the cerebral cortex of pilocarpine-

induced epileptic rats compared to their control littermates. These findings contrast with our data using human cerebral cortices obtained from drug-resistant MTLE and non-MTLE patients. Under similar experimental conditions, we found that the P2X7 receptor was upregulated in cortical nerve terminals isolated from epileptic patients compared to cadaveric controls. Moreover, increases in the P2X7 receptor density correlate with a higher potency of the P2X7 receptor agonist, BzATP, concerning inhibition of GABA, but not glutamate, uptake by nerve terminals isolated from the neocortex of MTLE patients. Thus, the absence of differences in the purinergic modulation between control and epileptic animals may be explained by shortage in the disease evolution after SE induced by pilocarpine, being this time insufficient to promote the morpho-functional changes observed in human patients with MTLE, where the disorder is allowed to develop for many years before last resource neurosurgery is indicated.

Taking into consideration that epileptic activity also gives rise to large quantities of adenosine released to the extracellular milieu as such (via equilibrative nucleoside transporters) or generated by the catabolism of released ATP (via the CD73 pathway), creating the ideal conditions to activate excitatory adenosine A_{2A} receptors counterbalancing the anti-epileptic role of the nucleoside via inhibitory A_1 receptors (see Cunha et al., 1996a), we thought it was appropriate to investigate for the first time the density of expression and cellular localization of the A_{2A} receptor in human hippocampal samples from control and MTLE patients exhibiting hippocampal sclerosis. The results obtained showed that A_{2A} receptors were upregulated in hippocampal astrocytes of MTLE patients. Interestingly, the astrocytic localization of surplus excitatory metabotropic A_{2A} receptors in the hippocampus of MTLE patients clearly differ from that found for the ionotropic P2X7 receptor, which was more abundant in neocortical nerve terminals of MTLE patients. Upregulation of astrocytic A_{2A} receptors in the hippocampus of MTLE human patients is not different from that verified in the brain of epileptic animal models (Huicong et al., 2013; Orr et al., 2015), where activation of the A_{2A} receptor promotes epileptiform activity under various disease conditions (Hosseinmardi et al., 2007; El Yacoubi et al., 2008, 2009; Fukuda et al., 2011; Rosim et al., 2011; Huicong et al., 2013). Interestingly, data from our group demonstrated that adenosine resulting from the extracellular catabolism of released ATP, via the CD73 pathway, activates preferentially excitatory A_{2A} receptors in the hippocampus (Cunha et al., 1996a),

and this enzyme is also significantly upregulated in rat models of temporal lobe epilepsy induced either by pilocarpine or kainate injections (Bonan et al., 2000).

Astrocytic A_{2A} receptors have been recently implicated in the control of the synaptic levels of glutamate by modulating both the release and the uptake of this neurotransmitter (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013). Moreover, activation of the A_{2A} receptor has been involved in the facilitation of GABA uptake by astrocytes, an effect requiring the synergism with BDNF (Vaz et al., 2011). Concerted actions of adenosine, via astrocytic A_{2A} receptors, may favor neuronal excitation due to synaptic glutamate accumulation, whose effects may rapidly become unbalanced due to the A_{2A} receptor-mediated promotion of GABA uptake by neighboring astrocytes.

The mechanism underlying the A_{2A} receptor-mediated downmodulation of glutamate uptake by astrocytes seems to involve partial disruption of the transmembrane Na^+ gradient due to the decrease of the Na^+/K^+-ATPase activity (Matos et al., 2013). These authors described a physical association between the A_{2A} receptor and the Na^+/K^+-ATPase in astrocytes of the cerebral cortex and suggested that there is a link to GLT-1 via the α2 subunit of the Na^+/K^+-ATPase, which may be crucial to regulate the glutamate transport in astrocytes. Furthermore, the prolonged activation of the A_{2A} receptor may result in a cAMP/protein kinase A-dependent reduction of GLT-1 and GLAST mRNA and protein levels, leading to a sustained decrease of glutamate uptake (Matos et al., 2013). Although the intracellular transduction elements implicated in the A_{2A} receptor-mediated inhibition of glutamate uptake may differ, the final result (i.e. partial collapse of the Na^+-driving force to take up glutamate) is quite similar to what we found to be involved in the modulation of glutamate uptake by activation of the ionotropic P2X7 receptor in cortical nerve terminals (Barros-Barbosa et al., 2015a; Barros-Barbosa et al., 2015b). To our knowledge, downmodulation of GABA uptake produced by the A_{2A} receptor-mediated decrease of Na^+/K^+ ATPase activity was not demonstrated yet, however it is plausible to assume that this modulation can also occur since the GABA transporters are expressed in astrocytes (Conti et al., 2004; Melone et al., 2015) and their function is also dependent on extracellular Na^+ levels (Allen et al., 2004; Kanner, 2006). However, other studies demonstrated that the A_{2A} receptor activation seems to be required to the potentiation of GABA uptake by BDNF in astrocytes (Vaz et al., 2011).

Taken together our results, in conjunction with the data from other authors, led us to anticipate that transient activation of P2X7 and A_{2A} receptors by released ATP during high-frequency neuronal firing and its subsequent catabolism into adenosine in the extracellular milieu (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013; Sims and Dale, 2014) may undertake facilitation of glutamatergic neurotransmission (Marcoli et al., 2008; Kanno and Nishizaki, 2012; Matos et al., 2013; Barros-Barbosa et al., 2015a; Barros-Barbosa et al., 2015b) while promoting, albeit to a lesser extent, the endurance of GABAergic neurotransmission ensuring tonic and more diffuse neuro-inhibition following an intense period of stimulation (Barros-Barbosa et al., 2015a; Barros-Barbosa et al., 2015b). This mechanism may be particularly relevant in processes such as learning and memory where transient glutamate endurance in the synaptic cleft may facilitate the induction of LTP while preventing excytotoxicity by the concomitant promotion of diffuse GABAergic inhibition. As a matter of fact, several studies demonstrated that both P2X7 (Chu et al., 2010; Campos et al., 2014) and A_{2A} receptors (Kessey et al., 1997; Almeida et al., 2003; Rebola et al., 2008) are essential players to promote long-term changes in synaptic plasticity.

While fine-tuning modulation of extracellular concentrations of GABA and glutamate by endogenously released purines, namely ATP and adenosine, appears to have a significant role under physiological conditions by potentiating neurotransmission, it might gain a different meaning during epileptic discharges where the density of both P2X7 and A_{2A} receptors is upregulated and the sequential activation of these two purinoceptors may constitute “an hazardous orchestra” (Rodrigues et al., 2015). Upregulation of P2X7 and A_{2A} receptors located respectively in nerve terminals and astrocytes of the brain of drug-resistant epileptic (MTLE) patients, contributes to amplify ATP- and adenosine-mediated signals resulting in the impairment of the transmembrane Na^+ gradient (see also Matos et al., 2013). This leads to a scenario where, besides the glutamatergic signaling endurance, the persistence of GABA-mediated neurotransmission owed to the decrease of GABA uptake may guide the $GABA_A$ receptor to rundown. Therefore, under pathological conditions, increases in the extracellular accumulation of GABA combined with impaired Cl^- gradient and altered $GABA_A$ receptor subunit composition that has been described in drug-resistant epileptic patients, may convert GABAergic inhibition into a pro-convulsive action instigated by GABAergic

rundown (Cohen et al., 2002; D'Antuono et al., 2004; Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010; Miles et al., 2012). The GABAergic rundown phenomenon is likely to occur during high-frequency neuronal stimulation and it has been linked either (1) to $GABA_A$ receptor desensitization (Ragozzino et al., 2005; Palma et al., 2007; Mazzuferi et al., 2010), or (2) to membrane depolarization stemming from changes in the way cells handle intracellular Cl^- homeostasis (D'Antuono et al., 2004; Miles et al., 2012) (see Figure 28).

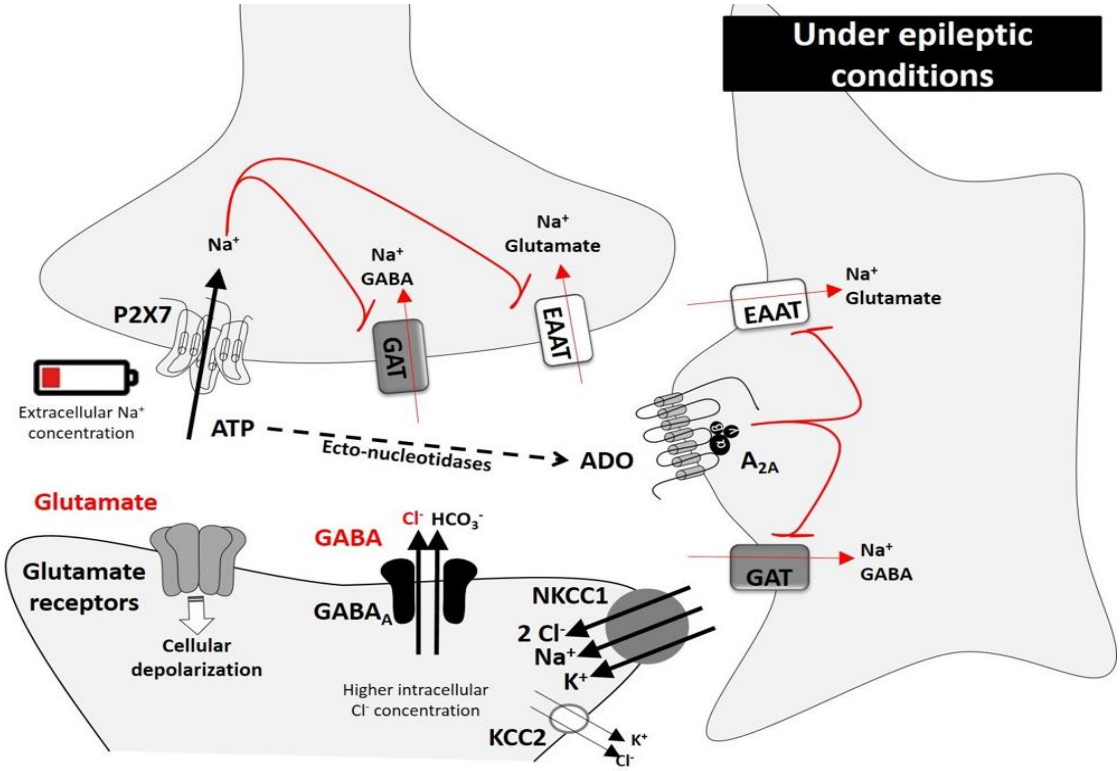


Figure 28 – Schematic diagram representing the role of purines released by high-frequency nerve discharges. During epileptic seizures, the extracellular concentrations of ATP and its metabolite, adenosine, increase drastically reaching levels high-enough to activate low-affinity ionotropic P2X7 and metabotropic A_{2A} receptors, respectively, which expression is upregulated in drug-resistant epileptic patients. Activation of both receptors favors the imbalance in the extracellular levels of GABA and glutamate, through downmodulation of their reuptake system operated by the disruption of the transmembrane Na^+ gradient. Under these conditions, inhibition of GABA uptake coupled to changes in Cl^- homeostasis can affect the inhibitory nature of $GABA_A$ receptor-mediated transmission, leading to GABA mediated excitation. On the other hand, parallel promotion of glutamatergic neurotransmission by excess of glutamate at the synapse may ultimately lead to excitotoxicity and neuronal degeneration. This highlights the inhibition of both P2X7 and A_{2A} receptors as relevant pharmacological targets to control drug-resistant human epilepsy.

Nowadays, increasing evidences have shown that altered neuronal Cl^- homeostasis in MTLE patients can affect the strength and even the signal of $GABA_A$ receptor-mediated neurotransmission, leading to a decrease in the efficacy of

GABAergic inhibition or even to GABA-mediated excitatory actions (Cohen et al., 2002; Huberfeld et al., 2007; Ben-Ari et al., 2012; Miles et al., 2012; Pavlov et al., 2013; Pallud et al., 2014). Interestingly, impairment of Cl^- homeostasis has been associated with the downregulation of KCC2 and often with the upregulation of NKCC1 in patients with drug-refractory temporal lobe epilepsies and in animals with acquired focal epilepsies (Köhling, 2002; Huberfeld et al., 2007; Blaesse et al., 2009; Miles et al., 2012). This might explain why, sometimes, increasing extracellular GABA levels (using, for instance, vigabatrin or tiagabine) or administering GABA mimetic drugs (like benzodiazepines) may result in paradoxical clinical effects in some epileptic patients (Ben-Ari, 2014; Khazipov et al., 2015).

Concurrently with the paradoxical excitatory effect resulting from the increase in extracellular GABA accumulation, parallel promotion of glutamatergic neurotransmission by decreases in the uptake of glutamate (Matos et al., 2013; Barros-Barbosa et al., 2015a; Barros-Barbosa et al., 2015b) may ultimately lead to excitotoxicity and neuronal degeneration. Findings from this study and other reports in the literature support the idea that the mechanisms underlying downmodulation of GABA and glutamate uptake by neuronal P2X7 and astrocytic A_{2A} receptors in epileptic patients are quite similar and involve the collapse of Na^+ gradient across plasma membranes (Matos et al., 2013; Barros-Barbosa et al., 2015a). Thus, activation of both purinoceptors lead to a common feature that is translated into (1) increases in the intracellular Na^+ concentration, which was already described to occur at the time of tissue injury (Yu et al., 2010; Romei et al., 2015), and (2) increases in the extracellular glutamate concentration that is characteristic of the epileptic situation (Janjua et al., 1992; During and Spencer, 1993; Soukupova et al., 2015).

In summary, this study fills a gap in the understanding of the human epileptic brain, regarding the pro-epileptic role of both ATP and adenosine in the extracellular milieu, in conditions where the synaptic levels of these purines rapidly increase, such as during intense neuronal discharges (Cunha et al., 1996b; Frenguelli et al., 2007; Heinrich et al., 2012; Wall and Dale, 2013). Altogether, the results obtained in this work and data from other authors (Kanno and Nishizaki, 2012; Matos et al., 2012; Matos et al., 2013) indicate that purines, ATP and adenosine, acting respectively on neuronal P2X7 and astrocytic A_{2A} purinoceptors can modulate the extracellular levels of GABA and glutamate, an effect that can dramatically increase

in drug-resistant epileptic patients where the expression of the two purinoceptors is upregulated. In view of this and in line with previous suggestions obtained from animal studies (Vianna et al., 2002; Hosseinmardi et al., 2007; El Yacoubi et al., 2008; Engel et al., 2012a; Huicong et al., 2013; Jimenez-Pacheco et al., 2013; Rodrigues et al., 2015), we now propose that the pharmacological targeting of neuronal ATP-sensitive P2X7 and astrocytic adenosine A_{2A} receptors with selective antagonists may constitute a novel and valuable therapeutic alternative to manage drug-resistant epileptic patients. We believe that the combined blockade of ionotropic P2X7 and metabotropic A_{2A} receptors in the epileptic brain will avoid neuronal excitability and excitotoxicity, improving the quality-of-life and longevity of drug-refractory MTLE patients by restoring the extracellular levels of GABA and glutamate.

Outside the epilepsy context, there is an explosion of data indicating that both P2X7 and A_{2A} receptors are involved in the pathophysiology of several neurological syndromes, including neurotrauma (Kimbler et al., 2012), multiple sclerosis (Gu et al., 2015), amyotrophic lateral sclerosis (Yiangou et al., 2006), Alzheimer's disease (McLarnon et al., 2006; Miras-Portugal et al., 2015; Orr et al., 2015; Sáez-Orellana et al., 2015), Parkinson's disease (Uchida et al., 2015), Huntington's disease (Díaz-Hernández et al., 2009) and psychiatric mood disorders (Kongsui et al., 2014). It appears that there is a common feature among all these pathological conditions, which may be the rapid increase in extracellular ATP levels that is characteristic of brain cells injury with its subsequent catabolism into adenosine, thus creating the conditions required to over activate damage receptors, like P2X7 and A_{2A} purinoceptors. Therefore, blockade of both P2X7 and A_{2A} purinoceptors with selective antagonists may also present clinical benefits in non-epileptic neurodegenerative conditions to curb "dangerous signals" emanating from an underlying dysfunction of these two purinoceptors.

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