University of Lisbon

Institute of Pharmacology and Neuroscience, Faculty of Medicine

Unit of Neuroscience, Institute of Molecular Medicine

Setting GABA levels: GABA transporters modulation by adenosine receptors

Ana Sofia Cristóvão Ferreira

Tese orientada pela Professora Doutora Ana Maria Sebastião

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Publications

The scientific content of the present thesis has been included in the publication of the following original articles:

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Only the experiments performed by the author of this thesis were included in the corresponding results chapter. Experiments performed by other co-authors (the minority of the papers content) are referred as such and discussed.

Other publications closely related to the content of this thesis:

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Vaz SH, **Cristóvão-Ferreira S**, Ribeiro JA, Sebastião AM (2008) Brain-derived neurotrophic factor inhibits GABA uptake by the rat hippocampal nerve terminals. Brain Res. 1219, 19-25.

Para a minha mãe,

"Tudo o que sabemos é uma impressão nossa, e tudo o que somos é uma impressão alheia"

In "O Livro do Desassossego",
Bernardo Soares

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Abbreviation list

AC – adenylate cyclase

Ach – acetylcholine

ADA – adenosine deaminase

ADK – adenosine Kinase

ADP – adenosinse diphosphate

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

AMP – adenosine 5'-monophosphate

ANOVA – analysis of variance

AOAA – aminooxyacetic acid

ATP – adenosine 5'-triphosphate

BAPTA – 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

BDNF – brain derived neurotrophic factor

bFGF – basic-fibroblast growth factor

BGT-1 – Betaine/GABA transporter 1

BRET – bioluminescence resonance energy transfer

$BRET_{50}$ – BRET constant (corresponds to the acceptor/donor ratio at which 50% of BRETmax is reached)

$BRET_{max}$ – BRET constant (maximal BRET signal)

BSA – bovine serum albumin

CADO – 2-chloro-adenosine

cAMP – adenosine3', 5' cyclic phosphate

cDNA – complementar DNA

CGS 21680 – 4-[2-[[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-yl]amino]ethyl] benzenepropanoic acid hydrochloride

CHO cells – chinese hamster ovary cells

ChTx – Cholera Toxin

CNS – central nervous system

CNT1 – concentrative nucleoside transporter 1

CNT6 – concentrative nucleoside transporter 6

CNTs – concentrative nucleoside transporters

CPA – N^6-cyclopentyladenosine

DAG – diacylglycerol

DDT1MF-2 cells – smooth muscle cell line

DMEM – Dulbecco's Modified Eagles Medium

DMSO – dimethylsulfoxide

DPCPX – 8-cyclopentyl-1,3-dipropylxanthine

DTT – DL-Dithiothreitol

EDTA – Ethylenediamine tetraacetic acid

eEPSCs – evoked excitatory post-synaptic currents

eIPSCs – evoked inhibitory post-synaptic currents

ENT1 – equilibrative nucleoside transporter 1

ENT2 – equilibrative nucleoside transporter 2

ENT4 – equilibrative nucleoside transporter 4

ENTs – equilibrative nucleoside transporters

ERK – extracellular signal-regulated kinase

FBS – foetal bovine serum

FITC – Fluorescein Isothiocyanate

FRET – fluorescent resonance energy transfer

GABA – gamma–aminobutyric acid

GABA-T – GABA transaminase

GAD – glutamate decarboxylase

GAT-1 – GABA transporter 1

GAT-2 – GABA transporter 2

GAT-3 – GABA transporter 3

GATs –GABA transporters

GDP – guanosine 5'- diphosphate

GF109203X – 2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide

GFAP – glial fibrillary acidic protein

GLAST–glutamate aspartate transporter

GLT-1 – glial glutamate transporter 1

GPCR – G protein coupled receptor

GS –glutamine synthetase

GTP – guanosine 5'- triphosphate

H-89 – N-[2-(p-Bromocinnamylamino)ethyl]- 5-isoquinolinesulfonamide dihydrochoride

HEK 293 cells – Human Embryonic Kidney 293 cells

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

[^{3}H]GABA – 4-amino-n-[2,3-^{3}H] butyric acid

[^{35}S]GTP – guanosine 5'-(g-[35S]-thio)triphosphate

IC_{50} – pharmacological constant (half maximal inhibitory concentration)

IMP–inosine monophosphate

IP_3–inositol trisphosphate

JNK – jun-N-terminal kinase

KHR – Krebs-Henseleit-Ringer

K_M – Michaelis-Menten constant

KO –knockout

L-DOPA – levodopa

LPS – lipopolysaccharide

MAP – mitogen-activated protein

MAPK –mitogen-activated protein kinase

mBU – mili BRET units (arbitrary units)

mRNA–messenger RNA

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

NECA – 5'-N-ethylcarboxamidoadenosine

NFκB – nuclear factor kappa-light-chain-enhancer of activated B cells

NGF – nerve growth factor

NGS – normal goat serum

NMDA –N-Methyl-D-aspartic acid

NOS – NO synthase

NP40 –nonyl phenoxypolyethoxylethanol

5'-NT – 5´-nucleotidase

p38 – protein 38

PAG – phosphate-activated glutaminase

PDD – phorbol-12,13-didecanoate

PIP_2 –Phosphatidylinositol-4,5-bisphosphate

PKA – protein kinase A (protein kinase cAMP dependent)

PKC – protein kinase C

PLA_2 –phospholipase A_2

PTx – Pertussis toxin

PLC – phospholipase C

PBS– phosphate buffer saline

RIPA – radioimmunoprecipitation assay

R-PIA – R-phenylisopropyladenosine

R-SNAP – R - Soluble NSF Attachment Protein

R-SNARE – R-SNAP receptor

RLuc – Renilla luciferase

Rp-cAMPs – Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate

SAH – S-adenosylhomocystein

SAHH – S-adenosylhomocystein hydrolase

SAPK – stress-activated protein kinase

SCH 58261 – 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine

SDS – Sodium dodecyl sulfate

SDS-PAGE– sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM – Standard error of the mean

sEPSCs – spontaneous excitatory post-synaptic currents

sIPSCs – spontaneous inhibitory post-synaptic currents

SKF 89976A – hydrochloride (1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride

SLMV – synaptic-like microvesicles

SNAP 5114 – (1-[2-[*tris*(4-methoxyphenyl)methoxy]ethyl]-(*S*)-3-piperidinecarboxylic acid),

TCA – tricarboxylic acids

TEA – triethylamine

TEMED – Tetramethylethylenediamine

TGF β – transforming growth factor β

TNF α – tumor necrosis factor-α

TRIS – tris(hydroxymethyl)-aminomethane

TrKB – tropomyosin related kinase B

TRITC – Tetramethylrhodamine isothiocyanate

U73122 – (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione)

VAMP3 – Vesicle-associated membrane protein 3

vGAT–vesicular GABA transporter

vGLUT – vesicular glutamate transporter

Vmax – maximal velocity

VRACs–volume regulated anion channels

YFP – yellow fluorescent protein

Abstract

Gamma-aminobutyric acid is the main inhibitory neurotransmitter in central nervous system. To assure a controlled GABAergic transmission GABA must be quickly removed from synapse, which occurs through specific transporters expressed both in pre-synaptic terminal (GAT-1) and surrounding astrocytes (GAT-1 and GAT-3). Adenosine, which is a well-known neuromodulator, promotes GABA release in the hippocampus (Cunha and Ribeiro, 2000). The main goal of this thesis was to identify a possible role of adenosine upon GABA uptake into pre-synaptic terminals and into astrocytes.

In pre-synaptic terminals, the removal of endogenous adenosine by ADA inhibited GABA uptake, an effect that was mimicked by the blockade of A_{2A} receptors. Thus, endogenous adenosine, through the activation of A_{2A} receptors, promotes GABA uptake into pre-synaptic terminals. Experiments with activators and inhibitors of transduction pathways revealed that AC/cAMP/PKA is the transduction pathway associated with this effect. In fact, the activation of PKA restrains an inhibitory tonic influence mediated by PKC, resulting in an increase of GABA uptake. The increase of transport rate occurs through the enhancement of the expression of GAT-1 at the surface membrane.

In astrocytes, adenosine has a biphasic effect upon GABA uptake. This biphasic effect is mediated by the adenosine A_1-A_{2A} receptors heteromers, whose presence in astrocytes was identified in this work. The adenosine A_1-A_{2A} receptor heteromer is coupled to both

$G_{i/0}$ and G_s protein, being AC/cAMP/PKA the intracellular pathway associated. At low concentration, adenosine activates the A_1 protomer, which through $G_{i/0}$ protein inhibit AC, decreasing PKA activity and consequently, GABA uptake mediated by both GAT-1 and GAT-3. When adenosine levels rise, adenosine activates preferentially the A_{2A} protomer, which through G_s activity enhances PKA activity, promoting GABA uptake into astrocytes. The adenosine A_1-A_{2A} receptors heteromer can be viewed as a unique entity since it reaches and leaves the membrane as an entire complex and all the components have to be available for the heteromer be functional. Moreover, this work clearly showed that the heteromer is associated with both G_s and $G_{i/0}$, being a tetramer of A_1-A_1-A_{2A}-A_{2A}, rather than an A_1-A_{2A} complex coupled to G_q as previously thought.

Globally, at tripartite synapse level, adenosine controls the final destination of GABA after its release into the synapse. At low levels, adenosine will inhibit uptake into astrocytes but promote the uptake of GABA into presynaptic terminals, therefore facilitating the inhibitory phasic and tonic transmission. At higher levels of adenosine, GABA uptake into astrocytes will be favoured, which may decrease GABAergic tonic transmission, therefore facilitating excitability. In conclusion, this work highlighted a yet unknown mechanism through which adenosine may contribute to the switch between inhibition and excitation, through a concerted cross-talk between astrocytes and inhibitory neurons.

Resumo

O ácido gama-aminobutírico (GABA) é o principal neurotransmissor inibitório do Sistema Nervoso Central (SNC). Uma vez na sinapse o GABA é rapidamente recaptado através de transportadores específicos expressos pelos neurónios mas também pelas células da glia, que envolvem a sinapse.

A rápida recaptação de GABA pelos transportadores permite um controlo adequado dos níveis de GABA na sinapse, o que é fundamental para a limitação temporal e espacial da transmissão inibitória. Este controlo é assegurado por transportadores específicos expressos no terminal pré-sináptico e nos astrócitos que envolvem a sinapse. Actualmente são conhecidos quatro transportadores de GABA: três de alta afinidade (GAT-1, GAT-2, GAT-3) e um de baixa afinidade (BGT-1). O GAT-1 é o principal transportador de GABA, sendo expresso pelos neurónios e também pelas células da glia. Por sua vez, O GAT-3 é o principal transportador das células gliais. Em conjunto, estes dois transportadores são os responsáveis pela recaptação de GABA nas sinapses do sistema nervoso central.

A adenosina é um conhecido neuromodulador, que desempenha, ao nível do sistema nervoso central, um vasto leque de acções, que resulta numa inibição tónica do SNC. De entre as funções desempenhadas, a adenosina modula a concentração de neurotransmissores da sinapse, regulando quer a sua libertação vesicular quer a sua recaptação por transportadores.

A adenosina exerce as suas acções através de receptores acoplados a proteínas G. Até ao momento foram identificados quatro subtipos diferentes de receptores: dois de alta afinidade (A_1 e A_{2A}) e dois de baixa afinidade (A_{2B} e A_3). Os receptores A_1 e A_3 são receptores inibitórios, estando classicamente acoplados a proteínas $G_{i/0}$. Por seu lado, os receptores do subtipo A_2 são receptores excitatórios, estando geralmente acoplados a proteínas G_s.

Tendo como ponto de partida o efeito modulador da adenosina através da activação dos receptores A_{2A}, sobre libertação de GABA no hipocampo (Cunha e Ribeiro, 2000), este trabalho teve como principal objectivo a identificação de um possível efeito modulador da adenosina sobre a recaptação de GABA para os terminais pré-sinápticos e também para os astrócitos, através dos transportadores GAT-1 e GAT-3.

Os resultados obtidos neste trabalham indicam claramente que a adenosina através da activação dos receptores A_{2A} promove a recaptação de [^{3}H]GABA para os terminais pré-sinápticos mediada pelo transportador GAT-1. Com o objectivo de determinar o efeito da adenosina endógena, os terminais pré-sinápticos (sinaptossomas) foram incubados com adenosina desaminase (ADA, 1U/ml), tendo sido observada uma diminuição da recaptação de [^{3}H]GABA. A ADA degrada a adenosina em inosina, um metabolito inactivo para os receptores de adenosina, permitindo assim inferir o efeito da adenosina endógena. O efeito inibitório causado pela remoção da adenosina endógena foi mimetizado pelo bloqueio dos receptores A_{2A} com o antagonista selectivo SCH 58261 (50 nM). O envolvimento

dos receptores A_{2A} foi ainda confirmado através do uso do agonista selectivo destes receptores (CGS 21680, 30 nM): a activação dos receptores A_{2A} pelo CGS 21680 promoveu a recaptação de [^{3}H]GABA, mediada pelo transportador GAT-1.

O envolvimento de outros subtipos de receptores de adenosina, especificamente os subtipos A_1 e A_{2B}, foi avaliado através do uso de agonistas e antagonistas selectivos. Tanto a activação como o bloqueio dos receptores A_1 ou A_{2B} não causaram qualquer efeito sobre a recaptação de [^{3}H]GABA mediada por GAT-1 em sinaptossomas.

Ensaios de biotinilação, realizados após incubação dos sinaptossomas com o agonista selectivo dos receptores A_{2A}, mostraram que o efeito excitatório da adenosina sobre a recaptação de [^{3}H]GABA ocorre através do aumento do número de transportadores GAT-1 na membrana celular. Este efeito foi confirmado por curvas de saturação do transportador GAT-1, que mostraram um aumento da velocidade máxima do transportador após incubação com o agonista selectivo dos receptores A_{2A}, o que é indicativo de um aumento do número de transportadores GAT-1 na membrana celular.

A via de transdução de sinal associada ao efeito dos receptores A_{2A} foi identificada através de ensaios de recaptação realizados na presença de bloqueadores e activadores de cinases intracelulares. O bloqueio da proteína cinase do tipo A (PKA) pelo H-89 (1μM) preveniu totalmente o efeito do CGS 21680, enquanto a activação da adenilato ciclase (AC) pela *forskolin* (10μM) imitou a acção do

agonista selectivo dos receptores A_{2A}. Assim, os resultados indicam que a adenosina, por activação dos receptores A_{2A} promove a recaptação de [^{3}H]GABA mediada por GAT-1, através da activação da via da PKA.

Foi ainda testado o envolvimento da via de sinalização mediada pela proteína cinase do tipo C (PKC). O bloqueio da PKC pelo GF109203X (1μM) promoveu a recaptação de [^{3}H]GABA mediada por GAT-1. Em concordância, a activação da PKC por ésteres de forbol (12,13-didecanoato de forbol – PDD 250nM) inibiu a recaptação de [^{3}H]GABA mediada por GAT-1, indicando que a PKC está endogenamente a inibir o transporte de [^{3}H]GABA para os terminais pré-sinápticos.

Estes resultados sugerem assim, o envolvimento das duas vias de sinalização na recaptação de [^{3}H]GABA mediada por GAT-1. Estudos de recaptação realizados com diferentes combinações de activadores e bloqueadores de ambas as proteínas cinases indicam que a activação dos receptores A_{2A} da adenosina, com consequente activação da PKA, restringe o efeito tónico inibitório da PKC sobre o transporte de [^{3}H]GABA, o que resulta numa potenciação da recaptação de GABA através do transportador GAT- 1 para o terminal pré-sináptico, por aumento do número de transportadores presentes na membrana celular.

O estudo de recaptação de [^{3}H]GABA em astrócitos sugere a existência de um efeito bifásico mediado pela adenosina sobre os transportadores GAT-1 e GAT-3. A incubação de culturas primárias de astrócitos com cloroadenosina (CADO), um análogo não-

metabolizável da adenosina teve um efeito bifásico sobre o transporte: a baixas concentrações (0,3µM), a CADO inibiu o transporte enquanto que concentrações mais elevadas (3 e 10 µM) de CADO promoveram a recaptação de [^{3}H]GABA.

Por outro lado, a remoção de adenosina endógena pela acção da adenosina desaminase (ADA, 1U/ml) diminuiu a recaptação de [^{3}H]GABA mediada por GAT-1 e por GAT-3, sugerindo que as concentrações de adenosina endógena presentes na cultura de astrócitos promovem o transporte de [^{3}H]GABA.

A realização de ensaios de recaptação com fármacos selectivos para os receptores de adenosina permitiu a identificação dos receptores envolvidos. Assim, o agonista selectivo dos receptores A_{2A} (CGS 21680, 30nM) promoveu a recaptação de [^{3}H]GABA mediada por GAT-1 e GAT-3. Surpreendentemente, este efeito para além de ter sido bloqueado pelo antagonista selectivo dos receptores A_{2A} (SCH 58261, 50nM) foi também prevenido pelo bloqueio dos receptores A_1 com o antagonista selectivo DPCPX (50nM). Por seu lado, a activação dos receptores A_1 com o agonista selectivo CPA (30nM) levou à diminuição da recaptação de [^{3}H]GABA mediada por GAT-1 e por GAT-3. Este efeito foi bloqueado quer pelo antagonista dos receptores A_1 quer pelo antagonista dos receptores A_{2A}. Estes resultados sugerem a existência de uma interacção entre os receptores A_1 e A_{2A} da adenosina nos astrócitos. De facto, a existência de uma interacção entre os receptores A_1 e A_{2A} da adenosina é conhecida desde há bastante tempo, tendo sido descrita inicialmente no hipocampo (Cunha *et al.,* 1994; Lopes *et al.,*

1999) e na junção neuromuscular (Correia-de-Sá e Ribeiro, 1994). A natureza desta interacção era desconhecida até 2006, quando se identificou a ocorrência de heterómeros de receptores da adenosina A_1-A_{2A} em células imortalizadas transfectadas (Ciruela *et al.,* 2006), através de ensaios de *bioluminesce ressonance energy transfer* (BRET). Recorrendo a esta tecnologia, bem como a ensaios de ligação, identificou-se neste estudo, pela primeira vez, a presença de heterómeros de receptores de adenosina A_1-A_{2A} nos astrócitos. O estudo reportado nesta dissertação foi pois o primeiro a identificar inequivocamente a presença de heterómeros A_1-A_{2A} em células não imortalizadas.

A identificação do subtipo de proteínas G acopladas ao heterómero de receptores de adenosina A_1-A_{2A} foi realizada através de ensaios de ligação de [^{35}S]GTPγS acoplados a imunoprecipitação e de ensaios de recaptação realizados na presença de toxinas que bloqueiam selectivamente a actividade das proteínas G_s e $G_{i/0}$. Os ensaios realizados indicam claramente que os heterómeros de receptores da adenosina A_1-A_{2A} estão acoplados a ambas as proteínas G_s e $G_{i/0}$, e não a uma única proteína Gq, como inicialmente se supunha, com base no que ocorre na heteromerização dos receptors da dopamina. O trabalho descrito nesta dissertação demonstrou ainda que o heterómero de receptores de adenosina A_1-A_{2A} é na verdade um tetrâmero formado por dois receptores A_1 e dois receptores A_{2A} (A_1-A_1-A_{2A}-A_{2A}), o que permite o acoplamento de duas proteínas G diferentes.

Ensaios de recaptação de [^{3}H]GABA realizados na presença do activador da AC, *forskolin*, e do inibidor competitivo da PKA, Rp-cAMPs, sugerem que o heterómero de receptores de adenosina A_1-A_{2A} sinaliza através da via AC/cAMP/PKA. O presente estudo permitiu ainda concluir que os heterómeros de receptores da adenosina A_1-A_{2A} funcionam como uma única entidade, que para ser funcional, necessita que todos os componentes estejam disponíveis para activação. Assim se entende que o bloqueio do receptor A_1 ou da proteína $G_{i/0}$ previna o efeito mediado pela activação dos receptores A_{2A}, bem como o bloqueio dos receptores A_{2A} ou da proteína G_s previna o efeito inibitório mediado pelos receptores A_1. Assim, nos astrócitos, o efeito bifásico da adenosina sobre a recaptação de [^{3}H]GABA é mediado pela activação dos heterómeros de receptores da adenosina A_1-A_{2A}. A baixa concentração, a adenosina activa preferencialmente os receptores A_1, levando à activação de proteínas $G_{i/0}$, que, por inibição da actividade da AC, provocam a redução dos níveis de cAMP, inibindo a PKA e reduzindo, consequentemente, a recaptação de GABA mediada por GAT-1 e GAT-3. Por outro lado, em concentrações superiores, a adenosina através da activação dos receptores $A_{2A,}$ e consequente activação de proteínas G_s, por activação da AC, eleva os níveis de cAMP, o que promove a actividade da PKA, e aumenta o transporte de GABA mediada por GAT-1 e GAT-3 em astrócitos.

Considerando a sinapse tripartida, os resultados sugerem que em baixas concentrações a adenosina conduz preferencialmente o GABA para o terminal pré-sináptico, favorecendo portanto a

transmissão fásica inibitória e impedindo o esgotamento das reservas pré-sinápticas. Em concentrações mais elevadas, a adenosina aumenta a recaptação de GABA pelos astrócitos, o que deverá diminuir a inibição tónica e permitir o aumento da excitabilidade. Assim, através da modulação dos transportadores de GABA, a adenosina pode ser considerada como um agente amplificador do tónus GABAérgico. Quando o tónus inibitório é prevalente, a adenosina direcciona a recaptação de GABA para os terminais pré-sinápticos, facilitando a transmissão fásica inibitória. Pelo contrário, a facilitação da recaptação de GABA pelos astrócitos reduz a inibição tónica, favorecendo a transmissão excitatória.

Em suma, este trabalho revela um mecanismo até agora desconhecido, através do qual a adenosina pode contribuir para a transição entre uma situação inibitória e outra excitatória, fenómeno que envolve a interacção entre astrócitos e neurónios inibitórios.

1 Introduction

1.1 GABA

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the central nervous system. Its presence in the central nervous system was described for the first time in 1950 (Awapara *et al.,* 1950; Roberts and Frankel, 1950; Udenfriend, 1950).

During the following years, several works identified the inhibitory role of GABA, particularly at the crustacean neuromuscular junction (Otsuka *et al.,* 1966; Potter, 1968). Later on, in 1970, GABA was localized to mammalian nerve terminals (Bloom and Iversen, 1970) and further studies revealed the association between GABA release and inhibition of cortical firing, clarifying the role of GABA as a neurotransmitter (Iversen *et al.,* 1971). At that time, several studies had reinforced this role of GABA in nervous system, namely the study on distribution of GABA and its related metabolic enzymes (Roberts and Eilderberg, 1960), the description of sodium-dependent GABA uptake mechanism (Elliott and van Gelder, 1960; Iversen and Neal, 1968) and the identification of GABA effects on post synaptic membranes (Krnjevic and Schwartz, 1967), which unequivocally supported the role of GABA as a neurotransmitter. This role was reinforced by the work of Curtis and colleagues, which showed that bicuculline, prevented the action of GABA and also the postsynaptic inhibition in the cerebral cortex (Curtis *et al.,* 1970), allowing the identification of receptor-mediated effects of GABA.

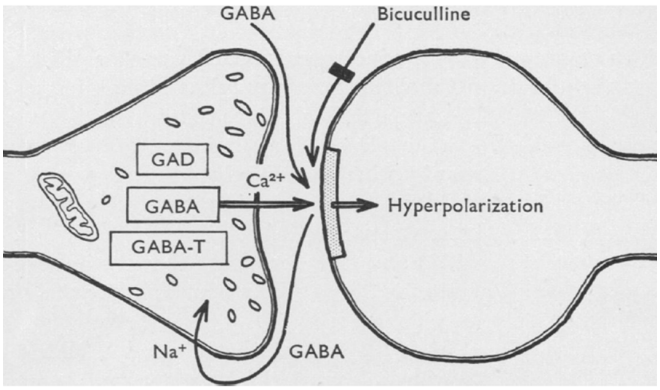

Figure 1.1 – Schematic representation of the evidence of GABA as a neurotransmitter in mammalian cerebral cortex (Iversen *et al.,* 1971)

1.1.1 GABA Receptors

GABA is estimated to be present in 60-75% of the synapses in the CNS (Durkin *et al.,* 1995). When it is released into the synapse, GABA binds to different receptors: $GABA_A$, $GABA_C$ and $GABA_B$. The first two are ionotropic receptors, mainly located in postsynaptic neurons while the later is a metabotropic receptor, localized both pre and postsynaptically.

1.1.1.1 *$GABA_A$ receptors*

$GABA_A$ is the main GABA receptor and its structure was identified in 1990 (Olsen and Tobin, 1990). $GABA_A$ receptors belong to the ligand-gated ion channel superfamily that includes nicotinic receptors for acetylcholine, glycine receptors and also serotonin receptors. $GABA_A$ is a pentameric receptor composed by distinct polypeptides. At the moment, several distinct peptides have been identified, namely six α-subunits, three β-subunits, three γ-subunits, one δ-subunit, one ε-subunit, one π-subunit and one θ-subunit (Schofield, 1987; MacDonald and Olsen, 1994; Mehta and Ticku, 1999).

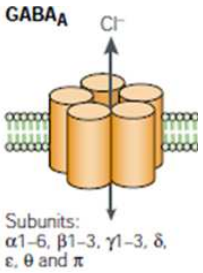

Figure 1.2 – Schematic representation of a $GABA_A$ receptor (adapted from Owens and Kriegstein, 2002).

Although a functional $GABA_A$ receptor always includes one α, one β and one γ subunit (Pritchett *et al.,* 1989), the $GABA_A$ subunits can be assembled in several possible combinations, with some of them being preferred. For example, $GABA_A$ receptors containing α1 subunits combined with β2 and γ2 subunits are the most abundant form in the brain (McKernan and Whiting, 1996). Furthermore, different subunits compositions of the receptors seem to be related with different cellular localizations, where they can mediate synaptic or extrasynaptic signaling (Mody, 2001).

The majority of $GABA_A$ receptors have affinity for benzodiazepines, which, allosterically, exacerbate the effect of GABA. This property is the basis for therapeutic effect of benzodiazepines as anxiolytic agents (for review, Bowery and Smart, 2006). The $GABA_A$ receptors contain other modulatory binding sites, sensitive to barbiturates, neurosteroids and ethanol (Macdonald and Olsen, 1994).

GABA binding to the receptor leads to a conformational change, which allows a net inward or outward flow through the channel, depending on the electrochemical gradient. $GABA_A$ receptors carry mainly chloride (Cl^-) ions but they can also carry other ions like

bicarbonate (HCO_3^-), although with lower efficiency (Bormann *et al*., 1987; Kaila, 1994).

1.1.1.2 *$GABA_C$ receptors*

A related ionotropic GABA receptor was named $GABA_C$ by Drew and colleagues, in 1984 (Drew *et al*., 1984). This receptor is a chloride-selective channel but is insensitive to the $GABA_A$ antagonist, bicuculline. $GABA_C$ are homo or hetero-pentomeric receptors composed by ρ subunits (Cutting *et al.,* 1991, 1992).

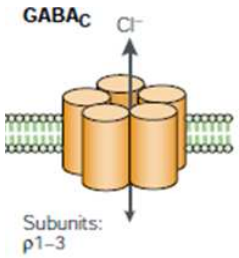

Figure 1.3 – Schematic representation of $GABA_C$ receptor (adapted from Owens and Kriegstein, 2002).

As the ρ subunits share extensive sequence homology with some $GABA_A$ subunits, $GABA_C$ receptors can be considered as pharmacological variants of $GABA_A$ receptors. However different studies involving $GABA_C$ receptors have strongly suggested that they have distinct pharmacology, structure, function, genetics and cellular localization, and consequently should be considered as an independent class of GABA receptors (Bormann, 2000). Although $GABA_C$ receptors were first identified in the retina (Feigenspan *et al.,* 1993; Qian and Dowling, 1993), posterior data suggests that $GABA_C$ receptor has a widespread distribution in the CNS (López-Chávez *et al.,* 2005).

1.1.1.3 *$GABA_B$ receptors*

In 1980, it was described the existence of a different GABA receptor ($GABA_B$), that was insensitive to bicuculline, chloride independent and responsible for the baclofen inhibitory effect upon neurotransmitter release in CNS (Bowery *et al.,* 1980). The $GABA_B$ receptor was insensitive for bicuculline and chloride independent and was termed $GABA_B$ receptor (Bowery *et al.,* 1980). $GABA_B$ is a metabotropic receptor which signals through $G_{i/0}$ proteins, decreasing adenylate cyclase activity and modifying calcium and potassium conductances. $GABA_B$ receptors are expressed either at pre-synaptic and pos-synaptic levels. Presynaptic activation of $GABA_B$ receptor results in inhibition of calcium channels, which leads to a reduction in neurotransmitter release. At the pos-synaptic level, the effect of $GABA_B$ is mediated by enhancement of potassium conductance, which leads to neuron hyperpolarization (Bormann, 1988).

Cloning of $GABA_B$ receptor showed a seven-transmembrane receptor that exists as a heterodimer composed of $GABA_{B1}$ and $GABA_{B2}$ subunits (White *et al*., 1998). Interestingly, the $GABA_B$ receptors were the first G-protein coupled receptors to be described as heteromeric receptors (Kaupmann *et al.,* 1998). However $GABA_B$ subunits cannot work separately: the $GABA_{B1}$ subunit binds to all known $GABA_B$ ligands (Kaupmann *et al.,* 1997), while $GABA_{B2}$ involved in different roles, namely: (1) to mask the retention signal of the $GABA_{B1}$ subunit, allowing that $GABA_B$ heteromer to reach the cell surface (Margeta-Mitrovic *et al.,* 2000; Calver *et al.,* 2001;

Pagano *et al.,* 2001), (2) to contain the molecular determinants required for G-protein coupling (Galvez *et al.,* 2001); (3) to play a critical role in G-protein activation within the heteromer (Margeta-Mitrovic *et al*., 2001; Robbins *et al*., 2001; Duthey *et al*., 2002; Havlickova *et al.,* 2002); and (4) to increase agonist affinity on $GABA_{B1}$ (Kaupmann *et al.,* 1998; White *et al*., 1998; Galvez *et al.,* 2001). Thus $GABA_B$ can be considered as a natural complementation of two non-functional receptors: one subunit recognizing the ligand and the other activating the G protein (Pin *et al.,* 2003).

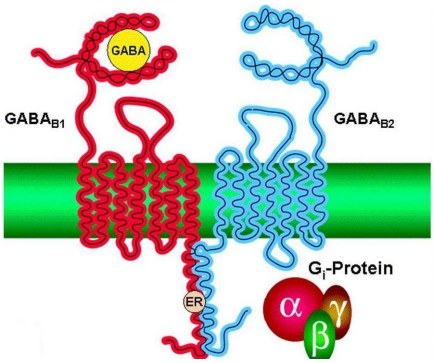

Figure 1.4 – Schematic representation of $GABA_B$ receptor (adapted from http://www.pharma.uzh.ch/research/neuropharmacology/researchareas/signaltransduction/introduction.html).

1.1.2 GABA metabolism

Neurons lack pyruvate carboxylase, so they are unable to *de novo* synthesis of glutamate and GABA (Patel, 1974; Shank *et al.,* 1985), which suggests the occurrence of a metabolic crosstalk between neurons and glial cells. In fact, the discovery of glutamine and glutamate pools in neurons and astrocytes was indicative of a

glutamate/glutamine cycle, working between neurons and glial cells (van den Berg and Garfinkel, 1971; Benjamin and Quastel, 1972).

Considering a GABAergic synapse, the majority of GABA is taken up into presynaptic terminals (see Schousboe, 2000), where without any metabolic changes, GABA can quickly be package into vesicles by a vesicular GABA transporter (vGAT), being available for dependent calcium exocytic release.

However, GABA can also be taken up into astrocytes (it has been estimated that 20% of released GABA is taken up into surrounding astrocytes, see Schousboe, 2000). By this path, GABA is firstly metabolized into glutamate and glutamine. Then, glutamine is redirected to the presynaptic terminal, where, GABA is resynthetized. This metabolic pathway is known as the aforementioned glutamate/GABA-glutamine cycle. Briefly, after being taken up into astrocytes, GABA is catabolised to succinate (a tricarboxylic acid (TCA) cycle intermediate via the concerned action of GABA transaminase and semialdehyde dehydrogenase. Succinate, through TCA cycle, will originate α-ketoglutarate, which is transaminated to glutamate by glutamate transaminase. Glutamate is then converted to glutamine by the glutamine synthetase (GS). Glutamine is then released to the extracellular space, from where is taken up into presynaptic neurons. After being taken up, glutamine is converted to glutamate by phosphate-activated glutaminase (PAG). Glutamate is finally converted into GABA, by glutamate decarboxylase (GAD) (see figure 1.5 and Bak *et al.,* 2006).

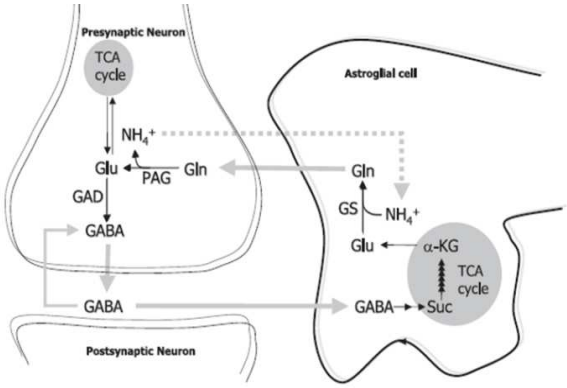

Figure 1.5 – Schematic representation of glutamate/GABA-glutamine cycle in a GABAergic synapse (adapted from Bak *et al*., 2006).

1.1.3 GABA transporters

Ambient levels of neurotransmitter in the synapse result from the balance between neurotransmitter release, diffusion within the synaptic cleft and the uptake through transporters. The main role attributed to neurotransmitter transporters is the shutdown of synaptic transmission. In the case of ionotropic receptors, both receptor desensitization and neurotransmitter uptake mediated by transporters contribute to the end of transmission, being unclear which is the principal phenomenon. Within these, extrasynaptic receptors desensitize slower than synaptic ones, so the relative contribution of transporters for shutting down GABA action at ionotropic receptors varies according to their location. In contrast, for metabotropic receptors, which desensitize slower than ionotropic receptors, neurotransmitter uptake is the predominant mechanism through which the synaptic transmission is finished (Borden, 1996).

Besides the temporal delimitation of synaptic transmission, transporters also contribute for synaptic spatial control by preventing the neurotransmitter spill over, assuring the precise location of transmission (see Krogsgaard-Larsen *et al.,* 1987). It was indeed demonstrated that the maintenance of appropriate levels of transmitter in the synaptic cleft by transporters is crucial for normal brain function (Giros *et al.,* 1996; Tanaka *et al*., 1997).

From a metabolic perspective, neurotransmitter uptake by transporters prevents the depletion of intracellular pools, preserving metabolic energy balance. The maintenance of these pools is critical for homeostasis, especially during periods of high activity. As mentioned above, neurotransmitter recycling can, however, be direct or indirect: the direct recycling occurs when the neurotransmitter is taken up directly to the pre-synaptic terminal, allowing the prompt replenishment of synaptic vesicles. The indirect recycling involves the surrounding astrocytes and the uptake by these cells implies metabolic conversion before the transmitter is available to be packaged into exocytic vesicles, as it was shown above.

GABA levels at the synapse are therefore mainly regulated by the specific transporters (GATs) expressed in neurons and astrocytes, which were identified in 1968 (Iversen and Neal, 1968). However, even before the recognition of GATs, Elliot and van Gelber (1958) had already demonstrated that GABA added to the incubation

medium can accumulate in slices of cerebral cortices (Elliot and van Gelber, 1958).

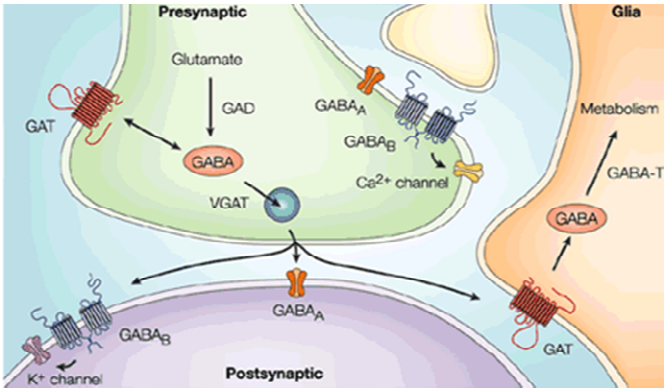

Figure 1.6 – Schematic representation of a GABAergic synapse. As illustrated, GATs are expressed in both presynaptic terminals and astrocytes (adapted from Owens and Kriegstein, 2002).

1.1.3.1 *GABA transporters family*

GABA transporters belong to the family of Na^+/Cl^- coupled receptors. In each transporter cycle, one uncharged GABA molecule is co-transported with two Na^+ ions and one Cl^- ion (see figure 1.7 and Kanner and Schuldiner, 1987). Thus GABA is carried up its concentration gradient using energy from the inward Na^+ gradient and the flow of one positive charge downs the electrical gradient (Richerson and Wu, 2003).

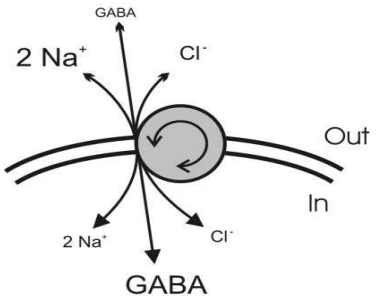

Figure 1.7 – Schematic representation of GABA transport. Each molecule of GABA is co-transported with two sodium ions and one chloride ion (adapted from Richerson and Wu, 2003)

Shortly, GABA binds the transporter in its resting state, facing extracellular space. After the binding of GABA, Na^+ and Cl^- ions, the transporter undergoes a conformational change enabling GABA and ions to face intracellular space, where they are released. Then, the unfilled transporter reverts to the resting state, waiting for a new cycle of transport (Borden, 1996).

Furthermore, it was shown that the magnitude of GAT-1 mediated currents is altered in response to changes in Na^+, Cl^- and GABA gradients (Lu and Hilgemann, 1999). Although these experiments were performed using nonphysiological concentrations, they provided initial evidence that transport can be modified (and possibly reverted) in response to ionic concentration changes.

So far, four distinct GABA transporters have been identified: GAT-1, GAT-2, GAT-3 and BGT-1. The first three are high affinity-transporters and the last is a low-affinity transporter.

1.1.3.2 *GAT-1*

GAT-1 was the first neurotransmitter transporter to be cloned (Guastella *et al.,* 1990). GAT-1 mRNA was found in all brain regions, being the highest levels of hybridization signal observed in brainstem nuclei, ventral tegmental area, pontine nuclei, cerebellum, thalamus, basal forebrain, hippocampus, olfactory bulb and neocortex. Concerning cellular localization, GAT-1 is expressed by both neurons and astrocytes. Although GAT-1 is mainly expressed at GABAergic neurons (which express concomitantly GAT-1 and GAD67), it can also be found in a small number of glutamatergic cells, suggesting that GABA uptake system mediated by GAT-1 is strongly expressed in the brain, being more widespread than the GABA synthesizing system (Minelli *et al.,* 1995). GAT-1 is a 67 kDa protein with twelve transmembrane regions, with both N- and C-termini in the intracellular space. GAT-1 has four putative glycosylation sites, being three of them located on the large extracellular loop that connects membrane segments 3 and 4. GAT-1 has also eight putative phosphorylation sites: three putative protein kinase C phosphorylation sites are intracellular, two at the N-terminal and one at C-terminal. The other three sites for PKC and the single site for PKA are located externally or within membrane segments (Guastella *et al.,* 1990), being therefore of questionable functional relevance.

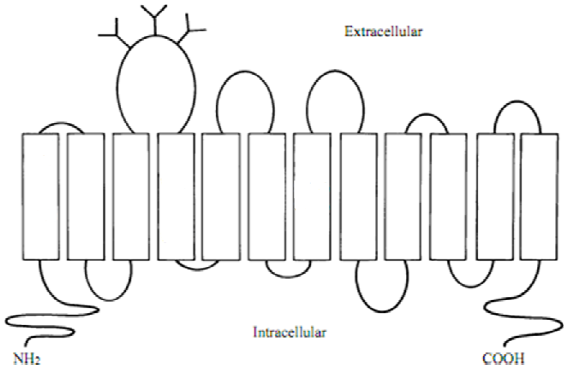

Figure 1.8 – Schematic representation of the GABA transporter GAT-1. The rectangles represent the twelve transmembrane domains, connected by extramembrane loops. The large extracellular loop, between membrane segments 3 and 4, contains three potential glycosylation sites, illustrated by the branched lines (adapted from Kanner, 1994).

1.1.3.3 *GAT-2*

GAT-2 mRNA was initially identified only at cells of the leptomeninges (pia and arachnoid - Durkin *et al.,* 1995). Later, a weak GAT-2 immunoreactivity was observed in the cortical parenchyma, where GAT-2 is expressed by neuronal and non-neuronal cells (Conti *et al.,* 1999). However, the predominant localization of GAT-2 to leptomeninges and blood vessels suggests that GAT-2 function is mainly related to the regulation of GABA levels in the cerebrospinal fluid (Minelli *et al.,* 2003).

1.1.3.4 *GAT-3*

In the early 90's, several studies indicated that GAT-3 was absent or very weakly expressed at the cerebral cortex (Clark *et al.,* 1992; Durkin *et al.*, 1995; Ikegaki *et al.,* 1994). Then, in 1996, Minelli and colleagues showed that GAT-3 is expressed in cerebral cortex, exclusively at astrocytes, being GAT-3 the main GABA transporter

expressed in glial cells (Minelli *et al*., 1996). In addition, Durkin and colleagues showed that GAT-3 mRNA is present in many brain areas, though it is less abundant the GAT-1 mRNA (Durkin *et al.,* 1995).

1.1.3.5 *BGT-1*

BGT-1, the fourth GABA transporter, was known as a result from renal physiology studies, where a betaine transporter was initially identified. This transporter had the ability to concentrate urine through the uptake of the betaine (glycine betaine), that was co-transported with Na^+ and Cl^- (see Borden, 1996). Then, Yamauchi and colleagues isolated a clone encoding a Na^+/Cl^- dependent transporter, which was able to utilize both GABA and betaine as substrates. Hence, the clone was named BGT-1 (Betaine/GABA transporter). Surprisingly, BGT-1 has higher affinity for GABA (K_M =93 µM) than for betaine (K_M =398 µM) (Yamauchi *et al.,* 1992).

The expression of BGT-1 as well as its role in CNS is still not clear although the presence of BGT-1 mRNA in human and mouse brain may suggest its involvement in GABAergic transmission. However, the distribution of BGT-1 mRNA does not correlate with GABAergic pathways, which corroborates the hypothesis that BGT-1 might not play a role in finishing GABA effects at synapses. Alternatively, betaine may be the primary substrate for BGT-1 in the brain (as it is in the kidney), which suggests an involvement of BGT-1 in osmoregulation, thus BGT-1 could contribute to the volume control in the CNS. Osmoregulation is particularly important for brain

function (see Borden, 1996), as the skull is a rigid and non-expandable container.

1.1.3.6 *GABA transporters modulation*

Considering that the control of GABA concentration at synapse is strongly regulated by GABA transporters, their modulation is a key factor to assure adequate GABA levels for brain function. GAT modulation occurs mainly by changes in their expression at the membrane level. In fact, this expression is not static and transporters can cycle to and from membrane on the time scale of seconds to minutes, being this cycling regulated by different factors, including extracellular signals (Zahniser and Sorkin, 2004; Sattler and Rothsein, 2006), protein phosphorylation (Foster *et al.,* 2006) and interactions with accessory proteins (Quick, 2006).

The average number of GAT-1 molecules in a cultured cortical inhibitory bouton is approximately 3000-4000 (Chiu *et al.,* 2002) and the size of the recycling pool is about 30% of total GAT-1 (Wang and Quick, 2005). Then, about 1000 GAT-1 molecules traffic to and from the membrane, with GAT-1 being inserted into the membrane in a calcium-dependent manner and internalized via a clathrin-dependent process (Deken *et al.,* 2003). The regulation of membrane expression levels can occur through changes in recycling pool size or modification of exocytosis and endocytosis rate. Changes at transporters surface membrane expression occur in a scale of minutes (Mennerick *et al*., 1999; Blakely and Bauman, 2000; Deken *et al*., 2000; Robinson, 2002).

Firstly, GAT-1 is modulated by its own substrate, GABA, being this a concentration dependent effect, involving changes in phosphorylation state, which consequently modify the transporter surface expression (Hu and Quick, 2008).

In fact, phosphorylation seems to be a critical cellular mechanism responsible for changes in the membrane expression of transporters. As aforementioned, GAT-1 has eight putative phosphorylation sites (Guastella *et al.,* 1990) and it was described that PKC activation decreases membrane GAT-1 levels (Corey *et al.,* 1994), in a way that is independent of protein synthesis. This action of PKC activators is mimicked by phosphatase inhibitors, suggesting that changes in membrane levels are dependent on protein phosphorylation. However, it was shown that the effect of PKC upon GAT-1 surface expression still occurs when consensus residues for PKC phosphorylation are removed (Corey *et al.,* 1994). Thus, it seems that there are other cellular mechanisms through which PKC can influence GAT-1 levels. In fact, a subsequent study showed that PKC effect upon GAT-1 function occurs through the regulation of syntaxin A1 availability to interact with the transporter. Further, a mutant transporter that fails to interact with syntaxin A1 is not regulated by the PKC (Beckman *et al.,* 1998).

Like PKC, Ca^{2+} depletion also decreases surface expression of GAT-1, while hypertonic sucrose treatment increases its surface expression (Beckman *et al.,* 1999; Deken *et al.,* 2003). Interestingly, these effects are not mediated by the same cellular mechanism: PKC and sucrose application modifies the endocytosis rate but does not

change the recycling pool size, which is dramatically diminished by extracellular calcium depletion (Wang and Quick, 2005).

1.1.3.7 *Reversal of GABA transporters*

It is known for a long time that neurotransmitters transporters can revert (Schwartz, 1987; Pin and Bockaert, 1989; O'Malley *et al.,* 1992; Attwell *et al.,* 1993; Levi and Reiteri, 1993; Cammack *et al.,* 1994; Lu and Hilgemann, 1999). For instance, after extracellular calcium removal, GABA is still released from cortical slices in response to: (1) an increase in K^+ to 50mM; (2) an exposure to veratridine (50 µM); (3) a high-frequency electrical stimulation (Szerb, 1979). GABA is also released from cultured striatal neurons, in the absence of calcium (Pin and Bockaert, 1989) and from cortical neurons in response to 55-56 mM of K^+ or 100 µM of glutamate (Belhage *et al.,* 1993). Neurotransmitter release in the absence of extracellular calcium occurs by the reversal of transporters since it is prevented by the blockade of transporters (Pin and Bockaert, 1989; Belhage *et al.,* 1993). As these studies were performed using non-physiological stimuli, the transporters reversal was initially associated with pathological conditions such as ischemia (Nicholls and Attwell, 1990; Levi and Raiteri, 1993). However, transporter-mediated GABA release can also occur in response to presynaptic stimulation (Schwartz, 1987; Bernath and Zigmond, 1988) or in response to an elevation in extracellular K^+ concentration from 3 to 12 mM (Gaspary *et al.,* 1998), a level of K^+ that is reached *in vivo* during neuronal firing (Krnjevic *et al.,* 1980; Somjen and Giacchino, 1985) and during seizures (Fisher *et al.,* 1976). In 2001, a study by

Wu and colleagues corroborate the hypothesis that GABA transporters reverse more easily than previously recognized: GABA reversal was induced by a small depolarization of membrane potential caused by 6mM K^+ in hippocampal cultures (Wu *et al*., 2001. This potassium increase is physiologically relevant as extracellular potassium concentration increases during neuronal firing *in vivo*. For example, an increase in $[K^+]$ to > 12mM occurs in the rat hippocampus *in vivo* in response to electrical stimulation of the angular bundle (Somjen and Giacchino, 1985). Then, instead of being associated with some pathological state, the reversal of GABA transporters seems to be related with modifications in the ratio between intra and extracellular concentrations of GABA. In fact, the rate of GABA uptake is governed by a driving force, which decreases as extracellular GABA concentration reduces. Then, the GABA uptake by transporters occurs until the equilibrium between intra and extracellular concentrations is achieved. Thus, GABA transporters are unable to uptake all the GABA, and extracellular GABA concentration fall around 0.1-0.8 µM (Hagberg *et al.,* 1985; Lerma *et al.*, 1986; Tossman *et al.,* 1986; Phillis *et al.,* 1994; Cavelier *et al*., 2005).

1.1.3.8 *GABA transport impair and neurological disorders*

As GATs are the main responsible for GABA clearance from synapse, a transporter dysfunction will consequently originate an unbalance in GABA concentrations, which has been associated with several pathologies, especially in epilepsy (e.g. Richerson and Wu, 2004), in which some therapeutic options are related with GABA transporter.

A possible therapeutic option in epilepsy is to increase the inhibitory tone in order to prevent seizures. This can be achieved through the blockade of GABA uptake or by the reversal of GABA transporters. Interestingly, in animal models, specific blockers of GAT-1 and blanket GABA transporter inhibitors have been shown to be selectively effective against different types of seizures. This can be due to a differential distribution of GABA transporters among brain regions. GAT-1 is predominantly expressed in the forebrain and limbic areas while GAT-3 is mainly presented in the brainstem. The kindled focal and generalised secondary seizures, and clonic convulsions, induced by chemoconvulsants and originated in limbic system or forebrain, are abolished by GAT-1 blockers. On the other hand, convulsions induced by electro shocks (originated in the basal ganglia and brainstem) are inhibited by non-selective GATs inhibitors (Nielsen *et al.,* 1991; Swinyard *et al.,* 1991; Dalby *et al.,* 1997). Actually, the discovery of a potential use of GABA transporter inhibitors in epilepsy occurred thirty years ago (Krogsgaards-Larsen *et al.,* 1981) and since then, several non-selective inhibitors have been experimentally demonstrated to possess anticonvulsant activity (Dalby *et al.,* 1997; Dalby, 2003; White *et al.,* 2005). Nowadays, tiagabine, a specific GAT-1 inhibitor (Borden *et al.,* 1994), is used as an adjuvant therapy for patients with focal seizures, namely with complex partial temporal lobe epilepsy and secondary generalised tonic-clonic seizures (Uthman *et al.,* 1998).

Other therapeutic approach involving the GATs reversal is the use of vigabatrine, which blocks GABA transaminase, the enzyme

responsible for GABA catabolism. The inhibition of GABA transaminase will increase GABA intracellular levels, favouring the reversal of GABA transporters (Wu *et al.,* 2001, 2003).

The reversal of GABA transporters was also observed during ischemic conditions, where the increase of extracellular K^+, by the inactivation of Na^+/K^+ ATPase, is enough to reverse GATs. In fact, GAT-1 inhibition reduced the amount of GABA released (and subsequently, cell swelling) during energy deprivation (Zeevalk and Nicklas, 1996). So, although it has been considered that the potentiation of GABAergic transmission contributes to reduce ischemia-induced damage (Green *et al.,* 2000), the neuroprotective effect mediated by GATs inhibition can be related with a decrease in the GABA released by the reversal of transporters (and consequently to a decrease in extracellular GABA levels) instead of being associated with a enhancement in extracellular GABA, caused by the blockade of GABA uptake, as some authors had suggested (Phillis, 1995; Green *et al.,* 2000). The reversal of GATs associated with an increase in intracellular GABA concentrations, which is also observed during ischemia (Erecinska *et al.,* 1984), contributes to an overactivation of $GABA_A$ receptors, which leads to an increased influx of Cl^- ions, and subsequently, to cell swelling and eventually cell death.

The enhancement in intracellular GABA levels is the result of three different aspects: first, the reduced ATP levels found under ischemia, stimulate GABA synthesis by GABA decarboxylase (Madl and Royer, 2000); second, the reduction of pH, that occurs under ischemia,

inhibits GABA transaminase, leading to an accumulation of intracellular GABA (Madl and Royer, 2000); third, the disruption of vesicular H^+-dependent GABA storage during ischemia rises cytoplasmic GABA, promoting GABA transporters reversal under ischemic conditions (see Allen *et al.,* 2004). Recently, Clarkson and colleagues identified an excessive tonic inhibition after stroke which was associated to the observed decrease in GAT-3 expression in peri-infarct cortex (Clarkson *et al.,* 2010). Thus, besides the reversal of the transporters, changes in transporters expression are also associated with ischemic conditions, which make GATs potential targets in treatment of stroke.

1.1.3.9 *Influence of GATs upon tonic and phasic GABAergic transmission.*

GABA acts through $GABA_AR$ and mediates both phasic and tonic inhibition. Phasic inhibition is a transient and point-to-point phenomena, as a consequence of the release of GABA following nerve terminal depolarization, leading to inhibitory postsynaptic currents (IPSCs). Tonic inhibition is associated with the spillover of GABA outside the synaptic areas leading to lower but sustained extracellular GABA levels. The main feature of phasic $GABA_AR$-mediated inhibition is the rapid synchronous opening of a relatively small number of channels that are clustered at the synaptic junction, whereas tonic inhibition results from random, temporally dispersed activation of receptors that are distributed over the neuronal surface. This distinction involves a deep difference in the control of

neuronal network activity by phasic and tonic forms of inhibition (see Farrant and Nusser, 2005).

At the central nervous system, the extracellular GABA concentration ranges from 0.2 to 2.5µM (Lerma *et al.,* 1986; Tossman *et al.,* 1986; Ding *et al.,* 1998; Kuntz *et al.,* 2004). At ambient GABA concentrations, the extra- and peri-synaptic $GABA_AR$ are preferentially activated as they have higher affinity for GABA, as compared to synaptic $GABA_AR$. These extra-synaptic receptors are then responsible for tonic inhibition (Brickley *et al.,* 1996; Farrant and Nusser, 2005), which is highly related with sustained changes in extracellular GABA concentration, therefore to changes in the activity of GABA transporters. In fact, GABA transporters are responsible for the maintenance of homeostatic GABA levels. This control can be achieved by changes in uptake rates- which are related with protein surface expression – but also by the reversal of GABA transporters (Gaspary *et al.,* 1998; Richerson and Wu, 2004).

Some electrophysiological studies have reported the influence of GATs in GABAergic transmission. For instance, in rat neocortex and in the hippocampus, GAT-1 blockers increase the decay time of evoked inhibitory post-synaptic currents (eIPSCs) without modifying the spontaneous IPSCs (sIPSCs) (Thompson and Gahwiler, 1992; Engel *et al.,* 1998; Overstreet and Westbrook, 2003; Keros and Hablitz, 2005), as well as enhance the amplitude of tonic $GABA_AR$ mediated currents (Nusser and Mody, 2002; Semyanov *et al.,* 2003; Keros and Hablitz, 2005; Marchionni *et al.,* 2007;), thus indicating that GAT-1 affects both tonic and phasic GABAergic transmission.

This influence of GAT-1 upon phasic and tonic transmission was further confirmed by Bragina and colleagues, using GAT-1 KO mice (first characterized by Jensen in 2003), who showed that a chronic impairment in GAT-1-mediated GABA uptake in the neocortex modifies both phasic and tonic $GABA_A$R-mediated inhibition. GAT-1 KO mice showed an increase in glutamate decarboxylase (GAD65/67) expression but no changes in GAT-3 expression or distribution (Bragina *et al*., 2008).

The influence of GABA transporters upon tonic transmission is probably related with transporters features, since GATs just take up GABA until reach equilibrium, being not able to remove all the extracellular GABA. This implies that a small (few micromolar) amount of GABA remains extracellularly, being enough to activate extra-synaptic $GABA_A$ receptors, which are the responsible for tonic inhibition.

The majority of studies about the influence of GABA transporters upon GABAergic transmission have been focused in the neuronal transporter, GAT-1. But, recently, a study had highlighted the role of glial transporter GAT-3 upon GABAergic transmission. It was observed that the GAT-3 blockade (just when GAT-1 was previously blocked) reduces the frequency of sIPSC (Kirmse *et al.,* 2009), attributing to GAT-3, a role in modulation of phasic GABAergic transmission, at least when GAT-1 is blocked, which means that the contribution of GAT-3 under basal conditions is still unclear.

1.2 The tripartite synapse and glial cells

For many years, glial cells were considered as supportive cells and unable to communicate with neurons or other glial cells. But, in the 90's years, several studies revealed the existence of bidirectional communication between neurons and astrocytes. These different studies led Araque and colleagues to propose that synapses are tripartite, consisting of synaptically associated glia as well as the presynaptic and the postsynaptic terminals (Araque *et al.,* 1999).

It was probably the discovery that astrocytes can be excited non-electrically that expanded the brain communication to non-synaptic elements and changed the paradigm of brain function based on neuronal activity to a function dependent on neuron-glia network.

There are four different types of glial cells: astrocytes, oligodendrocytes, microglia and Schwann cells. The first three are present in the central nervous system while the last one can be only found in the peripheral nervous system. Oligodendrocytes and Schwann cells are responsible for myelin production while microglia is related with immune defense of nervous system.

1.2.1 Astrocytes

The central nervous system is mainly composed of glia, in a proportion (in humans) of 1 neuron to 10 glial cells. These cells were identified by Rudolf Virchow in 1858. In his lecture, he described astrocytes as connective tissue, a kind of as "nervenkitt" or nerve-cement. Virchow named these cells as neuroglia (Virchow, 1858, quoted by Kettenmann and Verkhratsky, 2008). Although for him

neuroglia was a connective tissue, he admitted that it also contained a certain number of cellular elements. But Virschow was not the first to identify glial elements, Robert Remak had previously described, in 1838, a sheath around single nerve cells (R. Remak, PhD Thesis, University of Berlin, 1838, quoted by Kettenmann and Verkhratsky, 2008) and Heinrich Müller had previously discovered the radial fibers in the retina in 1851 (these cells were latter called as Müller glial cells).

During the following years, many different forms of glial cells were described and images were published (Deiters, 1865; Henle and Merkel, 1869; Golgi, 1903, quoted by Kettenmann and Verkhratsky, 2008). Later, in 1893, Michael von Lenhossek proposed the name astrocyte (von Lenhossek, 1893, quoted by Kettenmann and Verkhratsky, 2008). Astrocytes were then classified as fibrous or protoplasmic astrocytes by Kölliker (Kölliker, 1889, quoted by Kettenmann and Verkhratsky, 2008) and Andriezen (Andriezen, 1893, quoted by Kettenmann and Verkhratsky, 2008). Thus, although they are known since the 19th century, astrocytes were neglected for a long time, probably because they are not able to generate action potentials. They used to be considered as silent and supportive cells with relevant roles in metabolic pathways and also neurotransmitters uptake from synapse. In fact, it was early suggested that glial cells may remove and inactivate the products of neuronal activity (Lugaro, 1907, quoted by Henn and Hamberger, 1971). Later, it was shown that glial cells have the ability to accumulate amino acids (aspartic acid, threonine, serine, proline;

Hamberger, 1971) as well as neurotransmitters (glutamate, glycine, norepinephrine, serotonin, dopamine and γ-aminobutyric acid; Hamberger, 1971; Henn and Hamberger, 1971).

Then, in the early 90's, the arousal of fluorescence imaging techniques to monitor intracellular calcium levels, allow to identify a form of excitability based on variations of intracellular calcium concentration in astrocytes (Charles *et al.,* 1991; Cornell-Bell, 1990).

These studies suggested the involvement of non-neuronal elements to synaptic and non-synaptic transmission and came to revolutionize the field. Thus, nowadays astrocytes are considered as greatly polyvalent cells that are implicated in virtually all processes in the CNS. A single astrocyte can sense the activity and integrate the function of hundreds of neurons within its domain. Furthermore, each astrocyte extends processes to wrap blood vessels. Then, astrocytes work as local integration units and bridges between synaptic and non-synaptic communication (Volterra and Meldolesi, 2005), also control brain homeostasis.

1.2.1.1 *Astrocytes excitation*

The excitation of astrocytes in the form of calcium waves can be divided in two main categories: one is dependent on chemical signals generated by neuronal circuits (neuron-dependent excitation) and the other is independent of neuronal input (spontaneous excitation) (see Volterra and Meldolesi, 2005).

Neuron-dependent excitation of astrocytes is a widespread phenomenon that was firstly described in the hippocampus and

cerebellum (Bezzi and Volterra, 2001; Haydon, 2001). Different molecules are involved in this type of excitation: glutamate, GABA, acetylcholine, noradrenaline, dopamine, ATP, nitric oxide and brain derived neurotrophic factor (BDNF) (Khan *et al.,* 2001; Matyash *et al.,* 2001; Araque *et al.,* 2002; Rose *et al.,* 2003; Zhang *et al*., 2003; Zonta *et al.,* 2003; Bowser and Khakh, 2004; Fellin *et al.,* 2004). Astrocytes can discriminate neuronal inputs of different origins, and can integrate concomitant inputs. In fact, it was shown that astrocytes discriminate between the activity of cholinergic and glutamatergic synapses and also distinguish glutamatergic activity belonging to different axon pathways, for example, astrocytes have the ability to discriminate between the glutamate released by Schaffer collaterals or by alveus terminals (Perea and Araque, 2005).

Astrocytic spontaneous excitation was initially described in acute brain slices (Parri *et al.,* 2001; Aguado *et al.,* 2002; Nett *et al.,* 2002) and later during *in vivo* recordings (Hirase *et al.,* 2004; Nimmerjahn *et al.,* 2004). Spontaneous excitation occurs especially during development and decreases considerably during the first two postnatal weeks, when synaptic circuit formation occurs (Parri *et al.,* 2001). This form of astrocyte excitation decreases but does not disappear with adulthood (Nett *et al.,* 2002; Aguado *et al.,* 2002) and it is generated by calcium release from internal stores when IP_3 receptors are activated, supplemented by influx of extracellular calcium, possibly through voltage-gated channels (Parri *et al.,* 2001; Nett *et al.,* 2002; Aguado *et al.,* 2002). Importantly, spontaneous excitation of astrocytes can result in the excitation of neighbouring

neurons (Parri *et al.,* 2001). So, both neurons and astrocytes are sources of excitation, and operate in coordinated networks (Aguado *et al.,* 2002; Hirase *et al.,* 2004; Nimmerjahn et al., 2004). In fact, astrocytes are active players in synaptic transmission, and they express a range of neurotransmitter receptors (Kettenmann *et al.,* 1984; Verkhratsky and Steinhauser, 2000), which allow them to sense synaptic activity. On the other hand, astrocytes can modulate transmitter levels in the synapse through the release of gliotransmitters by exocytosis, transporters or membrane channels (Volterra and Meldolesi, 2005; Pankratov *et al.,* 2006), or by neurotransmitter uptake (Tzingounis and Wadiche, 2007).

1.2.1.2 *Gliotransmission*

According to Volterra and Meldolesi, a gliotransmitter is a substance that respects four criteria: (1) it is synthesized and/or stored in glial cells; (2) it shows a release regulated by physiological stimuli; (3) it promotes fast (milliseconds to seconds) responses in neighbouring cells; and (4) it has a role in physiological processes (Volterra and Meldolesi, 2005). Later, a fifth criterion was proposed: the release of gliotransmitter can be regulated by pathological stimuli (Martín *et al*., 2007).

Gliotransmitter release can occur by both exocytosis and non-exocytosis mechanisms, but for a long time, it was not clear that astrocytes expressed the necessary machinery to exocytose gliotransmitters. In 2004, a clear synaptic-like microvesicle (SLMV) compartment, which is competent for glutamate exocytosis was

identified in adult hippocampal astrocytes as well as a calcium dependent exocytic mechanism in cultured astrocytes (Bezzi *et al.,* 2004). The SLMV resembles glutamatergic synaptic vesicles, although less packed (Volterra and Meldolosi, 2005). This SLMV expresses exocytic proteins as R-SNAP, R-SNARE, VAMP3 and also vesicular glutamate transporters, vGLUTs (Bezzi *et al*., 2004). Furthermore, astrocytic exocytosis is quite different from neuronal exocytosis, since it is significantly slower than in neurons and the release machinery shows a higher affinity for calcium (Bezzi *et al*., 2004; Kreft *et al.,* 2004).

On the other hand, gliotransmitter exocytosis is obviously not triggered by the entry of extracellular calcium in response to action potentials but by the activation of GPCR, which lead to an IP_3-induced Ca^{2+} release from endoplasmic reticulum.

Further differences related with exocytic proteins expression have been described (see Volterra and Meldolosi, 2005).

Considering calcium changes, the increment of calcium is probably not the only mechanism involved in gliotransmitters release. In fact, other stimuli can evoke release of transmitters (Muyderman *et al.,* 2001; Joseph *et al.,* 2003). For example, it was described that GPCR-induced glutamate release was abolished by inhibiting the synthesis of prostaglandins, TNF-α or both (Bezzi *et al*., 1998, 2001; Pasti *et al.,* 2001).

Non-exocytic gliotransmitters release occurs through specialized proteins as channels or transporters present in the membrane of

astrocytes. Different plasma membrane channels have been associated with non-exocytic transport such as: (1) volume-regulated anion channels (VRACs); (2) GAP-junction hemichannels; (3) purinergic P2X receptors; and (4) cystine-glutamate antiporter.

Under hypo-osmotic conditions such ischemia, astrocytes swell and became permeable to anionic aminoacids as glutamate, aspartate and taurine, which are released through VRAC (Kimbelberg *et al.,* 1990).

GAP junctions form a pore between two adjacent cells, connecting their cytoplasm, and allowing molecules up to 1kDa to diffuse between adjacent cells (Malarkey and Parpura, 2008). GAP junctions are constituted by two connexins, usually named as hemichannels. These hemichannels can open directly to the extracellular space, allowing the release of different compounds. In fact, the opening probability of gap hemichannels is very low at physiological conditions but increases as the calcium concentration goes down, allowing the release of glutamate (Ye *et al.,* 2003) and ATP (Arcuino *et al.,* 2002; Stout *et al.,* 2002).

Purinergic $P2X_7$ ion channel could mediate the release of glutamate. Application of ATP to cultured astrocytes expressing $P2X_7$ receptors induced an inward current that was increased by low divalent cation external solution. The induced current was insensitive to voltage changes, which differentiates it from channels such as gap junctions. Release of glutamate was induced by ATP application. This release seems to be calcium independent since glutamate release was not

reduced after cell incubation with a membrane permeable calcium chelator (Duan *et al*., 2003).

Glutamate release has been also associated with Na^+-independent cystine/glutamate exchanger. Astrocytes are known as the main source of glutathione in central nervous system and its synthesis is dependent on the cystine uptake that occurs through both plasma membrane Na^+-independent cystine/glutamate exchanger and Na^+-dependent glutamate transporters (see McBean, 2002). Although under physiological condition, cystine/glutamate exchanger has no effect on glutamate release (Cavalier and Atwell, 2005); it can assume more relevance when cystine levels are modified.

Finally, gliotransmitter can also be released by the reversal of glial transporters. Regarding glutamate, once in the synapse, it is mainly taken up by glial cells through the specific transporters, GLAST and GLT-1 (Gadea and López-Colomé, 2001). In physiological conditions, and due to the fast conversion of glutamate into glutamine, the transporters do not usually revert. But, under pathophysiological conditions, such as ischemia, the ionic unbalance (increased K^+ levels) can favour the transporter reversal, allowing glutamate release. In fact, it was reported for the first time in 1990, that the reversal of glutamate transporters is triggered by an elevation of extracellular potassium levels (Szatkowski *et al.,* 1990). Although the range of potassium levels used in that work never occur under physiological conditions, several studies using transporter blockers have shown that reversal of glutamate transporters can occur during periods of ischemia or metabolic blockade (Longuemare and

Swanson, 1995; Zeevalk *et al.,* 1998; Seki *et al.,* 1999; Li *et al.,* 1999; Rossi *et al.,* 2000). Importantly, it was shown that the reversal of glutamate transporters can be a major event leading to astrocytic cell death (Re *et al.,* 2006). However, the extracellular increase of glutamate levels is not the cause of astrocytic death, being the result of depletion of cytoplasmic glutathione (Malarkey and Parpura, 2008). In fact, glutathione synthesis is dependent on the cystine uptake, which occurs through the above mentioned cystine/glutamate exchanger. The reversal of glutamate transporters depletes intracellular glutamate and abolishes the uptake of cystine, causing oxidative stress, which ultimately can result in cell death.

Comparing to glutamate, few studies about astrocytic GABA release have been done. But it was shown that activation of glutamate transporters results in GABA release through reversal of glial GABA transporters (Héja *et al.,* 2009). More recently, it was shown that besides potassium elevation and glutamate, glycine and d-serine are also able to induce astrocytic release of GABA (Lee *et al.,* 2011a). Further, GABA release was reduced when IP_3 receptors were previously blocked, suggesting that GABA release can be associated not only with calcium influx from outside but also from intracellular calcium stores, in response to metabotropic receptor activation (Lee *et al.,* 2011a). Furthermore, additional GABA was found to be released in the presence of BAPTA, a calcium chelator, corroborating previous results showing that GABA can be released from astrocytes in a calcium independent way, through transporter reversal.

So, astrocytes modulate synaptic transmission both by release of gliotransmitters and by uptake of neurotransmitters. The uptake is particularly relevant in the case of glutamate, as it is mainly taken up into astrocytes. On the other hand, GABA is mainly taken up by pre-synaptic terminals. However the role of glial GABA transporters in the clearance of synaptic GABA is not negligible (see Schousboe *et al.,* 2004).

Astrocytes are also involved in shaping synaptic connectivity in the brain by controlling the genesis and maintenance of synapses and also by affecting synaptic strength and plasticity (see Kettenmann and Verkhratsky, 2008). Furthermore, it seems that astrocytes can behave as a stem cell since they have the ability to re-enter in the cell cycle and produce all types of neural cells, from neurons to microglial cells (see Kettenmann and Verkhratsky, 2008).

Astrocytes are also involved in the control of brain homeostasis through several distinct mechanisms: regulation of volume and composition of extracellular space, namely K^+ and neurotransmitters levels and water movement (see Kettenmann and Verkhratsky, 2008); control of blood-brain barrier (see Kettenmann and Verkhratsky, 2008); release of reducing equivalents as glutathione (see Giaume *et al.,* 2007); chemoattraction of cells to their territory, for example, astrocytes coordinate the spatial positioning of oligodendrocytes (Tsai *et al.,* 2002), attract microglia and lymphocytes during inflammatory reactions and after injuries (Babcock et al., 2003; Marella and Chabry, 2004) and may drive reparative neural stem cells to lesion sites (Imitola *et al.,* 2004).

Astrocytes, through the release of GABA, can also regulate microglia function. It was recently shown that GABA can suppress the reactive response of both astrocytes and microglia to the inflammatory stimulants lipopolysaccharide (LPS) and interferon-γ by inhibiting induction of inflammatory pathways mediated by NFκB and p38 MAP kinase (Lee *et al.,* 2011b). These findings shed a new light upon the role of astrocytes on immunological responses in CNS but also a new role for GABA, which by becoming a player in inflammatory reactions, clearly exceeds its role as the classical main inhibitory neurotransmitter.

In fact, astrocytes respond to traumatic and chemical brain injury, gliosis and astrocytic swelling (see Aschner, 1998). Astrogliosis implies phenotypic alterations, astrocyte proliferation and growing, up-regulation of enzymes and cytoskeletal proteins, as glial fibrillary acidic protein (GFAP) and vimentin. An increase in the levels of transforming growth factor β (TGFβ), nerve growth factor (NGF), basic-fibroblast growth factor (bFGF) and growth factors receptors, as truncated forms of TrkB also occurs. These processes are usually regarded as neuroprotective, since they involve the production of neurotrophins and isolate damaged tissue (see Daré *et al.,* 2007).

Astrogliosis is also associated with an increase in extracellular adenosine. Moreover, the elevated levels of adenosine that follow brain injury could modulate astrogliosis, as suggested by the increase number of GFAP-positive cells after microinjection of an adenosine analogue into rat cortex (Hindley *et al.,* 1994).

1.3 Adenosine

Adenosine, which is a well-known neuromodulator, was first described in the heart, where it causes bradycardia and dilates the coronary arteries, causing a reduction in arterial pressure (Drury and Szeent-Györgi, 1929).

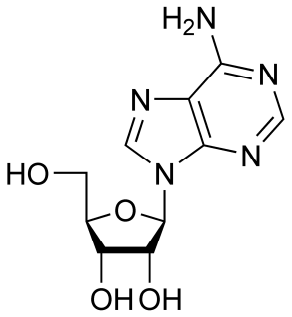

Figure 1.9 – Chemical structure of adenosine. Adenosine is a nucleoside, constituted by the purine adenine and a ribose ring.

The possibility of purinergic neurotransmission was first raised in 1954 when Holton and Holton suggested that ATP could be the transmitter released at central and peripheral terminals of sensory nerve fibers (Holton and Holton, 1954). Twenty years would pass before a specific role for adenosine in neurotransmission was hypothesized, after the discovery that iontophoretic applications of adenosine or its nucleotides caused a potent depressant effect on the responses of neurons (Phillips *et al.,* 1974, 1975, 1979a, b; Kostupoulos *et al.,* 1975; Kostupoulos and Phillis, 1977).

In addition to the description of its electrophysiological effects, in 1970, adenosine was classified as a potent activator of adenylate cyclase, capable of promoting cAMP production in the brain (Sattin and Rall, 1970). In 1976, it was suggested that adenosine effects

might be mediated by an adenylate cyclase-linked extracellular receptor (Mah, 1976).

The role of adenosine as a neurotransmitter or as a neuromodulator at the central nervous system was reinforced by different studies showing its release, in a calcium-dependent manner, from several in vivo and in vitro preparations (Pull and Mcllwain, 1972; Sulakhe and Phillis, 1975; Kuroda and Mcllwain, 1974; Schubert *et al.,* 1976; Daval *et al.,* 1980; Jhamandas and Dumbrille, 1980).

Although adenosine is not a classical neurotransmitter, there is some evidence that it may act as a retrograde messenger: when released by a postsynaptic neuron (that was loaded with adenosine by a patch pipette), adenosine was able to inhibit by 80% the excitatory postsynaptic potentials in that cell, suggesting that neurons, through the release of adenosine, can regulate their own excitatory afferents (Brundege and Dunwiddie, 1996).

Thus, without being a neurotransmitter, adenosine influences synaptic transmission, at the pre-synaptic level by inhibiting or promoting neurotransmitter release; pos-synaptically by hyperpolarizing or depolarizing neurons; and also non-synaptically by modulating glial cell activity.

In fact, one of the most prominent pre-synaptic effects of adenosine, through A_1 receptors activation, is the ability to inhibit the release of virtually every classical neurotransmitter. Still, adenosine most prominent effects occur upon excitatory glutamatergic synapses (e.g. Dunwiddie and Hoffer 1980; Kocsis *et al.,* 1984), where it can

often completely abolish synaptic transmission. Indeed, a major role of adenosine is to reduce excitability in the brain.

Adenosine can also promote neurotransmitter release (by A_{2A} receptors activation), although this type of modulation is much less frequent (Cunha *et al.,* 1994, Cunha and Ribeiro, 2000).

Adenosine is also regarded as an important neuroprotector, being these effects mainly mediated by the activation of A_1 receptors. Furthermore, adenosine efficiently connects synaptic activity, energy expenditure and nucleic acid metabolism, by acting as a sensor of the bioenergetic state of the cell (Newby *et al.,* 1985). Moreover, adenosine directly regulates transmethylation reactions, including DNA methylation (Boison *et al.,* 2002), which can lead to long-lasting epigenetic modifications.

Other than at the cellular level, the influence of adenosine upon cerebral blood flow enables it to further act as an energy balancing metabolite: when metabolism is increased, elevated ATP catabolism will produce higher amounts of adenosine, which (through the activation of A_{2A} receptors, Phillis, 1989) will induce vasodilation, allowing an improvement of oxygen and nutrient delivery via the cerebral vasculature.

1.3.1 Adenosine synthesis

In the central nervous system, the main source of adenosine is the dephosphorylation of 5'-AMP by 5'-nucleotidases (5'-NTs), (Meghji, 1993). These enzymes dephosphorylate non-cyclic nucleoside monophosphate to nucleosides and inorganic phosphate. So far,

seven distinct nucleoside monophosphate phosphohydrolases or 5'-nucleotidases (EC 3.1.3.5 and EC 3.1.3.6) have been cloned. Nucleotidases are responsible for both intracellular and extracellular synthesis of adenosine from the dephosphorylation of AMP. An alternative source of adenosine concerns the hydrolysis of S-adenylhomocysteine (SAH), which is catalyzed by SAH hydrolase (SAHH) (Palmer and Abeles, 1979).

1.3.1.1 *Extracellular synthesis of adenosine*

Adenosine found at the extracellular space can be released via equilibrative nucleotide transporters or be synthesized locally via ATP catabolism, which involves several enzymes, namely: ecto-nucleotidases, ecto-phosphodiesterases, and apyrases. These enzymes dephosphorylate all the adenine nucleotide into 5'-AMP, which is then dephosphorylated by ecto-5'-nucleotidase into adenosine. The entire catalytic pathway is complete in a few hundred milliseconds, and the rate-limitative step is the dephosphorylation of AMP into adenosine by ecto-5'-nucleotidase (Dunwiddie *et al.,* 1997).

1.3.1.2 *Ecto-nucleotidase*

After the vesicular release of ATP (which is co-secreted with neurotransmitters), ATP is metabolized by ecto-ATPase, ecto-ADPase and finally ecto-5'-nucleotidase, originating adenosine (Richardson *et al,.* 1987; Zimmermann *et al.,* 1986). Additionally, cAMP can also be released into the extracellular space, by probenecid-sensitive transporter (Rosenberg and Li, 1995),

contributing to increase extracellular adenosine concentrations (Dunwiddie *et al.,* 1992; Brundege *et al.,* 1997).

Ecto-nucleotidases are able to convert most nucleotides (except cAMP) into adenosine in less than a second (Dunwiddie *et al.,* 1997).

Ecto-nucleotidases are highly expressed in the brain, where they are mainly associated with glial cell membranes (Naidoo, 1962; Kreutzberg *et al.,* 1978; Schoen and Kreutzberg, 1995). Functionally, there is some evidence that ectonucleotidases are in close physical proximity with pre-synaptic inhibitory A_1 receptors (see Dunwiddie and Masino, 2001).

1.3.1.3 *Intracellular synthesis of adenosine*

For the net extracellular adenosine levels, the intracellular synthesis of adenosine is at least as significant as adenosine formation from breakdown of extracellular ATP (Lloyd *et al.,* 1993; Lloyd and Fredholm, 1995). Intracellular synthesis of adenosine occurs mainly by AMP dephosphorylation, which is catalyzed by cytoplasmic nucleotidases. The presence of cytosolic 5'-nucleotidase in the brain was firstly demonstrated in 1982 (Montero and Fes, 1982).

Another source of adenosine is the transmethylation pathway, where adenosine results from the hydrolysis of S-adenosylhomocysteine (SAH) catalyzed by SAH hydrolase (SAHH, E.C.3.3.1.1), which also originates L- homocysteine (Palmer and Abeles, 1979; Schrader *et al.,* 1981). This enzyme was firstly described in 1959, in rat liver. SAHH catalyses a reversible reaction, that preferentially evolves towards S-adenosylhomocysteine

synthesis (de la Haba and Cantoni, 1959). SAHH is expressed by most brain areas, being the higher expression levels present at cortex and cerebellum. Inside the cell, SAHH displays a nuclear expression where it is involved in transmethylation mechanisms. In detail, different methyl-transferases convert S-adenosylmethionine (SAM) into S-adenosylhomocysteine (SAH), which is then metabolized into adenosine and homocysteine by S-adenosylhomocysteine hydrolase (SAHH). So, adenosine is an obligatory end product of SAM-dependent transmethylation reactions and because of that is able to inhibit methylation reactions. To avoid this inhibition, adenosine is phosphorylated into AMP by a long isoform of adenosine kinase (ADK), which was described as a nuclear ADK (Cui *et al*., 2009). Under normal conditions SAHH has low impact upon neuronal excitability (Pak *et al.,* 1994), suggestive of a minor role in the control of cytoplasmic levels of adenosine in neurons.

1.3.2 Nucleoside transporters

Intracellular adenosine is not stored in vesicles nor released by exocytosis as a classical neurotransmitter. Instead, adenosine is released to the extracellular space through nucleoside transporters. Besides releasing adenosine, adenosine transporters also take it into the cell. The relevance of adenosine uptake by nucleoside transporters in terminating adenosine effects was first supported by different studies showing that nucleoside transporter blockade produce vasodilation, potentiate the ability of adenosine or decrease locomotor activity (Crawly *et al.,* 1983), depresses neuronal activity (Motley and Collins, 1983), increases nociceptive

thresholds (Yarbrough and McGuffin-Clineschmidt, 1981), and exerts anti-convulsive effects (Dragunow and Goddard, 1984). Therefore, nucleoside transporter inhibitors exacerbate the effects mediated by adenosine.

Nucleoside transporters can be divided in two main classes: the equilibrative (Na^+-independent) nucleoside transporters (ENTs) and the concentrative (Na^+-dependent) nucleoside transporters (CNTs) (Baldwin *et al.,* 1999). Six isoforms of CNTs (CNT1 - CNT6) and four isoforms of ENTs (ENT1 - ENT4) have been cloned, to date. Equilibrative transporters mediate nucleoside transport in both directions, depending on the nucleoside concentration gradient across the membrane. The four transporters are widely distributed and all of them are able to transport adenosine but they have different abilities to transport other nucleosides (Balwin *et al.,* 2004). The transport mediated by concentrative transporters is independent of nucleoside gradient, being coupled to sodium. Adenosine uptake in the brain occurs primarily by facilitated diffusion via equilibrative transporters, although some of it (10-20%) can be mediated by concentrative transporters (Geiger and Fyda, 1991; Parkinson *et al.,* 1994).

Nucleoside transporters are essential to facilitate the diffusion of adenosine produced in the cytoplasm to the extracellular space. They are also crucial to regulate the levels of extracellular adenosine as transporters are the main molecules responsible for removing adenosine from the extracellular space (Geiger and Fyda, 1991). They are thus responsible for restraining, both spatial and

temporally, purinergic modulation. As adenosine can be transported into and out the cells by equilibrative transporters, regulation of intracellular adenosine levels is critical to the regulation of extracellular adenosine concentration. Then, if the intracellular adenosine rises, ENTs will release adenosine for the extracellular space and when extracellular adenosine increases, ENTs will uptake the nucleoside into the cells. So, changes in the activity of enzymes involved in adenosine metabolism will modify the transporters activity. Transporter inhibitors can either increase (Sanderson and Scholfield, 1986; Phillis *et al.,* 1989; Dunwiddie and Diao, 1994) or decrease (Gu *et al.,* 1995) extracellular adenosine levels, depending on the transmembrane adenosine gradient and consequently depending on transport direction, into or out the cell. However, since the extracellular synthesis of adenosine from catabolism of nucleotides constitutes an alternative source of adenosine, the direction of transport is usually in the inward direction. Therefore, transporter inhibition usually leads to an increase in the extracellular levels of adenosine. Recent data suggests that, even under ischemic conditions, neuronal ENTs work in the inward direction, since adenosine uptake into neurons outweighs adenosine release (Zhang *et al.,* 2011). Thus, this result suggests that nucleoside transport activity restrains the neuroprotective effect of adenosine under ischemic conditions.

The source of adenosine may influence the activation of the different adenosine receptors subtypes. Thus, adenosine released by nucleoside transporters will activate preferentially A_1 receptors,

while adenosine derived from the extracellular catabolism of nucleotides will mainly act on A_{2A} receptors (Cunha *et al.,* 1996).

1.3.3 Adenosine degradation

Unlike its nucleotides, adenosine is usually not degraded outside the cell. So, adenosine must be first taken up through nucleoside transporters and then be inactivated either by deamination through adenosine deaminase (ADA) or by phosphorylation through adenosine kinase (ADK).

It is accepted that the catabolic reaction responsible for adenosine degradation is dependent on its intracellular concentration. As such, at low concentrations, adenosine is mainly inactivated by phosphorylation while at higher concentrations, ADK activity is saturated and adenosine is predominantly deaminated by adenosine deaminase (Meghji and Newby, 1990).

1.3.3.1 *Adenosine Kinase*

Adenosine Kinase (ADK, EC 2.7.1.20) is the major adenosine-metabolizing enzyme, being expressed mainly in astrocytes. ADK phosphorylates adenosine into AMP. In fact, its low K_M for adenosine suggests that ADK is the main enzyme responsible for adenosine catabolism, at least, for low adenosine concentration conditions. This premise is supported by several pieces of evidence: the fact that inhibition of ADK suppresses seizure activity in various models for epilepsy (Kowaluk and Jarvis, 2000); the release of higher amounts of adenosine by ADK-deficient fibroblasts in cultures, when compared to that released by ADA-deficient fibroblasts (Huber *et al.,*

2001); the ability of ADK inhibition to increase endogenous adenosine and depress neuronal activity in hippocampal slices, which is not affected by ADA inhibition (Pak *et al.,* 1994). In addition, the relevance of ADK for adenosine metabolism is also suggested by the existence of a substrate cycle between adenosine and AMP, which involves 5'-nucleotidase and ADK, functioning as an amplification system enabling small changes in ADK activity to result in significant changes in adenosine concentration. ADK and adenosine can be considered as key regulators of metabolism and cellular energetic balance. Both adenosine and ADK are involved in several physiological processes in most, if not all, invertebrate and lower species.

The regulation of ambient adenosine levels by ADK seems to have a key role in the susceptibility of brain tissue to ischemic injury. The increase in adenosine levels that is induced by ischemia has been previously attributed to a downregulation of ADK expression (Lynch *et al.,* 1998). Furthermore, transgenic mice overexpressing ADK, with decreased levels of adenosine, show a higher than expected cell death after a short period of ischemia (Pignataro *et al.,* 2007). Indeed, pharmacological inhibition of ADK in animal models is an effective strategy to suppress epileptic seizures as well as to protect strategy from stroke induced injuries (Kowaluk *et al.,* 1998). Moreover, ADK inhibitor has a better side-effect profile than the use of A_1 receptors agonists (Kowaluk *et al.,* 1998; Kowaluk and Jarvis, 2000; Jarvis *et al.,* 2002; Gouder *et al.,* 2004). As a result, a novel

generation of ADK inhibitors has been developed, but its therapeutic use in clinical practice has not been established yet.

1.3.3.2 *Adenosine Deaminase*

Adenosine deaminase (ADA, E.C.3.5.4.4) catalyzes the hydrolytic deamination of adenosine to inosine. The importance of ADA for inactivation of adenosine was demonstrated several years ago. In 1980, Dunwiddie and Hoffer described that ADA increases the size of evoked field responses in rat hippocampal slices (Dunwiddie and Hoffer, 1980). On the other hand, inhibition of ADA (by deoxycoformycin) causes adenosine-like sedative and hypnotic effects (Major *et al.,* 1981; Radulovacki *et al.,* 1983). Also, it was demonstrated that ADA inhibition prevents histological changes in the hippocampus by decreasing the infarct area and neuronal degeneration in animal models of global forebrain ischemia or focal ischemia (Lin and Phillis, 1992; Phillis and O'Regan, 1989).

Although the enzyme localization is mainly cytosolic, there are several evidences supporting the existence of an ecto-enzyme, bound to the extracellular side of membrane (Franco *et al.,* 1998). This ecto-ADA activity has been shown to have extra-enzymatic and co-stimulatory functional roles. It was, initially, described that ADA modulates ligand binding and signaling through A_1 receptors in DDT1MF-2 cells (a smooth muscle cell line). In fact, it was shown that binding of ADA to adenosine A_1 receptors increases their affinity for the ligand, suggesting that ADA was required for an effective coupling of G protein to A_1 receptor. Ecto-ADA seems therefore to

play a crucial role in adenosine signaling by facilitating signal transduction via A_1 receptors, an effect that was preserved even when ADA was enzymatically inactive (Ciruela *et al.,* 1996).

ADA and A_1 receptors have a high degree of colocalization both at cellular membrane level and at intracellular vesicles, which suggest that ADA and A_1 receptors can mutually regulate cell surface expression and also share a common endocytic pathway. Thus, in addition to its role upon A_1 receptor signaling, ADA is also involved in A_1 receptor desensitization (Ciruela *et al.,* 1996; Saura *et al.,* 1998). Furthermore, the co-internalization of ADA and A_1 receptor in the consequence of agonist stimulation is probably the molecular mechanism responsible for the enhancement of plasma adenosine levels found after caffeine and sulfophenyltheophylline consumption (Conlay *et al.*, 1997).

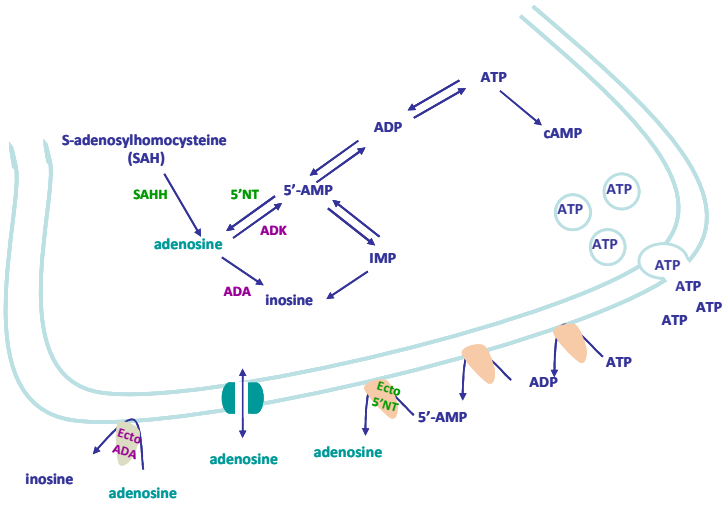

Figure 1.10 – Schematic representation of adenosine metabolism. Adenosine can be synthesized intra- and extra-cellulary. Inside the cell adenosine is formed from AMP metabolism through endo-5'-nucleotidase (5'NT) or by the transmethylation reaction catalyzed by s-adenosylhomocysteine hydrolase (SAHH), which converts s-adenosylhomocysteine (SAA) into adenosine and homocysteine. At the extracellular space, adenosine derives from the metabolism of ATP/ADP/AMP, being the last reaction catalyzed by the ecto-nucleotidase (Ecto 5'NT). The release of adenosine through the equilibrative nucleoside transporters is an alternative source of adenosine. Regarding clearance of extracellular adenosine, in some cases it can be converted into inosine by ecto-adenosine deaminase (ecto-ADA) in the extracellular space, but in most cases adenosine is taken up by the equilibrative nucleoside transporter into cells where adenosine can be phosphorylated to AMP by adenosine kinase (ADK) or deaminated to inosine by adenosine deaminase (ADA).

1.3.4 Adenosine levels regulation at brain

In the brain, adenosine concentrations are normally kept in the range of 25-250nM (Dunwiddie and Masino, 2001) by a steady-state expression of ADK, which is mainly expressed by astrocytes (Gouder *et al.,* 2004). Thus, physiological concentrations of adenosine are sufficient to tonically activate a substantial fraction of the high affinity A_1 and A_{2A} receptors (Dunwiddie and Masino, 2001).

The extracellular levels of adenosine are mainly regulated by an astrocyte-based adenosine cycle (Boison *et al.,* 2010). Astrocytes expressed both equilibrative nucleoside transporters – ENT1 and ENT2 (which were described above) - and the adenosine uptake into astrocytes is driven by intracellular phosphorylation of adenosine into 5´-adenosine monophosphate by adenosine kinase (Boison *et al.,* 2010).

In fact, under normal conditions, ADK phosphorylates intracellular adenosine and keeps the intracellular concentrations low, which sustains the concentration gradient that allows adenosine uptake into the cells by equilibrative nucleosides transporters. However, conditions prompting an increase in intracellular adenosine will reverse transport direction, leading to release of adenosine into the extracellular space (Gu *et al.,* 1995).

The levels of extracellular adenosine seems to be regulated by both adenosine A_1 and A_2 receptors since A_1 receptors blockade increases extracellular levels of adenosine in cardiac fibroblasts (Andresen *et al.,* 1999), and activation of A_{2A} receptors promotes adenosine transport in the hippocampus (Pinto-Duarte *et al.,* 2005) and at chromaffin cells (Delicado *et al.,* 1990).

1.3.5 Adenine nucleotides, adenosine and signaling

As mentioned before, at the extracellular space, adenine nucleotides are converted into adenosine. In the past, it was hypothesized that nucleotides, namely ATP, could directly activate adenosine receptors. Though the initial proposal of purinergic receptors

nomenclature made by Burnstock (1976) clearly postulated the existence of ATP and adenosine receptors separately, it is now plainly established that at the hippocampus, both ATP and ATP analogues have to be extracelullarly converted into adenosine to exert their inhibitory effects on synaptic transmission through the activation of adenosine A_1 receptors (Cunha *et al.,* 1998).

Although ATP is not able to activate adenosine receptors, it acts through the activation of two different classes of membrane receptors, the ionotropic P2X and the metabotropic P2Y (for review, Ralevic and Burnstock, 1998). Also cAMP evolved as relevant second messenger, which regulates several intracellular phenomena. Finally, adenosine regulates many physiological processes, especially in excitable tissues as heart or brain, through the activation of four distinct G protein coupled receptors.

In the brain, adenosine is involved in several physiological and pathological processes, namely, regulation of sleep, arousal, neuroprotection, epilepsy. The involvement of adenosine in a huge number of brain phenomena is related with a high expression of its receptors, although some adenosine effects are receptor-independent. Interestingly, although adenosine is involved in relevant physiological roles, including neuroprotection, the blockade of its receptors generally improves mental function and performance (Dunwiddie and Masino, 2001).

1.3.6 Adenosine receptors and signaling pathways

Adenosine acts on cell surface G-protein coupled receptors, which activate different intracellular pathways. G protein coupled receptors are formed by a single peptide channel, with seven alpha-helices transmembrane domains, a C- intracellular terminal and an N- terminal localized at the extracellular space. The N-terminal usually contains one or more glycosylation sites. The C- terminal contains phosphorylation and palmitoylation sites, which are involved in regulation of receptor desensitization and internalization (Perez and Karnik, 2005).

So far, four different adenosine receptors have been cloned: A_1, A_{2A}, A_{2B} and A_3. Adenosine receptors are widely distributed throughout the body, including the brain, being responsible for several distinct functions on the central nervous system as well as at the periphery.

The expression of adenosine receptors is not homogenous in different central nervous system areas. Adenosine A_1 receptors are particularly prevalent in the central nervous system, expressed in both neurons and glial cells. A_1 receptors can be expressed either pre- or post-synaptically. Higher expression levels were found in the cortex, hippocampus, cerebellum, thalamus, brain stem and spinal cord (see Ribeiro *et al.,* 2002). mRNA encoding A_1 receptor was also found in basal ganglia, where, A_1 receptors are present on both dopaminergic nigrostriatal and glutamatergic corticostriatal terminals (Wardas, 2002). Adenosine A_{2A} receptors are mostly expressed in the basal ganglia and olfactory bulb. However it is possible to find mRNA encoding A_{2A} receptor in other brain regions

where they are poorly expressed, namely in the hippocampus and the cortex (see Sebastião and Ribeiro, 1996). The A_{2B} receptors are mainly expressed in peripheral organs, being weakly expressed in the brain (Dixon *et al.,* 1996). Finally, A_3 receptors present an intermediate expression level in the human cerebellum and, as A_{2B} receptors, display a low expression in the entire brain (see Fredholm *et al.,* 2001).

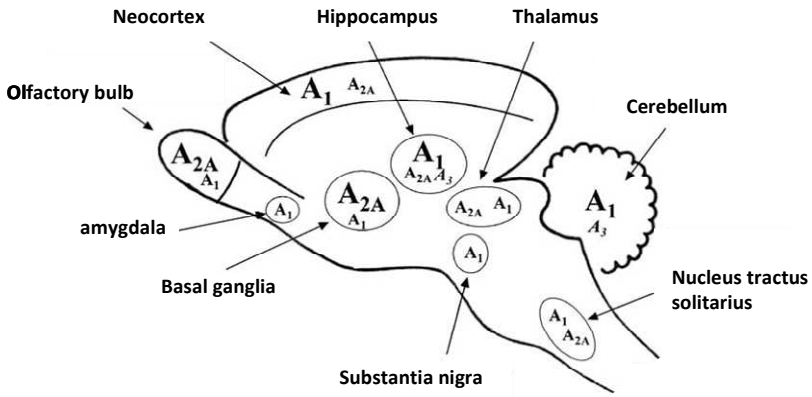

Figure 1.11 – Distribution of adenosine A_1 and A_{2A} receptors in the brain. Higher alphabets correspond to higher levels of expression (adapted from Ribeiro *et al.,* 2002).

A_1 and A_{2A} are high affinity receptors, with K_d values of 70 and 150nM, respectively, which allow its tonic activation by basal adenosine; A_{2B} and A_3 are considered low affinity receptors, with affinity constant values around 5100 and 6500 nm, respectively (see Dunwiddie and Masino, 2001). Interestingly, human A3 receptors show higher affinity for adenosine (see e.g. Fredholm *et al.,* 2001).

Classically, A_1 and A_3 receptors inhibit adenylate cyclase (AC) through the coupling to G_i or G_0. A_{2A} and A_{2B} receptors are coupled to G_s or G_{olf}, promoting adenylate cyclase activity. A_{2B} receptors are

also coupled to $G_{q/11}$, through which they can activate phospholipase C (PLC) (Ryzhov *et al.,* 2006). The A_3 receptor can also couple to $G_{q/11}$, also activating phospholipase C (Fredholm *et al.,* 2001). For the receptors coupled to AC, the increase of cAMP mediated by adenylate cyclase leads to the activation of cAMP dependent protein kinase (PKA), which in turn phosphorylates different targets such as ionotropic receptors or transmitter transporters. On the other hand, the activation of phospholipase C converts phosphatidylinositol 4, 5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP_3). Then DAG activates protein kinase C (PKC), which phosphorylates different substrates, while IP_3 stimulates calcium release from intracellular stores. Then, elevation of cytosolic Ca^{2+} can stimulate a variety of signaling pathways, including a family of phosphatidyl serine-dependent serine/threonine-directed kinases collectively called protein kinase C (PKC), phospholipase A_2 (PLA_2), Ca^{2+}-dependent K^+ channels, and nitric oxide synthase (NOS). IP_3 can also promote calcium influx from extracellular sources, if previous IP_3 stimulation depleted CA^{2+} intracellular store (see Ralevic and Burnstock, 1998).

The activation of G proteins can modify the activity of several enzymes and ion channels in a way that is independent of second messenger production. For example, A_1 receptors, via G_i activation, lead to activation of several types of K^+-channels and to blockade of N-, P- and Q-type Ca^{2+} channels (see Fredholm *et al.,* 2001). A_3 receptors are also able to modulate Ca^{2+} levels, through the

inhibition of adenylate cyclase. Both A_{2A} and A_{2B} receptors can modify the levels of intracellular calcium (see Fredholm *et al.,* 2001).

Aside from the involvement of AC/cAMP/PKA and PLC/IP_3-DAG/PKC, other transduction pathways are associated with adenosine receptor activation, namely the mitogen-activated protein kinase (MAPKs) (Schulte and Fredholm, 2003). The MAPK family is constituted by three main groups: extracellular regulated kinases (ERK) such as ERK1 and ERK2, stress-activated protein kinases (SAPK) such as p38 and jun-N-terminal kinase (JNK). These kinases are usually activated by tyrosine-kinases receptors (Seger and Krebs, 1995), but G protein coupled receptors can also signal through them (Gutkind, 1998; Liebmann, 2001; Marinissen and Gutkind, 2001). In fact, all adenosine receptors can be associated with MAPK pathway. First evidence for this came from a study with COS-7 cells transiently transfected with adenosine A_1 receptors, which could activate ERK1/2 via $G_{i/0}$ (Faure *et al.,* 1994).

Activation of A_{2A} receptors also increases MAPK activity (Sexl *et al*., 1997). Interestingly, the signal pathway used by A_{2A} receptors to activate MAPK can vary, depending on the cellular machinery availability. Thus, in CHO cells, A_{2A} receptors-mediated ERK1/ERK2 is mediated by Gs-AC-cAMP-PKA-MEK1, while in HEK 293 cells, MAPK activation by A_{2A} receptors involves PKC but not PKA, even though cAMP levels are found to be enhanced by G_s activity (Seidel *et al.,* 1999). Adding to the activation of MAPK, A_{2A} receptors can also inhibit ERK phosphorylation (Hirano *et al.,* 1996).

The activation of A_{2B} receptors can activate the three main branches of MAPK family (ERK1/2, p38 and JNK) (see Fredholm *et al*. 2001).

Finally, the A_3 receptors activate ERK1/2 in human fetal astrocytes (Neary *et al.,* 1998). Also, the phosphorylation of ERK1/2 was clearly demonstrated in CHO cells, transfected with A_3 receptors (see Schutle and Fredholm, 2000).

To conclude, MAPK activation by adenosine receptors is quite similar to that prompted by other G protein coupled receptors (Gutkind, 1998; Sugden and Clerk, 1998; Luttrell *et al.,* 1999). Interestingly, ERK1/2 phosphorylation is promoted either by receptors coupled to G_s (A_{2A}/A_{2B}) or to $G_{i/0}$ (A_1/A_3) proteins. The MAPK-mediated effects of adenosine receptors are mainly related to DNA synthesis, cellular differentiation, proliferation and apoptosis (see Schulte and Fredholm, 2003).

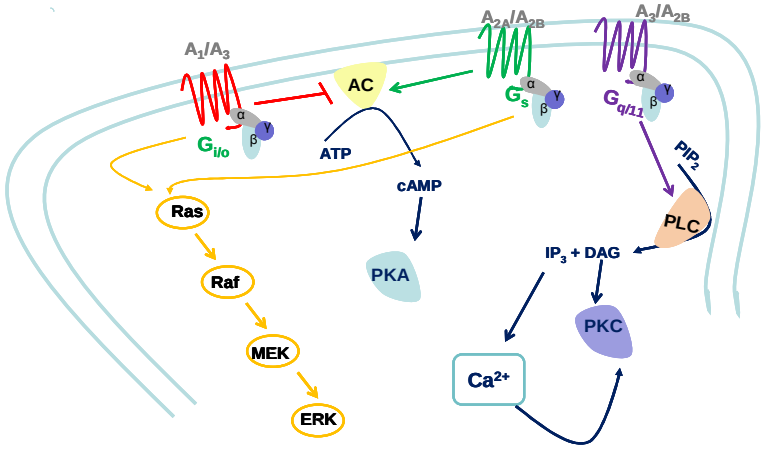

Figure 1.12 – Associated pathways to adenosine receptors. Adenosine A_1 and A_3 receptors are usually coupled to $G_{i/0}$ proteins, decreasing the activity of adenylate cyclase (AC). Adenosine A_{2A} and A_{2B} receptors are usually coupled to G_s, activating AC. Additionally, A_3 and A_{2B} receptors may couple to $G_{q/11}$ proteins, activating PLC pathway. Further, all adenosine receptors can activate the MAPK pathway.

Adenosine receptors activity is regulated by its expression at the membrane level which results from a balance between endocytosis and exocytosis rates. In turn, the endocytosis rate is related with the desensitization mechanism. It reduces receptor expression at membranes, consequently decreasing receptor activity and restraining the duration and intensity of signal. Desensitization can be classified as homologous or heterologous, with the first being dependent and specific for agonist binding and the second induced by receptor phosphorylation by PKA or PKC, in the presence or absence of ligand. Adenosine receptor subtypes desensitize differently: A_1 receptors are slowly phosphorylated and internalized (several hours are need to complete the process). A_{2A} and A_{2B} receptors desensitize faster, with down-regulation kinetics faster

than 1h. The A_3 receptors have the fastest desensitization profile, which often occurs within minutes (Klaasse *et al.,* 2008).

1.3.7 A_1-A_{2A} adenosine receptors interaction

Since the 90s that a close cross-talk between adenosine A_1 and A_{2A} receptors has been identified either at the central nervous system (Cunha *et al.,* 1994; Lopes *et al.*, 1999) or at the neuromuscular junction (Correia-de-Sá and Ribeiro, 1994). In fact, at the hippocampus, A_{2A} receptors are co-expressed and colocalized with A_1 receptors (Cunha *et al.*, 1994). Since then, the results strongly indicated that both receptors interact with each other. For example, the activation of A_{2A} receptors attenuates the ability of A_1 receptors to inhibit population spike at the hippocampus (Cunha *et al.,* 1994, O'Kane and Stone, 1998) as well as at the neuromuscular junction (Correia-de-Sá *et al.,* 1994). However these studies did not clarify the type of interaction between A_1 and A_{2A} receptors. Thus, in 1999, it was shown that the activation of A_{2A} receptors decrease presynaptic A_1 receptors binding as well as A_1 receptors functional responses in young adult rat hippocampus (Lopes *et al*., 1999).

More recently and taking advantage of BRET assays, Ciruela and colleagues have shown that adenosine A_1 and A_{2A} receptors can heteromerize in transfected HEK cells. Moreover, the A_{2A} receptors activation reduces the affinity of A_1 receptors agonists (Ciruela *et al.,* 2006). It has been suggested that under basal conditions, the relatively low extracellular levels of adenosine preferentially stimulate A_1 receptors, which has a higher affinity to adenosine

(Fredholm *et al.*, 2001). Then, when adenosine concentration is increased, it will mainly activate A_{2A} receptors in the heteromer. This activation will inhibit the A_1 receptor mediated effects (Ciruela *et al.*, 2006). According to Ciruela *et al*., (2006), the existence of adenosine A_1-A_{2A} receptors heteromers allows that adenosine can facilitate or inhibit glutamatergic transmission depending on concentration of adenosine.

1.4 Heteromers of G protein coupled receptors

For a long time, GPCR were considered to exist as monomers within the plasma membrane (Lefkowitz, 2004; Hill, 2006). However, in the last decade, studies have highlighted the existence of GPCR heteromers (Ciruela *et al*., 2001; Ferré *et al*., 2002; Ginés *et al.,* 2000; Hillion *et al*., 2002; Canals *et al.,* 2003; Milligan, 2004).

The $GABA_{B1}$-$GABA_{B2}$ heteromer is frequently considered as the first identified GPCR heteromer (White *et al.,* 1998). But, considering that the $GABA_{B1}$ and $GABA_{B2}$ molecules are not active when they are alone, this complex should be considered as a heteromeric receptor instead of a receptor heteromer. Thus, the δOR-κOR opioid receptor heteromer should be considered as the first reported neurotransmitter receptor heteromer (Jordan and Devi, 1999). Since then, an increasing list of GPCR heteromers have been identified (George *et al.*, 2002; Agnati *et al.,* 2003; Prinster *et al.,* 2005). This list includes both heteromers between GPCR activated by the same ligand (e.g. D_1-D_2, A_1-A_{2A}) and activated by different ligands (e.g. A_1-D_1, A_{2A}-D_2). The heteromerization can occur also between distant

related receptors, such as GPCR and ionotropic receptors heteromers (e.g. D_1-NMDA, D_5-$GABA_A$) (Ferré *et al.,* 2007).

A receptor heteromer can be defined as a complex molecule made up of different receptor molecules for the same or different ligand while a receptor homomer is composed by two identical receptor molecules. The terms receptor heteromer or homomer imply a direct physical interaction between the receptor molecules and it does not contemplate receptors indirectly linked by intermediate proteins (Ferré *et al.,* 2007). It is also important to clarify the difference between a receptor heteromer and a heteromeric receptor, being the last an oligomeric receptor for which the minimal functional unit is composed of different protein subunits, such as $GABA_A$, NMDA or AMPA receptors, for example. On the other hand, the protomer molecules of a heteromer are active by themselves.

The occurrence of neurotransmitter receptor heteromers was firstly suggested by radioligand binding assays, which demonstrated the existence of biochemical interaction between different GPCR (Gomes *et al*., 2000). They were initially named as "intramembrane receptor-receptor interactions" (see Agnati *et al.,* 2003). These interactions are characterized by the fact that the stimulation of one receptor changes the binding characteristics of another receptor of endogenous or exogenous ligands in crude membrane preparations (Agnati *et al.,* 2003). The use of crude membranes instead of intact cells allows the exclusion of intracellular signaling participation and advocates the existence of a kind of allosteric interaction between

adjacent receptors. In fact, it was already hypothesized in 1993 that these intramembrane interactions could imply an intermolecular crosstalk, which implies the occurrence of GPCR heteromerization (Zoli *et al.,* 1993).

Thus, GPCR heteromerization is quite related with allosterism as each protomer can exhibit several biochemical properties, which are modifiable by the presence of the other protomer ligand. In fact, the term allosterism is defined as a type of regulation mediated by an effector at one specific site (usually named as allosteric site) on a protein that has the ability to influence the ligand binding or protein active at a topographically different site. Interestingly, GPCR can display different properties when they are heteromerized. In fact, ligand binding alterations are a common property of neurotransmitter receptor heteromers, and are a useful tool to identify the presence of endogenous heteromers. For example, when dopamine D_1 and D_2 receptors are heteromerized, the specific ligands for D_1 receptor lose the selectivity and can also activate the D_2 receptor in the heteromer (Rashid *et al.,* 2007). Also, δ and κ opioid receptors display different affinities for several exogenous and endogenous ligands when they are heteromerized (George *et al.,* 2000). Changes on binding properties are probably related to some conformational alterations sensed by one protomer when a ligand binds to the other protomer (Ferré *et al.,* 2007). The activation of one protomer can both increase and decrease the affinity of the other protomer for its specific ligands. On the other hand, this interaction can be unidirectional, meaning that only one

protomer can change the other receptor affinity, or bidirectional, when both protomers have the ability to change the other receptor affinity. For example, considering the bidirectional effect, in the dopamine D_2- somatostatin SST_5 receptors heteromer, stimulation of D_2 receptors significantly enhances the affinity of SST_5 receptors for agonists and vice-versa (Rocheville *et al.,* 2000). In the view of unidirectional modifications, and looking for adenosine A_1-A_{2A} receptors and A_{2A}-D_2 receptors heteromers, in both cases, only the A_{2A} receptors activation has the ability to decrease ligand affinity for A_1 receptor or D_2 receptor (Ferré *et al.,* 1991; Hillion *et al.,* 2002; Canals *et al.,* 2003; Ciruela *et al.,* 2006).

Several biochemical mechanisms have been proposed to allow the heteromerization of GPCR, namely the covalent disulfide bonds of extracellular, coiled-coil interactions and epitope-epitope electrostatic interactions of intracellular domains and interactions of transmembrane domains (by contact or domain swapping) (Bouvier, 2001; Marshall, 2001; George *et al.,* 2002; Woods and Ferré, 2005). On the other hand, the lipid bilayer, namely some particular domains as the lipid rafts and the scaffold proteins involved in the stability of membrane network, should also be important for receptor heteromerization (Agnati *et al.,* 2003; Bockaert *et al.,* 2004; Woods and Ferré, 2005; Allen *et al.,* 2007).

It is recognized that GPCR heteromers can traffic, signal and internalize as a unique entity, existing as dimers through their life cycle (Gurevich and Gurevich, 2008; Milligan, 2008) but some studies have shown that, at least, some heteromers have an intrinsic

ability to self-associate at the membrane level (Rocheville *et al.,* 2000; Harding *et al.,* 2009). In fact, the D_2-SST_5receptors heteromer is formed at the plasma membrane level, after the stimulation of either protomer (Rocheville *et al.,* 2000). It was also shown by single molecule total internal reflectance that the average lifetime of association of M_1 muscarinic receptor homomer was around 0.5s and that no more than 30% of M1 receptors existed as M_1-M_1 homomers at a given time (Hern *et al.,* 2010). Thus, the stability of dimers at membrane level should be influenced by different factors, namely the presence of ligands and the level of receptor expression at membrane (Smith and Milligan, 2010).

Moreover, the stability could even change between closely related GPCR, for example, it was demonstrated that β_2-adrenoreceptor can form stable oligomers, whereas the β_1-adrenoreceptor shows only transient physical interactions (Dorsch *et al.,* 2009).

An important issue about GPCR heteromerization concerns its advantage for the cells. If GPCRs are capable of signaling by themselves, why do they form heteromers? The first answer can be related with an increase in signaling possibilities, allowing a fine tuning of responses. But, there are probably other reasons: (1) the heteromerization can modify cell surface delivery and retention of certain GPCRs (Pin *et al.,* 2003; Lopez-Gimenez *et al.,* 2007; Canals *et al.,* 2009); (2) can promote cross-activation (Carrillo *et al.,* 2003) or cross-inhibition (Lavoie *et al.,* 2002; Mercier *et al.,* 2002; Barki-Harrington *et al.,* 2003; Lavoie and Hébert, 2003; Breit *et al.,* 2004;) of signaling; (3) can modify desensitization profiles (Pfeiffer *et al.,*

2001); (4) can increase (Jordan *et al.,* 2001; McVey *et al.,* 2001; Pfeiffer *et al.,* 2002, 2003; Ramsay *et al.,* 2002; Perron *et al.,* 2003; Stanasila *et al.,* 2003; Xu *et al.,* 2003) or reduce (Breit *et al.,* 2004; Lavoie *et al.,* 2002; Lavoie and Hébert, 2003; Mercier *et al.,* 2002) internalization rate. Moreover, heteromerization can also modify the lateral diffusion of receptors in the plasma membrane. It was shown that dopamine D_1 receptors, which usually diffuse laterally in the plasma membrane, can be trapped in dendritic spines, after the activation of NMDA, promoting D_1-NMDA receptors heteromerization (Scott *et al.,* 2006).

Another feature of heteromerization is the modification of G protein-coupling (Banères and Parello, 2003; Jastrzebska *et al.,* 2006; Carriba *et al.,* 2007; Rashid *et al.,* 2007). For example, dopamine D_1-D_2 receptors heteromer is coupled to $G_{q/11}$ protein. When the receptors are alone, dopamine D_1 receptor is coupled to G_s while D_2 receptor is coupled to $G_{i/0}$ protein. Thus, the dopamine D_1-D_2 receptors heteromerization allows dopamine to signal through a different pathway, involving PLC activation (Rashid *et al.,* 2007).

Furthermore, the existence of heteromers as A_{2A}-D_2 provides a tight crosstalk between two different neurotransmitter systems. More specifically, the activation of A_{2A} receptors inhibits the effect mediated by D_2 receptors activation (Fuxe *et al.,* 2005). On the other hand, the heteromerization between receptors for the same ligand, such as A_1-A_{2A}, enables the heteromer to work as a function of the ligand concentration.

Taking advantage of some of their unique properties, GPCR heteromers arise as new possible therapeutic targets, and the development of drugs specifically designed to target them can, in the future, offer some clinical advantage. For example, A_{2A}-D_2 receptors heteromer may play a crucial role in Parkinson Disease treatment since results from Phase II clinical trials demonstrated that the administration of istradefylline (a novel adenosine A_{2A} receptor antagonist) attenuates diskinesias in Parkinson Disease patients treated with L-DOPA (Jenner, 2005).

As above mentioned, adenosine is a ubiquitous neuromodulator, whose tonic effect is to depress nervous system activity. Adenosine influences synaptic transmission through different mechanisms: by modulating neurotransmitter levels at synapse; by hyperpolarizing or depolarizing neurons; by influencing directly neurotransmitter receptors, by modulating astrocyte functions. Neurotransmitter availability at the synapse can be modulated by controlling the neurotransmitter release or by controlling the neurotransmitter uptake through specific transporters expressed by neurons and glia. Among the different neurotransmitters whose levels can be modulated by adenosine, GABA assumes particular relevance, since its main function is, as adenosine, to regulate the excitatory tonus in the nervous system. It is known that adenosine can modulate GABA release from the presynaptic terminal. So, it is particular interesting to understand if adenosine can also modulate the removal of GABA

from the synapse, namely its uptake into presynaptic terminals and surrounding astrocytes.

2 Aims

The main goal of this work was to identify the influence of adenosine receptors upon GABA transporters (GAT-1 and GAT-3) expressed both in pre-synaptic terminals and in surrounding astrocytes.

As a modulatory role for adenosine on GABA transport was identified in the course of the study, a second goal was the identification of the transduction system operated by the receptors to modulate GABA transporters.

As it was found a pharmacological interaction between adenosine A_1 and A_{2A} receptors, a third goal was the characterization of adenosine A_1 and A_{2A} receptors heteromerization.

3 Techniques

3.1 Isolated presynaptic terminals

Isolated nerve terminals were firstly isolated by Hebb and Whittaker in 1958 (Hebb and Whittaker, 1958) and by Whittaker in 1959 (Whittaker, 1959). The presynaptic terminals were isolated as a distinct fraction by differential and density gradient centrifugation and they present the most of the bound acetylcholine of the tissue (Gray and Whittaker, 1962). Later on, Whittaker and co-workers proposed the name synaptosomes for the isolated nerve terminals in order to emphasize their relatively homogeneity and their resemblance in physical properties to other subcellular organelles (Whittaker *et al.,* 1964). Synaptosomes are able to uptake and release neurotransmitters or related molecules. In 1968, Marchbanks showed that the uptake of choline by synaptosomes was qualitatively and quantitatively very similar to the transport observed in more organized tissue (Marchbanks, 1968). Synaptosomes have also able to synthesized proteins (Gordon and Deanin, 1968) and they contain at least one mitochondrion, which allow a complete glucose based metabolism (Marchbanks and Whittaker, 1967). Thus, synaptosomes have been considered a good model to perform different studies, namely the ones related to release and uptake of neurotransmitters, functioning as an *in vitro* model of the presynaptic component of a synapse.

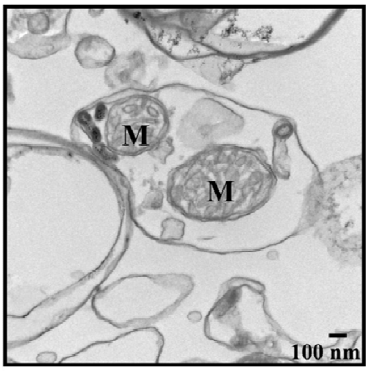

Figure 3.1 – Electronic microscopic visualization of a synaptosome. It is visible two mitochondria, marked with an M, which allow a complete glucose based mechanism; from Gredilla *et al.*, 2012.

3.2 Primary cultures of astrocytes

Studies using primary cultures of astrocytes had significantly contributed to reveal astrocyte main functions.

Although brain function results from the interaction between different types of cells, the use of isolated cultured cells allows the characterization of the function of each cell type in an independent way.

The first monotypic culture of astrocytes was described almost forty years ago (Booher and Sensenbrenner, 1972) and most laboratories used protocols with few modifications derived from this pioneer work or from the later modification by McCarthy and Vellis (1980). Primary cultures of astrocytes are easy to prepare and in theory can be prepared from organism in any age, including embryonic, fetal, neonatal, young, adult and old organisms. But concerning yield and purity, the optimal age is during astrogenesis peak, which is after the peak of neurogenesis and before the peak of oligodendrogenesis

(Saura, 2007). In rat or mice the astrogenesis peaks is around birth, being the ideal period from 2 days prenatal to 2 days postnatal for cortex and most CNS regions (Lim and Bosch, 1990), except cerebellum where time spans between post-natal days 4 and 7 (Hertz *et al.,* 1985).

Astrocytes in cultures can be different from those in vivo (see Hertz *et al.,* 1998), but still that, primary cultures are considered a good model to study astrocytes properties, since they allow to work in very defined and stable conditions for a prolonged period of time. Notwithstanding, the use of primary cultures should be complemented with subsequent studies in more integrative models, like slices or *in vivo* experiments, which can testify the obtained results. A problem with these more integrated approaches is to distinguish between functions of astrocytes or other cellular component, namely neurons, a problem easily overcomed with the cultures.

3.3 Biotinylation assays

A good way to study surface expression of proteins is taking advantage of the extraordinarily stable, non-covalent interaction between avidin and streptavidin with biotin. In fact, by surface biotinylation it is possible to isolate the proteins expressed at surface level from the intracellular fraction. The affinity of avidin for biotin is known for a long time (György *et al.,* 1941), and the same occurs in relation to the affinity of avidin for the bacterial protein streptavidin (Wilchek and Bayer, 1990). Since then several protocols

in biochemical fields has been used for different studies aiming to isolate surface membrane proteins.

All the chemical reagents available for biotinylation of surface proteins are formed by three building blocks: (i) the biotin moiety, for the subsequent interaction of biotinylated proteins to avidin beads, (ii) a spacer arm of sufficient length to allow protein capture by the beads, (iii) a reactive moiety to establish the covalent bond between biotin and the amine groups of surface proteins. For steric hindrance reasons, interaction between avidin and biotinylated proteins could be dramatically improved by increasing the spacer arm. It influences also the solubility of the reagent and can also be cleavable by chemical or physical agents to facilitate protein release after capture. In what concerns the covalent bound, the most common reactive groups employed include reactive esters, like the N-hydroxysuccinimide (NHS) group, which undergoes a nucleophilic substitution reaction in the presence of primary amines (Elia, 2008). Then, by the use of avidin beads, it is possible to separate surface biotinylated proteins from the intracellular fraction.

3.4 BRET – bioluminescence resonance energy transfer

Bioluminescence resonance energy transfer (BRET) has been used to study a wide range of protein-protein interaction in bacterial (Xu *et al.,* 1999), plant (Subramanian *et al.,* 2004) and mammalian cells (Angers *et al.*, 2000; Kroeger *et al.,* 2001). The use of BRET to study a protein dimerization was first described in 1999 (Xu *et al,.* 1999). Since then, hundreds of protein-protein interactions have been

studied by BRET assays. Although a significant part of these studies has been done with G-protein coupled receptors (GPCR), BRET has also been used to study other types of protein-protein interaction, as those involving other membrane receptors (Scaffidi *et al.,* 2004; Brown *et al.,* 2005), cytosolic receptors (Garside *et al.,* 2004; Michelini *et al.,* 2004), integrins (de Virgilio *et al.,* 2004; Scaffidi *et al.,* 2004), enzymes (de Virgilio *et al.,* 2004; Yung *et al.,* 2004), endophilins (Trevaskis *et al.,* 2005) and nuclear factors (Germain-Desprez *et al.,* 2003). The study of protein interaction by BRET implies heterologous coexpression of fusion proteins, which links proteins of interest to a bioluminescent donor enzyme or to an acceptor fluorophore. The non-radioactive transfer of energy from a donor enzyme to an acceptor fluorophore occurs after substrate oxidation, and its efficiency is inversely proportional to the sixth power of the distance between donor and acceptor. In fact, the energy released from the donor only excites the acceptor fluorophore if the distance between them is smaller than 10nm, resulting in light emission at a longer wavelength that can be detected and expressed relative to the donor light emission. Thus, a positive BRET signal is suggestive of a close physical interaction between the proteins under study.

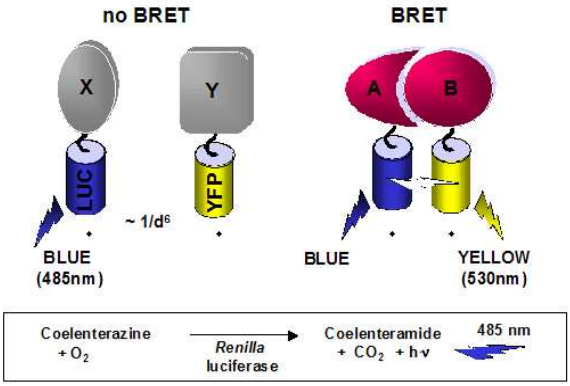

Figure 3.2 – BRET- bioluminence resonance energy transfer. BRET assays imply the expression of fusion protein, which results from the attachment of proteins of interest to a bioluminescence donor and to acceptor fluorophore. When the distance between the two fusion proteins are less than 10nm, the energy (that results from the added substrate oxidation by RLuc) will be transferred and accepted by the acceptor fluorophore. Image source: http://www.bio.utk.edu

BRET assays are also suitable to study ligand-modulated protein interactions. A modification of BRET signal may represent an increase or decrease in the number or rate of interactions between the proteins of interest, although it can also mean conformational changes that influence the relative positioning of the donor and acceptor molecules (Pfleger *et al.,* 2006).

An advantage of BRET technique is the possibility of studying proteins interaction in live cells, where they are expressed in a near-physiological environment in the correct cellular compartment. But, on the other hand, it is not possible to study endogenous proteins because of the need of fusion proteins (Pfleger *et al.,* 2006).

3.5 GTP-γ-[^{35}S]-assay

The evaluation of each G protein activity can be achieved through the quantification of specific activity of labeled GTP. The [^{35}S]GTP-γ-assay was first performed with purified receptor and G protein systems in phospholipid vesicles using adrenergic receptors (Asano *et al.,* 1984) and muscarinic receptors (Kurose *et al.,* 1986). Nowadays, the assay is commonly performed using cell membranes that express the receptor of interest, as was firstly described by Hilf and co-workers (Hilf *et al.,* 1989). More recently, it is also been used to identify the G protein family coupled to GPCR heteromers (Rashid *et al.,* 2007).

G proteins are heteromeric structures constituted by three different subunits: α, β and γ. At the resting state, GDP is bound to α subunit of the heteromeric G protein. The activation of a GPCR by the agonist leads to the dissociation of GDP from α subunit, allowing GTP binding to α subunit. This leads to the dissociation of Gα-GTP and Gβγ subunits that are able to interact with the downstream effectors.

The G protein heterotrimer is reformed by GTPase activity of the Gα subunit, forming Gα-GDP and so allowing Gα and Gβγ to recombine.

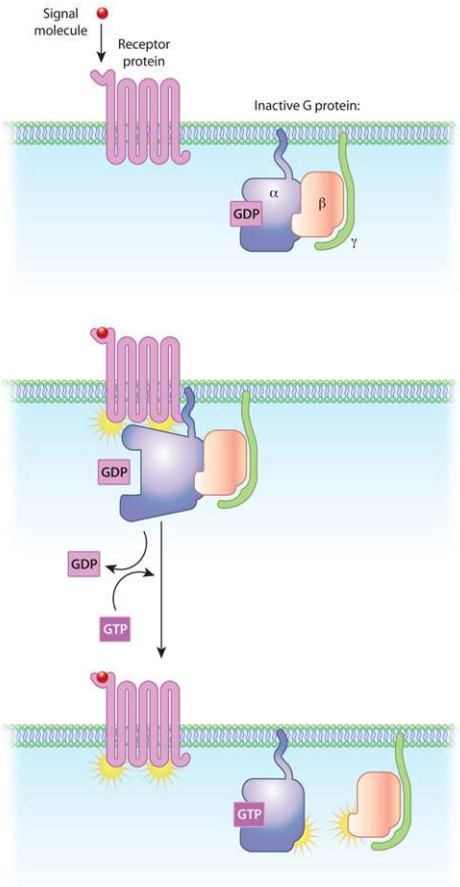

Figure 3.3 – G protein activation. At the resting state, GDP is bound to the α subunit of the heteromeric G protein. The binding of the agonist to the receptor leads to the dissociation of GDP from the α subunit, allowing the binding of GTP. After exchange of GDP with GTP on the α subunit, both the α subunit and the βγ complex may interact with other molecules to promote signaling cascades (from Nature Education www.nature.com/scitable).

The [^{35}S]GTP-γ-assay, using labeled GTP, can quantify G protein activity in response to the binding of ligands to GPCR. [^{35}S]GTP replaces the endogenous GTP and binds to the G_α subunit in response to receptor activation. As the thiophosphate bond established between G_α and [^{35}S]GTP is resistant to hydrolysis by the

GTPase, G proteins are not recycled and the [^{35}S]GTP-bound G_{α} subunits accumulate, which allows its quantification by measuring the amount of [^{35}S]GTP incorporated, which is easily performed through liquid scintillation spectroscopy. The GTP-γ-[^{35}S] assay has been used for studying a wide range of GPCR (see Harrison and Traynor, 2003). Although this assay is easier to perform for $G_{i/0}$ coupled receptors (this G protein family are more abundant and have higher rates of nucleotide exchange, and so a higher rate of labeled GTP incorporation), it is also been performed with receptors coupled to G_s and $G_{q/11}$ families. Further, by coupling [^{35}S]GTP-γ-assay to immunoprecipitation, it is possible to identify the family of G protein coupled to the receptor of interest.

The main feature of this assay is related with the possibility of measuring an earliest functional consequence of receptor occupancy. Thus, it is possible to evaluate traditional pharmacological parameters like efficacy or affinity. On the other hand, by measuring an earlier event, it avoids some amplification or modulation that may occur when analyzing parameters further downstream of the receptor (Harrison and Traynor, 2003).

4 Methods

4.1 Reagents

GABA (γ-aminobutyric acid) was obtained from Sigma and the **[^{3}H] GABA** (4-amino-n-[2, 3-^{3}H]butyric acid) specific activity 87.00 Ci/mmol and **[^{35}S] GTPγS** specific activity 1250Ci/mmol, were obtained from Perkin Elmer Life Sciences (Boston, MA, USA).

ADA (adenosine deaminase, E.C. 3.5.4.4, 200U/mg in 50% glycerol (v/v), 10mM potassium phosphate) was acquired from Roche (Germany).

CGS 21680 (4-[2-[[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-yl]amino]ethyl]benzenepropanoic acid hydrochloride), **SCH 58261** (2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine), **CPA** (N^{6}-cyclopentyladenosine), **DPCPX** (8-cyclopentyl-1,3-dipropylxanthine), **NECA** (5'-N-ethylcarboxamidoadenosine), **MRS 1706** (N-(4-acetylphenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purinyl)phenoxy]acetamide), **SKF 89976A** hydrochloride (1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride), (S)-**SNAP 5114** (1-[2-[*tris*(4-methoxyphenyl)methoxy]ethyl]-(*S*)-3-piperidinecarboxylic acid), **U73122** (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione), **GF109203X** (2-[1-(3-dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide) were obtained from Tocris (Bristol UK).

CADO (2-chloro-adenosine), **R-PIA** (R-phenylisopropyladenosine), **H-89** (N-[2-(p-Bromocinnamylamino)ethyl]- 5-isoquinolinesulfonamide dihydrochoride), **Rp-cAMPs** (Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate), **inosine**, **GDP** (guanosine 5'-diphosphate), **GTPγS** (guanosine 5'-O-[gamma-thio]triphosphate), **PDD** (4-β-phorbol 12, 13-didecanoate**), forskolin, ChTx** (Cholera Toxin from Vibrio cholerae), **PTx** (pertussis toxin from Bordetella pertussis) and Streptavidin were obtained from Sigma (MO, USA).

Sulfo-NHS-LC-biotin and **Avidin beads** were obtained from Pierce (Rockford, IL, USA).

Rat polyclonal **anti-GAT-1** (ab426), rabbit polyclonal **anti-beta actin** (ab8227), rabbit polyclonal **anti-alpha tubulin** (ab4074) were obtained from abcam (Cambridge, UK). Rabbit polyclonal **anti-A_1 adenosine receptor** (PA3-041) was obtained from Thermo Scientific (MA, USA). Rabbit polyclonal **anti-$G_{\alpha q/11}$** (sc-392), Goat polyclonal **anti-$G_{\alpha s}$** (sc-26766), rabbit polyclonal **anti-$G_{\alpha i-3}$** (sc-262) and **goat anti-Rabbit IgG-HRP** (sc-2004) were obtained from Santa Cruz (CA, USA). **Peroxidase anti-rabbit IgG**, **FITC-conjugated anti-rabbit IgG** (FI-1000) and **TRITC-conjugated anti-mouse IgG** (T-2762) were obtained from Vector (CA, USA). **Mouse anti-GFAP** was obtained from Sigma (MO, USA).

DMEM (Dulbecco's modified Eagle's medium), **Glutamine**, **FBS** (foetal bovine serum) were obtained from Invitrogen (NY, USA).

Antibiotic antimycotic was obtained from Sigma (St. Louis, MO)

All other reagents were purchased from Sigma (St. Louis, MO).

4.2 Experimental protocols

4.2.1 Synaptosomes isolation

Hippocampal synaptosomes were isolated as routinely performed in our laboratory (e.g. Vaz *et al.,* 2008) from halothane anaesthetized male Wistar rats with 3-4 weeks of age, except otherwise stated; in some experiments, and for comparison, adult (6-8 weeks) rats were used; animal handling was according to the European guidelines (86/609/EEC) and Portuguese Law. Briefly, the hippocampi were homogenized in 15ml ice-cold sucrose solution (sucrose 0.32M, EDTA 1mM, HEPES 10mM, BSA 1mg/ml, pH 7.4) and centrifuged at 3000g for 10min at 4ºC; the supernatant was then centrifuged at 14000g for 12min at 4ºC and the pellet resuspended in 3ml of a Percoll 45 %(v/v) in KHR solution (NaCl 140mM, EDTA 1mM, HEPES 10mM, KCl 5mM, glucose 5mM, pH 7.4). After centrifugation at 14000g for 2min at 4ºC, the top layer (which corresponds to the synaptosomal fraction) was washed with KHR solution. The synaptosomal fraction was resuspended in Krebs-HEPES buffer (mM: NaCl 125, KCl 3, glucose 10, $MgSO_4$ 1.2, NaH_2PO_4 1, $CaCl_2$ 1.5, AOAA 0.1, HEPES 10, pH 7.4). The amount of protein was quantified by Bradford´s Method (Bradford, 1976) and the protein concentration adjusted to 1.5 mg protein/ml.

4.2.2 Cell lines and primary astrocytic cultures

The astrocytes were prepared from the cortex of newborn (P1-P2) Wistar rats of either sex, according to the European guidelines (86/609/EEC). Briefly, rat brains were dissected out of pups and cortex were isolated and the meninges and white matter were removed. Cortex was dissociated gently by grinding in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen), filtered through a cell strainer and centrifuged at 200g, 10min. The pellet was resuspendend in DMEM and filtered. The cells were then seeded and kept for 4 weeks in DMEM supplemented with 10% (v/v) of heat inactivated FBS (foetal bovine serum), glutamine 2mM and antibiotic (Sigma) in a humidified atmosphere (5% CO_2) at 37°C.

CHO cells clones expressing A_1 receptors, A_{2A} receptors or both were obtained and cultured as previously indicated (Orru *et al*., 2011). HEK-293T cells were grown in DMEM supplemented with 2mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% (v/v) of FBS. Cells were maintained at 37ºC in an atmosphere of 5% CO_2, and were passaged when they were 80-90% confluent, i.e. approximately twice a week.

4.2.3 [^{3}H]GABA uptake assays

In synaptosomes - [^{3}H]GABA uptake assays were performed as previously indicated (Vaz *et al*., 2008) with minor modifications. Briefly, synaptosomes (0.5 mg protein/ml) were added to Krebs-HEPES buffer at 37ºC and, except where otherwise specified, incubated with adenosine deaminase (ADA 1U/ml), to remove

endogenous extracellular adenosine) for 15min before the addition of the test drugs; test drugs were then added and incubation continued for further 20 min. Control samples were incubated for the same amount of time but in the absence of test drugs. Whenever the influence of a drug over another drug was assayed, the first (modifier) drug was allowed to equilibrate with the synaptosomes for 10 minutes before the addition of the test drug; in the corresponding control assays, the synaptosomes were incubated for the same amount of time in the absence of the modifier drug. GABA uptake was initiated by the addition of 5µM [^{3}H]GABA (except otherwise specified) at a specific activity of 2x10^{-4}Ci. This working [^{3}H]GABA solution was obtained by addition of cold GABA to the purchased [^{3}H]GABA into Krebs-HEPES buffer. The transport was stopped after 40seconds with 10ml of ice-cold Krebs-HEPES buffer, followed by rapid filtration. The amount of [^{3}H]GABA taken up by synaptosomes was quantified by liquid scintillation counting. All assays were performed in three replicates.

The non-specific, non-GAT-1 mediated GABA uptake was assayed in all experiments, in the presence of SKF89976a (20µM), a specific blocker of GAT-1, which was added 20 min before [^{3}H]GABA. For 40s incubation with [^{3}H]GABA (5µM), the radioactivity taken up by the synaptosomes in the presence of SKF89976a corresponded to 9.8 ± 3.2% (n=9) of total radioactivity taken up by the synaptosomes in the absence of the GAT-1 inhibitor. In each experiment, the non-specific uptake was subtracted from total uptake to calculate the GAT-1 mediated GAT-1 uptake.

In astrocytes - Assays were performed in a non supplemented low-glucose DMEM. The astrocytes were incubated with ADA (1U/ml) for 15 min before the addition of the test drugs; test drugs were added and incubation continued for further 20 min. GABA uptake was initiated by the addition of 30µM [^{3}H]GABA (except otherwise specified) at a specific activity of 2x10^{-7}Ci. The transport was stopped after 40s with 2 ml of ice-cold PBS. The amount of [^{3}H]GABA taken up by astrocytes was quantified by liquid scintillation counting. The GAT-1- and GAT-3-mediated transports were calculated through the subtraction of the amount of GABA taken up in the presence of the specific blocker of GAT-1, SKF 89976a (20µM) or the specific blocker of GAT-3, SNAP 5114 (40 µM), respectively to the total transport.

4.2.4 Biotinylation assays

In synaptosomes - The synaptosomes (3-4mg protein/ml) were incubated with drugs for 35min (initially with ADA (1U/ml) for 15min, followed by addition of test drug in the remaining 20min); equal incubation times were used in the parallel control assays, but in the absence of drugs. Synaptosomes were then washed three times with PBS/Ca^{2+}/Mg^{2+} (1.37M NaCl, 21mM KCl, 18mM KH_2PO_4, 100mM $Na_2HPO_4.2H_2O$, 1M $CaCl_2$, 0.5mM $MgCl_2$) and then incubated for 1h with 1mg/ml Sulfo-NHS-LC-biotin (Pierce) in TEA buffer (0.01M TEA (triethylamine), 1M $CaCl_2$, 150mM NaCl) with gentle shaking. The biotin reaction was quenched with 100mM glycine dissolved in PBS/Ca^{2+}/Mg^{2+} for 30min. The synaptosomes were then lysed with radioimmunoprecipitation assay (RIPA) buffer

(Tris base 50mM, EDTA 1mM, NaCl 150mM, SDS 0.1%, NP40 1%) supplemented with protease inhibitor cocktail and centrifuged at 14000g, 4ºC, 10min. The pellet was discharged. Biotinylated surface proteins were immunoprecipitated with Streptavidin (2µl/10 µg protein) (Sigma) overnight at 4ºC and centrifuged at 14000g, 10min at 4ºC. The pellet (biotinylated fraction) was separated from the supernatant (intracellular fraction). The biotinylated fraction was washed 3 times in 500 µl RIPA (14000g, 10min). Then, 70 µl of Laemmli buffer [Tris-HCl 70mM pH 6.8; glycerol 6%; SDS 2%; DTT (DL-Dithiothreitol) 120mM; bromophenol blue 24 ppm] was added to the pellet and heated to 95ºC for 5min. The supernatant was collected (surface membrane expression) for Western Blot analysis.

In astrocytes **-** Astrocytes were initially incubated with ADA (1U/ml) and then incubated for 30 min without (control) or with the agonists or antagonist of adenosine A_1 receptors or A_{2A} receptors or both. When antagonist and agonist were tested together, the antagonist was added 15 min before. After, they were incubated for 1 h with 1 mg/ml Sulfo-NHS-LC-biotin (Pierce) in PBS/Ca^{2+}/Mg^{2+} buffer with gentle shaking. The biotin reaction was quenched with 100 mM glycine for 30 minutes. The astrocytes were mechanically lysed with sucrose-containing buffer (sucrose 0.32M, EDTA 1mM, HEPES 10mM, BSA 1mg/ml, pH 7.4) supplemented with protease inhibitor cocktail and centrifuged at 14,000 g, 4ºC, 10 min. Biotinylated surface proteins were immunoprecipitated with avidin beads (Pierce) overnight at 4ºC and centrifuged at 14,000 g, 10 min at 4ºC. The avidin beads were pelleted by centrifugation at 3,000g, 4ºC,

10min. The pellet (biotinylated fraction) was separated from the supernatant (intracellular fraction). Then, 150µl of Laemmli buffer [Tris-HCl 70mM pH 6.8; glycerol 6%; SDS 2%; DTT (DL-Dithiothreitol) 120mM; bromophenol blue 24 ppm] was added to the pellet and heated to 37ºC for 30 min. The avidin beads were removed by filtration. Equal volumes of each sample was loaded on gel and resolved by SDS-PAGE.

4.2.5 Western Blot

For GAT-1 detection in synaptosomes **-** After denaturation (by Laemmli buffer heated at 95ºC for 5min), the synaptosomes extracts were run on an 8% acrylamide gel. Protein was transfected to a nitrocellulose membrane by electroblotting. Western blotting was performed using the anti-GAT-1 (1:5000), ab426 (abcam) and visualized using ECL reagents (Amersham). The protein amount was normalized by the anti – β-actin (1:7500), ab8227 (abcam).

For GAT-1 and GAT-3 detection in primary astrocytic cultures – The astrocytes were mechanically lysed with sucrose-containing buffer (sucrose 0.32M, EDTA 1mM, HEPES 10mM, bovine serum albumin 1mg/ml, pH 7.4). To clarify, the homogenate was centrifuged (13,000g, 10 min, at 4ºC) and the supernatant was collected. After denaturation (by Laemmli buffer heated at 95ºC for 5 min), the extracts were run on a 10% acrylamide gel. Protein was transferred to a nitrocellulose membrane by electroblotting. Western blotting was performed using the anti-GAT-1 (1:100) and anti-GAT-3(1:200), kindly provided by N. Brecha, UCLA. After exposure to secondary

antibody (peroxidase anti-rabbit (1:250) Vector; Burlingame, CA), bands were visualized by BioRad Chemidoc and Quantity One software.

4.2.6 Immunocytochemistry

Primary astrocytes were incubated with ADA (1U/ml) for 15min, fixed in 4% paraformaldehyde for 20 min and washed with PBS containing 20 mM glycine (buffer A) to quench the aldehyde groups. Then, after permeabilization with 10% normal goat serum (NGS) containing 0.3% Triton X-100 for 5 min, cells were treated with PBS containing 1% bovine serum albumin. After 1 h at room temperature, astrocytes were incubated with antibodies rabbit anti-GAT-1 (1:100; kindly provided by N. Brecha, UCLA) and mouse anti-GFAP (1:800; Sigma) or rabbit anti-GAT-3 (1:200; kindly provided by N. Brecha, UCLA) and mouse anti- GFAP (1:800; Sigma) antibodies for 3 h at RT. After washes, astrocytes were stained with the secondary antibodies for 1.5 h at room temperature (FITC-conjugated anti-rabbit IgG (FI-1000) and TRITC-conjugated anti-mouse IgG (T-2762; Vector Laboratories). Dishes were then mounted, air-dried, and coverslipped using Vectashield mounting medium (H-1000; Vector).

4.2.7 BRET

Primary astrocytes or HEK-293T cells were transiently co-transfected with a constant amount of the cDNA encoding for receptors fused to RLuc, and with increasingly amounts of the cDNA corresponding to receptors fused to YFP, To quantify receptor-YFP expression, cells

(20 µg protein) were distributed in 96-well microplates (black plates with a transparent bottom) and fluorescence was read in a Fluo Star Optima Fluorimeter (BMG Labtechnologies, Offenburg, Germany) equipped with a high-energy xenon flash lamp, using a 10nm bandwidth excitation filter at 400nm reading. Receptor-fluorescence expression was determined as fluorescence of the sample minus the fluorescence of cells expressing the BRET donor alone. For BRET, the equivalent of 20µg of cell suspension was distributed in 96-well microplates (Corning 3600, white plates; Sigma) and 5µM coelenterazine H (Molecular Probes) was added. One minute later, the readings were collected using a Mithras LB 940 that allows the integration of the signals detected in the short-wavelength filter at 485nm (440-500nm) and the long-wavelength filter at 530nm (510-590nm). To quantify receptor-RLuc expression luminescence readings were also performed after 10 minutes of adding 5µM coelenterazine H. The net BRET is defined as [(long-wavelength emission)/(short-wavelength emission)]-Cf where Cf corresponds to [(long-wavelength emission)/(short-wavelength emission)] for the donor construct expressed alone in the same experiment. BRET is expressed as miliBRET units, mBU (net BRET x 1000).

4.2.8 [^{35}S] GTP-γ- S assay

For quantification of GTP activity, GDP (10µM) was added to the primary astrocytic membranes and incubated on ice for 10 minutes. Membranes were incubated at 37ºC for 10 minutes with ADA (1U/ml) before the addition of DPCPX or SCH 58261 antagonists and after 10min minutes, the [^{35}S]GTP-γ-S (1nM) and the tested drugs

were added and incubate for 30 minutes at 37ºC. Membranes were collected and solubilised and the antibodies were added: 5µg of anti–$G_{\alpha\ i\text{-}3}$ (sc-262), 10µg of anti–$G_{\alpha\ S}$ (sc-26766) or 10µg of anti–$G_{\alpha\ q/11}$ (sc-392), for G_i, G_S and G_q studies, respectively. After an overnight incubation at 4ºC, protein G-sepharose was added and incubated for 90 min at 4ºC. The sepharose was washed 5 times with the solubilisation buffer and the incorporation of [^{35}S]GTP-γ-S was measured by liquid scintillation.

4.3 Statistical analysis

All data are present in mean ± SEM to test for statistical significance, the data were analysed by Students't-test or by *one way* ANOVA followed by Bonferroni's multiple comparison test. Values of $p \leq 0.05$ were considered to represent statistically significant differences.

5 Results

5.1 Adenosine modulation of GAT-1-mediated GABA uptake by synaptosomes

5.1.1 Rationale

To assure a controlled GABA signaling, GABA is quickly removed from the synaptic cleft, and this occurs through Na^+ and Cl^- dependent GABA transporters (GATs) present in pre-synaptic nerve endings and surrounding astrocytes (Borden, 1996). The predominant GAT in GABAergic nerve endings is GAT-1 (Iversen and Kelly 1975). These terminals also possess adenosine A_1 and A_{2A} receptors. A_1 receptors do not directly modulate GABA release (Cunha and Ribeiro, 2000) but influence the action of other neuromodulators upon GABA release (Cunha-Reis *et al.*, 2008). A_{2A} receptors facilitate GABA release (Cunha and Ribeiro, 2000). In spite of this detailed information on how release of GABA is controlled by the two high affinity adenosine receptors, no study so far has evaluated how the uptake of GABA into nerve endings is modulated by adenosine. I therefore investigated the influence of adenosine A_1, A_{2A} and A_{2B} receptors upon GAT-1-mediated GABA transport.

5.1.2 Adenosine A_{2A} receptors tonically enhance GAT-1 -mediated GABA transport

Before evaluating the influence of any drug on GABA uptake by synaptosomes, experiments were designed to define the time of

incubation with [^{3}H]GABA, as well as the GABA concentration to be used in subsequent GABA uptake assays. [^{3}H]GABA (5 μM) uptake was linear (correlation coefficient = 0.95) for incubation times ranging from 0 to 80 s. For 40 s incubation, the K_M of GAT-1-mediated GABA transport, determined in one experiment where [^{3}H]GABA concentrations ranged between 0 and 10 μM, was 5 μM. This value is similar to that previously reported by others (1.57 μM < K_M < 13 μM (Wood and Sidhu, 1986). An incubation time of 40 s and a [^{3}H]GABA concentration of 5 μM were therefore used in all the subsequent experiments. Under these conditions and in the absence of test drugs the amount of [^{3}H]GABA taken up by the synaptosomes through GAT-1, i.e., after subtraction of the uptake in the presence of SKF 89976A (20 μM), was 2.86 ± 0.34 nmol/mg protein (n= 25). To know if extracellular adenosine endogenously released by the synaptosomes could influence [^{3}H]GABA uptake, synaptosomes were incubated for 15 min with adenosine deaminase (1 U/ml), an enzyme that catabolises adenosine into inosine. [^{3}H]GABA was then added, and its uptake was compared with the uptake by synaptosomes incubated for the same period of time in the absence of ADA. As shown in figure 5.1.A, ADA decreased [^{3}H]GABA uptake by 17 ± 2.6% (n = 4, $p<0.01$). This inhibition cannot be attributed to the formation of extracellular inosine as this adenosine metabolite even at a concentration (10 μM) higher than that expected to occur because of deamination of extracellular endogenous adenosine, had no effect on [^{3}H]GABA uptake (n = 3,$p > 0.05$) (Fig. 5.1.A). As also shown in Fig. 5.1.A, blockade of A_{2A} receptors with their selective

antagonist, SCH 58261 (50 nM), mimicked the effect of ADA (% inhibition of GABA uptake in the presence of SCH 58261: 25 ± 2.2%, n= 4, $p < 0.01$). When applied to synaptosomes where endogenous adenosine had been removed by ADA, SCH 58261 (50 nM) was devoid of effect upon [^{3}H]GABA uptake (Fig. 5.1.B). Taken together the above results strongly indicate that GAT-1-mediated GABA transport is tonically facilitated by adenosine A_{2A} receptors activation with endogenous adenosine. To avoid A_{2A} receptors occupancy by endogenous extracellular adenosine, which would mask the action of added agonists, ADA (1 U/ml) was added to the incubation medium in all subsequent experiments, except otherwise indicated.

Adenosine A_{2A} receptors activation with the selective agonist CGS 21680 (30 nM) (Jarvis *et al*., 1989) enhanced [^{3}H]GABA uptake by 26 ± 1.0% (n=4, $p< 0.01$), an effect fully prevented by the presence of SCH 58261 (50 nM) (Fig. 5.1.B), a selective A_{2A} receptor antagonist (Zocchi *et al.,* 1996). When the effects of different concentrations of the A_{2A} receptor agonist were compared in the same experiments, it was apparent that a maximum increase was attained with 30 nM of CGS 21680; an higher concentration caused a lower effect (Fig. 5.1.C), probably because of receptor desensitization (Mundell *et al*., 1997).

The activation of adenosine A_{2A} receptors leads to the same effect in adult rats (6–8 weeks), where CGS 21680 (30 nM) increased [^{3}H]GABA uptake by 20 ± 3.4% (n=4, $p < 0.001$) (Fig. 5.2). This effect

was completely banned by the pre-incubation of the A_{2A} receptor antagonist, SCH 58261 (50 nM).

As can be concluded from the saturation curve shown in Figure 5.1.D, the Vmax of GAT-1 was enhanced from 3.91 ± 0.18 nmol/mg protein in the control condition to 5.34 ± 0.20 nmol/mg protein in the presence of 30 nM of the adenosine A_{2A} receptors agonist ($p < 0.005$, $n = 3$), while the K_M value was not significantly modified (4.01 ± 0.65 μM in the presence of CGS 21680 and 4.15 ± 0.63 μM in its absence, $p > 0.05$, $n= 3$), suggesting an increase in the number of membrane GAT-1 transporters without a change in the transporter affinity for GABA. An increase in the expression of GAT-1 in surface membranes (16 ± 3.1%, $p= 0.0024$, $n= 5$) was found in biotinylation assays with synaptosomes treated with CGS 21680 (30 nM) for 20 min (Fig. 5.1.E). This increase in GAT-1 density at surface membranes was accompanied by a decrease of 14 ± 2.7% ($p= 0.0033$, $n= 4$) in the amount of GAT-1 in the intracellular fraction (Fig. 5.1.F).

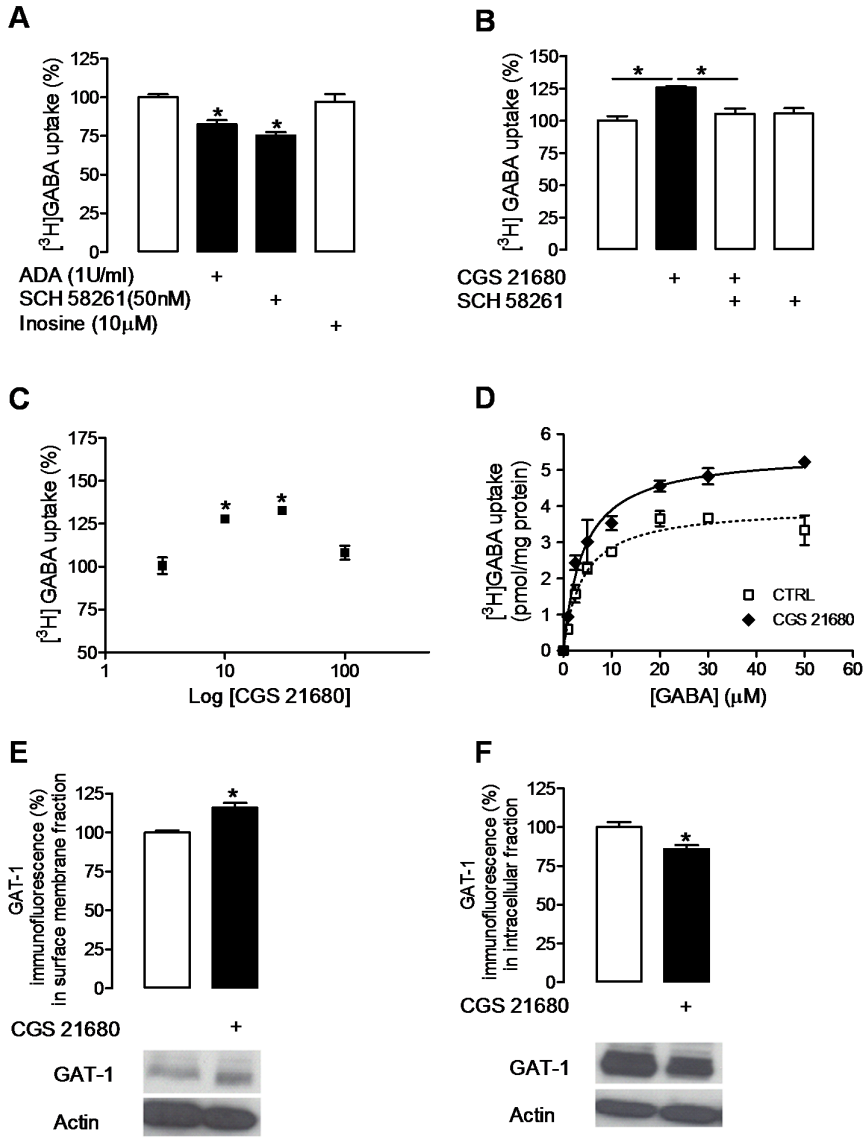
A
[3H]GABA uptake (%)
ADA (1U/ml)
SCH 58261(50nM)
Inosine (10μM)
B
[3H] GABA uptake (%)
CGS 21680
SCH 58261
C
[3H] GABA uptake (%)
Log [CGS 21680]
D
[3H]GABA uptake (pmol/mg protein)
CTRL
CGS 21680
[GABA] (μM)
E
GAT-1 immunofluorescence (%) in surface membrane fraction
CGS 21680
GAT-1
Actin
F
GAT-1 immunofluorescence (%) in intracellular fraction
CGS 21680
GAT-1
Actin

Figure 5.1 – Adenosine, through adenosine A_{2A} receptors, enhances GABA transport into nerve endings by increasing the surface density of GAT-1 and maximum transport rate. Panel (A) illustrates the inhibition of GABA uptake caused by removal of endogenous extracellular adenosine with adenosine deaminase (ADA, 1 U/ml), which deaminates adenosine into inosine, or by preventing the action of endogenous adenosine over A_{2A} receptors with a selective antagonist SCH 58261 (50 nM); the absence of effect of inosine (10 μM), the metabolite formed by adenosine deamination by ADA is also shown. Panel (B) illustrates the facilitation of GAT-1 mediated GABA transport by activation of A_{2A} receptors with a selective agonist, CGS 21680 (30 nM), and the ability of the A_{2A} receptors antagonist, SCH 58261 to prevent this effect. By itself SCH 58261 did not influence GABA transport as endogenous adenosine has been removed with ADA. The concentration-response curve for the effect of CGS 21680 is illustrated in panel C. Panel (D) shows a saturation curve, depicting the amount of GABA taken up as a function of the concentration of GABA. Panels (E) and (F) show results from western blots with a GAT-1 antibody, performed in the membrane sediment after biotinylation (E), to assess GAT-1 surface density or in the supernatant (F), to assess the density in the intrasynaptosomal pool. Note that the surface GAT-1 density was enhanced by the A_{2A} receptors agonist, CGS 21680 whereas intrasynaptosomal GAT-1 density was decreased. The density of GAT-1 (ordinates) is expressed in immunofluorescence arbitrary units, after normalization against b-actin, which was used as a loading control; 100% corresponds to GAT-1 density in the absence of CGS 21680. Representative western blots are showed in each panel below the corresponding column. In all panels the results are expressed as mean ± SEM, with n = 3–5. The statistical significance was calculated through *one way* ANOVA followed by Bonferroni's multiple comparison test, (*$p < 0.01$). All tested drugs were incubated for 20 min before addition of [^{3}H]GABA; while testing CGS 21680 in the presence of SCH 58261, the antagonist was added 10 min before the agonist. In all experiments, except in those illustrated in (A), endogenous adenosine was removed by incubation with ADA (1 U/ml).

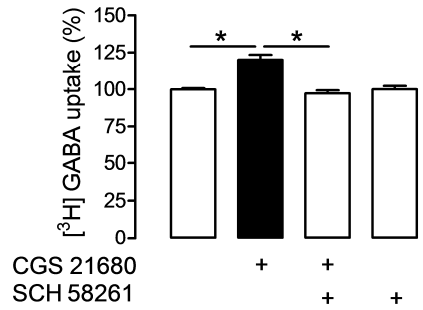

Figure 5.2 – Adenosine A_{2A} receptors activation promotes GAT-1 mediated GABA uptake in adult rats. The activation of A_{2A} receptors by its selective agonist (CGS 21680, 30 nM) promoted GABA uptake. This effect was fully prevented by the presence of the adenosine A_{2A} receptors selective antagonist (SCH 58261, 50 nM). All assays were performed after the removal of endogenous adenosine with ADA (1 U/ml). Results are as mean ± SEM with n = 4. The statistical significance was calculated by *one way* ANOVA followed by Bonferroni's multiple comparison test (*p < 0.001, n = 4).

5.1.3 Adenosine A_1 and A_{2B} receptors do not affect GAT-1 mediated GABA transport

To evaluate the tonic influence of adenosine A_1 and A_{2B} receptors upon GAT-1 mediated GABA transport, experiments were performed in synaptosomes in the absence of ADA. As illustrated in figure 5.3.A, neither the blockade of the A_1 receptors with its selective antagonist DPCPX, at a concentration of 50 nM, one hundred times higher than its K_D for A_1 receptors (Bruns *et al.,* 1987) nor A_1 receptors activation with its selective agonist, CPA at a concentration (30 nM) more than ten times higher than its K_D for A_1 receptors (Williams *et al.*, 1986) have any effect on GAT-1-mediated GABA uptake. To evaluate the role of A_{2B} receptors upon GABA transport and due to the lack of selective agonists of A_{2B} receptors, a blanket agonist (NECA) was used in the presence of A_{2A} and A_1 receptors selective antagonists,

SCH 58261 (50 nM) and DPCPX (50 nM). NECA was used at a concentration (3 μM) previously shown to induce A_{2B} receptors-mediated responses in astrocytes (Peakman and Hill, 1994) but it was devoid of effect on [^{3}H]GABA uptake (Fig. 5.3.B). The adenosine A_{2B} receptor antagonist, MRS 1706 (50 nM) (Kim *et al*., 2000) was also devoid of effect upon [^{3}H]GABA uptake ($p > 0.05$, $n = 4$) (Fig. 5.3.B) in synaptosomes that were not pre-incubated with ADA. Altogether, these results indicate that neither endogenous nor exogenous activation of adenosine A_1 nor A_{2B} receptors affect GAT-1 mediated GABA transport into nerve terminals.

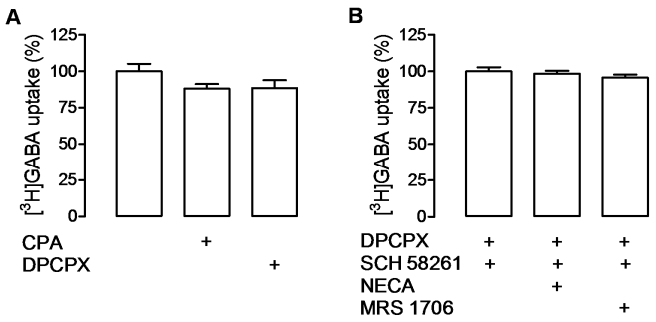

Figure 5.3 – Adenosine A_1 and A_{2B} receptors do not affect GAT-1 mediated GABA transport into nerve endings. In Panel (A) it is shown the absence of influence of A_1 receptors. Both the activation of A_1 receptors by the selective agonist (CPA, 30 nM) and the blockade by the selective antagonist (DPCPX, 50 nM) failed to modify GABA uptake. Panel (B) illustrates the absence of influence by adenosine A_{2B} receptors. The blanket adenosine receptors agonist NECA (3 μM) was used as an A_{2B} receptors agonist, as the adenosine A_1 and A_{2A} receptors were previously blocked by DPCPX (50nM) and SCH 58261 (50nM), respectively. Note that neither NECA, nor A_{2B} receptors blockade with the selective antagonist MRS 1706 (50 nM), influenced GABA uptake. Results are mean ± SEM from four experiments. Statistical differences were evaluated by one way ANOVA followed by Bonferroni's multiple comparison test and no statistical differences emerged ($p > 0.05$). Adenosine deaminase was not added. The antagonists were added 10 min before the agonists, when tested together.

5.1.4 Activation of adenylate cyclase mimics adenosine A_{2A} receptors activation and PKA blockade prevents the action of the A_{2A} receptors agonist

To investigate the involvement of the adenylate cyclase (AC)/PKA transduction pathway in the enhancement of GABA transport into the nerve endings, a selective activator of AC, forskolin (Awad *et al.,* 1983) and a selective PKA inhibitor (Chijiwa *et al.*, 1990) were used. Blockade of PKA with H-89 (1μM), *per se*, had no effect on [^{3}H]GABA uptake ($n = 3$, $p > 0.05$, Fig. 5.4.A), but prevented the facilitatory action of CGS 21680 (30 nM) upon GABA transport (Fig. 5.4.B). On the other hand, AC activation with forskolin (10 μM) increased [^{3}H]GABA uptake by 29 ± 5.1% ($n = 6$, $p< 0.001$, Fig. 5.4.A), therefore mimicking the effect of A_{2A} receptors activation by CGS 21680 (30 nM). As expected, the effect of forskolin (10 μM) on [^{3}H]GABA uptake was prevented by H-89 (1 μM) ($n = 3$, $p < 0.001$, fig. 5.4.A). The facilitatory actions of forskolin and CGS 21680 upon GABA uptake were not additive (Fig. 5.4.B), suggesting that they operate a common pathway.

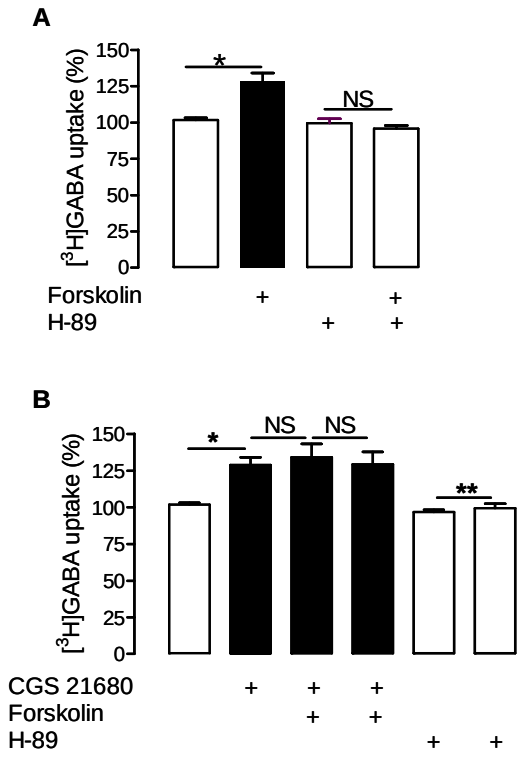

Figure 5.4 – Influence of the AC/PKA transduction pathway. Panel (A) shows the modifications of GAT-1 mediated GABA transport caused by the adenylate cyclase (AC) activator, forskolin (10 μM), and by the PKA inhibitor H-89(1 μM). Note that forskolin enhanced GABA uptake ($p < 0.05$), an action prevented by H-89, although H-89 per se did not affect GABA transport into the nerve endings. Panel (B) illustrates how the manipulation of the AC/PKA pathway influences the adenosine A_{2A} receptors mediated enhancement of GABA transport. Note that AC activation mimicked the effect of the A_{2A} receptors agonist, CGS 21680, and that both effects were not additive; the PKA inhibitor, H-89, fully prevented the effect of CGS 21680. All assays were performed after the removal of endogenous adenosine with ADA (1 U/ml). Results are as mean ± SEM with n = 3–6. The statistical significance was calculated by *one way* ANOVA followed by Bonferroni's multiple comparison test, *$p < 0.05$. The incubation period with test drugs was 20 min; whenever forskolin or H-89 were used together with CGS 21680, they were added 10 min before CGS 21680; when testing the ability of H-89 to prevent the effect of forskolin, H-89 was added 10 min before forskolin.

5.1.5 PKC constitutively inhibits GABA transport, and prevents A_{2A} receptors -mediated facilitation of GABA transport

The influence of PKC upon GABA uptake was investigated by using its selective inhibitor, GF109203X (Toullec *et al.*, 1991) and its activator, PDD (Castagna *et al.,* 1982). Upon blockade of PKC with GF109203X (1 μM) [^{3}H]GABA uptake was enhanced by 26 ± 5.8% ($p < 0.05$, n = 5, Fig. 5.5.A), suggesting that PKC is tonically activated and inhibits GAT-1-mediated GABA transport. Further activation of PKC by PDD (250 nM) decreased [^{3}H]GABA uptake by 17 ± 1.6% ($p < 0.05$, n = 4, Fig. 5.5.A), which indicates that tonic PKC activation is submaximal and reinforces the conclusion that PKC activation promotes the inhibition of GAT-1 mediated GABA transport into nerve endings.

To figure out the mechanism associated to the effect of PKC activation upon GABA transporter, saturation assays were performed. As it can be seen in Fig. 5.5.B the activation of PKC with PDD (250 nM) led to a decrease in the Vmax of GAT-1, from 1.81 ± 0.08 nmol/mg protein in the control condition to 1.19 ± 0.06 nmol/mg protein in the presence of PKC activator, PDD ($p = 0.0013$, n = 3). On the other hand, the value of K_M was not significantly modified (4.38 ± 0.78 μM in the presence of PDD (250 nM) and 3.89 ± 0.80 μM in its absence, $p > 0.05$, n = 4), suggesting a decrease in the number of membrane GAT-1 transporters without a change in the transporter affinity for GABA. A decrease in the expression of GAT-1 in surface membranes (12 ± 2.1%, $p = 0.0092$, n = 3) was found in biotinylation assays with synaptosomes treated with PDD

(250 nM) for 20 min (Fig. 5.5.C). This reduction in GAT-1 density at surface membranes was accompanied by an augment of 12 ± 2.9% ($p = 0.01$, $n = 3$) in the amount of GAT-1 in the intracellular fraction (Fig. 5.5.C). Tonic inhibition of GABA transport by PKC might result from its constitutive activity, rather than from tonic activation of a membrane receptor coupled to phospholipase C (PLC), as blockade of PLC with U73122 (3 μM) had no effect on [$_3$H]GABA uptake ($p > 0.05$, $n = 3$, Fig. 5.5.E).

When PKC was activated by PDD (250 nM), the A_{2A} receptors agonist, CGS 21680 (30 nM), was no longer able to facilitate GABA transport (Fig. 5.6.A). On the other hand, the facilitatory action of CGS 21680 upon GABA transport was not additive with the facilitatory action of PKC blockade with GF109203X (Fig. 5.6.B). Taken together the above results suggest that to observe a facilitatory action of adenosine A_{2A} receptors upon GAT-1- mediated GABA transport, PKC has to be submaximally activated.

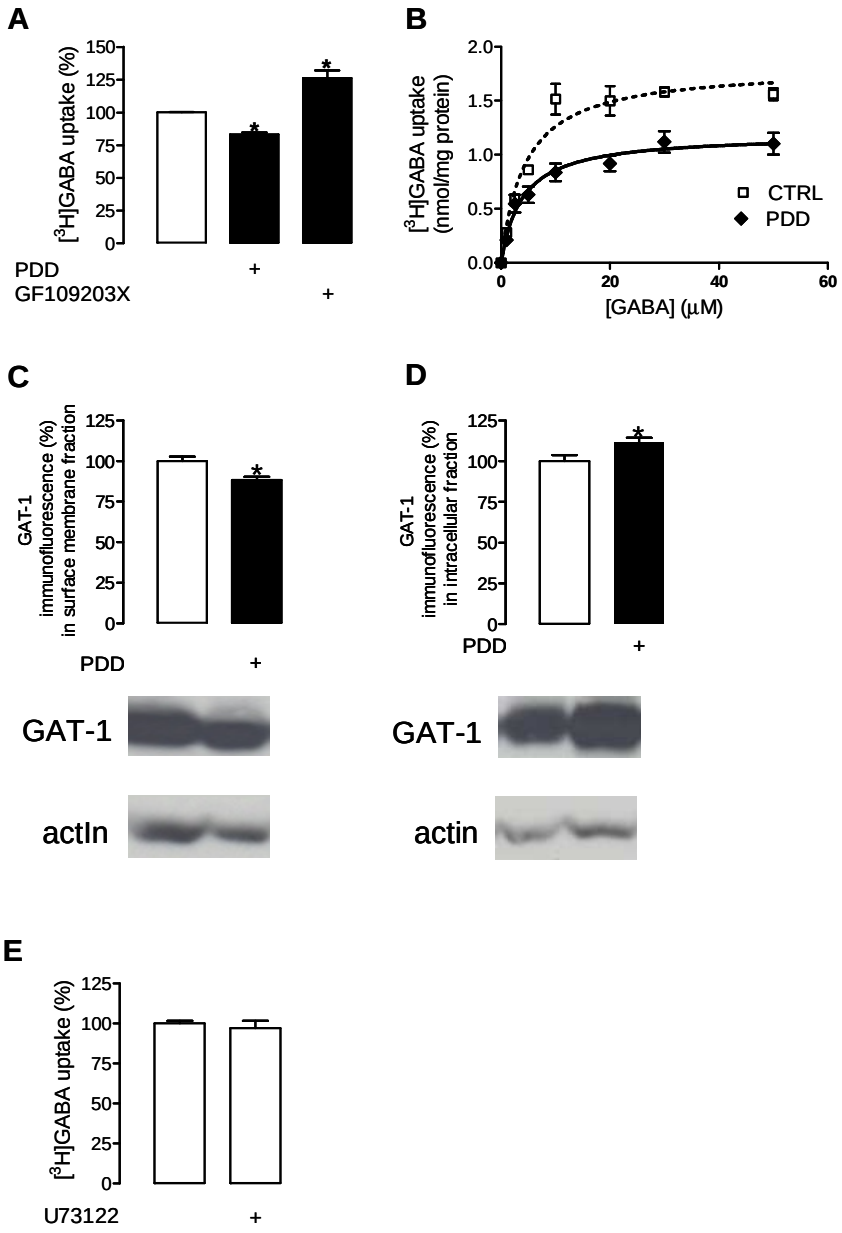
A
[3H]GABA uptake (%)
150
125
100
75
50
25
0
PDD
GF109203X
+
+
B
[3H]GABA uptake (nmol/mg protein)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
[GABA] (μM)
CTRL
PDD
C
GAT-1 immunofluorescence (%) in surface membrane fraction
125
100
75
50
25
0
PDD
+
GAT-1
actIn
D
GAT-1 immunofluorescence (%) in intracellular fraction
125
100
75
50
25
0
PDD
+
GAT-1
actin
E
[3H]GABA uptake (%)
125
100
75
50
25
0
U73122
+

Figure 5.5 – Influence of the PLC/PKC transduction pathway upon GABA transport. Panel (A) shows the consequences of manipulating PKC activity upon GAT-1 mediated GABA transport. Note that PKC activation with PDD (250 nM) decreased uptake, and that PKC inhibition with GF190203X (1 μM) enhanced uptake. Panel (B) shows a saturation curve, depicting the amount of GABA taken up as a function of the concentration of GABA. Panels (C) and (D) show results from western blots with a GAT-1 antibody, performed in the membrane sediment after biotinylation (C), to assess GAT-1 surface density or in the supernatant (D), to assess the density in the intrasynaptosomal pool. Note that the surface GAT-1 density was decreased by the PKC activator, PDD whereas intrasynaptosomal GAT-1 density was enhanced. The density of GAT-1 (ordinates) is expressed in immunofluorescence arbitrary units, after normalization against b-actin, which was used as a loading control; 100% corresponds to GAT-1 density in the absence of PDD. Representative western blots are showed in each panel below the corresponding column. Panel (E) shows the absence of modification of transport caused by blockade of PLC with U73122 (3 μM). All assays were performed after the removal of endogenous adenosine with ADA (1 U/ml). In all panels the results are expressed as mean ± SEM from four to five experiments. The statistical significance was calculated through one way ANOVA followed by Bonferroni's multiple comparison test (*$p < 0.001$). All tested drugs were incubated for 20 min before addition of [^{3}H]GABA.

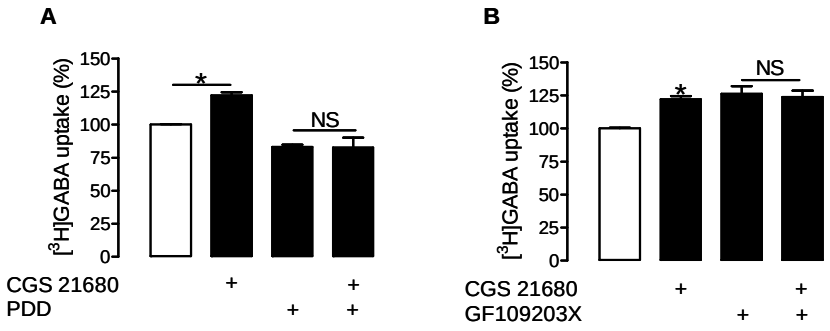

Figure 5.6 – Influence of PKC activity upon the A_{2A}R mediated facilitation of GABA transport. Panel (A) illustrates how activation of PKC with PDD (250 nM) affects the action of the A_{2A} receptors agonist, CGS 21680 (30 nM) upon GAT-1 mediated GABA transport. Note that PKC activation with PDD fully prevented the effect of CGS 21680. Panel (B) shows how inhibition of PKC with GF109203X (1 μM) influenced the effect of the A_{2A} receptors agonist CGS 21680 (30 nM). Note that both facilitatory effects were not additive. All assays were performed after the removal of endogenous adenosine with ADA (1 U/ ml). Results are as mean ± SEM from four experiments. The statistical significance was calculated through *one way* ANOVA followed by Bonferroni's multiple comparison test, *$p < 0.05$. The incubation period with test drugs was 20 min; whenever PDD or GF109203X were used together with CGS 21680, those PKC influencing drugs were added 10 min before CGS 21680.

5.1.6 PKA and PKC interaction

The results described above clearly indicate that both PKA and PKC are involved in the enhancement of GABA transport induced by adenosine A_{2A} receptors activation. In an attempt to identify which mechanism would occur first, it was next investigated how both kinases cross-talked to modulate GAT-1-mediated GABA transport. So, experiments were designed in which activators or inhibitors of one transduction system were pre-incubated with the synaptosomes for 10 min before the addition of drugs towards the other

transduction system; the amount of GABA taken up only in the presence of the first (modifier) drug was taken as 100%. When PKC was previously inhibited by GF109203X (1 μM), the AC activator, forskolin (10 μM), was no longer able to enhance GABA uptake (Fig. 5.7.A) whereas in the same synaptosomal batch but in the absence of GF109203X, forskolin caused its usual facilitation of GABA uptake (Fig. 5.7.B). On the other hand, previous blockade of PKA with H-89 (1 μM) failed to influence the inhibition of GABA transport caused by PKC activation as PDD (250 nM) caused a similar inhibition of GABA uptake in the presence (Fig. 5.7.C) and in the absence (Fig. 5.7.D) of H-89, both effects being obtained from the same synaptosomal batch. Taken together these results suggest that modulation of GABA uptake by PKC can occur independently of PKA activity, and that PKC has to be active to see an action of PKA upon GAT-1 mediated GABA uptake. Therefore, the direct modulator of GABA uptake appears to be PKC, PKA acting as a modifier of PKC-induced modulation. Reinforcing this conclusion was the observation that activation of AC with forskolin caused a pronounced change in the influence of the PKC blocker, GF109203X, upon GABA uptake (Fig.5.7.E and 5.7.F). Moreover, forskolin was able to counteract the inhibitory effect of PDD on GABA transport as GABA transport in the presence of Forskolin (10 μM) + PDD (250 nM) was similar ($p > 0.05$, $n = 4$) to that observed in the same synaptosomal batch in the control conditions (in the absence of any drug).

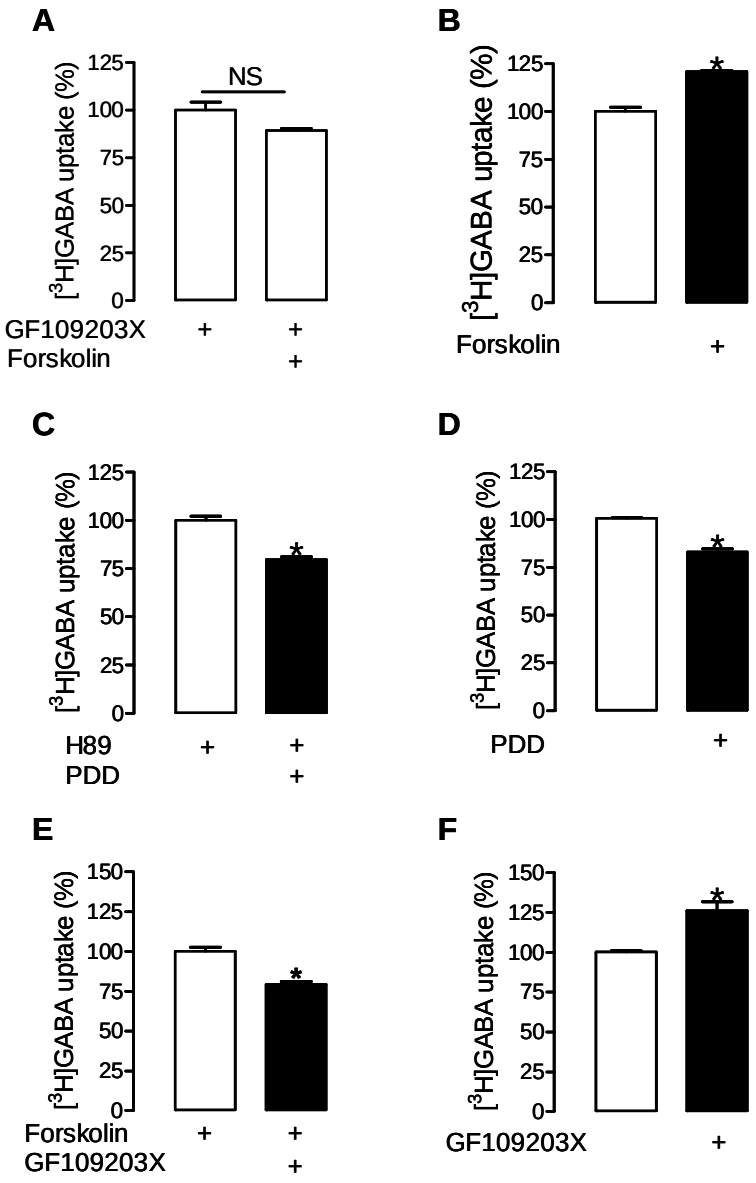
A
[3H]GABA uptake (%)
125
100
75
50
25
0
NS
GF109203X + +
Forskolin +
B
[3H]GABA uptake (%)
125
100
75
50
25
0
*
Forskolin +
C
[3H]GABA uptake (%)
125
100
75
50
25
0
*
H89 + +
PDD +
D
[3H]GABA uptake (%)
125
100
75
50
25
0
*
PDD +
E
[3H]GABA uptake (%)
150
125
100
75
50
25
0
*
Forskolin + +
GF109203X +
F
[3H]GABA uptake (%)
150
125
100
75
50
25
0
*
GF109203X +

Figure 5.7 – PKC and PKA interaction. Panels (A) and (B) illustrate how the inhibition of PKC with GF109203X (1 μM) affects the facilitatory action of the AC activator, forskolin (10 μM), upon GAT-1-mediated GABA transport. Note that the facilitatory effect of forskolin was lost when PKC was previously blocked with GF109203X. Panels (C) and (D) show how inhibition of PKA with H-89 (1 μM) influences the action of the PKC activator PDD (250 nM). Note that the inhibitory effect of PDD was preserved when PKA was previously blocked with H-89. Panels (E) and (F) show how forskolin influenced the effect of GF109203X. Note that, when added alone the PKC inhibitor promoted GABA uptake into nerve endings (*$p < 0.001$, $n = 4$), while when AC was previously activated with forskolin, GF109203X decreased GABA uptake (*$p < 0.01$, $n = 3$). All assays were performed after the removal of endogenous adenosine with ADA (1 U/ml). The results are presented as mean ± SEM from three- four experiments (*$p < 0.0005$). The statistical significance was calculated through the Student's t-test. The incubation period with test drugs was 20 min; when testing more than one drug, GF109203X (A), H-89 (C), or forskolin (E) were added 10 min before the corresponding test drugs in those experiments [forskolin in (A), PDD in (C) or GF109203X in (E)].

5.1.7 Discussion

The main findings reported in this subchapter are that endogenous adenosine, through A_{2A} receptor activation, promotes GAT-1-mediated GABA transport into hippocampal nerve endings. This action might be mediated by the AC/PKA transduction system as the action of the A_{2A} receptors agonist is mimicked by AC activation and is prevented by PKA inhibition. Furthermore, the results with drugs that manipulate the PLC/PKC pathway indicate that this transduction system is tonically restraining GAT-1-mediated GABA transport and that it has to be submaximally activated to allow the facilitatory actions of A_{2A} receptors or AC. Altogether, these results indicate that adenosine, through A_{2A} receptors activation and subsequent enhancement of AC activity, restrains the inhibition of GAT-1 by PKC (see Fig. 5.8). Results from biotinylation assays allowed the conclusion that the enhancement of GAT-1 activity promoted by A_{2A} receptors activation is due to an increase in the surface membrane expression of GAT-1 transporters. This conclusion is corroborated by data from saturation curves, as the maximum velocity of transport (Vmax) was enhanced in the presence of A_{2A} receptors agonists without appreciable changes in the affinity of the transporter for GABA (K_M).

In the hippocampus, A_{2A} receptors activation promotes GABA release (Cunha and Ribeiro, 2000) and, as now showed, promotes GAT-1-mediated GABA uptake. Therefore A_{2A} receptors influence GABAergic transmission in the hippocampus by fastening GABA signaling turnover. Indeed, through facilitation of GABA removal

from the synaptic cleft and enhancement of its uptake into nerve endings, A_{2A} receptors may contribute to a reduction of tonic inhibition by GABA. On the other hand an enhancement of GABA transport will minimize GABA receptor desensitization and accelerate the replenishment of the GABA releasable pool at GABAergic nerve terminals. This will most probably lead to an enhancement in the efficiency of the phasic inhibitory signaling at the hippocampus, therefore contributing to a better control of hippocampal excitability.

The influence of adenosine A_{2A} receptors on GABAergic transmission in the hippocampus may contrast with what occurs in the basal ganglia. Thus, in striatum, adenosine A_{2A} receptors inhibit GABA release (Kirk and Richardson, 1995). It was also shown that A_{2A} receptors agonist, CGS 21680, decreases GABA uptake, though this inhibition was observed at high and non-A_{2A} receptor selective concentrations (100 nM–10 μM) of the agonist (Gonzalez *et al.,* 2006). Furthermore, full inhibition of transport was only attained by high (0.1–1 mM) concentrations of SKF 89976A (Gonzalez *et al.,* 2006), and so one cannot be sure if the transport was only occurring through GAT-1.

The opposite effects mediated by A_{2A} receptors into GAT-1 mediated GABA uptake in both hippocampus and basal ganglia can be related with the main transmission in each brain area. Then, hippocampal transmission is mainly glutamatergic while basal ganglia transmission is mainly GABAergic. So, at the hippocampus, A_{2A} receptors facilitate the excitatory transmission as they promote

GABA uptake, reducing extracellular GABA levels while at basal ganglia, A_{2A} receptors assist the inhibitory transmission by inhibiting GABA uptake, which will lead to an enhancement in ambient GABA levels. Then, adenosine, through A_{2A} receptors activation, acts as an amplifier of the main transmission (excitatory at hippocampus and inhibitory at basal ganglia).

Adenosine A_{2A} receptors can couple to both PKA and PKC and this has been identified either at the hippocampus (Cunha and Ribeiro, 2000) or at the striatum (Gubitz *et al.,* 1996). These two transduction systems can cross-talk, as it has been shown in Aplysia sensory neurons where activation of PKC attenuates PKA-mediated actions of serotonin (Sugita *et al.*, 1997). In the rat tail artery, adenosine A_{2A} receptors mediated enhancement of noradrenaline release involves both PKA and PKC, PKA is operating downstream to PKC, as the consequences of manipulating PKC are blunted by PKA inhibition whereas the consequences of manipulating AC/PKA are not influenced by PKC inhibition (Fresco *et al.,* 2004). The now reported influence of A_{2A} receptors upon GAT-1 transporters also involves PKA and PKC, though PKC might operate downstream to PKA. In fact, the effect of forskolin was prevented by PKC inhibition, but the blockade of PKA did not modify the consequences of PKC activation; thus, these results strongly suggest that PKC operate downstream to PKA.

In cortical nerve terminals, PKA and PKC act synergistically to facilitate the release of glutamate (Millán *et al*., 2003). In what concerns modulation of GABA transport through GAT-1, it became

clear that PKA and PKC operate in opposed ways, with PKA facilitating GABA uptake and PKC inhibiting it. Moreover, the activation of PKC by PDD decreased the Vmax of GAT-1 but did not change the K_M value of the transporter. These results indicate that the number of GAT-1 is reduced at the surface membrane level while the affinity of GAT-1 for GABA is not affected by PKC activation. Biotinylation assays corroborated these results as the membrane expression of GAT-1 is decreased while the GAT-1 levels are augmented in the intracellular fraction. PKC-induced internalization of GAT-1 has been shown by others using nerve terminals from the *substantia nigra pars reticulata* or cultured cortical neurons (Bahena-Trujillo and Arias-Montaño 1999; Wang and Quick, 2005). To my knowledge, the data herein reported were the first to show that GAT-1 mediated GABA uptake into nerve endings can be facilitated through a cAMP dependent process, and that this results from an increase in the surface expression of GAT-1 transporters. It was also showed that the PKA-induced facilitation is due to decreased PKC-mediated inhibition. This counteracting activity does not necessarily result from a direct phosphorylation of PKC by PKA; instead, cAMP/AC/ PKA signaling may be affecting any PKC substrate involved in GAT-1 recruitment (see Fig. 5.8).

As mentioned above, the reported facilitation of GAT-1 mediated transport into nerve endings might contribute to fasten neurotransmitter recycling. Considering that A_{2A} receptors activation also promote GABA release (Cunha and Ribeiro, 2000), both effects of adenosine A_{2A} receptors lead to an enhancement of phasic

GABAergic signaling in the hippocampus. As disturbances in GABAergic transmission and, specifically, in GABA transport have been associated to several diseases, namely epilepsy and mood disorders (Conti *et al.,* 2004; Gether *et al.,* 2006), the data now reported expand the knowledge about the ways adenosine uses to influence neuronal functioning and reinforces the interest of using adenosine A_{2A} receptors related drugs to correct brain dysfunctions.

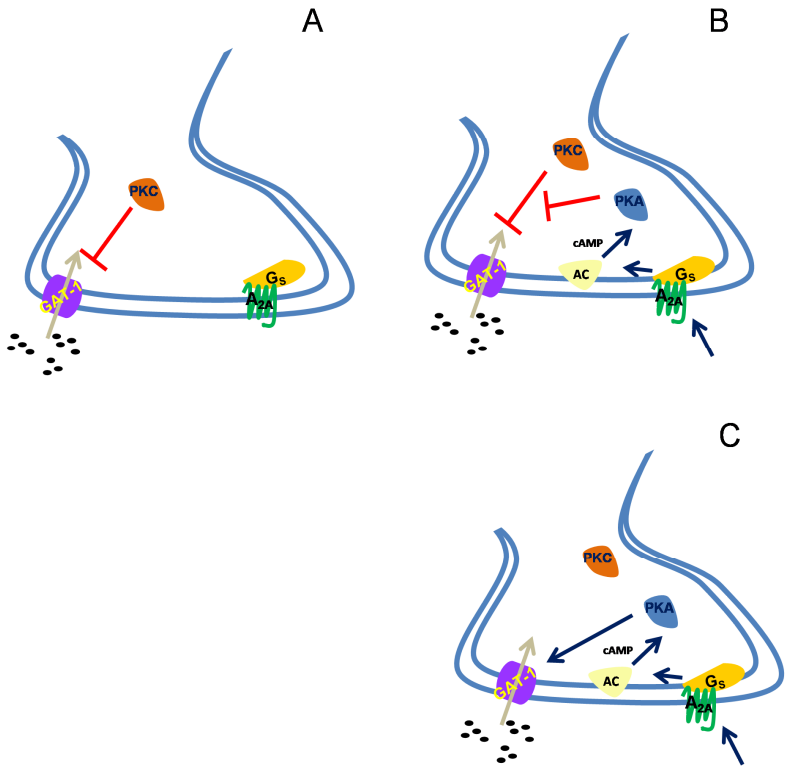

Figure 5.8 – Schematic representation of the influence of A_{2A}R upon GAT-1 mediated GABA transport into nerve endings. PKC is tonically restraining GABA uptake by hippocampal nerve endings through GAT-1 (A). The activation of A_{2A} receptors leads to the activation of AC/PKA pathway, which reduces PKC-mediated tonic inhibition upon GAT-1 (B), resulting in an enhancement of GAT-1-mediated GABA uptake (C).

5.2 Modulation of astrocytic GABA Transport by Adenosine A_1–A_{2A} Receptor Heteromers

5.2.1 Rationale

Astrocytes modulate synaptic transmission because they can release and uptake neurotransmitters (Hamilton and Attwell, 2010) and, therefore, fine tune the balance between excitation and inhibition. GABA is the main inhibitory neurotransmitter in the CNS, playing a crucial role in the control of excitability (Krnjevic and Schartz, 1967), plasticity (Artola and Singer, 1987), and network synchronization (Blatow *et al.,* 2003). These actions depend on changes in the extracellular concentrations of GABA, which are under control of GABA transporters (GATs) expressed in both neurons and astrocytes (Minelli *et al.,* 1995, 1996). Cortical astrocytes express GAT-1 and GAT-3 subtypes, and it has been estimated that ≈ 20% of extracellular GABA may be taken up into astrocytes (Hertz and Schousboe, 1987). Astrocytes release large amounts of ATP, which is then hydrolyzed into adenosine by the action of ecto-nucleotidases (Hamilton and Attwell, 2010). Extracellular adenosine operates through G-protein-coupled receptors. In the case of neural cells, the adenosine A_1 and A_{2A} receptors are those that are most likely activated by basal levels of extracellular adenosine. The A_1 receptors are often inhibitory and couple to $G_{i/o}$-proteins, whereas the A_{2A} receptors are usually coupled to G_s-proteins, enhancing cAMP accumulation and PKA activity (Fredholm *et al.,* 2001). A_1 and A_{2A}

receptors may closely interact in such a way that activation of A_{2A} receptors can lead to inhibition of A_1 receptors-mediated responses (Correia-de-Sá and Ribeiro, 1994; Cunha *et al.,* 1994; Lopes *et al.,* 1999). Some interactions may occur at the functional and transduction system levels (Sebastião and Ribeiro, 2000), but energy transfer assays in the form of bioluminescence (BRET) and fluorescent (FRET) resonance energy transfer have identified the presence of adenosine A_1–A_{2A} receptor heteromers in immortalized transfected cells (Ciruela *et al.,* 2006). In addition, coimmunoprecipitation experiments with presynaptic membranes showed that A_1–A_{2A} receptors heteromers modulate glutamate release from presynaptic nerve terminals (Ciruela *et al.,* 2006). Together, these data suggest a putative role of adenosine A_1–A_{2A} receptors heteromers in neurons. However, direct evidence for adenosine A_1–A_{2A} receptor heteromerization in neural cells is still lacking, since direct BRET evidence was obtained in immortalized cell lines and coimmunoprecipitation indicates strong interaction but not necessarily heteromerization of proteins. The main aim of the present chapter was to clarify whether adenosine A_1 and A_{2A} receptors modulate GAT-1- and/or GAT-3-mediated GABA transport into astrocytes, due to the role of these cells in overall GABA transport.

5.2.2 Endogenous adenosine tonically modulates GABA uptake

To assess the role of adenosine during GABA uptake, the astrocytes were incubated with different concentrations of CADO, an adenosine analogue with similar affinity for adenosine A_1 and A_{2A}

receptors that is resistant to hydrolysis or uptake by the cells. As can be observed in panel A of figure 5.9, at a relatively low CADO concentration (0.3 μM), there was an inhibition of total [^{3}H]GABA uptake by astrocytes (21 ± 3.8%, n=5, *p<0.05), whereas at higher concentrations (3–10 μM), CADO facilitated total [^{3}H]GABA uptake (33 ± 9.5%, n=5, *p<0.05 and 24 ± 2.8%, n=4, *p< 0.05, for 3 and 10 μM respectively). This biphasic influence on GABA transport could be either attributable to activation of different adenosine receptors, namely adenosine A_1 and A_{2A} receptors, or a differential influence over the two GATs present in astrocytes, GAT-1 and GAT-3 (Fig. 5.9.F, 5.9.G). Hence, GAT-1 or GAT-3 activity was independently assayed. The removal of endogenous adenosine with ADA (1U/ml) led to a decrease in GABA transport, and this decrease was highly significant when transport was mediated by either GAT-1 (24 ± 5.0%, n=4, *p<0.01; Figure 5.9.B) or GAT-3 (18 ± 3.6%, n=4, *p<0.01; Figure 5.9.C), suggesting that extracellular adenosine is tonically facilitating GAT-1 and GAT-3 activity. To avoid occupation of adenosine receptors with the endogenous ligand, all subsequent transport assays were performed in cells preincubated with ADA (1 U/ml).

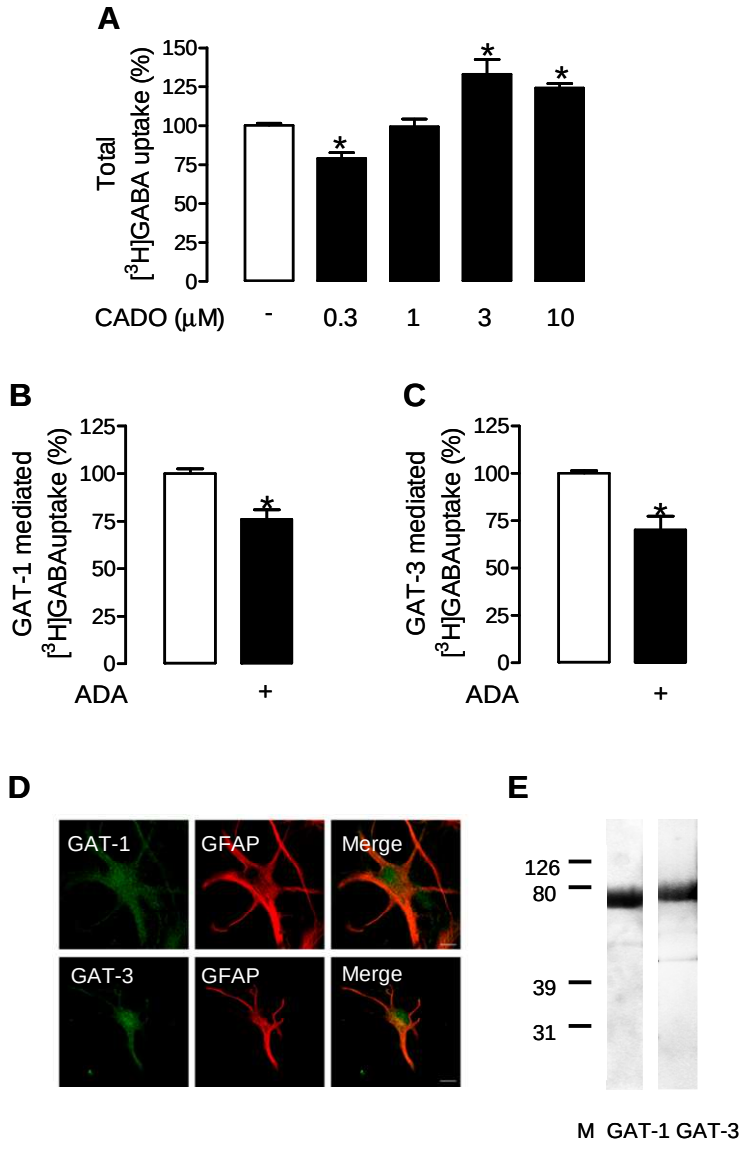
A
Total [3H]GABA uptake (%)
150
125
100
75
50
25
0
CADO (μM)
-
0.3
1
3
10
B
GAT-1 mediated [3H]GABAuptake (%)
125
100
75
50
25
0
ADA
+
C
GAT-3 mediated [3H]GABAuptake (%)
125
100
75
50
25
0
ADA
+
D
GAT-1
GFAP
Merge
GAT-3
GFAP
Merge
E
126
80
39
31
M GAT-1 GAT-3

Figure 5.9 – Adenosine receptor activation modulates [^{3}H]GABA uptake in astrocytes. Panel A illustrate the effect mediated by CADO upon [^{3}H]GABA uptake. Astrocytes were incubated with medium or with Increasing CADO concentrations and the total [^{3}H]GABA uptake was determined. CADO had a biphasic effect, at 0.3µM, CADO decreased [^{3}H]GABA uptake (21 ± 3.8%, n=5, *p<0.05) while at higher CADO concentration (3-10µM, [^{3}H]GABA uptake was facilitated (33 ± 9.5%, n=5, * p<0.05 and 24 ± 2.8%, n=4, * p< 0.05, for 3 and 10 µM respectively. In panels B and C, it is shown the consequence of endogenous adenosine removal by ADA (1U/ml). Astrocytes that were incubated with (ADA 1U/ml) showed a decreased GABA transport both by GAT-1(24 ± 5.0%, n=4, * p<0.01) and by GAT-3 ((18 ± 3.6%, n=4, * p<0.01). In D, for immunohistochemistry analysis of GAT-1 (green, top row) and GAT-3 (green, bottom row) expression by astrocytes, GFAP (red) was used as astrocyte marker. In E, solubilized astrocytes were analyzed by SDS-PAGE and immunoblotted using rabbit anti-GAT-1 antibody (1:100) or rabbit anti-GAT-3 antibody (1:200) (M, molecular mass markers). Results in A–E are shown as mean ± SEM of four to five independent experiments. Statistical significance was calculated by one-way ANOVA, followed by Bonferroni's multiple comparison test or by student T test *p<0.05 compared with control (white bars).

5.2.3 Adenosine A_1 receptors activation decreased and adenosine A_{2A} receptors activation enhanced GABA uptake

Selective agonists of adenosine A_1 and A_{2A} receptors were used to assess the influence of the adenosine receptors on GABA transporters. The selective adenosine A_1 receptor agonist CPA (30 nM) decreased maximal velocity (V_{max}) of GABA transport mediated by GAT-1 (Figure 5.10.A) or GAT-3 (Figure 5.10.B), whereas the selective agonist for adenosine A_{2A} receptors, CGS 21680 (30 nM), enhanced Vmax for GAT-1 (Figure 5.10.A) and GAT-3 (Fig. 5.10.B), without affecting transport K_M values ($p < 0.05$, n = 6). These data indicate that adenosine receptor activation modified maximum

transport capacity rather than in the affinity of the transporters for GABA and that inhibition of GAT-1 and GAT-3 is mediated by adenosine A_1 receptors, whereas facilitation requires adenosine A_{2A} receptors activation.

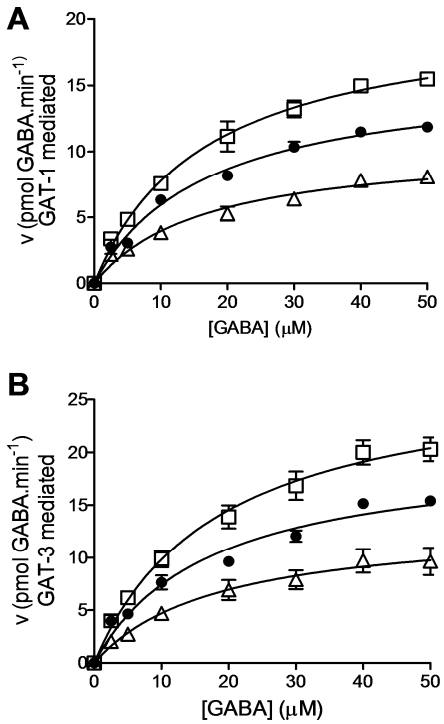

Figure 5.10 – Adenosine A_1 receptors activation decreased Vmax of GAT-1 and GAT-3 while adenosine A_{2A} receptors activation led to an enhancement of Vmax of both GAT-1 and GAT-3. The transport kinetics of GAT-1 (Panel A) and GAT-3 (Panel B) were determined using increasing [^{3}H]GABA concentrations. The adenosine A_{2A} receptors agonist CGS 21680 (30 nM, squares) enhanced Vmax of both GAT-1 and GAT-3 without modify the K_M. On the other hand, the adenosine A_1 receptors agonist CPA (30 nM, triangles) decreased Vmax of GAT-1 and GAT-3, with no changes in the K_M. statistical significance was calculated by *one-way* ANOVA followed by Bonferroni's multiple comparison test (n=6). Results are expressed in the tables 5.1 and 5.2 present in the next page.

Table 5.1 – GAT-1 transport kinetic constants (Vmax and K_M) in control conditions and after the activation of adenosine A_1 receptors (by CPA, 30nM) and the adenosine A_{2A} receptors (by CGS 21680 30nm). Results are present as Mean ± SEM. statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test (* $p < 0.05$, n=6).

	Control	A_1R Activation	A_{2A}R activation
Vmax (pmol GABA/min)	15 ± 0.9	8 ± 0.3 *	25 ± 1.7 *
K_M (μM)	5.0 ± 0.8	6 ± 0.9	4.9 ± 0.5

Table 5.2 – GAT-3 transport kinetic constants (Vmax and K_M) in control conditions and after the activation of adenosine A_1 receptors (by CPA, 30nM) and the adenosine A_{2A} receptors (by CGS 21680 30nm). Results are present as Mean ± SEM of six independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test (* $p < 0.05$).

	Control	A_1R Activation	A_{2A}R activation
Vmax (pmol GABA/min)	23 ± 1.6	14 ± 1.1 *	31 ± 1.6 *
K_M (μM)	18 ± 2.7	18 ± 1.9	18 ± 2.2

To further confirm that adenosine A_1 and A_{2A} receptors affect GABA transport in opposite ways, combinations of selective agonists and antagonists for either receptor (Table 5.3) were used. For these experiments, the concentration of each compound was chosen to act in a selective way. Results are summarized in Figures 5.11 and 5.12. The effect of adenosine A_1 receptor selective agonist (CPA,

30nM) and of the adenosine A_{2A} receptor selective agonist (CGS 21680, 30nM) effect were tested in the presence of the adenosine A_1 receptor selective antagonist (DPCPX, 50 nM) or the adenosine A_{2A} receptor selective antagonist (SCH 5826, 50 nM). Surprisingly, the effect of the adenosine A_1 receptor agonist was fully prevented not only by previous blockade of the adenosine A_1 receptors with DPCPX but also by the blockade of adenosine A_{2A} receptors with SCH 58261. Analogously, facilitation of GABA transport by the adenosine A_{2A} receptors agonist CGS 21680 was completely abolished by the blockade of either adenosine A_{2A} or A_1 receptors. These results strongly indicate that adenosine A_1 and A_{2A} receptors are tightly interacting and represent a clear example of cross antagonism between the two receptors. Such antagonism may be attributable to heteromerization (Ferrada *et al.,* 2009; Moreno *et al.,* 2011); thus, it was decided to test whether adenosine A_1 and A_{2A} receptors may form heteromers in astrocytes.

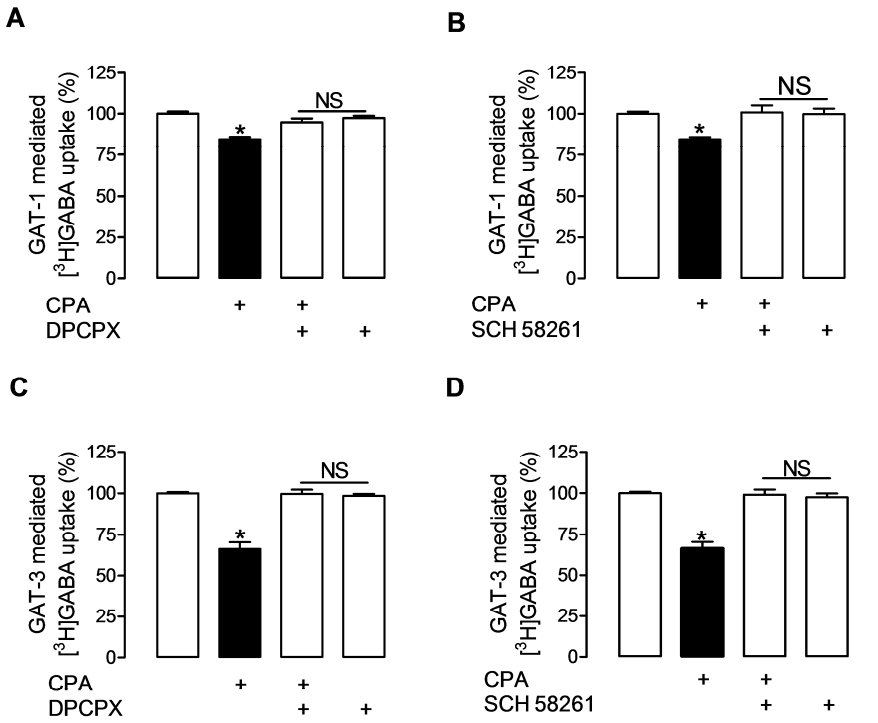

Figure 5.11 – Inhibition of [^{3}H]GABA uptake is promoted by adenosine A_1 receptors. Astrocytes were treated for 15 min with ADA (1 U/ml) before the addition of medium, the adenosine A_1 receptor antagonist DPCPX (50 nM), or the adenosine A_{2A} receptor antagonist SCH 58261 (50 nM). After 20 min, the adenosine A_1 receptor agonist CPA (30 nM) was added, and the GAT-1 (A, B) or GAT-3 (C, D) mediated [^{3}H]GABA uptake was measured as indicated in Methods chapter. Adenosine A_1 receptors activation decreased [^{3}H]GABA uptake by GAT-1 (16 ± 1.5%) and by GAT-3 (34 ± 4.3%). The adenosine A_1 receptors mediated effect was abolished by the previous blockade of adenosine A_1 receptors and also A_{2A} receptors. Results are mean ± SEM of six to eight independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test; * $p < 0.001$ compared with control (white bar); NS, $p > 0.05$.

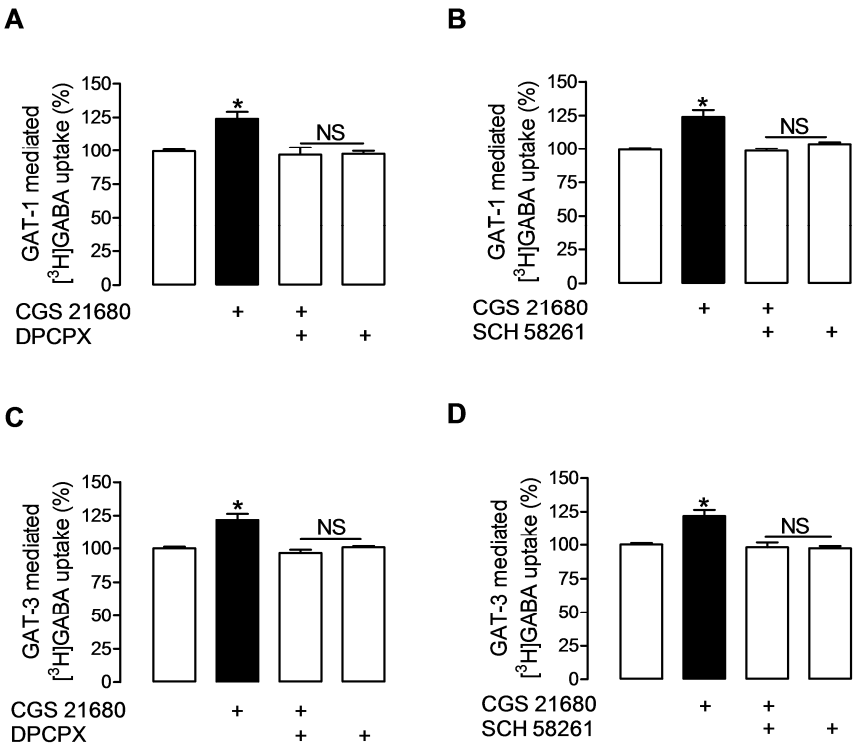

Figure 5.12 – Adenosine A_{2A} receptors activation facilitates [^{3}H]GABA uptake. Astrocytes were treated for 15 min with 1 U/ml ADA before the addition of medium, the adenosine A_1 receptor antagonist DPCPX (50 nM), or the adenosineA_{2A} receptor antagonist SCH 58261 (50 nM). After 20 min, the adenosine A_{2A} receptor agonist CGS 21680 (30 nM) was added, and the GAT-1 (A, B) or GAT-3 (C, D) mediated [^{3}H]GABA uptake was measured as indicated in Methods. Adenosine A_{2A} receptors activation increased [^{3}H]GABA uptake by GAT-1 (24 ± 5.2%) and by GAT-3 (22 ± 4.6%). The blockade of adenosine A_1 or A_{2A} receptors totally prevented GABA uptake facilitation mediated by adenosine A_{2A} receptors activation. Results are mean ± SEM of six independent experiments. Statistical significance was calculated by one-way ANOVA followed by Bonferroni's multiple comparison test; * $p < 0.001$ compared with control (white bar); NS, $p > 0.05$.

Table 5.3 – Binding affinity of agonists and antagonists of adenosine receptors (Ki values with 95% confidence intervals or ± S.E.M in parentheses) (adapted from Fredholm *et al.,* 2001).

Drug	A_1R	$A_{2A}R$	$A_{2B}R$	A_3R
CPA	2.3 (1.5-3.4) [a]	790 (470-1,360) [a]	34,400 (±11,100) [b] 21,000 (±4,300) [d]	43 (30-61) [a]
CGS 21680	290 (230-360) [a]	27 (12-59) [a]	361,000 (±21,100) [b]	67 (50-90) [a]
DPCPX	3.9 (3.5-4.2) [a]	129 (35-260) [a]	50 (±3.7) [b] 51 (±6.1) [d]	4,000 (2,600-6,000) [a]
SCH 58261	290 (210-410) [c]	0.6 (0.5-0.7) [c]		>10,000 [c]

a-Klotz *et al.*, 1998; b- Linden *et al*., 1999; c -Ongini *et al.,* 1999; d-Ji and Jacobson, 1999

5.2.4 Adenosine A_1 -A_{2A} receptor heteromers in astrocytes

The Bioluminescence Resonance Energy Transfer (BRET) approach was used to evaluate the ability of adenosine A_1 receptors to heteromerize with adenosine A_{2A} receptors in astrocytes. Adenosine A_1 and A_{2A} receptors expression is relatively low at 2 weeks of cell culture but increases later on. Thus, to avoid competition with endogenous receptors, BRET measurements were performed using

2-week cultured astrocytes transiently co-transfected with a constant amount of A_{2A}R-RLuc (7.5 µg cDNA) and increasing amounts of A_1R-YFP (4 to 15µg cDNA). Fusion of RLuc to A_{2A} receptor or to YFP to A_1 receptor did not modify receptor function as determined by cAMP assays (Canals *et al.,* 2003). A positive and saturable BRET signal was found for the pair A_{2A}R-RLuc and A_1R-YFP (Figure 5.13.A). From the saturation curve, a $BRET_{max}$ of 94 ± 15 mBU and a $BRET_{50}$ of 16 ± 2 were calculated. As a negative control the A_{2A}R-RLuc and serotonin $5HT_{2B}$R-YFP pair was used. As shown in Figure 5.13.A the negative control gave a linear non-specific BRET signal, thus confirming the specificity of the interaction between A_{2A}R-RLuc and A_1R-YFP in astrocyte primary cultures.

Ligand binding assays to receptor heteromers in isolated membranes usually reveal a "biochemical fingerprint" which consists of changes in ligand binding characteristics of one receptor when the partner receptor is occupied by agonist (Ferré *et al.,* 2009). No intracellular cross-talk can occur in disrupted membranes and therefore it can be assumed that the "fingerprint" results from intramembrane receptor-receptor interactions. Although an indirect approach, it is accepted as identifier of receptor heteromers in native tissues or in cells expressing the natural non-heterologous receptors (Ferré *et al.,* 2009). Therefore, binding experiments were performed in collaboration with Prof. Rafael Franco's group (University of Barcelona) to identify native adenosine A_1-A_{2A} receptors heteromers in 4-week cultured astrocytes. As shown in Figure 5.13.B, the displacement of adenosine A_1 receptors agonist

[^{3}H]R-PIA binding by the adenosine A_{2A} receptor agonist, CGS 21680 (but not by the adenosine A_{2A} receptor antagonist, SCH 58261) was significantly (p<0.01) better represented by a biphasic than by a monophasic curve. It is not expected that the adenosine A_{2A} receptors agonist, at concentrations lower than 500 nM, would significantly bind to adenosine A_1 receptors (<1% binding to adenosine A_1 receptors, according to known K_D value). However, 500 nM CGS 21680 significantly (p< 0.05) displaced the binding of the selective adenosine A_1 receptor agonist, [^{3}H]R-PIA, with an IC_{50} value of 90 $\pm$ 30 nM. Obviously, higher concentrations of CGS 21680 caused a further displacement of [^{3}H]R-PIA binding that, according to its IC_{50} value (8 $\pm$ 4 μM), reflects the binding of CGS 21680 to the adenosine A_1 receptors. As expected, the adenosine A_{2A} receptors antagonist, SCH 58261, only displaced the adenosine A_1 receptors agonist binding (IC_{50} of 500 $\pm$ 120 nM) at concentrations known to lose adenosine A_{2A} receptors selectivity and to bind to adenosine A_1 receptors (Fig. 5.13.B). Taken together these data indicate that the biphasic [^{3}H]R-PIA binding displacement curve observed in the presence of the A_{2A} receptor agonist constitutes the fingerprint of the adenosine A_1-A_{2A} receptor heteromer in non-transfected primary cultured astrocytes.

To evaluate whether adenosine A_1-A_{2A} receptors heteromerization could be influenced by agonist or antagonist binding, a series of experiments was performed in transiently co-transfected HEK-293T cells using a constant amount of A_{2A}R-RLuc (1.5μg cDNA) and increasing amounts of A_1R-YFP (1 to 8μg cDNA). In agreement with

previous results (Ciruela *et al.,* 2006), a positive and saturable BRET signal was found. Stimulation (20min) with the adenosine A_{2A} receptor agonist (CGS 21680, 30nM) (Figure 5.13.C), or with the adenosine A_1 receptor agonist (CPA, 30nM) (Figure 5.13.D) did not promote any consistent ($p>0.05$) change in $BRET_{max}$ or $BRET_{50}$ values. Similar BRET values were also obtained in the presence or absence of adenosine A_{2A} receptor (Figure 5.13.E) or adenosine A_1 receptor (Figure 5.13.F) antagonists indicating that neither agonist nor antagonist binding affected the receptor oligomerization state.

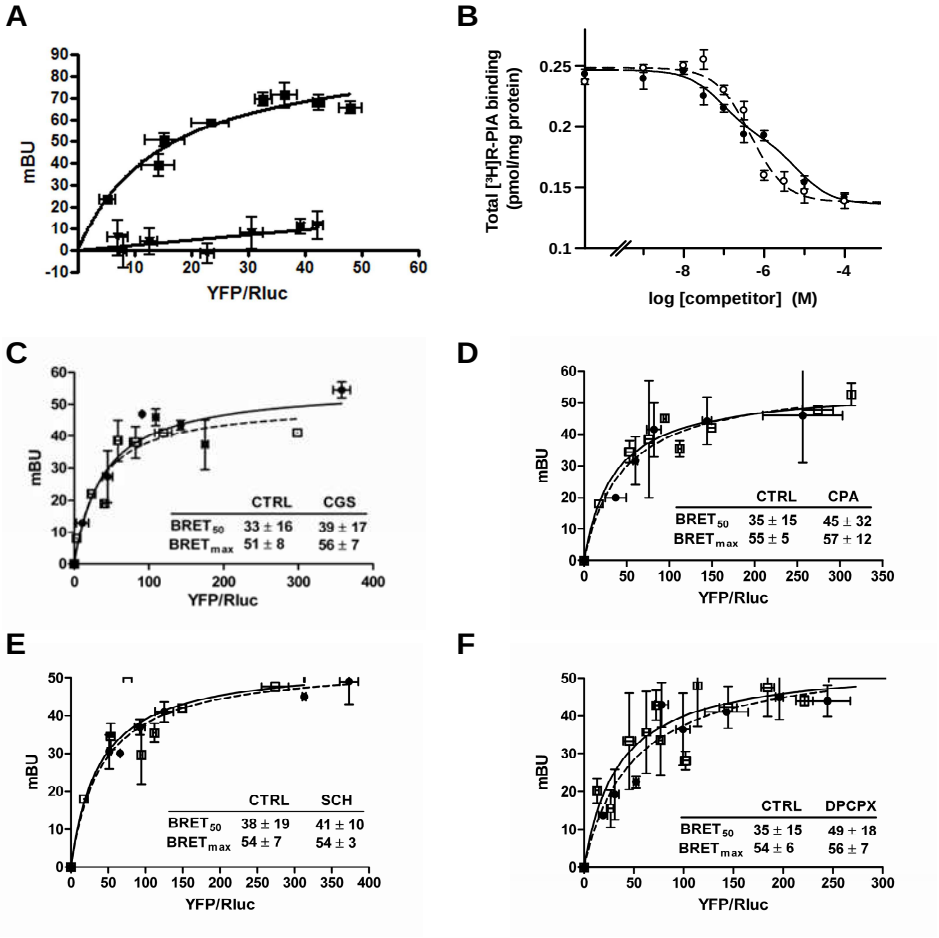
A
mBU
YFP/Rluc
B
Total [3H]R-PIA binding (pmol/mg protein)
log [competitor] (M)
C
mBU
YFP/Rluc
CTRL CGS
BRET50 33 ± 16 39 ± 17
BRETmax 51 ± 8 56 ± 7
D
mBU
YFP/Rluc
CTRL CPA
BRET50 35 ± 15 45 ± 32
BRETmax 55 ± 5 57 ± 12
E
mBU
YFP/Rluc
CTRL SCH
BRET50 38 ± 19 41 ± 10
BRETmax 54 ± 7 54 ± 3
F
mBU
YFP/Rluc
CTRL DPCPX
BRET50 35 ± 15 49 ± 18
BRETmax 54 ± 6 56 ± 7

Figure 5.13 – Adenosine A_1-A_{2A} receptor heteromers in astrocytes. In (A, C, D, E, F) BRET saturation experiments were performed using 2 weeks cultured astrocytes (A) or HEK-293 cells (C-F) co-transfected with 1.5 µg (A) or 1µg (C-F) cDNA corresponding to A_{2A}R-Rluc and increasing amounts of cDNA corresponding to A_1R-YFP (squares) or $5HT_{2B}$-YFP (triangles, as negative control) constructs. In (C to F) cells were treated for 10 min with medium (squares, solid line) or with 30nM CGS 21680 (C), 30nM CPA (D), 50nM SCH 58261 (E) or 50nM DPCPX (F) (circles, dotted lines). The $BRET_{max}$ and $BRET_{50}$ values are shown in the inserts. Both fluorescence and luminescence of each sample were measured before every experiment to confirm similar donor expressions (about 100,000 luminescent units) while monitoring the increase acceptor expression (500 to 10,000 fluorescent units). Data are means ± S.D. of 3 different experiments grouped as a function of the amount of BRET acceptor. In (B) competition experiments of 0.8 nM [^{3}H]R-PIA versus increasing concentrations of the A_{2A} receptor agonist, CGS 21680 (solid line), or the A_{2A} receptor antagonist, SCH 58261 (dotted line), were performed using astrocytic membranes (0.18 mg protein/ml). Experiments in B have been performed by a collaborator (Vicent Casadó) at University of Barcelona. Data are mean ± S.E.M. of a representative experiment (n = 3) performed in triplicates.

5.2.5 Adenosine A_1 or A_{2A} receptors activation, but not its blockade, leads to internalization of the A_1-A_{2A} receptor heteromers

Heteromerized receptors are expected to internalize together. To test this possibility, agonist-mediated internalization of adenosine A_1 receptors and A_{2A} receptors was studied in astrocytes. Western blot data clearly showed that adenosine A_1 receptors immunoreactivity at the cell surface did not only decrease after incubation of astrocytes with the adenosine A_1 receptor agonist (21 ± 0.6%), but also after incubation with the adenosine A_{2A} receptor agonist (20 ± 1.5%), as can be seen in figure 5.14.A. This decrease was

accompanied by an increase in A_1 receptors immunoreactivity in the intracellular fraction (36 ± 4.9%, after adenosine A_1 receptors activation; 28± 4.2%, after adenosine A_{2A} receptors activation), which is illustrated in the figure 5.14.B. No significant changes in surface (Figure 5.14.C) or intracellular (Figure 5.14.D) adenosine A_1 receptors immunoreactivity were detected upon incubation with either adenosine A_1 or A_{2A} receptors antagonists. Interestingly, when the adenosine A_1 receptor agonist was added after previous blockade of either adenosine A_1 or A_{2A} receptors by the selective antagonists, it was no longer able to modify adenosine A_1 receptors immunoreactivity at the cell surface (Figure 5.14.E) or in the intracellular fraction (Fig. 5.14.F). Similarly, adding the adenosine A_{2A} receptor agonist after a previous blockade of adenosine A_1 or A_{2A} receptors did not promote any modification of adenosine A_1 receptors immunoreactivity at cell surface (Figure 5.14.E) or in the intracellular fraction (Fig. 5.14.F). It therefore becomes clear that blockade of either adenosine A_1 or A_{2A} receptors prevents adenosine A_1 receptors internalization induced by exposure to adenosine A_1 or A_{2A} receptor agonists.

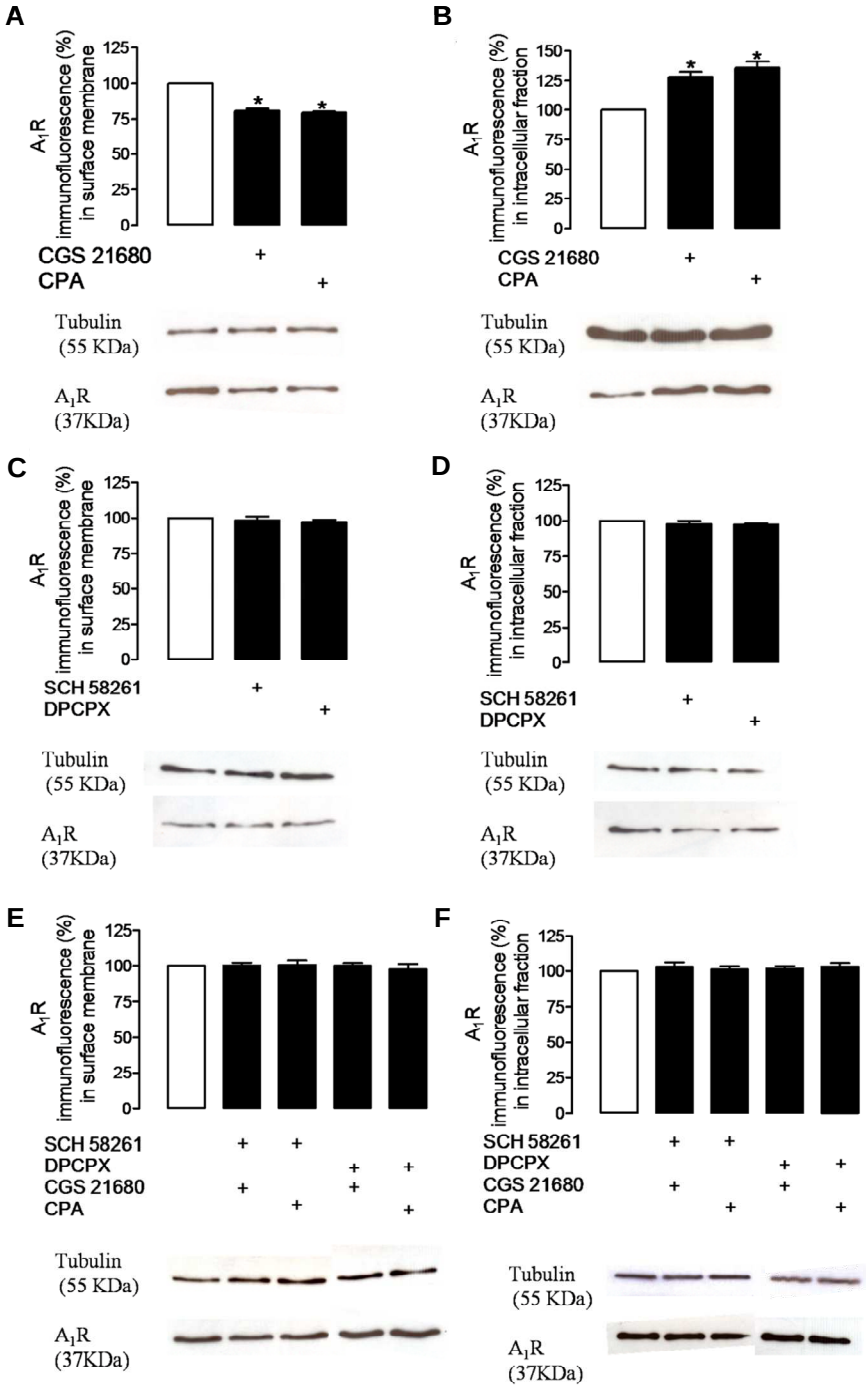
A
A1R immunofluorescence (%) in surface membrane
125
100
75
50
25
0
*
*
CGS 21680
CPA
+
+
Tubulin (55 KDa)
A1R (37KDa)
B
A1R immunofluorescence (%) in intracellular fraction
150
125
100
75
50
25
0
*
*
CGS 21680
CPA
+
+
Tubulin (55 KDa)
A1R (37KDa)
C
A1R immunofluorescence (%) in surface membrane
125
100
75
50
25
0
SCH 58261
DPCPX
+
+
Tubulin (55 KDa)
A1R (37KDa)
D
A1R immunofluorescence (%) in intracellular fraction
125
100
75
50
25
0
SCH 58261
DPCPX
+
+
Tubulin (55 KDa)
A1R (37KDa)
E
A1R immunofluorescence (%) in surface membrane
125
100
75
50
25
0
SCH 58261
DPCPX
CGS 21680
CPA
+ +
+ +
+ +
+ +
Tubulin (55 KDa)
A1R (37KDa)
F
A1R immunofluorescence (%) in intracellular fraction
125
100
75
50
25
0
SCH 58261
DPCPX
CGS 21680
CPA
+ +
+ +
+ +
+ +
Tubulin (55 KDa)
A1R (37KDa)

Figure 5.14 – Adenosine A_1 or A_{2A} receptors activation (but not their blockade) in astrocytes promotes internalization of adenosine A_1-A_{2A} receptors heteromers. Astrocytes were incubated for 30 min with the adenosine A_1 receptor agonist CPA (30 nM) or with the adenosine A_{2A} receptor agonist CGS 21680 (30 nM), alone (A and B) or in the presence of either the adenosine A_1 receptor antagonist DPCPX (50 nM) or the adenosine A_{2A} receptor antagonist SCH 58261 (50 nM) (E and F), or only with DPCPX (50 nM) or SCH 58261 (50 nM) (C and D), before starting the biotinylation protocol. When testing the action of agonists in the presence of antagonists, the antagonists were added 15 min before the agonists. Adenosine A_1 receptors expression at surface membranes (left panels) and intracellular fraction (right panels) was determined as indicated in Methods. Results are mean ± S.E.M of 5 independent experiments. Statistical significance was calculated by *one way* ANOVA followed by Bonferroni's multiple comparison test; *p<0.001 compared with control (100%, white bar).

5.2.6 The adenosine A_1-A_{2A} receptor heteromer is coupled to $G_{i/0}$ and G_s proteins

To figure out which G protein(s) is (are) coupled to the adenosine A_1-A_A receptors heteromer, assays of GTP-γ-[^{35}S] binding followed by immunoprecipitation using antibodies against different G proteins (G_s, $G_{i/0}$ and $G_{q/11}$) were performed. The approach is similar to that reported by Rashid and colleagues to identify G_q coupling to the dopamine D_1-D_2 receptor heteromer (Rashid *et al.,* 2007). As illustrated in Figure 5.15.A, the adenosine A_1 receptor selective agonist (CPA, 30nM) but not the adenosine A_{2A} receptor selective agonist CGS 21680 (30nM) significantly increased the $G_{i/0}$ activity, an effect unpredictably prevented by the adenosine A_{2A} receptor selective antagonist. In what concerns G_s activity (Figure 5.15.B), it was enhanced by the adenosine A_{2A} receptor selective agonist CGS 21680 (30nM), but not by the adenosine A_1 receptor agonist, CPA (30nM); again, and unpredictably, the effect of the adenosine A_{2A}

receptor agonist was fully abolished by the adenosine A_1 receptor antagonist, DPCPX (50nM). None of the adenosine receptor agonists affected $G_{q/11}$ activity, which was enhanced by acetylcholine (10μM), used as a positive control in the same batch of astrocytic membranes (Figure 5.15.C). These data suggest that adenosine A_1-A_{2A} receptor heteromers are coupled to both $G_{i/o}$ and G_s proteins and not to a unique $G_{q/11}$ protein.

G protein activity may be permanently modified by the binding of several toxins; thus these are useful tools to dissect out a differential receptor-G protein coupling in intact cells and to evaluate the functional consequences of G-protein-mediated-signaling blockade. GABA uptake assays were therefore performed using cholera toxin (ChTx), which uncouples G_s from the receptors due to ADP-ribosylation and permanent activation of the α_S-subunit (Gill and Meren, 1978), as well as using pertussis toxin (PTx), which catalyzes the ADP-ribosylation of the α $G_{i/0}$-subunit and locks it in the GDP-bound inactive state thus preventing $G_{i/0}$ protein activation (Bokoch and Gilman, 1984). Inhibition of either GAT-1- or GAT-3-mediated GABA uptake induced by the adenosine A_1 receptor agonist, CPA, was fully prevented by PTx, but, interestingly, this toxin also prevented adenosine A_{2A} receptor-mediated facilitation of GAT-1- and GAT-3-mediated GABA uptake (Figure 5.16). Similar results were obtained in the reciprocal experiment using ChTx. In fact the toxin prevented not only the facilitation of GAT-1- and GAT-3-mediated transport caused by the adenosine A_{2A} receptors agonist, CGS 21680, but also the inhibition of GABA transport mediated by the

adenosine A_1 receptors agonist, CPA (Figure 5.17). The ChTx and PTx data strongly suggest that the adenosine A_1-A_{2A} receptors heteromer is coupled to both G_s and G_i proteins. The results also indicate that if one G protein (G_s or G_i) is blocked or receptor-uncoupled, both adenosine A_1 and A_{2A} receptors agonists lose their effect upon GABA uptake. It seems therefore that the adenosine A_1-A_{2A} receptor heteromer coupled to both $G_{i/0}$ and G_s is the mediator of both the inhibitory and the excitatory effects triggered by, respectively, CPA and CGS 21680.

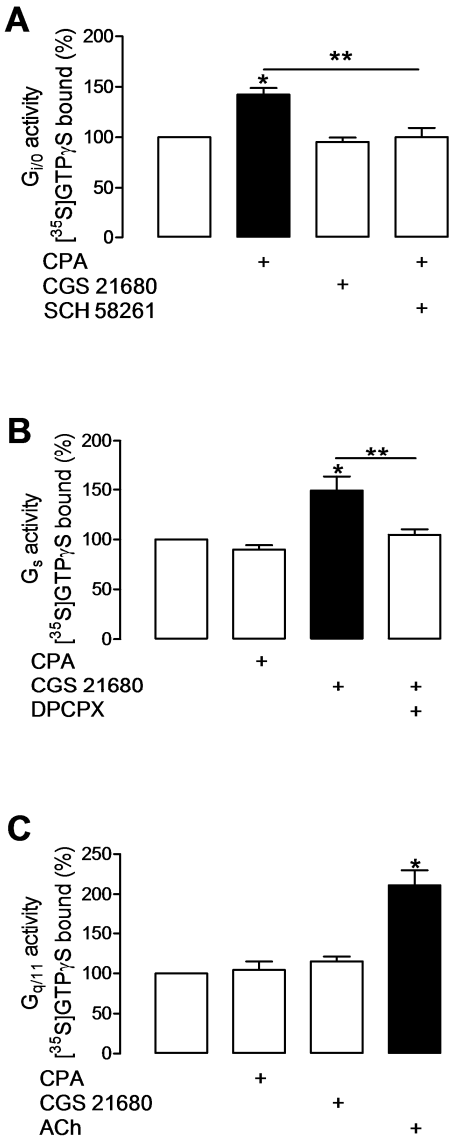
A
$G_{i/0}$ activity
[^{35}S]GTPγS bound (%)
200
150
100
50
0
*
**
CPA
CGS 21680
SCH 58261
+
+
+
+
B
G_s activity
[^{35}S]GTPγS bound (%)
200
150
100
50
0
*
**
CPA
CGS 21680
DPCPX
+
+
+
+
C
$G_{q/11}$ activity
[^{35}S]GTPγS bound (%)
250
200
150
100
50
0
*
CPA
CGS 21680
ACh
+
+
+

Figure 5.15 – [^{35}S]GTP-γ-S assays suggest the involvement of both G_s and $G_{i/0}$ in the adenosine A_1-A_{2A} receptors heteromer. GTP-γ-[^{35}S] assays was performed as described in Methods to test $G_{i/0}$ activity (A), G_s activity (B) or $G_{q/11}$ activity (C) using membranes from astrocytes treated for 10 min with medium, the adenosine A_{2A} receptor antagonist SCH 58261 (50 nM) or the adenosine A_1 receptor antagonist DPCPX (50 nM) prior the activation with adenosine A_{2A} receptor agonist CGS 21680 (30 nM) or adenosine A_1 receptor agonist CPA (30 nM) or Ach (10 μM) as positive control. In Panel A, it is shown that only the activation of adenosine A_1 receptors (CPA, 30nM) led to an increase in $G_{i/0}$ activity (42 ±6.4%). This effect was totally abolished by the previous blockade of adenosine A_{2A} receptors. The activation of adenosine A_{2A} receptors was unable to modify $G_{i/0}$ activity. In panel B is illustrated the modulation of Gs activity. The adenosine A_1 receptor agonist did not modify the G_s activity. The adenosine A_{2A} receptor activation led to an enhancement of G_s activity (49 ± 14%). The previous blockade of adenosine A_1 receptors prevented this increase mediated by adenosine A_{2A} receptors activation. The panel C illustrates that only acetylcholine can increase $G_{q/11}$ activity (111 ±19%). Both adenosine A_1 and A_{2A} receptors activation were unable to modify $G_{q/11}$ activity. Results are as mean ± S.E.M from 5-8 independent experiments. Statistical significance was calculated by *one way* ANOVA followed by Bonferroni's multiple comparison test; *p<0.001 compared with control (100%, white bar), ** p<0.001 compared with cells treated only with the agonist.

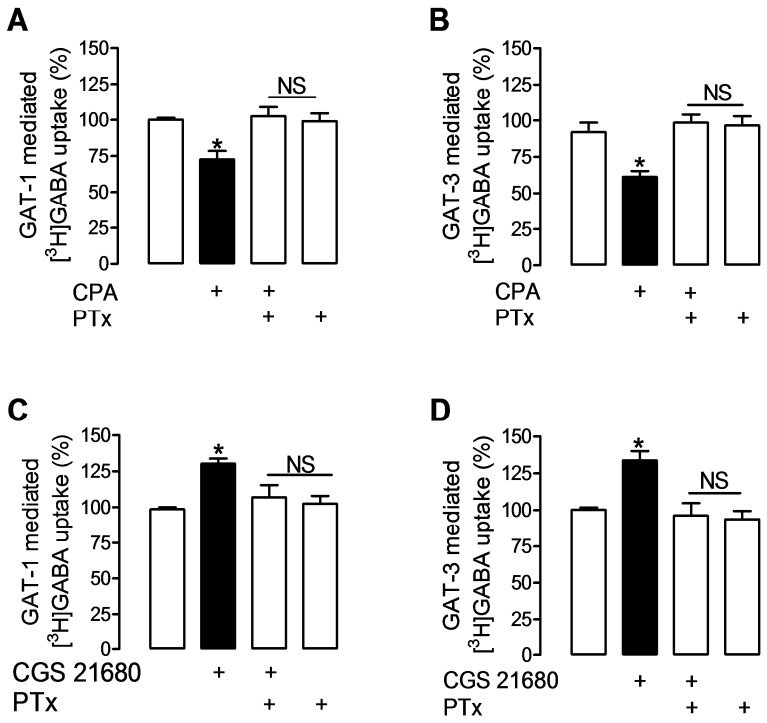

Figure 5.16 – Blockade of $G_{i/0}$ proteins by PTx prevents both effects mediated by adenosine A_1 and A_{2A} receptors. Astrocytes were treated with medium, PTx (5μg/ml) prior to stimulation with CPA (30 nM) or CGS 21680 (30 nM) and GAT-1 and GAT-3 mediated [^{3}H]GABA uptake was measured as indicated in Methods. PTx were pre-incubated with the astrocytes for 4h and then removed prior to uptake assays. The inhibitory effect mediated by adenosine A_1 receptors (27 ± 5.8%, for GAT-1; 39 ± 3.9% for GAT-3) was totally prevented by the previous incubation of astrocytes with the toxin. PTx was also able to prevent the facilitatory effect mediated by adenosine A_{2A} receptors (30 ± 3.5%, for GAT-1; 33 ± 6.4% for GAT-3). Results are as mean ± S.E.M from 4-6 independent experiments. Statistical significance was calculated by *one way* ANOVA followed by Bonferroni's's multiple comparison test; *p<0.01; NS: P>0.05.

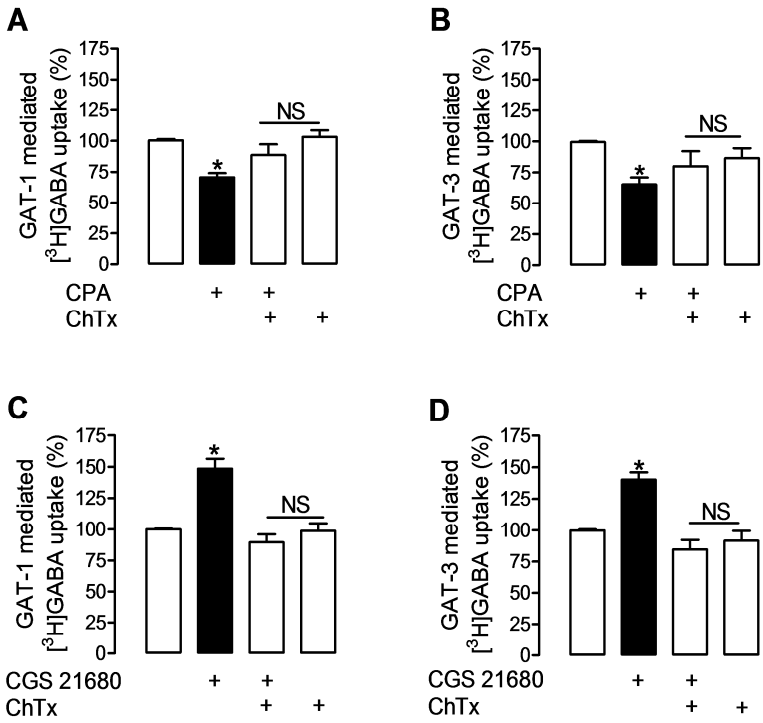

Figure 5.17 – Blockade of G_s proteins by ChTx prevents both effects mediated by adenosine A_1 and A_{2A} receptors. Astrocytes were treated with medium, ChTx (5μg/ml) prior to stimulation with CPA (30 nM) or CGS 21680 (30 nM) and GAT-1 and GAT-3 mediated [^{3}H]GABA uptake was measured as indicated in Methods. ChTx was pre-incubated with the astrocytes for 4h and then removed prior to uptake assays. The excitatory effect mediated by $A_{2A}R$ (48 ± 8.0%, for GAT-1; 40 ± 5.8% for GAT-3) was totally prevented by the previous incubation of astrocytes with the toxin. ChTx was also able to prevent the inhibitory effect mediated by A_1R (29 ± 3.3%, for GAT-1; 35 ± 5.6% for GAT-3). Results are as mean ± S.E.M from 4-6 independent experiments. Statistical significance was calculated by one way ANOVA followed by Bonferroni's multiple comparison test; *p<0.01; NS: P>0.05.

5.2.7 The A_1-A_{2A} receptor heteromer signals through AC/PKA pathway

The coupling of both G_s and $G_{i/0}$ to the adenosine A_1-A_{2A} receptors heteromer predicts that the transduction system operated by adenosine heteromer to modulate GABA transport into astrocytes is centered in the adenylate cyclase/cAMP/PKA cascade. To address the question of whether a single cAMP/PKA-centered transduction unit is able to both inhibit and facilitate GABA transport, it was tested the influence of drugs known to interfere with this transduction pathway upon the effect of adenosine A_1 and A_{2A} receptors agonists on GABA transport. In addition, phospholipase C (PLC) blocker was also tested, in order to evaluate a putative transduction pathway classically associated to $G_{q/11}$. The inhibitory action of adenosine A_1 receptors agonist upon GAT-1 (Figure 5.18.A) and GAT-3 (Figure 5.18.C) still occurred in the presence of PLC blocker U73122 (3µM, Smith *et al.,* 1990), but it was totally abolished by the blockade of PKA by Rp-cAMPs (100 µM, Wang *et al.,* 1991). The activation of adenylate cyclase (AC) with a supramaximal concentration of forskolin (10µM, Awad *et al.,* 1983), *per se,* increased GABA transport and occluded the inhibitory effect of the adenosine A_1 receptors agonist (CPA) upon GABA transport (Fig. 5.18.A and 5.18.C). The facilitatory effect of the adenosine A_{2A} receptors agonist, CGS21680, was not affected by the PLC inhibitor, U73122, but was totally impaired by the PKA blocker, Rp-cAMPs. The adenylate cyclase activator, forskolin, mimicked the action of the adenosine A_{2A} receptor agonist and its facilitatory effect was not

additive with that of CGS 21680, indicating a common mechanism (Fig. 5.18.B and 5.18.D).

It though appears that the adenosine A_1-A_{2A} receptor heteromer signals through the AC/cAMP/PKA cascade, which is in accordance with data obtained in the experiments designed to identify the G-proteins involved in heteromer signaling.

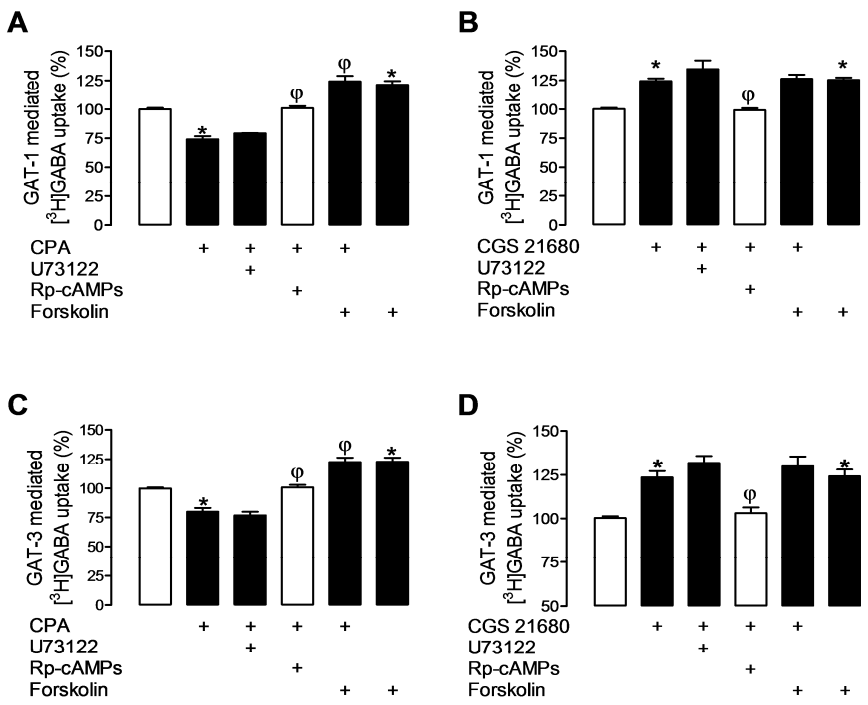

Figure 5.18 – Adenosine A_1-A_{2A} receptor heteromer signaling. Astrocytes were treated for 15min with 1U/ml ADA before the addition of medium, the PLC inhibitor U73122 (3 μM), the PKA inhibitor Rp-cAMPs (100 μM) or the adenylate cyclase enhancer forskolin (10 μM). After 20 min, the adenosine A_1 receptor agonist CPA (30 nM) (A and C) or the adenosine A_{2A} receptor agonist CGS 21680 (30 nM) (B and D) were added and the GAT-1 (A and B) or GAT-3 (C and D) mediated [^{3}H]GABA uptake was measured as indicated in Methods. Panel A and C show that the inhibitory effect mediated by CPA (26 ± 2.7% for GAT-1; 20 ± 3.3% for GAT-3) persisted when PLC was previous blocked by U73122 (21 ± 0.4% for GAT-1; 23 ± 3.1% for GAT-3) but was totally abolished by the PKA inhibition. The activation of AC by forskolin increased *per se* GABA uptake (21 ± 3.1% for GAT-1; 23 ± 3.6% for GAT-3) and avoided the inhibitory effect mediated by CPA. Panel B and D illustrate that the facilitatory effect mediated by CGS 21680 (24 ± 2.3% for GAT-1; 24 ± 3.7% for GAT-3) persisted when PLC was previous blocked by U73122 (35 ± 7.2% for GAT-1; 32 ± 3.9% for GAT-3) but was totally abolished by the PKA inhibition. The activation of AC by forskolin increased *per se* GABA uptake (26 ± 3.6% for GAT-1; 30 ± 5.6 for GAT-3). Both effects mediated by adenosine A_{2A} receptor agonist and by AC activator are not additive, suggesting a shared mechanism. Results are mean ± S.E.M from 4-10 independent experiments. Statistical significance was calculated by *one way* ANOVA followed by Bonferroni's multiple comparison test; *P<0.001 vs control (100%, white columns), $^{\varphi}$P<0.001 vs cells treated with the agonist alone.

5.2.8 Discussion

This work clearly shows that GABA uptake by astrocytes is under modulation by extracellular adenosine, which, by interacting with a functional unit constituted by adenosine A_1-A_{2A} receptor heteromers coupled to two distinct G proteins, $G_{i/o}$ and G_s, can either boost or depress the amount of inhibitory neurotransmitter available to neurons. Using an adenosine analogue, CADO, with high structural similarity to adenosine but with the advantage of not being taken up by the cells nor metabolized by ecto-enzymes, it was observed that submicromolar concentrations of this agonist inhibit GABA uptake while at low micromolar concentrations there is an enhancement of GABA transport. Considering that the affinity of adenosine for the adenosine A_1 receptors is slightly higher than for the adenosine A_{2A} receptors (Fredholm *et al.,* 2001), it is likely that the inhibition was mediated by adenosine A_1 receptors and facilitated by adenosine A_{2A} receptors. Accordingly, the adenosine A_1 receptor selective agonist, CPA, inhibited GABA uptake into astrocytes, while the adenosine A_{2A} receptor selective agonist, CGS 21680 facilitated it. Unexpectedly, the blockade of either receptor with selective antagonists prevented the effects mediated by either agonist, a strong indication that adenosine A_1 and A_{2A} receptors are interacting at the molecular level in primary cortical astrocytes. Abundant evidence of adenosine A_1-A_{2A} receptors functional crosstalk has been described, namely, adenosine A_{2A} receptors activation attenuates adenosine A_1 receptors-mediated responses in the hippocampus (Cunha *et al*., 1994; Lopes *et al*., 1999), at the neuromuscular junction (Correia-de-

Sá and Ribeiro, 1994), and in transfected cells (Ciruela *et al.*, 2006); however, no attempt has been made to unequivocally and directly identify adenosine A_1-A_{2A} receptors heteromerization in neural cells. Therefore, to figure out the presence of adenosine A_1-A_{2A} receptor heteromers in neural cells, BRET (bioluminescence resonance energy transfer) assays were performed in primary cultures of astrocytes that were transfected with cDNAs for A_{2A}R-RLuc and A_1R-YFP. In these assays a positive, specific and saturable BRET signal for the energy transference between A_{2A}R-RLuc and A_1R-YFP was detected in living primary astrocytes. Moreover the heteromer in native astrocytes was detected by looking for a "fingerprint" which consists of changes in ligand binding characteristics of adenosine A_1 receptor when the adenosine A_{2A} receptor is activated. These results complemented and strengthened the evidence for adenosine A_1-A_{2A} receptor heteromerization in non-transfected astrocytes.

Biotinylation assays showed that exposure to either adenosine A_1 or A_{2A} receptors agonists led to similar decreases in surface expression of adenosine A_1 receptors, and to similar increases in the adenosine A_1 receptors levels in intracellular fractions. It then appears that binding of a single ligand to the heteromer is sufficient to promote internalization of the two receptors. The previous blockade of adenosine A_1 or A_{2A} receptors prevents the heteromer internalization mediated by both adenosine A_1 and A_{2A} receptors agonists, suggesting that internalization of heteromer in response to agonists is a consequence of heteromer activity. Complementary immunohistochemistry assays in HEK cells coexpressing A_1R-YFP and

A_{2A}R-Rluc performed in collaboration with Prof. Rafael Franco group (University of Barcelona) corroborate this correlation (see Cristóvão-Ferreira *et al.,* 2011).

Agonist exposure did not affect BRET, suggesting that agonist binding does not induce pronounced allosteric modifications in the receptors. It therefore appears that the adenosine A_1-A_{2A} receptor heteromer in astrocytes mostly works as an integral entity, leaving and probably also reaching the cell surface as a heteromeric structure. Furthermore, since antagonists did not modify the receptor levels at the membrane or the BRET signal, the loss of effect of one agonist upon previous blockade the other receptor cannot be attributed to heteromer disruption or formation. Cross-antagonism, which is considered a heteromer fingerprint (Ferré *et al.,* 2009), is likely due to conformational changes induced by the antagonist and leads to a non-functional state of the signaling receptor by uncoupling it from G-protein-mediated signaling.

Heteromers may couple to G-proteins different from those to which each individual receptor partner usually couple. This is indeed the case of dopamine D_1-D_2 receptor heteromer, which couple to G_q (Rashid *et al.,* 2007) in a clear shift from the canonical D_1 coupling to G_s and D_2 coupling to $G_{i/0}$. To identify the transduction system operated by the adenosine A_1-A_{2A} receptor heteromer in astrocytes. I first used an approach similar to that used by Rashid and colleagues (Rashid *et al*., 2007), i.e. GTP-γ-[^{35}S] binding followed by differential immunoprecipitation. Data obtained allowed to conclude that the adenosine A_1-A_{2A} receptor heteromer in astrocytes seems to be

coupled to both $G_{i/0}$ and G_s proteins. Interestingly, the cross-antagonism was also evident in these assays. Thus, the adenosine A_1 receptor agonist, but not the adenosine A_{2A} receptor agonist, increased $G_{i/0}$ activity, but the enhancement was also prevented when the adenosine A_{2A} receptor antagonist was present. Reciprocally, G_s activation, which was restricted to the adenosine A_{2A} receptor agonist, was prevented when the adenosine A_1 receptors were blocked with the antagonist. Similar conclusions could be drawn from GABA uptake assays since toxin-induced prevention of coupling of receptors to either G_s or $G_{i/0}$ led to reciprocal impaired function of adenosine A_1 or A_{2A} receptors agonists to modulate GABA transport. Altogether these data provide strong evidence for a heteromeric functional entity regulating GABA uptake by astrocytes. This functional unit consists of an adenosine A_1-A_{2A}-$G_{i/0}$-G_s complex, which signals through G_s when the adenosine A_{2A} protomer is activated and through G_i when the adenosine A_1 protomer is activated. Most importantly, the blockade of a single partner in the complex led to adjustments in the whole unit. Due to size constrictions (Han *et al.,* 2009), the coupling of two different G proteins suggest that the minimal structure of adenosine A_1-A_{2A} receptor heteromer should be a tetramer, as two GPCR molecules cannot bind to more than a single G protein (Han *et al.,* 2009). Work done in collaboration supports this hypothesis. In detail, co-workers of this study, using BRET assays with a double luminescence/fluorescence molecular complementation approach, have showed that adenosine A_1-A_{2A} receptor heteromers appear as

heteromers of homomers with a minimal structure consisting of an A_1-A_1-A_{2A}-A_{2A} complex (Cristóvão-Ferreira *et al.,* 2011). The heterotetramer makes it possible to accommodate the two different G proteins. Data obtained also in collaboration using CellKey label-free assays (Cristóvão-Ferreira *et al.,* 2011) corroborate data acquired by me with GTP-γ-[^{35}S] binding assays followed by differential immunoprecipitation of G proteins and also with functional data with cholera and pertussis toxin, as described in this chapter. Furthermore, the transduction system operated by the heteromer seems to involve the adenylate cyclase, consistent with the G protein data and the data obtained in the presence of forskolin or of the inhibitor of the PKA (Rp-cAMPs), both of which occluded the effects of the A_1 or A_{2A} receptors selective agonists upon GABA uptake. Altogether data obtained strongly suggest that functional unit is an A_1-A_1-A_{2A}-A_{2A}-G_s-$G_{i/0}$ complex (Cristóvão-Ferreira *et al.,* 2011).

At submicromolar concentrations, the adenosine analogue CADO activated the inhibitory component of the heteromer, inhibiting GABA uptake. A ten-fold rise in concentration of the non-selective ligand, CADO, was enough to gate adenosine A_{2A} receptors activation and engages a completely opposite modulation of GABA uptake. Assuming a near 10 fold higher potency of CADO as compared to adenosine (Ribeiro and Sebastião, 1987), the shift from inhibition to enhancement of GABA uptake might occur at low micromolar concentrations of extracellular adenosine. These concentrations are easily attained at a tripartite synapse, where

astrocytes and neurons release considerable amounts of ATP, which are degraded into ADP, AMP and adenosine by ecto-5'-nucleotidases. The higher the release of ATP, as at high neuronal firing rates in reciprocal neuron-to-astrocyte communication at the tripartite synapse (see Fields and Burnstock, 2006), the higher the expected concentration of extracellular adenosine. It is therefore likely that sustained neuronal firing promotes activation of the adenosine A_{2A} protomer of the adenosine A_1-A_{2A} receptor heteromer leading to facilitation of GABA uptake. Activation of GABA uptake by astrocytes will lead to a decrease in ambient GABA and a subsequent depression of tonic GABAergic inhibition resulting in enhanced excitatory tonus. Conversely, at submicromolar adenosine concentrations, there is a preferential activation of the A_1 protomer of the adenosine A_1-A_{2A} receptors heteromer and GABA uptake by astrocytes would be inhibited and tonic inhibition by GABA would be enhanced. Thus, through an adenosine action upon adenosine A_1-A_{2A} receptor heteromers, astrocytes might behave as dual amplifiers, facilitating excitation at intense astrocytic to neuronal signaling and increasing inhibition at low neuronal firing rates. This switch in neural activity may require a highly efficient control to avoid sudden state transitions, and this seems to be the main advantage of heteromerization of adenosine A_1 and A_{2A} receptors in astrocytes. Indeed, overstimulation of just one of the receptor protomers leads to internalization of the whole functional unit, therefore allowing a double brake in the system and avoiding an abrupt inhibitory signaling and a sudden switch from excitation to

inhibition as a consequence of desensitization of only the excitatory protomer.

To conclude, the present work provides strong and complementary evidence that, upon GABA uptake, adenosine has a biphasic effect, which is mediated by adenosine A_1-A_{2A} receptor heteromers coupled to both $G_{i/o}$ and G_s proteins. Extracellular adenosine acting on these adenosine A_1-A_{2A} receptor functional units operates in a concerted way to balance a PKA-dependent action on GABA uptake as indicated in the scheme of Figure 5.19. The neural output would be inhibitory at low firing rates and facilitatory at high firing rates. This work therefore discloses a so far unknown way through which adenosine by acting on adenosine receptors in astrocytes may significantly contribute to neurotransmission in a dual manner, which depends on the concentration of the nucleoside that is in turn dependent on neuronal firing activity. This adds a novel conceptual way to understand the fundamental role of astrocytes at tripartite synapses.

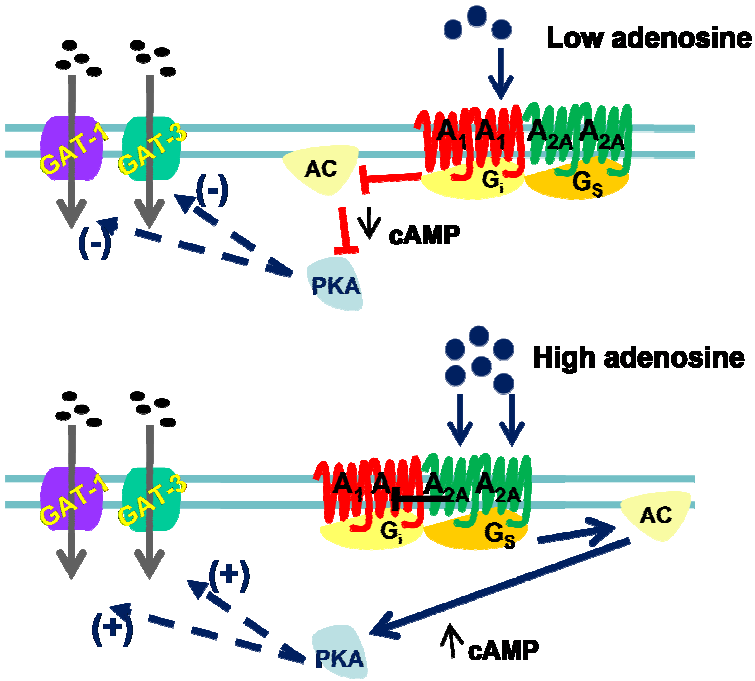

Figure 5.19 – Schematic representation of adenosine A_1-A_{2A} receptor heteromer function. At low levels, adenosine binds preferentially to the A_1 protomer of the heteromer, which will activate $G_{i/0}$ protein, and through a mechanism that involves adenylate cyclase (AC), and PKA activity, leads to a decrease (-) in GABA uptake mediated by GAT-1 and GAT-3. At higher concentrations, adenosine activates the A_{2A} protomer of the heteromer inhibiting A_1 and, through G_s protein, couples to the AC/cAMP/PKA pathway, leading an enhancement (+) of GABA uptake.

6 General Conclusions

The present work clearly demonstrates that adenosine, in a concentration dependent way, modulates GABA uptake into presynaptic terminals and astrocytes.

According to the tripartite synapse model, the study of GABA transport should include both presynaptic terminals and astrocytes, since both components are active partners of the synapse, having the ability to influence each other. Although, GABA is mainly taken up into neurons, glial uptake can assume an important role, at least in some conditions. Here, it is postulate, that adenosine, depending on its concentration, drives the uptake of GABA into presynaptic terminals or into glial cells, which affects the intracellular releasable pool of GABA, and consequently the phasic inhibitory transmission.

Results described in chapter 5.1 show that endogenous adenosine through activation of adenosine A_{2A} receptors promotes GAT-1-mediated GABA uptake into presynaptic terminals, an effect that occurs through activation of AC/PKA pathway. The increase of GABA uptake into presynaptic terminal accelerates the replenishment of GABA releasable pool at GABAergic nerve terminals, which is important, especially at periods of intense neuronal activity. On the other hand, the quick removal of GABA from the synapse minimizes GABA receptor desensitization at pos-synaptic neurons. Both effects contribute to the efficiency of the phasic inhibitory transmission.

At the subchapter 5.2, I showed that, in astrocytes, low concentrations of adenosine inhibit both GABA uptake mediated by

GAT-1 and by GAT-3. In contrast, at higher concentrations adenosine promotes the GAT-1 and GAT-3 mediated-GABA transport. The adenosine effects in astrocytes are mediated by the activation of adenosine A_1-A_{2A} receptors heteromers, which are coupled to both $G_{i/0}$ and G_s proteins. The inhibitory effect is mediated by the activation of the A_1 protomer, whose effects are due to the activation of $G_{i/0}$ proteins. The A_{2A} protomer is activated by higher adenosine levels, and trough the activation of Gs proteins is the responsible for the excitatory effects. The entire heteromer works as a unique entity, being functional just when all the components (receptors and G proteins) are available. Furthermore, results herein reported indicate that the heteromer also reach and leave the membrane as an unique entity. This is probably a protective effect, which avoids sudden state transitions, as the two protomers mediate opposite effects. In detail, the activation of adenosine A_1 receptors by low levels of adenosine mediates inhibitory effects and leads to the internalization of both adenosine A_1 and A_{2A} receptors. When adenosine levels rise, the A_{2A} protomer will be activated, mediating excitatory effects. But as the membrane expression of the A_{2A} receptor is already decreased (in response to previous activation of A_1 receptor), the amplitude of A_{2A} receptor-mediated effect will be reduced. In case of an activation of the A_{2A} protomer the whole unit will be further internalized, avoiding sudden state transition due to overactivation of inhibitory A_1 receptor in the absence of the compensatory A_{2A} receptor.

Putting all the results together, at low concentrations, adenosine decreases astrocytic uptake, driving GABA to presynaptic terminal, thus facilitating phasic inhibitory transmission, as the uptake to presynaptic terminals will accelerate the recycling of intracellular releasable GABA pool. At higher adenosine concentrations, GABA is driven to astrocytes, which will shunt GABA from the presynaptic terminal, delaying the recycling of neuronal intracellular pools. Besides, the increase of astrocytic uptake reduces ambient GABA levels, decreasing tonic inhibition. Both actions contribute to facilitation of excitatory transmission.

Due to adenosine modulation upon GABA transporters, adenosine can be seen as an amplifier of GABAergic tonus. For instance, in situations where the inhibitory tonus is predominant, adenosine drives GABA uptake into nerve terminals, facilitating phasic inhibition. In opposite, the adenosine excitatory mediated effect promotes GABA uptake into astrocytes, which reduces tonic inhibition, facilitating excitatory transmission. Thus, adenosine and GABA work synergistically in order to achieve brain homeostasis.

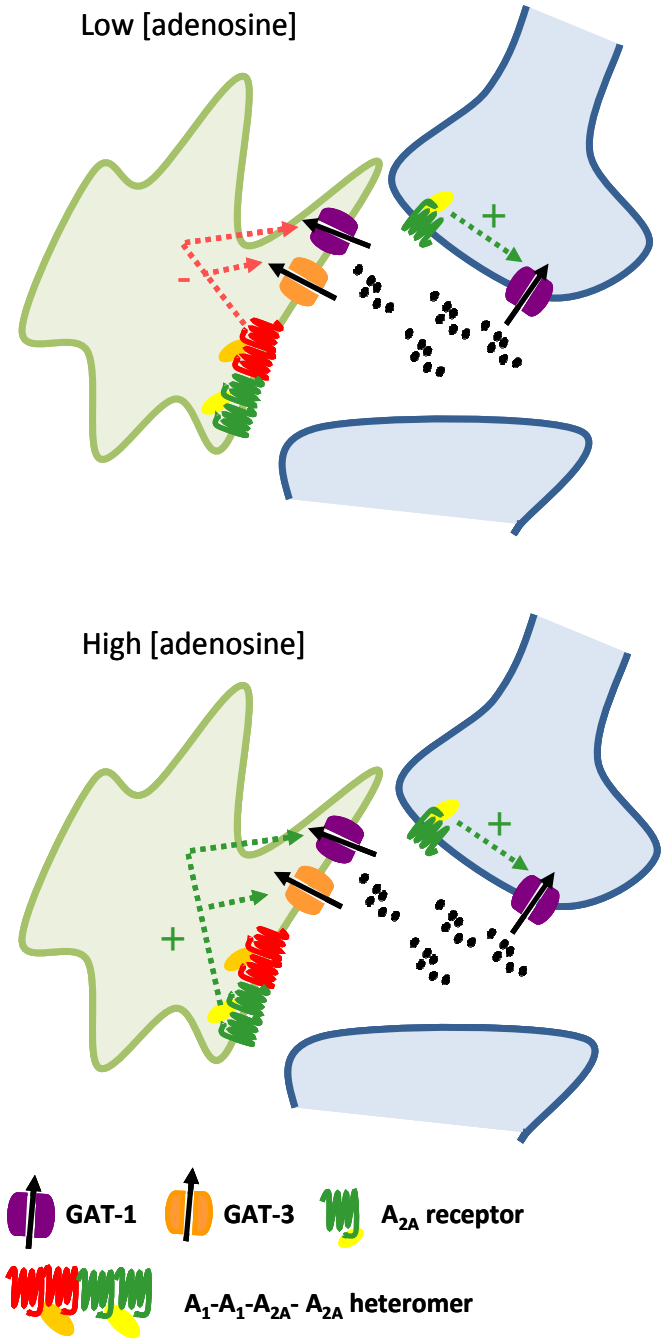

Figure 6.1 – Schematic representation of adenosine effect upon GABA transport at tripartite synapse. At low concentration, adenosine will decrease astrocytic uptake, driving GABA to presynaptic terminal. At higher adenosine concentrations, GABA will be driven to astrocytes.

7 Future perspectives

After neurotransmitter receptors, transporters start to be seen as key components of transmission. It is nowadays accepted that transporters can release transmitters, even in physiological conditions, which modify the synapse scenario and justify the study of transporters modulation by different endogenous agents. On the other hand, transporters, by the clearance of transmitters from synapse, modify the expression of receptors, namely by reducing desensitization. Then, the evaluation of transporters modulation assumes particular relevance for understanding brain function.

In what concerns GABA transporters, their involvement (specifically GAT-1) in tonic and phasic transmission has been established during the last few years. In future work, it will be important to clarify the role of GAT-3 (and glial uptake) in the control of GABA levels, and consequently in tonic but also phasic inhibition.

The work presented in this thesis clearly shows that adenosine modulates both GAT-1 and GAT-3 function. It therefore drives the interest of evaluating the consequences of this modulation on phasic and tonic transmission.

Furthermore, GABA transporters, adenosine dysfunction and astrogliosis have been associated with epileptogenic status. In fact, the epileptogenic foci feature with low levels of adenosine. Being adenosine a pleiotropic factor, it is not clear to figure out by which mechanism the lack of adenosine influences the epileptic status.

One possible hypothesis is the interference with GABA transporters system, since dysfunction of GAT-1 is closely connected with seizures occurrence.

In fact, GABA transporters have been associated with epilepsy, specially with seizures control, since a GAT-1 blocker (tiagabine) is used as adjunctive treatment for focal and secondarily generalized seizures in patients aged 12 or older (e.g. Genton *et al.,* 2001). The therapeutic effect of tiagabine is related with an enhancement of extracellular GABA levels that occurs as consequence of GAT-1 blockade; therefore the enhancement of extracellular GABA contributes to the control of overexcitation associated with seizure occurrence.

On the other hand, a GAT-1 dysfunction was recently associated to the genesis of absence epilepsy (Cope *et al*., 2009). Interestingly, systemic or intrathalamic administration of agents that promote GABAergic inhibition, including the anti-epileptic drugs vigabatrine and tiagabine, initiate or exacerbate seizures in patients and animals (Hosford and Wang, 1997; Hosford *et al.,* 1997; Danober *et al*., 1998; Perucca *et al.,* 1998 Ettinger *et al.*, 1999). Thus, increased rather than impaired $GABA_AR$ inhibition may be a feature of absence seizures (Cope *et al.,* 2009).

Furthermore, it was shown in a model of absence epilepsy (GAERS – genetic absence epilepsy rats from Strasbourg) that the enhancement of $GABA_A$ receptors-mediated tonic currents was due to a impaired GABA uptake by GAT-1. This impaired GABA uptake could not be related with the protein levels, which is unchanged

and, interestingly, is restricted to thalamocortical neurons, which participate in absence seizure genesis (Cope *et al.,* 2009). In addition, it was also shown that GAT-1 KO mice exhibited spontaneous absence seizures that were blocked by systemic administration of the anti-absence drug ethosuximide (Cope *et al.,* 2009).

On the other hand, epilepsy can be considered as a disorder of astrocytes dysfunction (see Boison, 2010). In fact, astrogliosis has been associated to the epileptic brain as well as some modifications in distinct astrocytic membrane receptors and channels (Binder and Steinhauser, 2006). A direct involvement of astrocytes on epilepsy was firstly proposed in 2005, when a study showed that some anti-epileptic drugs, namely valproate, gabapentine and phenytoin reduced the ability of astrocytes to propagate calcium signaling, suggesting that pathological activation of astrocytes may play a central role in the genesis of epilepsy (Tian *et al.,* 2005). Furthermore, it was also shown that the paroxysmal depolarization shifts (abnormal prolonged depolarizations) were triggered by glutamate release from a non-synaptic source (Tian *et al.,* 2005), proposing that glutamate release from astrocytes can trigger epileptic seizures. So, taken together, these results suggest that astrocytes may amplify, maintain and expanded neurogenic seizure activity as well as may be the source of epileptic activity (Tian *et al.,* 2005).

In addition to being a source of glutamate, astrocytes are also an important source of ATP and consequently adenosine, which is an

endogenous anticonvulsant agent (see Boison, 2010). In fact, it was recently hypothesized that ATP released from astrocytes in response to neuronal activity inhibits adjacent neurons, a mechanism that could prevent seizures propagation (Kumaria *et al*., 2008).

Then, taking together all the "puzzle pieces" and the knowledge about each one in the context of epilepsy, it is very pertinent to draw a project, whose main aim would be to evaluate how astrocytes-adenosine-GATs-GABA axis operates in physiological and also pathological conditions, namely epilepsy. The identification of possible astrocytes-adenosine-GATs-GABA axis dysfunctions may allow the development of new potential therapeutic avenues.

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10 Annex

The scientific content of the present thesis was published in the form of two original articles (see Annex).