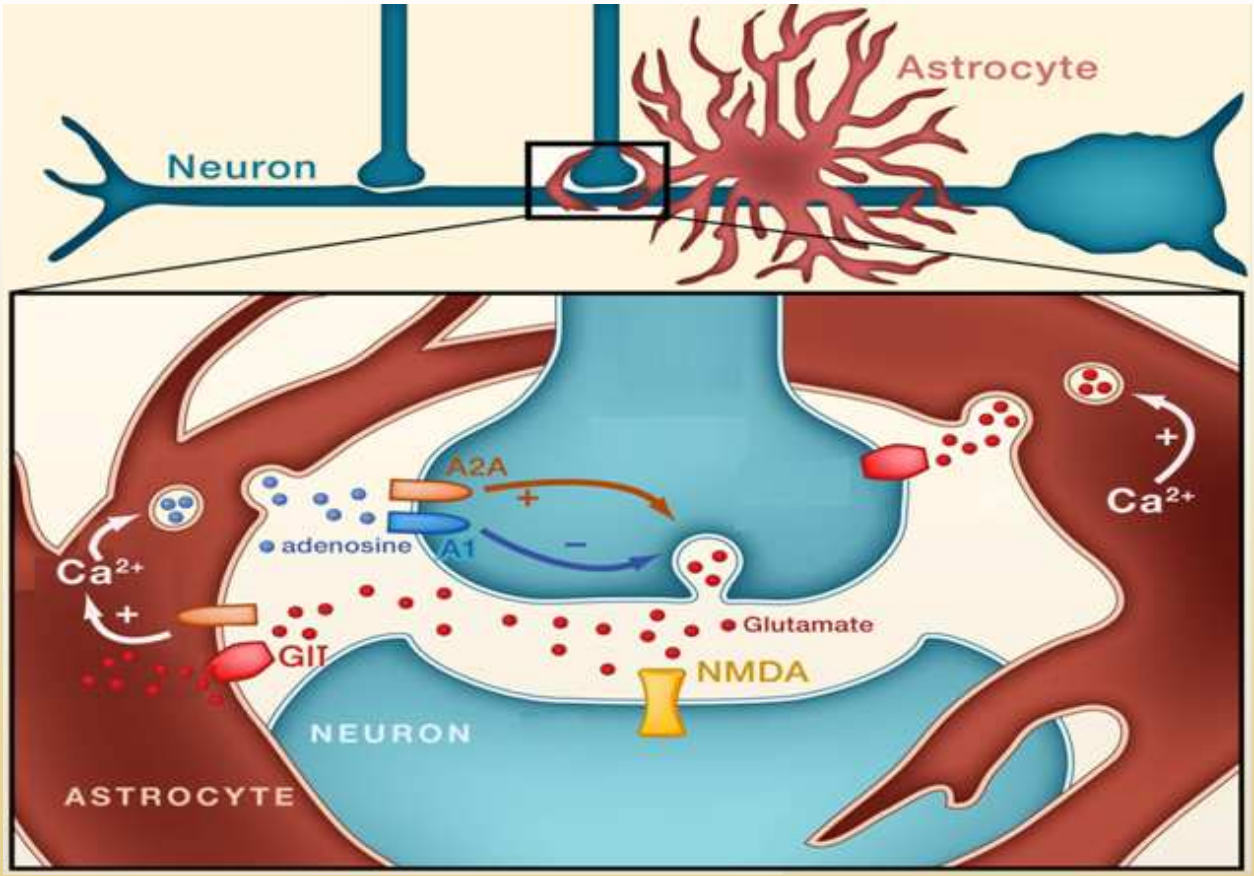


Marco António Paisana de Matos

Role of adenosine A_{2A} receptors in astrocytes - implications for glutamatergic activity

2013

· U C ·

Universidade de Coimbra

Front cover: Astrocytes Detect and Control Synaptic Transmission. Adapted from Navarete and Araque, 2011.

Role of adenosine A_{2A} receptors in astrocytes - implications for glutamatergic activity

O papel dos receptores de adenosina A_{2A} em astrócitos - implicações para a atividade glutamatérgica

Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra, para prestação de provas de doutoramento na área das Ciências da Saúde, especialidade Ciências Biomédicas, conduzido sobre a orientação da Dra. Paula Maria Garcia Agostinho e do Prof. Dr. Rodrigo Antunes Cunha.

Marco António Paisana de Matos

Faculdade de Medicina da Universidade de Coimbra

The work conducing to this dissertation was performed at:

Centre for Neuroscience and Cell Biology, associated laboratory to the Faculty of Medicine of the University of Coimbra, Coimbra, Portugal.

Department of Neurology, Boston University School of Medicine, Boston, USA.

The work was primarily financed through a PhD grant attributed to Marco Matos from the Foundation for Science and Technology of Portugal with the reference ***SFRH/BD/36289/2007***, supported by POPH - NSRF - Type 4.1 - Advanced Training, subsidized by the European Social Fund and national funds from the Portuguese Ministry of Education and Science.

The work was additionally financed by the National Institutes of Health (NIH, grant NS041083-07) and Defense Advanced Research Projects Agency (DARPA, grant 09-68-ESR-FP-010).

Table of Contents

Agradecimentos / Acknowledgements xii
List of Publications xiv
Lista de abreviaturas / List of Abbreviations xv
Resumo xix
Abstract xxii

CHAPTER 1. GENERAL INTRODUCTION AND OBJECTIVES 2-63

1. Astrocytes 4-24
1.1. Astrocytes and the tripartite synapse 4
1.2. Heterogeneity in astrocyte morphology and physiology 7
1.3. Astrocytes functions in the CNS 10
1.3.1. Energy and metabolism 10
1.3.2. Fluid, ion, pH and transmitter homeostasis 11
1.3.3. Synaptic modulation 12
1.3.4. Regulation of blood flow 13
1.3.5. Immune/inflammatory response 13
1.3.6. Role in development 14
1.4. Astroglial transmitter release and regulation of synaptic plasticity 14
1.4.1. Glutamate release 15
1.4.2. D-Serine release 17
1.4.3. ATP and adenosine release 18
1.5. Astrocyte networks modulate neuronal activity: role in cognition 20

2. Glutamate transport 25-41
2.1. Glutamate homeostasis in the CNS 25
2.2. Astrocytic glutamate transporters 26
2.3. Regulation of astrocytic glutamate transporters 29
2.4. Glutamate transporters and synaptic function 32
2.5. Involvement of glutamate transporters in neurologic diseases 35
2.5.1. Involvement in neurodegenerative diseases - Alzheimer's disease 36
2.5.2. Involvement in neuropsychiatric disorders - Schizophrenia 38

3. Adenosine receptors 42-60
3.1. Purinergic signaling - overview 42
3.2. Adenosine receptors 46
3.3. Adenosine receptors and motor function 49
3.4. Adenosine receptors in astrocytes 52
3.5. Adenosine receptors and cognitive function 56
3.6. Involvement of adenosine receptors in neurologic diseases 57
3.6.1. Involvement in neurodegenerative diseases - Alzheimer's disease 59
3.6.2. Involvement in neuropsychiatric disorders - Schizophrenia 60

4. Objectives and outline 61-63

CHAPTER 2. ADENOSINE A_{2A} RECEPTORS MODULATE GLUTAMATE UPTAKE IN CULTURED ASTROCYTES AND GLIOSOMES 64-97

1. Abstract 66
2. Introduction....66
3. Material and methods 68
3.1. Materials 68
3.2. Animals....69
3.3. Preparation of primary astrocytes cultures 70
3.4. Preparation of glial plasmalemmal vesicles (gliosomes)....70
3.5. Drug treatments 71
3.6. D-[^{3}H] aspartate uptake 72
3.7. A_{2A}R binding to gliosomal and synaptosomal membranes....73
3.8. Western blotting 74
3.9. Immunocytochemical analysis....74
3.10. Quantitative PCR....75
3.11. Statistical analysis 76
4. Results 77
4.1. A_{2A}R decreases D-Aspartate uptake in cultured astrocytes and gliosomes 77
4.2. Impact of A_{2A}R on the relative activities of GLT-I, GLAST and EAAC1....79
4.3. Co-localization between A_{2A}R and glutamate transporters 83
4.4. A_{2A}R decreases D-aspartate uptake through an increase on cAMP/PKA 89
4.5. A_{2A}R activation decreases GLT-I and GLAST expression 90
5. Discussion 92
6. Acknowledgements 96

CHAPTER 3. ANTAGONISTIC INTERACTION BETWEEN ADENOSINE A_{2A} RECEPTORS AND Na^+/K^+-ATPase-α_2 CONTROLLING GLUTAMATE UPTAKE IN ASTROCYTES 98-125

1. Abstract 100
2. Introduction 101
3. Material and methods 102
3.1. Animals....102
3.2. Preparation of total membranes 103
3.3. Preparation of gliosomes and synaptosomes....103
3.4. Na^+/K^+-ATPase (NKA) activity assay....104
3.5. D-[^{3}H] aspartate uptake 104
3.6. Drug treatments....105
3.7. Co-immunoprecipitation (Co-IP)....105
3.8. Western blotting....106
3.9. Immunohistochemistry 106
3.10. *In situ* Proximity Ligation Assay (PLA)....107
3.11. Statistical analysis 108
4. Results 108
4.1. Activation of A_{2A}R decreases Na^+/K^+-ATPase (NKA) activity in gliosomes 108
4.2. Comparison of the effect of A_{2A}R on the NKA activity and on D-aspartate uptake 110

4.3. Low concentrations of NKA-inhibitor ouabain blunt the A_{2A}R-mediated inhibition of D-aspartate uptake in astrocytes 111
4.4. NKA activity is increased selectively in astrocytes from Gfa2-A_{2A}R-KO mice....113
4.5. GLT-I and NKA-α2 immunoreactivities are increased in Gfa2-A_{2A}R-KO mice....115
4.6. A_{2A}R is physically associated with NKA-α2....117
5. Discussion....120
6. Acknowledgements....125

CHAPTER 4. ASTROCYTIC ADENOSINE A_{2A} RECEPTORS CONTROL THE AMYLOID-β PEPTIDE INDUCED DECREASE OF GLUTAMATE UPTAKE 126-147

1. Abstract....128
2. Introduction....128
3. Material and methods....130
3.1. Materials....130
3.2. Animals....132
3.3. D-[^{3}H] aspartate uptake....132
3.4. Quantitative PCR....133
3.5. Western blotting....134
3.6. A_{2A}R binding to primary astrocyte membranes....134
3.7. *Ex vivo* analysis of gliosomes from $A\beta_{1-42}$-icv injected rats....135
3.8. Statistical analysis....136
4. Results....137
4.1. A_{2A}R blockade prevents $A\beta_{1-42}$-induced astrogliosis and reduced D-aspartate uptake.137
4.2. A_{2A}R blockade prevents $A\beta_{1-42}$-induced downregulation of glutamate transporters....139
4.3. $A\beta_{1-42}$ increases the expression and density of astrocytic A_{2A}R....139
4.4. $A\beta_{1-42}$-icv injection causes memory deficits, astrogliosis and decreased D-aspartate uptake - role of A_{2A}R....142
5. Discussion....145
6. Acknowledgements....148

CHAPTER 5. DELETION OF ADENOSINE A_{2A} RECEPTORS IN ASTROCYTES ALTERS GLUTAMATERGIC SYNAPSES AND TRIGGERS PSYCHOMOTOR AND COGNITIVE DYSFUNCTIONS CHARACTERISTIC OF SCHIZOPHRENIA 150-183

1. Abstract....152
2. Introduction....152
3. Material and methods....154
3.1. Animals....154
3.2. Preparation of total membranes....154
3.3. Preparation of gliosomes and synaptosomes....156
3.4. D-[^{3}H] aspartate uptake....157
3.5. L-[^{3}H] glutamate release....157
3.6. Biotinylation assay of surface receptors....158
3.7. Western blotting....158
3.8. Working memory analysis....159

3.9. Reference memory analysis 160
3.10. Locomotion in home cage 160
3.11. Drug administration 161
3.12. Statistical analysis 162
4. Results 162
4.1. Opposite modulation of MK-801-induced psychomotor activity by astrocyte *versus* forebrain neuronal A_{2A}R 162
4.2. Spatial working memory is impaired in Gfa2-A_{2A}R-KO, in contrast to Fb-A_{2A}R-KO mice 164
4.3. Glutamate uptake is increased in Gfa2-A_{2A}R-KO but not in Fb-A_{2A}R-KO mice 167
4.4. Selective GLT-I inhibition prevents the exacerbation of MK-801-induced psychomotor activity and reverses the working memory deficits in Gfa2-A_{2A}R-KO mice 169
4.5. The evoked glutamate release from nerve terminals is increased in Gfa2-A_{2A}R-KO mice 171
4.6. Increased NMDA-NR2B and decreased AMPA-GluR1/2 surface levels in GFAP-A_{2A}R-KO mice 172
4.7. Selective blockade of regulated AMPAR internalization reverts the cognitive impairment in GFAP-A_{2A}R-KO mice 174
5. Discussion 177

CHAPTER 6. GENERAL CONCLUSIONS **184-201**

1. Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes 186
2. Antagonistic interaction between astrocytic adenosine A_{2A} receptors and Na^+/K^+-ATPase-α2: implications for glutamate uptake 187
3. Astrocyte adenosine A_{2A} receptors control the amyloid-β induced decrease of glutamate uptake 188
4. Deletion of adenosine A_{2A} receptors in astrocytes alters glutamatergic synapses and triggers psychomotor and cognitive dysfunctions characteristic of schizophrenia 190
5. Glutamate transporters and adenosine A_{2A}R in astrocytes as therapeutic targets 194

REFERENCES 202

Agradecimentos /Acknowledgements

Esta dissertação resulta de um esforço coletivo. Sendo assim, gostaria de expressar os meus agradecimentos a todos aqueles que, por um motivo ou outro, contribuíram para a realização deste trabalho:

À Doutora Paula Agostinho por aceitar supervisionar o trabalho experimental conducente à realização da minha tese de doutoramento e pela confiança depositada em mim desde o início. Pela sinceridade e frontalidade com que abordou todo o meu trabalho e orientação do meu doutoramento. Obrigado!

Ao Professor Doutor Rodrigo Cunha por me ter dado uma oportunidade no seu laboratório e por ter incentivado o meu crescimento científico durante os 4 anos que estive sobre a sua orientação. Apenas a sua capacidade singular de dominar e integrar com destreza conhecimento proveniente de diversas áreas me poderia permitir realizar com sucesso um trabalho que não fazia parte do seu domínio directo de investigação. Por me ter incutido o instinto de crença e perseverança nas minhas ideias que sei hoje ter sido o meu maior ganho profissional nesta experiência. Obrigado!

To Professor Jiang-Fan Chen for trusting me with a project without knowing me and for all openhanded instruction he gave me while I was in the lab. I'm particularly indebted to you and proud of the work we managed to accomplish while I was in Boston. Thank you very much!

Agradeço também à Doutora Catarina Oliveira, por me ter proporcionado realizar parte do trabalho apresentado nesta dissertação no Centro de Neurociências e Biologia Celular.

Aos meus colegas e amigos do grupo "Purines at CNC" que partilharam parte desta epopeia comigo, em especial ao Alexandre "jóia de moço" Rodrigues, por ter abreviado alegremente a sua esperança média de vida tantas (demasiadas) vezes comigo no laboratório da radioctividade. Valeu, garotão! Ao Pablo, Manu (eu prefiro "Nelita"), Bibi (eu prefiro "Cachoeira"), Giana, Chico, Vasquinho, Sanches (eu prefiro "Sanchez"),

Carla, Henrique (o “Lorde! ou melhor, o “Conde!), Dani, Ardais, Samira, Paula Canas, Patrícia, Prof. Ângelo e Caroline, muito obrigado!

Aos membros e amigos do CNC, que, de uma forma ou outra (principalmente esta última) contribuíram para a realização deste trabalho. Em especial, ao Carreira, Joana Salgado, Rita Perfeito, Ana Silva, Inês Morte, Catarina Oliveira, Rui Nobre, Teresa Girão (especial obrigado!), Isabel Nunes, Luísa Cortes, obrigado!

To all the colleagues in “Jiang-Fan’s lab”, including Yu Mei, Ji Hoon, Wei Li, Marie, Ping Li, Melissa, Jessica, but also Michael and Andy, thanks so much for all the companionship! Special thanks to Olivia Whitman from the International Office, for generously helping us with all the bureaucracies and problems that came to arise.

A todos os meus companheiros de Boston, em especial ao Rui, Gonçalo, Dário, Tellechea, Ermelindo, Sousa Lima, Sofia, Azatuhi, obrigado!

Aos amigos de sempre que tudo tentaram para sabotar este trabalho com vagabundagem e alcoolismo saudável: Sá, Patrick, Teixeira, Viegas, Vitorino, Jorginho, Kaly boy, obrigado!

À minha família, por ter sido sempre o meu abrigo: Mãe, Pai, Hugo, muito obrigado!

Betty…este trabalho é tanto teu como meu. É parte da nossa aventura e sem ti seria uma ilusão (e uma desilusão:)). “Cenas e coisas” em abundância para nós. Obrigado, obrigado, obrigado. *

Dedicado ao meu Pai.

“Touch a scientist and you touch a child.”

Ray Bradbury

List of publications

Part of the scientific work presented in this dissertation resulted in the publication/ acceptance of the next manuscripts in peer-reviewed international scientific journals:

- Matos M, Augusto E, Agostinho P, Cunha RA, Chen JF. (2013) Antagonistic interaction between adenosine A_{2A} receptors and Na^+/K^+-ATPase-α_2 controlling glutamate uptake in astrocytes. *J Neurosci*. (accepted)

- Matos M, Augusto E, Machado NJ, dos Santos-Rodrigues A, Cunha RA, Agostinho P. (2012) Astrocytic adenosine A_{2A} receptors control the amyloid-β peptide-induced decrease of glutamate uptake*. J Alzheimers Dis.* 31: 555-567.

- Matos M, Augusto E, Santos-Rodrigues A, Schwarzschild MA, Chen JF, Cunha RA, Agostinho P. (2012) Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes. *Glia* 60: 702-716.

During the course of the work, active participation in the development of colleagues' work and other projects resulted in the publication of the next manuscripts in peer-reviewed international scientific journals:

- Augusto E, Matos M, Sévigny J, El-Tayeb A, Müller CE, Cunha RA, Chen JF. (2013) Ecto-5'-nucleotidase (CD73)-mediated formation of adenosine is critical for the striatal adenosine A2A receptor functions. *J Neurosci*. 33:11390-11399.

- Matos M, Augusto E, Oliveira CR, Agostinho P. (2008) Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: involvement of oxidative stress and mitogen-activated protein kinase cascades. *Neuroscience* 156: 898-910.

Lista de abreviaturas / List of abbreviations

Aβ - Amyloid-beta peptide

$Aβ_{1-42}$ - Aβ isoform of 42 aa residues

A_1R - Adenosine A_1 receptor subtype

A_{2A}R - Adenosine A_{2A} receptor subtype

A_{2A}R-GKO - A_{2A}R global KO mice

A_{2B}R - Adenosine A_{2B} receptor subtype

A_3R - Adenosine A_3 receptor subtype

ACh - Acetylcholine

AChE - Acetylcholinesterase

AD - Alzheimer's disease

ADA - Adenosine deaminase

ADK - Adenosine kinase

Ado - Adenosine

ADP - Adenosine 5'-diphosphate

ALS - Amyotrophic lateral sclerosis

AMP - Adenosine 5'-monophosphate

AMPA-R - α-amino-2, 3-dihydro-5- methyl-3-oxo-isoxazolepropanoic acid receptor

AMPc - Adenosina 5'-monofosfato cíclica

ANOVA - Analysis of variance

ApoE - Apoliprotein E

APP - Amyloid precursor protein

AQ-4 - Aquaporin 4

AR - Adenosine receptores

ARNm - Ácido ribonucleico mensageiro

Asp - Aspartate/ Aspartic Acid

ATP - Adenosine 5'-triphosphate

BBB - Blood brain barrier

BD - Bipolar disorder

BDNF - Brain-derived neurotrophic factor

bFGF - Basic fibroblast growth factor

BSA - Bovine serum albumin

C1q - Complement component 1, q subcomponent

CA1/3 - Cornu Ammonis areas1/3

Ca^{2+} - Calcium

$[Ca^{2+}]i$ - Free intracellular calcium concentration

CADO - 2-chloroadenosine

cAMP - cyclic adenosine monophosphate

CB_1R - Cannabinoid receptor type 1

CD11b/OX-42 - Cluster of differentiation 11b / Complement receptor 3

CD73 (or 5'-NT) **-** Ecto-5'-nucleotidase

CGS 21680 - 6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2yl]amino]ethyl]benzene propanoic acid hydrochloride

Cl-IB-MECA - 2-chloro-N6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide

CNS - Central nervous system

Co-IP - co-immunoprecipitation

COX-2 - Cyclooxygenase 2

CPA - N^6**-**cyclopentyladenosine

CPu - Caudate Putamen

CSF - Cerebrospinal fluid

Ct - Cycle threshold

Cx-43 - Connexin 43

D-[^{3}H]aspartate - Tritium-labeled D-aspartate

D_1R - D_1 dopamine receptor subtype

D_2R - D_2 dopamine receptor subtype

DA - Dopamine

dbcAMP - Dibutyryl-cAMP

DG - Dentate gyrus
DHK - Dihydrokainate
DIV - Days *in vitro*
DMEM - Dulbecco's Modified Eagle Medium
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DPM - Disintegrations *per* minute
EAAC1 - Excitatory amino-acid carrier 1
EAAT1/2/4 or 5 - Excitatory amino acid transporter types 1/2/4 or 5
ECM - Extracellular matrix
EDTA - Ethylenediamide tetraacetic acid
EGF - Epidermal growth factor
EGTA - Ethylene bis(oxyethylenenitrilo)tetraacetic acid
ENT1/2 - Equilibrative nucleoside transporter types 1/2
E-NTPDase - Ecto-nucleotide triphosphate diphosphohydrolase
EphA3/4 - Ephrin-A3/4
EPSCs - Excitatory postsynaptic currents
ER - Endoplasmic reticulum
ERK - Extracellular signal-regulated protein kinase
Fb-A_{2A}R KO - Forebrain selective A_{2A}R KO mice
FBS - Fetal Bovine Serum
FDA - Food and Drug Administration
FGF - Fibroblast growth factor
GABA - Gamma-aminobutyric acid
GAD - Glutamate decarboxylase
GDNF - Glial cell line-derived neurotrophic factor
Gfa2-A_{2A}R KO - GFAP-driven A_{2A}R KO mice
GFAP - Glial fibrillary acidic protein
GLAST - Glutamate-Aspartate transporter
Gln - Glutamine
GLT- I - Glutamate transporter-1
Glu - Glutamate
GluR1/2/3/4 - AMPA-R subunits 1/2/3/4
GluT - Glutamate transportes
GPe - Globus Pallidus external segment
GPi - Globus Pallidus internal segment
GS - Glutamine synthetase
H1 - Histone H1
H89 - H89 dihydrochloride
HBM - HEPES buffered medium
HBS - Hanks buffered saline
HBSS - Hank's balanced salt solution
HD - Huntington's disease
HEK - Human Embryonic Kidney (cells)
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
i.p - Intraperitoneal
ICV - Intracerebroventricular
IDE - Insulin-degrading enzyme
IFN-γ - Interferon-gamma
IGF - Insulin-like growth factor
iGluR - ionotropic glutamate receptor
IL-1β -Interleukin-1 beta
IL-6 - Interleukin-6
iNOS - inducible Nitric Oxide synthase
IP_3 - Inositol triphosphate
K^+ - Potassium
KA - Kainate
K_M - Michaelis-Menten constant
KO - Knockout

L-[^{3}H] glutamate - Tritium-labeled L-glutamate
LME - L-leucine methyl ester
LTD - Long-term depression
LTP - Long-term potentiation
MAP - Microtubule Associated Protein
MAPK - Mitogen-activated protein kinase
MCI - Mild cognitive impairment
MCP-1/ CCL2 - Monocyte chemotactic protein-1/ chemokine ligand 2
MDD - Major depressive disorder
MEM - Minimum essential medium
mGluR1/2/5 - metabotropic glutamate receptor subtypes 1/2/5
MK-801 - Dizocilpine
MSN's - Medium-sized spiny neurons
mV - milivolt
Na^+ - Sodium
NAc - Nucleus Accumbens
NFκB - Nuclear factor kappa B
NFT - Neurofibrillary Tangle
NGF - Nerve growth factor
NGS - Normal goat serum
NHS - Normal horse serum
NKA - α2 - NKA alpha *2* isoform
NKA - Na^+/K^+-ATPase
NMDA - N-methyl-D-aspartate
NMDA-R - N-methyl-D-aspartate receptor
NMG - N-Methyl-D-glucamine
NO - Nitric oxide
NR1 - NMDA-R subunit 1
NR2A - NMDA-R subunit 2A
NR2B - NMDA-R subunit 2B
P_{2X}R - P2X purinergic receptor
P_{2Y}R - P2Y purinergic receptor
PBS - Phosphate Buffered Saline
PCR - Polymerase chain reaction
PD - Parkinson's disease
PFC - Prefrontal cortex
PHF - Paired Helical Filament
PKA/B/C - Protein Kinase types A/B/C
PLA - Proximity ligation assay
PLC- Phospholipase C
PCP - Phencyclidine
PPI - Prepulse inhibition
PSD-95 - Post synaptic density protein of 95 kDa
qPCR - Quantitative PCR
RNA - Ribonucleic acid
ROS - Reactive oxygen species
rpm - Rotations per minute
S100β - S100 calcium-binding protein beta
SCH 58261 - 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo-[1,5 c]pyrimidine
SCZ - Schizophrenia
SD - Standard deviation
SDS - Sodium dodecyl sulfate
SEM - Standard error of the mean
SICs - Slow inward currents
SLC1 - Solute carrier family 1
SNAP-23/25 - Synaptosomal-associated protein types 23/25
SNc - Substantia nigra pars compacta
SNr - Substantia nigra pars reticulata
St-A_{2A}R KO - Striatum selective A_{2A}R KO mice
STN - Subthalamic nucleus

STR - Striatum

Tat- GluR2$_{3Y}$ - GluR endocytosis inhibitor

Tat-GluR2$_{3S}$ - inactive Tat-GluR2$_{3Y}$

TBOA - DL-threo-β-benzoyloxyaspartate

TCA - Tricarboxylic acid / Krebs cycle

TEMED - Tetramethylethylenediamine

TGFβ -Transforming growth factor beta

THAL - Thalamus

TNFα -Tumor necrosis factor alpha

UTP - Uridine 5′-triphosphate

VGLUT - Vesicular glutamate transporter

V_{max} - Maximum reaction velocity

VNUT - Vesicular nucleotide transporter

VTA - Ventral Tegmental area

WM - Working memory

WT - Wild type

Resumo

Nos últimos anos, a visão clássica de astrócitos como simples células de suporte de neurónios tem sido substituída por um novo paradigma em que os astrócitos são considerados elementos activos de processamento cerebral. Tal concepção é baseada na existência de uma comunicação bidirecional entre astrócitos e neurónios ao nível sináptico que foi denominada como "sinapse tripartida". Além da libertação de gliotransmissores, os astrócitos são capazes de modular sincronicamente a actividade sináptica na sinapse tripartida através de um processo dinâmico de transporte e reciclagem de glutamato, o principal neurotransmissor do Sistema Nervoso Central (SNC).

No cérebro de mamíferos, a captação do glutamato é sustentada principalmente através do transportador de glutamato-1 (GLT-I) e pelo transportador de glutamato-aspartato (GLAST), ambos transportadores de alta afinidade do glutamato presentes em astrócitos, que desempenham um papel crucial na prevenção da acumulação de glutamato extracelular. A importância desta função é realçada pelo facto da perturbação da expressão ou atividade destes transportadores resultar na ativação excessiva de receptores de glutamato, resultando numa atividade neuronal anormal e numa eventual morte neuronal por excitotoxicicidade. Em conformidade, um grande número de estudos tem demonstrado que a disfunção no transporte do glutamato está associada a diversas doenças neurológicas (por exemplo, doença de Alzheimer; epilepsia) e vários distúrbios neuropsiquiátricos (por exemplo, esquizofrenia; doença bipolar), para além de estar implicado em alterações de plasticidade sináptica em que se estabelecem muitas alterações mnemónicas e cognitivas. Assim, um esforço considerável tem sido despendido para compreender os mecanismos responsáveis pela regulação dos transportadores, particularmente em processos patológicos. Neste contexto, o papel desempenhado pela adenosina, um modulador clássico e ubíquo do SNC que actua principalmente através da activação de receptores inibitórios do subtipo A_1 (A_1R) ou receptores

excitatórios do subtipo A_{2A} (A_{2A}R), foi aqui investigado na regulação da actividade dos transportadores de glutamato.

No presente estudo mostrámos que os A_{2A}R controlam/inibem a captação de glutamato através de um mecanismo dual: i) a activação sistemática (24 h) dos A_{2A}R leva a uma activação da via AMPc/ proteína cinase A que conduz a uma redução dos níveis de ARN mensageiro (ARNm) e proteicos de GLT-I e GLAST, provocando assim a uma redução prolongada da captação de glutamato; ii) por outro lado, a ativação aguda (10 min) dos A_{2A}R inibe imediatamente a captação de glutamato, um resultado que se verificou ser dependente de um acoplamento físico entre o A_{2A}R astrocítico com a ATPase Na^+/K^+ do subtipo α2 (NKA-α2), que está associada ao GLT-I, num processo de regulação rápida do transporte de glutamato. Em resumo, este duplo mecanismo de inibição dos transportadores de glutamato pelos A_{2A}R astrocíticos proporciona um novo mecanismo que permite compreender a capacidade dos A_{2A}R modularem a actividade glutamatérgica rapidamente e prolongadamente, num processo estreitamente relacionado com a etiologia de diversas doenças cerebrais.

Posteriormente foram investigadas as implicações na inibição da captação de glutamato pelos A_{2A}R num modelo de doença de Alzheimer (DA), uma doença neurodegenerativa associada a uma deficiente captação de glutamato, a excitotoxicidade e morte celular. O estudo aqui apresentado examinou a capacidade do bloqueio dos A_{2A}R prevenir a diminuição da captação de glutamato em diferentes preparações de astrócitos cerebrais (*in vivo* e *ex vivo*) sujeitas ao peptídeo de beta-amilóide ($A\beta_{1-42}$). Em conformidade, tanto o bloqueio farmacológico, bem como a excisão genética dos A_{2A}R, conseguiram prevenir eficientemente a diminuição da captação de glutamato em astrócitos em cultura tratados com $A\beta_{1-42}$ e em vesículas da membrana plasmática de astrócitos (gliossomas) proveniente de ratos injectados intracerebroventricularmente (ICV) com $A\beta_{1-42}$. No seu todo, estes resultados mostram que os A_{2A}R astrocíticos desempenham um papel crucial no desenvolvimento da disfunção na

captação de glutamato induzida pelo Aβ e proporcionam uma justificação suplementar para os benefícios terapêuticos promissores do bloqueio selectivo dos A_{2A}R na patogénese da DA.

Finalmente examinámos as consequências comportamentais de uma captação hiperactiva de glutamato causada pela eliminação seletiva dos A_{2A}R astrocíticos (em murganhos Gfa2-A_{2A}R-KO). Assim, mostramos que murganhos Gfa2-A_{2A}R-KO apresentam uma variedade de endofenótipos característicos da esquizofrenia, ou seja, uma diminuição no desempenho da memória operacional (WM) e um aumento significativo da resposta psicomotora ao MK-801, um antagonista dos receptores do glutamato do tipo N-metil-D-aspartato (NMDA), ambos revertidos pela inibição seletiva dos GLT-I. Além disso, estes resultados sugerem também que os A_{2A}R neuronais e astrocíticos podem ter efeitos neuromodulatórios opostos, dado que a supressão da actividade dos A_{2A}R neuronais mostrou ser pro-cognitiva, enquanto a eliminação selectiva dos A_{2A}R em astrócitos potenciou défices na WM. Finalmente demonstrou-se que a disfunção do sistema glutamatérgico observada estava associada a uma alteração de tráfico membranar das subunidades GluR1/GluR2 dos receptores AMPAR induzido pelos receptores NMDAR2B, sugerindo que a supressão genética dos A_{2A}R astrocíticos pode conduzir a extensas e drásticas alterações no sistema glutamatérgico que conferem susceptibilidade a um tipo de características presentes em esquizofrenia.

Em resumo, os resultados aqui apresentados indicam que os A_{2A}R astrocíticos, ao regularem a actividade dos transportadores de glutamato e de possivelmente mecanismos glutamatérgicos adicionais, podem ser mediadores importantes da comunicação entre astrócitos e neurónios, em particular nas disfunções glutamatérgicas subjacentes à doença de Alzheimer e esquizofrenia. Uma compreensão mais detalhada da fisiologia dos transportadores de glutamato e do papel regulador fundamental operado pelos A_{2A}R astrocíticos poderá levar ao potencial desenvolvimento de estratégias terapêuticas para modulação da função dos transportadores de glutamato em diversas doenças cerebrais.

Abstract

In the last years, the classical view of astrocytes as simple supporting cells for neurons has been replaced by a new paradigm in which astrocytes are active processing elements of the brain. Such novel conception is based on the existence of a bidirectional communication between astrocytes and neurons at the synaptic level in what was termed the “tripartite synapse”. In addition to the release of gliotransmitters, astrocytes are able to synchronously modulate synaptic activity within the tripartite synapse by a dynamic process of glutamate transporting and recycling, the most important neurotransmitter of the Central Nervous System (CNS).

In mammals, basal glutamate uptake in the brain is maintained essentially by glutamate transporter-1 (GLT-I) and glutamate-aspartate transporter (GLAST), both astrocyte Na^+-dependent high-affinity glutamate transporter types, which play a crucial role in preventing the accumulation of extracellular glutamate. Disrupting the expression or activity of these transporters results in excessive activation of glutamate receptors, abnormal neuronal activity, and eventual excitotoxic neuronal death. In accordance, a great body of work has shown that glutamate transport dysfunction is associated with many neurological diseases (e.g. Alzheimer’s disease; epilepsy, etc) and neuropsychiatric disorders (e.g. schizophrenia; bipolar disease, etc), in addition to being implicated in synaptic plasticity alterations which underlie changes in memory and cognition. Therefore, considerable effort is being expended to understand the mechanisms responsible for transporter regulation and dysfunction in disease. In this context, the role of adenosine, a classical and ubiquitous modulator of the CNS acting mainly via activation of high-affinity inhibitory A_1 receptors (A_1R) or excitatory A_{2A} receptors (A_{2A}R), was here investigated as a promising candidate regulating glutamate transporter activity.

We now report that A_{2A}R control/inhibit the glutamate uptake by a dual mechanism: i) the prolonged (24h) activation of A_{2A}R led to a cAMP/protein kinase A-dependent reduction of GLT-I

and GLAST mRNA and protein levels which, in turn, elicits a sustained decrease in glutamate uptake; ii) On the other hand, the acute (10min) activation of A_{2A}R immediately inhibited glutamate uptake, an effect that was found to be dependent on a physical coupling between astrocyte A_{2A}R with Na^+/K^+-ATPase α_2 subtype (NKA-α_2), linked to GLT-I in order to rapidly regulate astrocytic glutamate transport. In resume, this dual mechanism of inhibition of glutamate transporters by astrocytic A_{2A}R provides a novel candidate mechanism to understand the ability of A_{2A}R to both rapidly and prolongabily modulate glutamatergic activity, a process intricately associated with the etiology of several brain diseases.

Subsequently, we investigated the implications of A_{2A}R inhibiton of glutamate uptake in an animal model of Alzheimer's disease, a neurodegenerative condition associated with the contribution of a deficient glutamate uptake and ensuing excitotoxicity and cell death. The present study demonstrated the ability of A_{2A}R blockade to prevent the amyloid-beta ($A\beta_{1-42}$)-induced decrease in glutamate uptake in different (*in vivo* and *ex vivo*) brain astrocyte preparations. In accordance, both the pharmacological blockade as well as the genetic excision of A_{2A}R efficiently prevented the decline in glutamate uptake in cultured astrocytes treated with $A\beta_{1-42}$ and in glial plasmalemmal vesicles (gliosomes) from intracerebroventricularly (ICV) injected $A\beta_{1-42-}$ rats. Together, these results show that astrocyte A_{2A}R play a crucial role in the development of Aβ-induced glutamate uptake dysfunction and provide an additional justification for the promising therapeutic benefits of selective blockade of A_{2A}R in early Alzheimer's disease pathogenesis.

Finally we examined the behavioral consequences of an exacerbated glutamate uptake caused by selective deletion of A_{2A}R in astrocytes (in Gfa2-A_{2A}R KO mice). We here show that Gfa2-A_{2A}R-KO mice exhibit an array of endophenotypes characteristic of schizophrenia, namely a decrease in working memory (WM) performance and an enhanced psychomotor response to MK-801, which were reverted by GLT-I selective inhibition. In addition, the present results also suggest that neuronal and astrocytic A_{2A}R may have opposite neuromodulation effects, since

the suppression of neuronal A_{2A}R activity was shown to be pro-cognitive while the selective deletion of astrocytic A_{2A}R potentiated working memory deficits. Finally, we demonstrated that the glutamatergic dysfunction observed was associated with an alteration of NMDAR2B-mediated AMPAR GluR1/GluR2 subunit membrane trafficking, suggesting that genetic deletion of astrocytic A_{2A}R may lead to drastic and extensive modifications in the glutamatergic system that confer susceptibility to a schizophrenia-type of traits and reveal a novel mechanistic view of the fundamental role of the neuron-astrocytic communication in cognitive processes affected.

In resume, the results presented here indicate that astrocyte A_{2A}R, by controlling GLT-I activity and possibly additional glutamatergic mechanisms, may be critical mediators of the astrocyte-neuron communication and glutamatergic dysfunction underlying Alzheimer's disease and schizophrenia. The complete understanding of glutamate transporter physiology and the crucial regulatory role operated by A_{2A}R may lead to the future development of strategies for modulating glutamate transporter function in the treatment of different brain disorders.

CHAPTER 1

GENERAL INTRODUCTION

1. Astrocytes

1.1. Astrocytes and the tripartite synapse

The term neuroglia, or “nerve glue”, was coined in 1859 by the German pathologist Rudolph Virchow, who proposed that the neuroglia was as an inactive “connective tissue” holding neurons together in the central nervous system (CNS) (Bullock et al., 1977). Later on in 1895, Lenhossek (1895) coined the term “astrocytes” as a replacement for the term glial cells in higher vertebrates. His intention was to circumvent the expression glia=glue which implied a passive function for these cells. With a new name, he intended to indicate that these cells were functionally as important as neurons. The name was based on morphology, in particular, to the presence of multiple processes depicting a start shaped appearance (Matyash and Kettenmann, 2010).

Currently the glia is known to be divided into two major classes: i) macroglia, a group of cells derived from neuroectodermal precursors, which include astrocytes, oligodendrocytes and ependymal cells (Matyash and Kettenmann, 2010) and, ii) microglia, an assortment of phagocytic cells derived from mesodermal bone marrow macrophage precursors, recognized as the brain’s representatives of the immune system (Streit et al., 1999). Today, it is particularly difficult to define astrocytes as a specific cellular class. In fact, oligodendrocytes are characterized by their expression of myelin proteins, microglia by their macrophage-related markers, and neurons by their electrical properties and ability to make synapses, and thus, these cells can be well defined on a molecular level. In contrast, there is no unique marker that can label all astrocytes and not other cell types in the brain. Astrocytes are an ubiquitous type of star-shaped glial cell that is defined in part by what it lacks: axons, action potentials, and synaptic potentials. In addition, astrocytes greatly outnumber neurons, often 10:1 and occupy 25% to 50% of brain volume (Volterra and Meldolesi, 2005). The membrane potential (V_m) of astrocytes is more negative than that of neurons. For example, astrocytes have a V_m of about -

85 millivolt (mV), whereas neuronal membrane potential is typically -65 mV. In addition, owing to the high density of potassium (K^+) channels in setting the resting potential, the V_m of astrocytes is more sensitive to extracellular K^+ fluctuations than that of neurons (Magistretti and Ransom, 2002).

Although astrocytes are anatomically obvious, their functions have been difficult to determine. Discoveries in the last 25 years, however, have revealed some of their functions and established the essential nature of interactions between neurons and astrocytes for normal brain function. Thirty-years after the discovery of Rudolph Virchow, Camillo Golgi and Santiago Ramón y Cajal, using various primary staining and microscopic techniques, reported a considerable diversity of glial cells in the brain and found that astrocytes contacted blood vessels (Ramon and Cajal 1899). Further enhancements in morphological characterization of astrocytes, thanks to the improvements in cellular labeling and imaging technologies, showed that astrocytic morphology is far more complex than previously thought (**Fig. 1**).

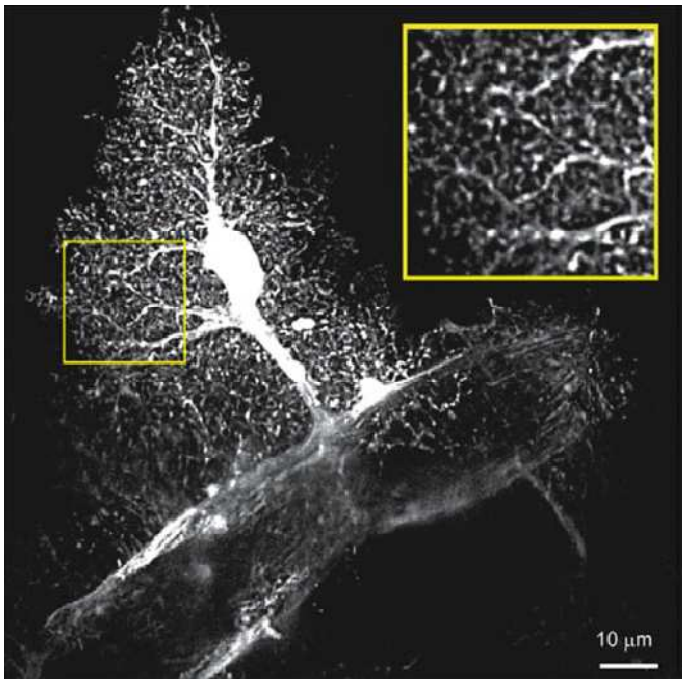


Fig.1 - Structural complexity of an astrocyte. A single astrocyte labelled with enhanced green fluorescent protein (eGFP) contacting a large blood vessel. Insert shows astrocytic processes at higher magnification (adapted from Nedergaard et al. 2010)

Cajal was indeed the first to systematically study astrocytes from a structural standpoint (Garcia-Marin et al., 2007), and until very recently, the view of astrocytic morphology has been based both on Cajal's metal impregnation methods and on glial fibrillary acidic protein (GFAP) labelling staining. However, these techniques provided a limited imaging of astrocytes. In fact, by filling single astrocytes with fluorescent dyes, it was shown that GFAP staining reveals only 15% of the astrocytic volume, and that astrocytes extend fine processes that occupy the surrounding neuropil (Bushong et al., 2002). Indeed, astrocytes occupy non-overlapping spatial territories in which a single astrocyte contacts hundreds of neuronal processes and multiple neuronal cell bodies (Bushong et al., 2002; Halassa et al., 2007). The processes from one astrocyte contact tens of thousands of synapses; with more than 50% of hippocampal excitatory synapses, for example, being closely opposed to an astrocytic process (Ventura et al., 1999). This structural relationship was later termed the "tripartite synapse", a characterization of the structural and functional relationship between the astrocyte and the pre- and post-synaptic terminals (Araque et al., 1999).

The concept of "tripartite synapse" began with a series of evidences obtained by different laboratories during the 90s that revealed the existence of a bidirectional communication between neurons and astrocytes (Bezzi and Volterra 2001; Perea et al. 2009). The signalling between neurons and astrocytes at the "tripartite synapse" is reciprocal; astrocytes sense neuronal activity by increasing intracellular levels of calcium (Ca^{2+}) and respond by releasing a variety of different molecules, the so-called gliotransmitters (Bezzi and Volterra 2001). However, the tripartite synapse concept has been further debated because not all astrocytes display a coupling of increased intracellular Ca^{2+} with the release of gliotransmitter release (Fiacco et al. 2007; Petravicz et al. 2008).

This possibility that perisynaptic astrocytic processes communicate with neurons is a new concept in synaptic physiology wherein, in addition to the information flow between the pre- and post-synaptic neurons, astrocytes exchange information with synaptic elements by responding

to synaptic activity with the overall goal of modulating synaptic transmission (Haydon et al., 2001; Volterra et al., 2005).

Astrocytes are also intimately associated with the cerebral microvasculature, onto which they extend several end-feet (Simard et al., 2003). Thus, thanks to their strategic position between synapses and blood vessels, astrocytes are thought to be mediators of neurovascular coupling, the process by which neuronal activity is coupled to cerebral blood flow and the putative cellular substrate of functional brain imaging (Mulligan et al., 2004; Takano et al., 2006).

The use of molecular genetics has revolutionized the study of astrocytic physiology and has provided an unprecedented understanding of how these cells can impact brain function at the levels of synapses, circuits and behavior (Fiacco et al., 2009).

1.2. Heterogeneity of astrocyte morphology and physiology

In the nineteenth century, two classes of astrocytes were described using an anatomical nomenclature that largely survives today: i) fibrous astrocytes of the white matter and ii) protoplasmic astrocytes of the grey matter (Matyash and Kettenmann, 2010). Their distinct morphological differences were first appreciated by Golgi staining, which revealed that protoplasmic astrocytes are complex cells with numerous fine processes, while fibrous astrocytes are less complex, with fewer branching processes (**Fig.2**). Whereas protoplasmic astrocytes appear distributed relatively uniformly within cortical gray matter, fibrous astrocytes are organized along white matter tracts, where they are oriented longitudinally in the plane of the fibre bundles (Oberheim et al, 2012).

In spite of the simplistic anatomical classification, characterizing the diverse cells that collectively are referred to as astroglia continues to be a challenge in neurobiology (Matyash and Kettenmann, 2010). In addition to the morphological nomenclature, other classifications have been proposed based on different aspects of astrocyte biology such as development,

proliferation or factors controlling proliferation. Many studies have described sub-regional differences in astroglial morphology, on GFAP levels, on the capacity of transporting glutamate, on voltage-dependent current patterns, on receptor expression, on gap junctions, on surface and intracellular proteins and on RNA (Reichenbach and Wolburg, 2005). Thus, it is now being increasingly perceptible that, similarly to neurons, there is a remarkably diverse and regionally diverse array of astrocytic populations throughout the CNS (Emsley and Macklis, 2006), with the categorization of different astrocytes shifting towards a functional definition of each astrocyte projection (which act as functional units) instead of a classical morphological classification (Naverette and Araque, 2011)**.**

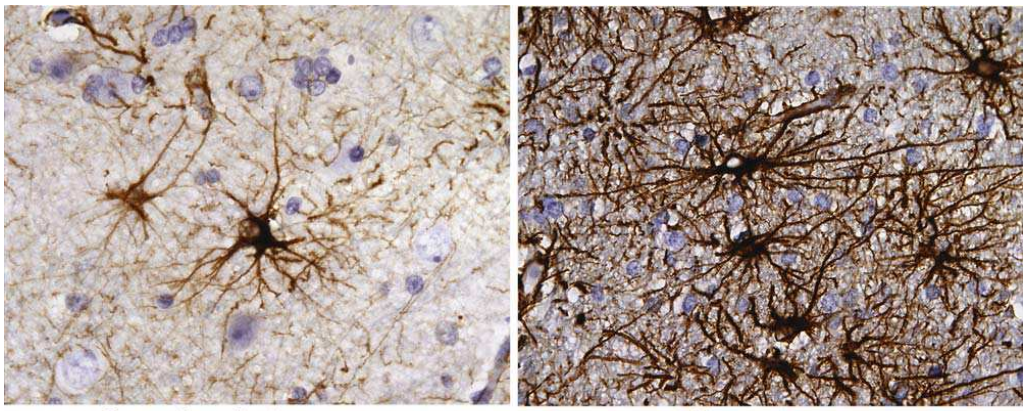


Fig.2 - Human astrocytes stained for GFAP with a hematoxylin counter-stain. Current studies of astrocytic morphologies use immunohistochemical techniques to reveal their structure. Depicted here are protoplasmic astrocytes from the gray matter and fibrous astrocytes from the white matter stained for GFAP (brown) with a light hematoxylin counterstain (blue). (Taken from Levinson et al. 2005)

Recent studies suggest that the morphology, density and proliferation rate of astroglia can even independently define the discrete cytoarchitecture of the adult mammalian CNS, supporting the concept that regional astroglial heterogeneity reflects important molecular and functional differences between distinct classes of astroglia, much like the long-accepted heterogeneity of neuronal populations (Emsley and Macklis, 2006). Moreover, mRNA microarray results have highlighted only a small number of genes that are ubiquitously

expressed by astrocytes, whereas several other genes have been found only in astrocytes from particular brain regions (Bachoo et al., 2004). In this context, marginal subclasses of astrocytes coexisting in the same brain region have been described, including tanycytes, 'radial' cells, Bergmann glia, protoplasmic astrocytes, fibrous astrocytes velate glia, marginal glia, perivascular glia, and ependymal glia (for review see, Reichenbach and Wolburg, 2005) (see **Fig.3**).

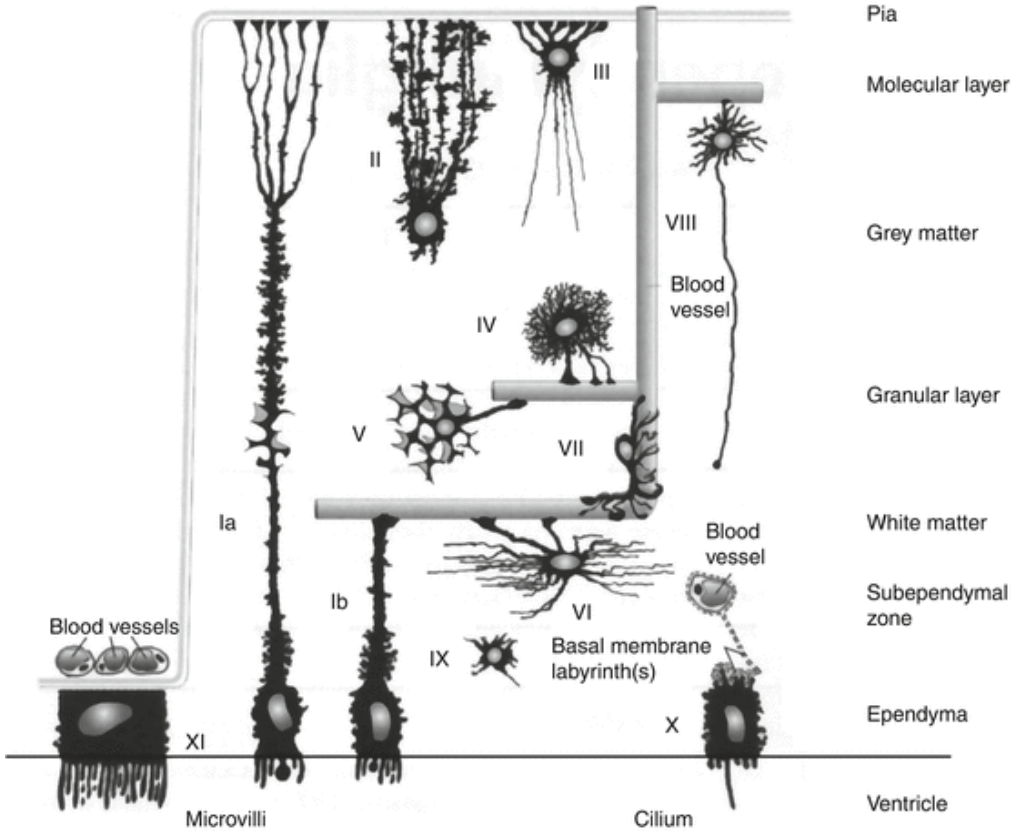


Fig.3 - Morphological diversity of astrocytes. Ia - pial tanycyte; Ib - vascular tanycyte; II - radial astrocyte (Bergmann glial cell); III - marginal astrocyte; IV - protoplasmic astrocyte; V – velate astrocyte; VI - fibrous astrocyte; VII - perivascular astrocyte; VIII - interlaminar astrocyte; IX – immature astrocyte; X- ependymocyte; XI - choroid plexus cell. (Taken from Reichenbach and Wolburg, 2005)

In addition to the morphological and functional diversity of astrocytes, more recent studies have revealed inter-species differences in astrocytic form and function, which highlight the potential importance of astrocytic function in complex brain processing. For example, human

protoplasmic astrocytes manifest a threefold larger diameter and have tenfold more primary processes than those of rodents (Oberheim et al., 2009). Therefore, human cortical evolution has been accompanied by increasing complexity in the form and function of astrocytes, which may reflect an expansion of their functional roles in synaptic modulation and brain circuitry.

1.3. Astrocytes functions in the CNS

In accordance with the multitude of different astrocyte populations, astrocytes are known to play many different functions (see **Fig.4**). First and foremost, astrocytes represent the main cellular element of homeostatic system, which is responsible for all aspects of metabolic support, nutrition, control of ion and neurotransmitter environment, regulation of brain-blood barrier (BBB) and control of oxidative stress in the brain (Parpura et al., 2012). Because astrocytic processes are highly dynamic subcellular elements capable of mobility, retraction and extension, astrocytes can dynamically shape the extracellular space, which may have a strong impact on the neuronal network by influencing the extracellular diffusion of neurotransmitters (Sofroniew and Vinters, 2012). In addition, astrocytes express a multitude of signalling cascades, are endowed with trans-cellular communication routes represented by gap junctions and are able to release a wide array of gliotransmitters via several regulated pathways (Matyash and Kettenmann, 2010). This complex signalling machinery may involve astroglia in a variety of information processing routines existing in the CNS and make astrocytes indispensable elements in shaping higher cognitive functions of the brain.

1.3.1. Energy and metabolism

Astrocytes make important contributions to CNS metabolism. Astrocytes, which have processes that on the one hand contact blood vessels and on the other hand contact neuronal perikarya, axons (at nodes of Ranvier) and synapses, are well positioned to take up glucose from blood vessels and deliver energy metabolites (e.g. lactate) to neurons (Rouach et al., 2008). In addition, being the principal storage sites of glycogen in the CNS, astrocytes can

sustain neuronal activity during hypoglycemia and during periods of high neuronal activity (Brown et al., 2007).

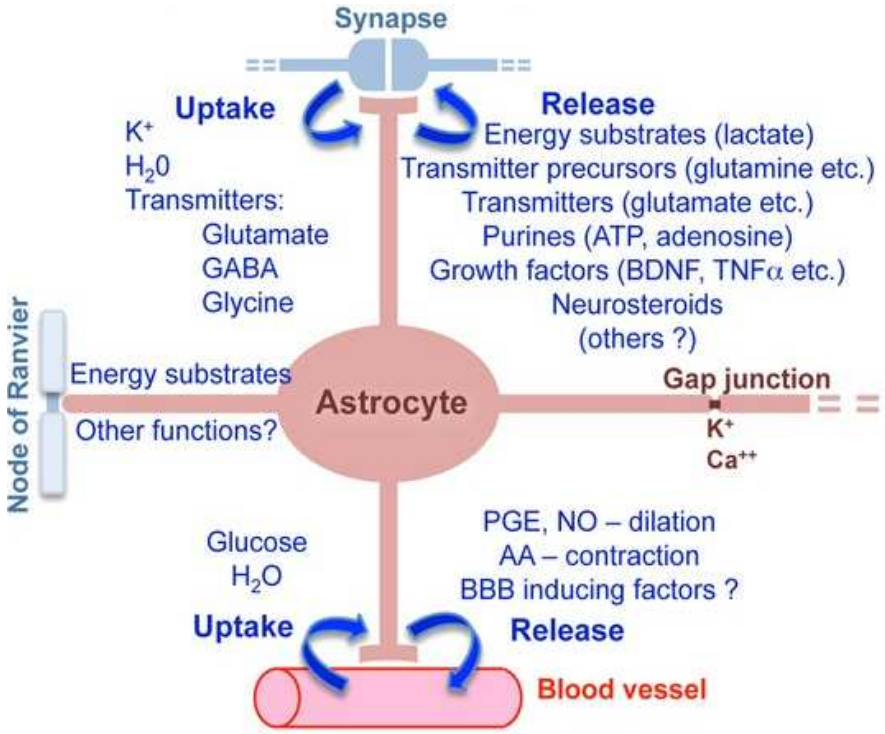


Fig.4 - Astrocyte functions in the CNS. (adapted from Sofroniew and Vinters, 2005)

1.3.2. Fluid, Ion, pH, and transmitter homeostasis

Astrocytic processes envelop almost all central synapses and exert essential functions in maintaining the homeostasis of fluids, ions, pH and transmitters in the synaptic interstitial fluid, in a manner that is critical for healthy synaptic transmission (Sofroniew and Vinters, 2012). Astrocytic membranes are endowed with a vast array of proteins (channels, receptors and transporters) critical for K^+, Na^+ and H^+ clearance and pH homeostasis. In addition, water channels such as aquaporin 4 (AQP4) are densely clustered along astrocytic processes that contact blood vessels and play a critical role in regulating fluid homeostasis in the healthy CNS (Simard and Nedergaard et al., 2004). Astrocyte processes at synapses also play essential roles in transmitter homeostasis by expressing high levels of transporters for neurotransmitters

such as glutamate (see chapter 2), GABA and glycine that serve to clear the neurotransmitters from the synaptic space (Seifert et al., 2006; Bergles et al., 2010). Absence or malfunction of these membrane proteins affects the activity of neuronal networks and leads to important brain dysfunctions. For instance, impairment of K^+ buffering by a reduced expression of these channels has been proposed to contribute to neuronal hyperexcitability and epilepsy (Seifert et al. 2006).

1.3.3. Synaptic modulation

Accumulating evidence shows that astrocytes can play direct roles in synaptic transmission through the regulated release of synaptically active molecules including glutamate, purines (ATP and adenosine), GABA, and D-serine (Halassa et al., 2007; Perea et al., 2009). The release of such gliotransmitters occurs in response to changes in neuronal synaptic activity, and can alter neuronal excitability through a modification of astrocytic excitability as reflected by increases in astrocyte $[Ca^{2+}]_i$ (Perea et al., 2009). Such evidence has given rise to the previously mentioned conception of the 'tripartite synapse', which posits that astrocytes play direct and interactive roles with neurons during synaptic activity in a manner that is essential for information processing by neural circuits (Araque et al., 1999), thus acting as spatial and temporal filters of the synaptic input (see sections 1.4, 1.5 and 2.4). In accordance, a recent study suggested that the amplitude of astrocyte Ca^2 signals reflects the number of activated hippocampal synapses (Honsek et al., 2010). In addition to having direct effects on synaptic activity via the release of gliotransmitters, astrocytes have also the potential to exert powerful and long-term influences on synaptic function through the release of growth factors (e.g. GDNF, VEGF) and related molecules (Barres et al., 2008). Finally, astrocytes play a role in the formation, maintenance, and pruning of synapses by releasing signals that induce expression of complement C1q in synapses and thereby tag them for elimination by microglia (Christopherson et al., 2005; Stevens et al., 2007).

1.3.4. Regulation of blood flow

Astrocytes make extensive contacts with and have multiple bidirectional interactions with blood vessels, including regulation of local CNS blood flow. Recent findings show that astrocytes produce and release various molecular mediators, such prostaglandins, nitric oxide (NO), adenosine and arachidonic acid, that can increase or decrease CNS blood vessel diameter and blood flow in a coordinated manner (Iadecola and Nedergaard, 2007). Moreover, astrocytes may be the primary mediators of changes in local CNS blood flow in response to changes in neuronal activity (Simard et al., 2003).

1.3.5. Immune/inflammatory response

Astrocytes have been shown to initiate, regulate, and amplify immune-mediated mechanisms involved in different human CNS diseases (Farina et al., 2007), representing an important source of cytokines (e.g. TNFα, IL-6, IL-1β) and chemokines (e.g., MCP-1/ CCL2) and express major histocompatibility (MHC) class I, that is proposed to stimulate T cells (Th2) responses, providing homeostatic mechanisms to limit brain inflammation (Dong and Benveniste, 2001). Another remarkable feature of astrocytes is their ability to respond to pathological situations, where they engage in a series of structural and functional changes, collectively, referred to as reactive astrogliosis (Pekny and Nilsson, 2005). The hallmarks of reactive gliosis include an increase of astrocyte cell number increase at the site of injury, hypertrophy of cellular processes and upregulation of many metabolic proteins and components of the cytoskeleton, such as GFAP and Vimentin (Pekny and Nilsson, 2005). Depending on the nature and extent of the insult, reactive astrocytes may assist or harm surrounding tissue, being able to provide neural protection and repair, glial scarring and confinement, but can also mediate opposite effects leading to up-regulation of CNS inflammation and contribute to tissue damage (Hamby and Sofroniew, 2010).

1.3.6. Role in development

The development of astrocytes tends to occur after the initial generation of neurons in many CNS regions. Nevertheless, astrocytes exert a number of important functions during development of both gray and white matter. Molecular boundaries formed by astrocytes take part in guiding the migration of developing axons and of certain neuroblasts (Powell et al., 1999). In addition, substantive evidence is accumulating indicating that astrocytes are essential for the formation and function of developing synapses by releasing molecular signals such as thrombospondin (Barres et al., 2008).

1.4. Astroglial transmitter release and regulation of synaptic plasticity

There is a growing body of evidence demonstrating a role for astrocytes in regulating long-term synaptic plasticity at central synapses. Such contribution involves the release of transmitters ("gliotransmitters") from astrocytes (Parpura and Zorec, 2010) by diverse mechanisms including, reverse-transporters, hemichannels, volume-activated channels and through exocytosis (Montana et al., 2006; Stigliani et al., 2006). The process of vesicular release (see **Fig.5**) requires the docking and fusion of transmitter-filled vesicles with the plasma membrane, which is mediated via the formation of the SNARE complex; a multi-protein complex that is formed by vesicular and plasma membrane proteins (Wilhelm et al., 2004). Indeed, astrocytes were consistently shown to be equipped with a plethora of different SNARE proteins involved in the mechanisms of transmitter release (Maienschein et al., 1999; Malarkey and Parpura, 2009).

Gliotransmitters can be divided into two broad types: (i) amino acids and their derivatives, such as glutamate, aspartate, homeocysteic acid, D-serine, GABA and taurine; and (ii) nucleotides and their derivatives, like ATP, uridine 5′-triphosphate (UTP), adenosine and uridine diphosphate-glucose (UDP-glucose) (Malarkey and Parpura, 2009). These mediators have

been reported to act pre- and/or postsynaptically at nearby synapses affecting transmission in a transient or long-lasting manner (Panatier et al. 2006; Serrano et al. 2006).

1.4.1. Glutamate release

Glutamate is probably the best characterized gliotransmitter able to modulate synaptic transmission. In accordance, in the hippocampal CA1 region, astrocytes in the *stratum radiatum* sense the activity of Schaffer collateral afferents and respond to increased activity with Ca^{2+} elevations and release of glutamate (see **Fig.5**). The astrocytic-derived glutamate then acts on extrasynaptic NR2B-containing NMDA receptors located on the dendrites of CA1 pyramidal cells. Activation of such receptors results in large, slow inward currents (SICs) in the pyramidal cells able to significantly depolarize the cells and even to trigger their firing (Angulo et al. 2004; Fellin et al. 2004; Jourdain et al. 2007*;* Navarrete and Araque 2010; Han et al., 2012). For example, cannabinoid exposure *in vivo* has been shown to activate astroglial cannabinoid metabotropic CB_1 receptors (CB_1Rs), eliciting glutamate release, which, in turn, induces long-term depression (LTD) via the activation of N-methyl-D-aspartate receptors (NMDA-Rs) containing the subunit 2B (NR2B) (see **Fig.6**). The importance of this mechanism is highlighted by its requirement for the cannabinoid-induced alteration of working-memory (Han et al., 2012).

In addition, astrocyte-derived glutamate release might also activate receptors localized at the presynaptic level. Through activation of group I metabotropic glutamate receptors (mGluRs) astrocytes can enhance the frequency of spontaneous and evoked excitatory synaptic currents (Perea and Araque 2007; Navarrete and Araque 2010). Alternatively, astrocytes can induce the potentiation or depression of inhibitory synaptic transmission by activation of presynaptic kainate or mGluRs, respectively (Liu et al. 2004a; b). In addition, it has been recently shown that glutamate release from cortical astrocytes is also able to broaden action potentials and therefore to facilitate ensuing synaptic transmission (Sasaki et al. *2011*). Therefore, a single gliotransmitter can exert multiple effects depending on the sites of action and the recruted

receptor subtypes, which provides a high degree of complexity to the astrocyte-to-neuron communication. This complexity becomes even higher when considering that other gliotransmitters, such as GABA, ATP, adenosine (a metabolic product of ATP), or D-serine, could have converging actions on the same neuron or, on the contrary, divergently act on several cells (both neurons and astrocytes), thus evoking distinctive responses (Perea et al. 2009).

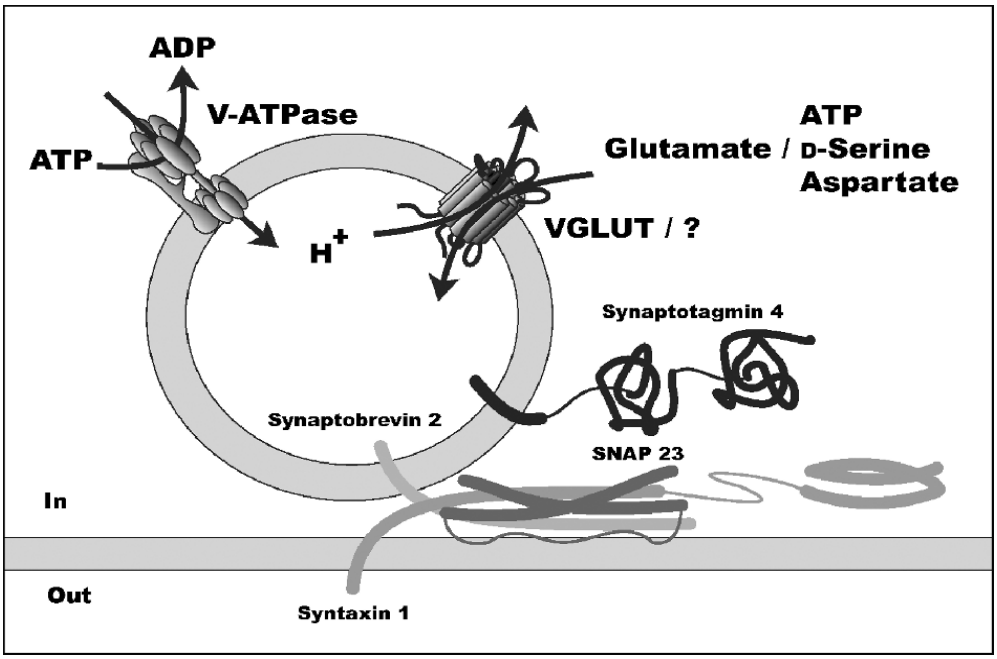


Fig.5 - Gliotransmitter release by Ca^{2+}-dependent vesicular exocytosis. Transmitters are packaged in vesicles that are released from the astrocyte when the vesicle fuses with the plasma membrane. This fusion process is mediated by synaptotagmin 4 and SNARE proteins, namely by: syntaxin 1, synaptobrevin 2 and SNAP 23. The transmitters are packaged into the vesicle by vesicular transporters, which that use the proton gradient generated by vacuolar type H^+ -ATPases (Taken from Malarkey and Parpura, 2009).

Apart from the structural-functional differences, it is interesting to note that at synapses in both CA1 and dentate gyrus synapses, astrocyte-derived glutamate is able to directly activate NMDA-Rs. This is probably because NMDA-Rs have much higher affinity for glutamate than all other glutamate receptors; therefore, they could be particularly suited for nonsynaptic

communication that implies wider diffusion and lower local accumulation of glutamate (Navarrete and Araque 2010).

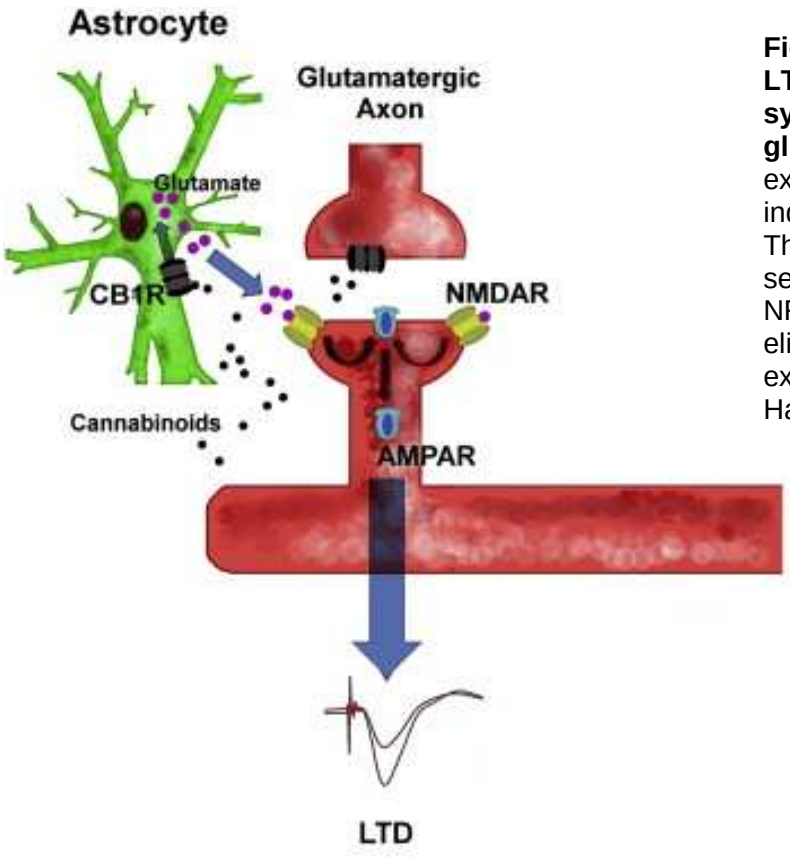


Fig.6 - Proposed model for *in vivo* LTD induction at CA3-CA1 synapses elicited by astrocyte glutamate release. Cannabinoid exposure activates astroglial CB_1R, inducing the release of glutamate. This perisynaptic glutamate, in turn, selectively activates postsynaptic NR2B-containing NMDA-R, which elicits AMPA-R endocytosis-mediated expression of LTD (Adapted from Han et al., 2012).

1.4.2. D-Serine release

The most common forms of synaptic plasticity found in the brain depend on the activation of NMDA-Rs. This includes NMDA-R-mediated LTP and LTD, which involve the insertion or the removal of -α-amino-2, 3-dihydro-5- methyl-3-oxo-isoxazolepropanoic acid (AMPA) receptors from the postsynaptic membrane, respectively (Malenka and Bear 2004). NMDA-Rs are peculiar glutamate receptors since they require the binding of an agonist, glutamate, and a co-agonist that was identified to be glycine (Johnson and Ascher 1987). In the last decade, however, several studies have challenged this idea by showing that another amino acid, D-serine, was indeed serving as an NMDA-R endogenous co-agonist (Mothet et al. 2000) and that it was

released from astrocytes (Yang et al. 2003; Mothet et al. 2005). Recordings obtained from cultures (Yang et al. 2003; Mothet et al. 2005) and from acute slices (Panatier et al. 2006; Henneberger et al. 2010) have demonstrated that astrocytes were important for supporting NMDA-R activity at glutamatergic synapses through the supply of D-serine, thereby enabling NMDA-R -mediated synaptic plasticity.

1.4.3. ATP and adenosine release

It has been consistently demonstrated that ATP can be released *in vitro* (Guthrie et al., 1999; Maienschein et al., 1999; Newman et al., 2003; Zhang et al., 2003; Coco et al., 2003) and *in vivo* (Pascual et al., 2005; Gordon et al., 2005; Lee et al., 2013; Cao et al., 2013) from astrocytes, mediating the coupling between astrocytes and between astrocytes and neurons. In culture and *in vivo*, elevation of the Ca^{2+} signal within one astrocyte leads to a Ca^{2+} wave that propagates through the coupled astroglial network. High-resolution cell culture studies showed that ATP is able to mediate Ca^{2+} waves, the specialized form of astrocyte communication (Guthrie et al., 1999). Indeed, considerable evidence shows that ATP is an important extracellular signalling molecule that is utilized by astrocytes to signal with one another as well as to neurons. A previous study has shown that ATP is enriched in astrocytic dense-core vesicles that are positive for secreteogranin II (Coco et al., 2003). This study also showed that the release of ATP from astrocytes is Ca^{2+} dependent and is inhibited by tetrodotoxin. The recent discovery of the vesicular nucleotide transporter (VNUT) (Sawada et al., 2008) and its enrichment in brain astrocytes reinforces the evidence for a Ca^{2+}-dependent, vesicular pathway of ATP release from astrocytes. The vesicular release of ATP from astrocytes is followed by its extracellular degradation to its metabolite adenosine through the crucial action of ectonucleotidases such as ecto-5'-nucleotidase (CD73), a key GPI-anchored glycoprotein with a major astrocytic localization in the adult brain (Kreutzberg et al., 1978; Augusto et al., 2013). Adenosine is then recaptured by astrocytes via bi-directional nucleoside transporters (Peng et

al., 2005) and rapidly converted to AMP (and then back to ATP) via adenosine kinase (ADK), another key enzyme of the "ATP-adenosine-cycle", which is also predominantly expressed in astrocytes in the adult brain (Boison et al., 2009).

Mounting evidence has demonstrated that astrocytic purines may serve to adjust synaptic efficacy so that it can be modulated according to various plasticity events taking place in different brain areas. This balanced modulation requires that astrocytes can increase and decrease basal synaptic transmission. Inhibition of excitatory transmission has been shown in different paradigms where ATP released by astrocytes is converted extracellularly into adenosine, which then acts presynaptically on adenosine A_1 receptors (A_1R) to inhibit excitatory transmission. Interestingly, this form of heterosynaptic depression has been shown in different brain regions, from the hippocampus (Manzoni et al. 1994; Zhang et al., 2003; Pascual et al., 2005; Serrano et al. 2006), retina (Newman et al., 2003) or the neuromuscular junction (Todd et al., 2010). The importance of this mechanism is underscored by the recent findings demonstrating its involvement in sleep regulation (Halassa et al., 2009), cognitive impairment (Stone et al., 2009) and depressive-like behaviors (Cao et al., 2013). In addition, astrocytes have been shown to increase basal synaptic transmission through activation of facilitatory presynaptic A_{2A} receptors ($A_{2A}R$) (Panatier et al., 2011). Single action potentials in the hippocampal CA1 region elicit the activation of astrocyte metabotropic glutamate subtype 5 receptors (mGlur5) which lead to ATP release following Ca^{2+} elevation (**Fig.7**).

Thus, astrocytes use a balanced A_1R-$A_{2A}R$ modulation to differentially influence synaptic plasticity. Because this adenosinergic mechanism operates over significantly longer timescales than does synaptic transmission, it is an ideal cellular candidate for mechanisms underlying the control of slowly evolving behaviors such as memory or sleep (Halassa and Haydon, 2011). While the relevance of all these processes for network activity and cerebral communication remains to be unravelled, they all point to a pivotal role played by astrocytes in the regulation of synaptic strength.

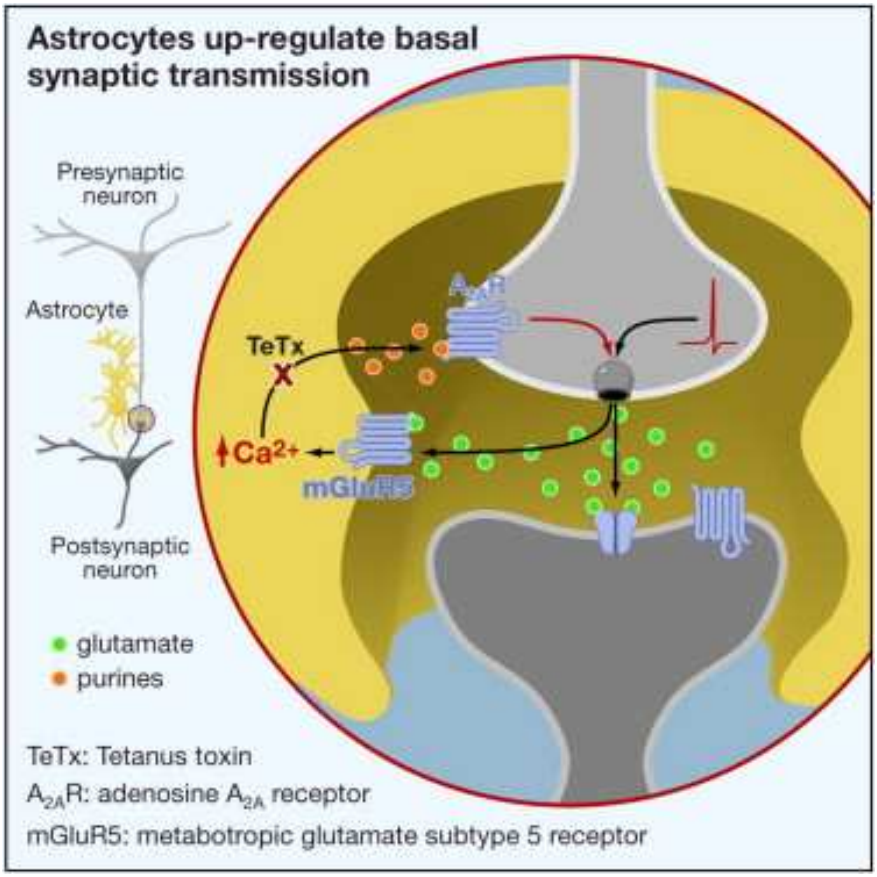


Fig.7 - Astrocytes in the hippocampal CA1 region detect synaptic activity induced by single-synaptic stimulation. Astrocyte activation occurs at functional compartments found along astrocytic processes and involves mGluR5 induced-increase in $[Ca^{2+}]i$, as revealed by the blockade of their activity with a Ca^{2+} chelator TeTx. Astrocytic modulation and stimulation of basal synaptic transmission is mediated by the release of ATP and the activation of presynaptic A_{2A} receptors by ATP catabolite adenosine (Taken from Panatier et al. 2011).

1.5. Astrocyte networks modulate neuronal activity: role in cognition

Untill now contemporary neuroscience has considered neuronal networks as the only substrate of memory and learning. However, information processing in the neuronal networks might not offer adequate complexity and precision to explain how the brain produces, processes and manipulates information for reasoning, comprehension and learning (Bezzi and Volterra, 2011). In contrast, the astroglial syncytium network conceptually allows more diverse spatial

and temporal routes for informational exchange and modulation. By projecting extensive contacts to multiple synaptic membranes within their domains, every astrocyte can integrate the information flowing through neuronal networks, and is capable of regulating these neuronal networks through the release of neurotransmitters, regulation of the extracellular environment and by affecting neuronal metabolism (Verkhratsky and Parpura, 2010). This observation forms the basis of the concept that each astrocyte territory represents an island made up of many thousands of synapses (about 140,000 in the hippocampal region of the brain, for instance) whose activity is controlled by only one astrocyte. The individual microdomains are further integrated through intercellular contacts, which multiply the processing capabilities. As a result, astrocytes are increasingly perceived as critical participants of the processing of higher brain functions. Release of ATP-derived adenosine (Pascual et al., 2005; Serrano et al. 2006), D-serine (Panatier et al., 2006), glycogenolysis and lactate transport (Suzuki et al., 2011), water channel AQP4 levels (Li et al., 2012) and cannabinoid receptor CB1 function (Han et al., 2012) are among the astrocyte-specific mechanisms recently associated with cognitive processes (see **Fig.8**). In addition, striking structural modifications of the morphology of the astrocyte surrounding synaptic contacts occur in parallel to neuronal plastic adaptations in brain areas activated by simple behavioral paradigms of learning. Thus, the size, the density and the extension of GFAP-immunoreactive astrocytes are increased in the cortex (Sirevaag et al., 1987; Jones et al., 1996), hippocampus (Wenzel et al., 1991; Huang and Lee, 1997; Gomez-Pinilla et al., 1998) and cerebellum (Anderson et al., 1994) following spatial and motor-skill learning tasks.

Holger Hydén demonstrated almost 50 years ago that learning changes the base composition of nuclear RNA, i.e. it induces an alteration in gene expression (Híden and Eghyázi, 1963). An equally revolutionary observation at that time was that changes in the base composition of the RNA occurred in both neurons and astrocytes during learning paradigms. From these findings, Holger Hydén concluded that the establishment of memory is correlated

with protein synthesis, and he demonstrated the *de novo* synthesis of several high-molecular protein species after learning (Hertz et al., 2001). In accordance, the protein, S-100β, which is mainly found in astrocytes, was increased during learning, and antibodies towards this protein inhibited memory consolidation. S-100β belongs to a family of Ca^{2+}-binding proteins, and Hydén soon realized the importance of Ca^{2+} in brain function. He established that astrocytes showed more marked and earlier changes in RNA composition in Parkinson's disease (PD) than neurons. Hydén also had the vision to suggest that ''mental diseases could as well be thought to depend upon a disturbance of processes in glia cells as in the nerve cells'', and he showed that antidepressant drugs cause profound changes in astrocytic RNA (Híden and Eghyázi, 1963; Hertz et al., 2001). It is thus not surprising that psychiatric disorders with a strong impairment of cognitive and emotional processes, namely major depressive disorder (MDD) and schizophrenia, have been consistently associated with selective changes in astrocytic function (Webster et al, 2005; John et al., 2012; Hines et al., 2013; Cao et al., 2013). Accordingly, different studies have shown altered gene expression of GFAP and S-100β, glutamate transporters, glycogen metabolism, and glutamine synthetase in MDD and schizophrenia (for review see: Schwarz and Myint, 2011; Rajkowska and Stockmeier, 2013; Takahashi and Sakurai, 2013).

Deficits in learning and memory are closely correlated with impairments of synaptic plasticity. A key brain region for storage of memories is the hippocampus, where synaptic connections exhibit activity-dependent plasticity changes such as LTP, the sustained increase in synaptic strength associated with memory formation (Verkhratsky and Parpura, 2010). Recently, the astrocyte-derived neuromodulator D-serine, an endogenous co-agonist of NMDA-R (see section 1.4.2), was shown to be required for the induction of synaptic plasticity and memory processing (Henneberger et al., 2010). During aging, the content of D-serine and the expression of its astrocyte specific synthesizing enzyme serine racemase are significantly decreased in the hippocampus, highlighting the critical role of astrocytes, through the availability of D-serine, in

the deficits of synaptic mechanisms of learning and memory that occur in the course of aging (Mothet et al., 2006; Panatier et al., 2006; Henneberger et al., 2010).

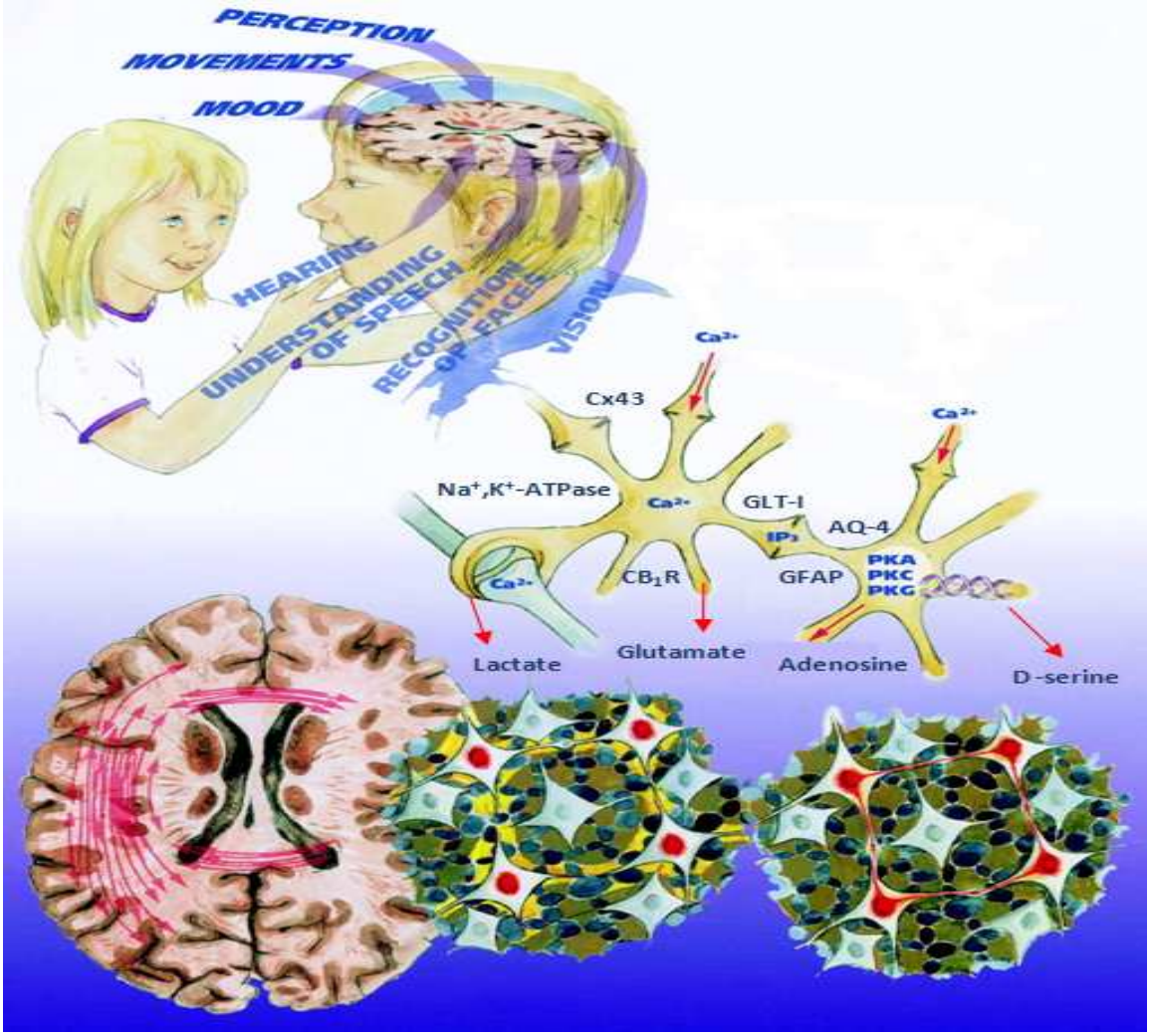


Fig.8 - **Astrocyte involvement in cognition**. The widespread astrocyte network enables the long-range integration of the information. An assortment of gliotransmitters, astrocyte molecules and Ca^{2+} signaling modulate the information flowing though neuronal networks. The progression across the astrocytic syncytium through Ca^{2+} waves, constitutes the neuronal-astrocytic-neuronal impulse transmission system. The lower right conglomerate of neurons and astrocytes illustrates conventional synaptic impulse transmission from neuron to neuron, whereas the neurons in the left conglomerate are functionally linked by astrocytic Ca^{2+} waves (in yellow). (Adapted from Hertz et al., 2001).

In addition, it has been consistently shown that lactate produced by glycogenolysis in astrocytes is required for memory processing and retention (Gibbs et al., 2006 and 2008; Suzuki

et al., 2011; Newman et al., 2011). Recently, astroglial-derived glutamate elicited by activation of astroglial CB_1R has been shown to induce working memory impairment in mice, upon cannabinoid administration (Han et al., 2012), whereas astrocyte-specific knnockout mice for Apolipoprotein E (ApoE), were shown to have a profound impairment on working memory in the absence of typical Alzheimer's disease pathology (Hartman et al., 2001). Finally, astrocytic interleukin-1 receptor (IL-1R) (Ben Menachem-Zidon et al., 2011), water channel AQ-4 (Mitterauer et al., 2010; Skucas et al, 2011; Li et al, 2012), glutamate transporters (Bechtholt-Gompf et al., 2010; John et al., 2012), astrocyte-specific Na^+/K^+-ATPase (NKA) (Moseley et al., 2007) and astrocytic connexin 43 (Cx-43) hemichannels (Stehberg et al, 2012) were revealed to modulate spatial and fear memory processing.

In addition to memory, several astrocyte-specific transgenic mice models have shed some light on the control of different behaviors by astrocytes. Selective disruption of exocytosis in astrocytes showed the involvement of adenosinergic gliotransmission in sleep regulation and cognitive impairment following short-term sleep loss (Halassa et al., 2009). Impairment of the function of the astrocyte-specific Na^+/K^+ Atpase (NKA) results in an increased anxiety-related behavior and impaired spatial learning in mice (Moseley et al., 2007) and mice lacking astrocytic-specific connexin-43 (Cx-43) exhibited exacerbated locomotor activity (Theis et al., 2003). Finally, the influence of astrocytes on synaptic functions is important in reward circuits, and may contribute to drug addiction (Miguel-Hidalgo et al., 2009) and depressive-like behaviors (Cao et al., 2013).

In the attempt to understand how neurons process information in the neuronal network responsible for memory and learning, the observation that astrocytes listen and talk to synapses by exerting both excitatory (Araque et al., 1999; Brockhaus and Deitmer, 2002; Di Castro et al., 2011; Fellin et al., 2004; Jourdain et al., 2007; Pascual et al., 2005; Pasti et al., 1997, Pasti et al., 2001; Perea and Araque, 2007; Lee et al., 2013) and inhibitory (Parpura et al., 1994; Kang et al., 1998; Zhang et al., 2003; Panatier et al., 2006; Serrano et al., 2006; Panatier et al., 2011)

actions on neurons represents one of the most relevant finding in brain research over the last decades. This discovery literally revolutionized our view of a brain function based ‘‘only’’ on billions of neurons interacting dynamically in the neuronal network. Against this background, it is not surprising that an integrated view of the role of astrocytes not only in the processing of sensory information, but also in the genesis of cognitive functions is now gradually emerging (Mothet et al., 2006; Panatier et al., 2006; Henneberger et al., 2010). Therefore, a full understanding of the astrocyte role in cognititive functions represents, indeed, a formidable challenge in neurobiological research.

2. Glutamate Transport

2.1. Glutamate homeostasis in the CNS

L–glutamate is an essential amino acid for every cell and plays a unique role as the major excitatory neurotransmitter in the communication within the brain (Headley and Grillner, 1990). Because of this critical function, the homeostasis of glutamate is tightly regulated by a highly dynamic cycle between the neurons and astrocytes (Danbolt, 2001). As the major excitatory neurotransmitter in the CNS, the levels of extracellular glutamate have to be tightly controlled for both physiological and pathological reasons (Gether et al., 2006). Physiologically, during glutamatergic neurotransmission, the extracellular concentration of glutamate released into the synaptic cleft determines the extent of receptor stimulation on postsynaptic neurons (Sattler and Rothstein, 2006). It is thus critical to maintain a low concentration of extracellular glutamate to reduce baseline stimulation of these receptors. This helps producing a high signal-to-noise ratio during the synaptic transmission to convey the adequate signal (Danbolt, 2001). Low concentrations of extracellular glutamate also reduce the chance for glutamate to leak out of the synapse and spill out to neighbouring synapses, which helps avoiding subsequent nonspecific

stimulation of receptors (Huang and Bergles, 2004). Pathologically, numerous *in vivo* and *in vitro* studies have shown that excessive amounts of glutamate are highly toxic to neurons, which is referred as glutamate excitotoxicity (Choi, 1988). Multiple downstream signal cascades have been demonstrated in glutamate induced excitotoxicity (Sattler and Rothstein, 2006). In particular, the binding of glutamate to the ionotropic glutamate receptors can induce the influx of Ca^{2+} through receptor-coupled ion channels. High concentrations of intracellular Ca^{2+} then activates Ca^{2+}-dependent proteases or phospholipases, and produces free radicals that are toxic to the neurons (Arundine and Tymianski, 2003). Because of these reasons, released extracellular glutamate has to be removed, especially from the synaptic cleft. No known enzymes (or other molecules) have been identified to extracellularly metabolize or inactivate glutamate. Instead, most of the extracellular glutamate is transported into mainly the surrounding astrocytes as well as by postsynaptic neurons.

2.2. Astrocytic glutamate transporters

All glutamate transporters (GluT) belong to the solute carrier family 1 (SLC1) (Kanai and Hediger, 2004). So far, five glutamate transporter subtypes have been identified in the CNS based on their cell- or region- specific distribution (see **Table I**). They are named as excitatory amino acid transporters 1 [EAAT1; rodent analog, L-glutamate/ L-aspartate transporter (GLAST)], 2 [EAAT2; rodent analog, L -glutamate transporter 1 (GLT-I)], 3 (EAAT3; rodent analog, excitatory amino acid carrier 1 (EAAC1)], 4 (EAAT4), and 5 (EAAT5) (Anderson and Swanson, 2000). Although GluTs are expressed in both neurons and astrocytes, two astroglial transporters, GLT-I/EAAT-2 and GLAST/EAAT-1, are primarily responsible for the uptake of extracellular glutamate and maintenance of glutamate homeostasis, together accounting to up to ~90% of all the glutamate transport in the CNS (Tanaka et al., 1997).

There are many reasons for astrocytes being the cellular type primarily responsible for glutamate uptake: i) the astrocytic membrane potential is more stable than the excitable

neuronal membrane potential; ii) the increase of the intracellular sodium (Na^+) concentration that occurs during a neuronal action potential can negatively affect Na^+/glutamate co-transporters; iii) astrocytes maintain more efficiently the physiological Na^+ and K^+ gradients than neurons during ATP depletion, which make them better suited to maintain low extracellular glutamate levels via uptake mechanisms; iv) astrocytes have a lower intracellular glutamate concentration than neurons, due to its rapid conversion to glutamine and α-ketoglutarate, which create a transmembrane glutamate gradient that favours its uptake by the astrocytes (Kanai et al., 1994; Anderson and Swanson, 2000).

Table I - Glutamate transporter subtypes (adapted from Sattler and Rothstein, 2006)

Glutamate transporter subtype	Human homologue	Cell type	Anatomic localization
GLAST	EAAT1	Astrocytes, oligodendrocytes	Cerebellum, cortex, spinal cord
GLT1	EAAT2	Astrocytes	Throughout brain and spinal cord
GLT1b	EAAT2b	Astrocytes and neurons	Throughout brain and spinal cord
EAAC1	EAAT3	Neurons	Hippocampus, cerebellum, striatum
EAAT4	EAAT4	Purkinje cells	Cerebellum
EAAT5	EAAT5	Photoreceptors and bipolar cells	Retina

The importance of astrocytic GluTs is underscored by studies with *in vivo* administration of antisense DNA (Rothstein et al., 1996) or knockout mice (Rothstein et al., 1996; Tanaka et al., 1997; Watase et al., 1998) for GLT-I and GLAST, showing that disruption of the uptake leads to an array of CNS pathologies, including epilepsy, lethal spontaneous seizures, motor discoordination and hippocampal CA1 neuron loss (Rothstein et al., 1996; Tanaka et al., 1997; Watase et al., 1998).

GluTs are classified as "Na^+-dependent high-affinity transporters," since the translocation of glutamate across the membrane is coupled to the Na^+, H^+, and K^+ to utilize the free energy associated with these ionic gradients (Anderson and Swanson, 2000). Because of the high

intracellular glutamate concentration (mM), GluTs work against a steep concentration gradient to maintain low extracellular concentrations (μM). Current stoichiometric models indicate that GluTs operate via an "alternate access mechanism" whereby the translocation of one molecule of glutamate into the cell is coupled with the inward co-transport with 3 Na^+ and 1 H^+ ion (**Fig.9**). In turn, the return of the empty carrier to the extracellular side of the plasma membrane is coupled to the export of 1 K^+ ion. Thus, there is a net entrance of one positive charge and one ATP is hydrolysed to trigger the NKA (Danbolt, 2001). In addition, GluTs, particularly EAAT4 and EAAT5 may function as glutamate-gated chloride (Cl^-) channels, generating an anion current that is not stoichiometrically coupled to the uptake of glutamate (Sattler and Rothstein, 2006).

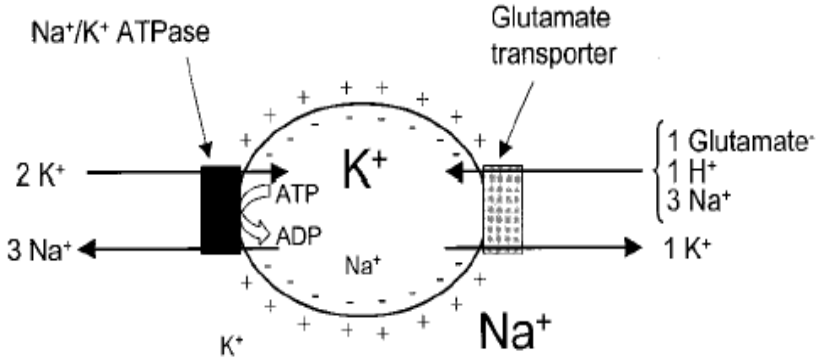


Fig.9 - Schematic representation of astrocytic glutamate uptake. Uptake of glutamate and H^+ is driven by the gradient driving force of Na^+ and K^+ movement down their respective concentration gradients, enabled by the coupled transport of a Na^+/ K^+ - ATPase. Three Na^+ and one H^+ enter, while one K^+ is transported out of the cell during glutamate uptake. (Anderson and Swanson, 2000)

Astroglial GluTs have several important roles, including: 1) termination of the synaptic effects of glutamate; 2) prevention of potentially toxic accumulation of extracellular glutamate; 3) supply of glutamate for synthesis of glutamine, which is involved in ammonia detoxification and in the crucial glutamine-glutamate cycle; and 4) warning of the metabolic demand by nearby neurons and thus activation of glycogenolysis in astrocytes, for production of carbohydrate

metabolites. In addition to these functions, GLT-I and GLAST are also expressed in astrocyte end-feet surrounding brain capillaries, allowing the critical efflux of extracellular glutamate from the brain to the blood and thus providing an additional neuroprotective mechanism against toxic accumulation of glutamate (Benarroch et al., 2010).

Neuronal GluTs, including EAAT3 and EAAT4, have a functional significance at certain specialized glutamatergic synapses, where they control the activation of postsynaptic metabotropic glutamate receptors (Anderson and Swanson, 2000). For example, EAAT4 is specifically located in Purkinje cells of the cerebellum and has a role in limiting the activation of metabotropic glutamate receptors involved in mechanisms of synaptic plasticity in these cells (Benarroch et al., 2010). EAAT3 is the primary glutamate transporter in forebrain neurons and, by allowing glutamate uptake, regulates the availability of the GABA precursor and thus GABA synthesis in inhibitory interneurons (Sattler and Rothstein, 2006).

2.3. Regulation of astrocytic glutamate transporters

GLAST and GLT-I are 65% identical at the amino acid level and have similar hydropathy plots (Anderson and Swanson, 2000). Proposed models suggest that the first 338 or 340 amino acids form the six α-helical transmembrane domains of GLT-I or GLAST, with two or four additional transmembrane domains and an aqueous medium-facing a reentrant loop, spanning the membrane from position 392-415, similar to the reentrant loops postulated for several ion channels (see **Fig.10**). Although some structural discrepancies remain, several studies provided independent confirmation that this pore-loop structure is responsible for Na^+, K^+, glutamate or Cl^- translocation (Kanai and Hediger, 1992; Pines et al., 1992; Beart and O'Shea, 2007).

Despite the crucial role of astroglia in the maintenance of glutamate homeostasis in the brain, the regulation of astrocytic GluTs is still poorly understood. Glutamate, itself, is probably one of the first actors responsible for the regulation of GluTs, not only through its receptors, but also by direct interaction with its transporters through a mechanism that involves cytoskeleton-

dependent protein trafficking (Gegelashvili et al., 1997). Moreover, many studies have confirmed the role played by diverse factors released by neurons (neuropeptides, growth factors and neurotransmitters) in the control of GLAST and/or GLT-I expression (Gegelashvili et al., 2000). Several factors have been identified in the regulation of glutamate transporter (Sattler and Rothstein, 2006). Astrocytic GluTs possess multiple protein phosphorylation sites in their primary amino acid sequences, and protein kinases, particularly protein Kinase C (PKC), A (PKA) and B (PKB or AKT), cyclic adenosine monophosphate (cAMP) or even the extracellular signal related kinases (ERK1/2) have been shown to dynamically regulate glutamate uptake (Gegelashvili et al., 2000, Figiel et al., 2004; Matos et al., 2008). The use of selective inhibitors of these signaling kinase pathways revealed that depending on the extracellular stimuli and glutamate transporter subtype, the expression of GluT may be regulated distinctly by different signaling proteins/pathways (Anderson and Swanson, 2000).

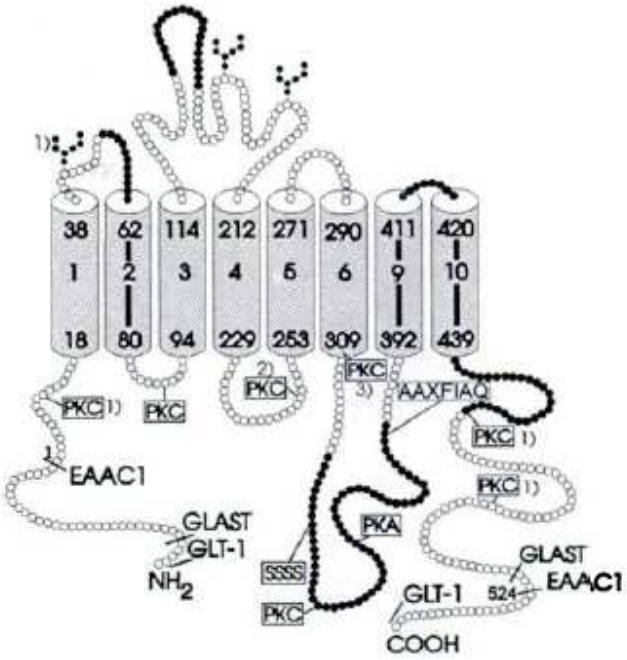


Fig.10 - Proposed model for the astrocytic GluTs according to Pines et al. (1992). Potential phosphorylation sites PKC and PKA are marked and potential N-glycosylation sites are indicated by the hexagons (Taken from Kanai et al., 1992).

Another mechanism involved in the regulation of glutamate uptake, at the transporter level, results from the interaction of redox agents with transporter sulfhydryl groups. Hydrogen peroxide (H_2O_2), nitric oxide (NO), radical superoxide anion ($O_2^{\cdot-}$) and peroxynitrite anion

($ONOO^-$) were all shown to inhibit glutamate uptake (Gegelashvili et al., 2000). Both astrocytic transporter subtypes, GLT-I and GLAST, contain functional cysteine residues that are sensitive to oxidative formation of cystine bridges, which inhibit glutamate flux through the transporters (Anderson and Swanson, 2000).

Recently, ephrin-A3, an astrocyte ligand of ephrin-A4 receptor tyrosine kinases which are implicated in synapse formation and plasticity, has been found to actively regulate glutamate uptake, preventing the upregulation of GLT-I expression to non-physiologically high levels (Carmona et al., 2009; Filosa et al., 2009). Therefore, dendritic EphA4 and asytrocyte ephrin-A3 interact at the synapse vicinity controlling the abundance of perisynaptic GluT and, by this way, regulate synaptic function promoting LTP (Filosa et al., 2009).

In addition to the external modulators and signalling pathways, recently it was shown that glutamate transporting in astrocytes is coupled to the activity of Na^+/ K^+-ATPases (NKA), which generates electrochemical gradients to drive transmitter uptake (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011). NKA are ubiquitous plasma membrane enzymes responsible for maintaining the membrane potential of cells using the energy of adenosine triphosphate (ATP) hydrolysis (Reinhard et al., 2013). A functional NKA consists of a large catalytic α-subunit harbouring the ATP-binding sites and a smaller β-subunit required for full enzymatic activity and also functioning as an intercellular adhesion protein (Reinhard et al., 2013). In the brain, three different α-subunits isoforms are present in a cell-specific manner: the low-affinity α_1 is present in all cell types, the high-affinity α_2 isoform is restricted to astrocytes and the high-affinity α_3 isoform is expressed exclusively in neurons (Benarroch et al., 2011). It is thus not surprising that NKA activity and specifically the α_2-isoform has emerged as a robust modulator of glutamate uptake in astrocytes as heralded by the observations that: i) ATP depletion leads to a reversal of glutamate uptake (Longuemare et al., 1999); ii) inhibitors of NKA such as ouabain impair glutamate transporter activity (Pellerin and Magistretti, 1997; Kawahara et al., 2002; Rose et al., 2009; Genda et al., 2011) and lead to glutamate transporter clustering and redistribution

(Nakagawa et al., 2008; Nguyen et al., 2010); iii) and the α_2 subunit of the NKA co-localizes with GluT (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011) (see **Fig.11**). These findings demonstrate that in addition to multiple GluT modulatory signalling pathways, GluT and NKA are likely part of the same macromolecular complexes and operate as a functional unit to regulate glutamatergic neurotransmission.

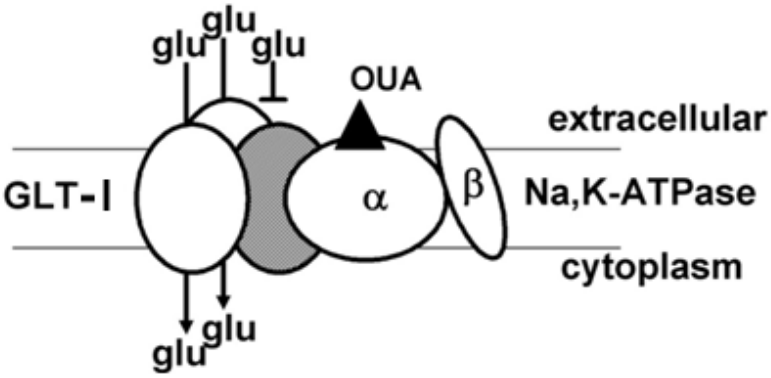


Fig.11 - Proposed model for coupling between GLT-I and NKA in the astrocyte membrane (Adapted from Rose et al., 2009).

2.4. Glutamate transporters and synaptic function

Studies completed during the last decade indicate that GluTs do more than maintaining low extracellular glutamate levels. Because these proteins are abundant near synapses and bind glutamate rapidly, they compete with receptors for glutamate that is released thus shaping the concentration transient that receptors are exposed to. As a result, transporter activity influences receptor occupancy at individual synapses, and prevents further promiscuous activation of receptors at neighboring synapses (Huang and Bergles, 2004). Although a general picture of GluT function has emerged from these studies, it is clear that the contribution of transporters to synaptic modulation varies considerably among synapses; it is highly dependent on their structure, their association with astrocytes, and the properties and locations of their glutamate receptors. Furthermore, this interaction is not fixed, but often changes depending on the

frequency of release, the local ionic environment, and the structure of the synapse (Tzingounis and Wadiche, 2007).

GluTs have an affinity comparable to that of *N*-methyl-D-aspartate (NMDA) receptors and metabotropic glutamate (mGluR) receptors (GluT EC_{50}: 4 - 30 μM) and seem to bind glutamate as rapidly as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (Wadiche et al., 1995). Since the number of GluTs is higher than the number of receptors in the extrasynaptic region, it seems conceivable that GluTs indeed govern the level and rate of glutamate binding to glutamate receptors (Huang and Bergles, 2004). This idea is supported by recent studies of excitatory synapses in the hippocampus, cerebellum, striatum, cortex and amygdale (for review see Huang and Bergles, 2004). In the hippocampus, blocking astrocyte glutamate uptake has been shown to increase NMDA-R (Arnth-Jensen et al., 2002; Angulo et al., 2004), AMPA-R (Mennerick and Zorumski, 1994; Tong and Jahr, 1994; Tsukada et al., 2005), kainate (Min et al., 1998) and mGluR (Huang et al., 2004; Oliveira et al., 2008; Bellesi et al., 2010) activation and bolster excitatory postsynaptic currents (EPSCs). In the cerebellum it was shown that, in response to stimulation of single and multiple adjacent neurons, astrocyte GluTs were able to limit postsynaptic AMPA-R (Marcaggi et al., 2003; Takayasu et al., 2006) and extrasynaptic mGluR (Reichelt and Knopfel, 2002) activation, desensitization and LTD. Similarly, in the striatum inhibition of GLT-I decreased the corticostriatal synaptic transmission through desensitization of AMPA receptors (Goubard et al., 2011). In the neocortex it was shown that NMDA-R -induced EPSCs were enhanced by selective blocking GLT-I (Campbell and Hablitz, 2004). Finally, in the amygdala, blocking GluTs leads to the breakdown of synapse independence and LTP input-specificity (Tsvetkov et al., 2004). These observations suggest a close interaction between astrocytic GLT-I and perisynaptic glutamate receptors regulating the synaptic glutamate concentration and postsynaptic depolarization which, ultimately, modulates the induction of LTD (Luscher et al., 1998; Wang et al., 2006; Pita-Almenar et al., 2006; Filosa et al., 2009) and LTD (Omrani et al, 2009; Bellesi et al., 2010) at excitatory synapses.

Additionally, GluTs not only restrict the "spillout"' of glutamate and consequent activation of homosynaptic receptors, but also shield receptors from glutamate that is released at neighboring synapses (heterosynapses) (Asztely et al., 1997; Arnth-Jensen et al., 2002).

In addition to the previous mentioned mechanisms of GluT modulation of glutamatergic synaptic efficacy, synaptic activity itself can regulate GluT transport density. In the hippocampus, astrocytic glutamate uptake was markedly increased following high-frequency stimulation that results in LTP (Pita-Almenar et al., 2006). Furthermore it was demonstrated that an increase in sensory activity (whisker stimulation) caused a twofold increase in astrocyte GluT levels, with an accompanying astrocytic retraction in the barrel cortex (Genoud et al., 2006). In addition, changes in neuronal activity through kindling (Miller et al., 1997), seizures (Zhang et al., 2004) and drugs of abuse (Xu et al., 2003) also led to changes in GluT levels.

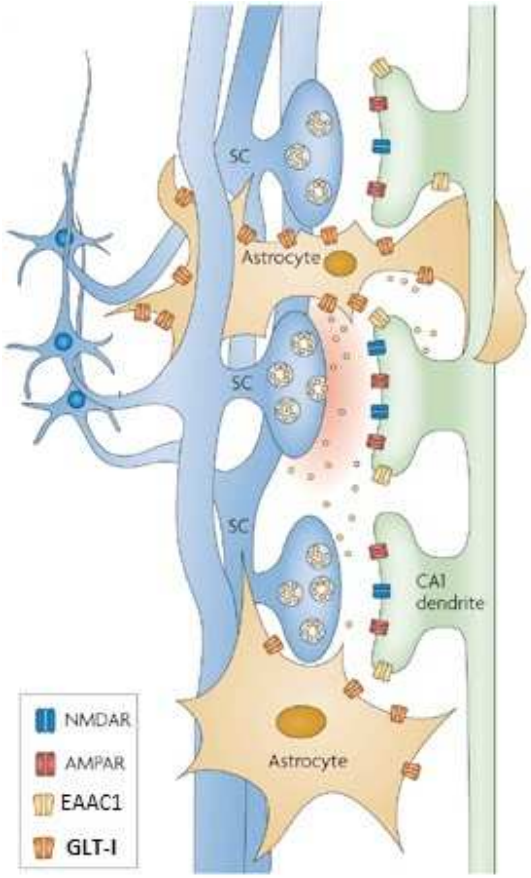


Fig.12 - A representation of the involvement of astrocytic GluTs in hippocampal synaptic transmission. Schaeffer collateral (SC) fibres from CA3 pyramidal neurons (in blue) make non-terminal synapses with CA1 pyramidal neurons (in green). These synapses are partially enveloped by astrocytic processes (in yellow) which actively remove glutamate from the synaptic cleft and thus promote homosynaptic independence, input specificity and restrict heterosynaptic plasticity. Neuronal GluTs are located perisynaptically, whereas astrocyte transporters face the synapse. (Adapted from Tzingounis and Wadiche, 2007).

Together, these data suggest that astrocytic GluTs are dynamically influenced by neuronal activity and, in turn, are able to modulate neuronal activity by promoting synapse independence, input specificity and restricting heterosynaptic plasticity (Tzingounis and Wadiche, 2007). Importantly, these studies suggest that there is a finite window for the beneficial effect of GluT activity. Alterations in transporter activity, either an increase (Omrani et al., 2009; Filosa et al., 2009) or decrease (Yang et al., 2005; Potier et al., 2010), outside of this window, may lead to altered synaptic plasticity phenomena (e.g. LTP; LTD) which underlie changes in memory and cognition (Katagiri et al., 2001; Malenka et al., 2004; Pita-Almenar et al., 2012).

2.5. Involvement of glutamate transporters in neurologic diseases

Given the critical role of glutamatergic neurotransmission in mechanisms of excitotoxicity, it is not surprising that impairment of astrocytic GluTs has been linked to a wide range of neurological disorders. The direct links between astroglial GluTs and neurological diseases were first observed in animals with reduced expression levels of GLT-I or GLAST. Both antisense knockdown and the GLT-I null mouse provided insight into the major contribution of GLT-I to total glutamate transport in the CNS (Rothstein et al., 1996; Tanaka et al., 1997). GLT-I knockout (KO) mice retained only less than 10% of total glutamate transport in the cortex and developed seizures and hippocampal pathology, the majority eventually dying by several weeks of age. In addition, subacute removal of GLT-I by antisense methods led to hind limb paralysis (Rothstein et al., 1996). Furthermore, the knockout of the GLAST transporter leads to motor discoordination and increased susceptibility to traumatic brain injury (TBI) (Watase et al., 1998).

Given the critical functions of astroglial GluTs in the CNS, it is not surprising that dysfunction of these transporters and glutamate-induced excitatory toxicity have been implicated in many neurological diseases, including amyotrophic lateral sclerosis (ALS) (Rothstein et al., 1995), Alzheimer's disease (AD) (Masliah et al. 1996), stroke and ischemia (Martin et al., 1997), trauma (Watase et al., 1998), epilepsy (Tanaka et al., 1997), Parkinson's

disease (PD) (Hazell et al., 1997), Huntington's disease (HD) (Lievens et al., 2001), demyelinating diseases (Korn et al., 2005), HIV dementia (Pappas et al., 1998), gliomas (Ye et al., 2009) and neuropsychiatric disorders such as schizophrenia (SCZ) (Simpson et al, 1992) and bipolar disorder (Smith and Meador-Woodruff, 2002). Abnormalities of GluT expression as a result of altered transcription or splicing, increased turnover of the transporter, altered trafficking of GluTs, abnormal phosphorylation or cleavage of the protein, and reduced transport capacity are all potential mechanisms leading to GluT dysfunction (Maragakis and Rothstein, 2004).

2.5.1. Involvement in neurodegenerative diseases - Alzheimer's disease

AD is a neurodegenerative disorder which is clinically characterized by progressive memory loss and dysfunction of higher cognitive domains (Querfurth and LaFerla, 2007). Pathologically, the aggregation and deposition of β-amlyoid (Aβ) peptides, as amyloid plaques, and the formation of neurofibrillary tangles represent the classical hallmarks of the disease (Hardy and Higgins, 1992).

Considerable evidence (see review, Querfurth and LaFerla, 2007) suggests that an initial glutamatergic synaptic dysfunction in cortical regions precedes Aβ deposition, neurofibrillary tangle formation, neuronal cell death and subsequent deterioration of brain function (Selkoe et al., 2002). Disturbance of glutamatergic neurotransmission and consequent excitotoxicity is also believed to be deeply implicated in the progression of this dementia (Francis, 2003; Hynd et al., 2004). The best illustration of this idea is that memantine (1-amino-3,5-dimethyl-adamantance) , a low-affinity, non-competitive NMDA-R antagonist has been approved to treat moderate to severe AD by the Food and Drug Administration (FDA) in 2003 (Sonkusare et al, 2005). In addition, the neuronal dysfunctions are accompanied by modifications in astrocytes surrounding the amyloid-plaques, including astrogliosis, process retraction, dysfunctional degradation and clearance of Aβ, production of inflammatory mediators and ultimately cell death (Nagele et al.,

2004; Wyss-Coray, 2006). In line with the initial glutamatergic dysfunction, several studies have shown a significant reduction of glutamate transporter activity in the AD brain. Brain cortical tissue (Scott et al., 1991; Masliah et al., 1996; Liang et al., 2006), synaptosomes (Hardy et al., 1987), platelets (Ferrarese et al., 2000) or fibroblasts (Zoia et al., 2005) derived from AD patients all showed an exacerbated decrease of glutamate uptake. Down-regulation or abnormal expression of GluTs, GLAST/EAAT1 and GLT-I /EAAT2, has also been detected in *post-mortem* human tissue (Masliah et al., 1996; Scott et al., 2002) and in transgenic mice developing AD-like pathology (Dabir et al., 2006), which were closely associated with tau deposition and neurofibrillary changes (Thai, 2002). A significant decrease in V_{max} and K_M for glutamate uptake and a decrease in protein expression of GLT-I and GLAST were also observed in AD transgenic mice (Li et al., 1997; Masliah et al., 2000; Scott et al., 2002) and in animals injected intraceberoventriculary (icv) with $A\beta_{1-42}$ (Piermartiri et al., 2010). Finally, *in vitro* studies have also consistently shown that Aβ reduces astrocytic glutamate uptake (Harris et al., 1996; Parpura-Gill et al., 1997; Lauderback et al., 1999; Mattson and Chan, 2003; Fernández-Tomé et al., 2004; Matos et al., 2008). A study using gene chips and immunohistochemistry also showed a sharp impairment in the expression of GLT-I and GLAST at both gene and protein levels in hippocampus and *gyrus frontalis medialis* of AD patients, even in early clinical stages of disease (Jacob et al., 2007).

The mechanisms for the altered expression and activity pattern or loss of GLT-I and GLAST in AD are still unclear although several pathological factors have been hypothesized. Oxidative damage has been discussed as a possible mechanism, and Aβ may be involved in the reaction sequence (Lauderback et al., 1999, 2001) since reactive oxygen species (ROS) are elevated in the AD brain (Lauderback et al., 1999). Furthermore, pro-inflammatory cytokines, which are increased in the AD brain, may also cause a reduction in glutamate transporter activity (Liang et al., 2002). Finally, the reduction of neuronal soluble factors in the AD brains, such as BDNF, might also affect the expression pattern of GluTs (Rodriguez-Kern et al., 2003).

Whether these alterations os GluTs constitute the triggers of synaptic dysfunction or neuronal injury in AD or are a result of an ongoing process is still not clear. The dysregulation of astroglial GluTs is unlikely to be the primary pathogenic mechanism; instead, it may accelerate the progress of the disease through an altered control of extracellular glutamate levels and triggering massive excitatory toxicity (Francis, 2003; Hynd et al., 2004). Therefore, maintaining glutamate homeostasis by increasing the expression of astroglial GluTs, especially GLT-I could potentially slow down the progression of the disease. Therefore, of future importance are functional manipulations of GluTs, for example, under expression of AD-associated genes in AD transgenic models (Querfurth and LaFerla, 2007).

2.5.2. Involvement in neuropsychiatric disorders - Schizophrenia

While the focus on GluT dysfunction in the neurologic disorders has been studied most extensively in neurodegenerative diseases, recent work also suggests that GluT biology may play a role in neuropsychiatry disorders, such as schizophrenia (Nanitsos et al., 2005).

Schizophrenia is an incurable and debilitating mental disorder with a presumed neuro-developmental origin (Ross et al., 2006). In addition, the existing pharmacotherapy is mostly directed to alleviate the positive symptoms of the disorder, the pathological hallmarks which are exclusive to schizophrenic patients, including delusions, hallucinations, and disorganized thinking. However, recent clinical data now suggest that the negative symptoms, such as anhedonia, depression and social withdrawal, and the cognitive symptoms, characterized by working memory and executive deterioration (van Os and Kapur, 2009), contribute more to the poor quality of life expectancy and functional disability in schizophrenia, than do positive symptoms (Velligan et al., 2002; Goff et al., 2008). In addition, patients with prominent negative symptoms often have a history of poor adjustment before the onset of illness and response to medication is often limited (Smith et al., 2010). Existing pharmacotherapy and conventional models have long emphasized dopaminergic (DA) dysfunction as the prime neurochemical

basis of schizophrenia, building upon the pharmacological evidence that striatal DA hyperfunction seems associated with the positive symptoms (Snyder, 1976). However, the limitations of the DA model to account for negative and cognitive symptoms led to the emergence of glutamatergic models, based on the observation that psychotomimetic agents such as phencyclidine (PCP) and dizocilpine (MK-801) induce psychotic and cognitive disturbances in human and animals similar to those observed in schizophrenic patients by blocking N-methyl-D-aspartate receptors (NMDA-R) (Moghaddam and Javitt, 2012). In fact, cortical glutamatergic hypofunction is well positioned to explain not only the positive and negative symptoms, but also the cognitive decline that is at the premorbid stage of schizophrenia (Ranganath et al., 2008). Therefore, cortical NMDA-R glutamate-hypofunction is currently suggested to trigger and integrate most neurochemical dysfunctions found in schizophrenia (Kristiansen et al., 2007; de Bartolomeis et al., 2012). Accordingly, the reduction in glutamatergic activity from mesocortical projections, leads to a reduction in the activity of GABAergic and dopaminergic neuronal activity in the ventral tegmental area (VTA), a crucial region of the midbrain which projects to numerous areas of the brain and is widely implicated in the regulation of cognition, motivation and emotional processes (Kristiansen et al., 2007) (see **Fig.13**). Thus, a decrease in the activity of the dopamine projections from the VTA to the cortex, leading to low cortical dopamine levels, is now considered to elicit the negative symptoms (Moghaddam and Javitt, 2012). In addition, lower negative regulation from the VTA GABA projections, has been proposed to incite the nigrostriatal dopaminergic and the thalamocortical pathways, leading, respectively, to an increased dopamine release in the striatum and the subsequent development of the positive symptoms and an increased glutamate release in the cortex and ensuing cognitive symptoms (Moghaddam and Javitt, 2012). Thus, the ability of NMDA-R projections to integrate the multitude of impaired interactions between multiple brain regions, rather than a particular locus of dysfunction, allows it to be the ideal candidate to explain the mechanistic dysfunctions underlying the slowly evolving and broad

cognitive and emotional dysfunctions found in schizophrenia (Lisman et al., 2008; Field et al., 2011) (see **Fig.13**).

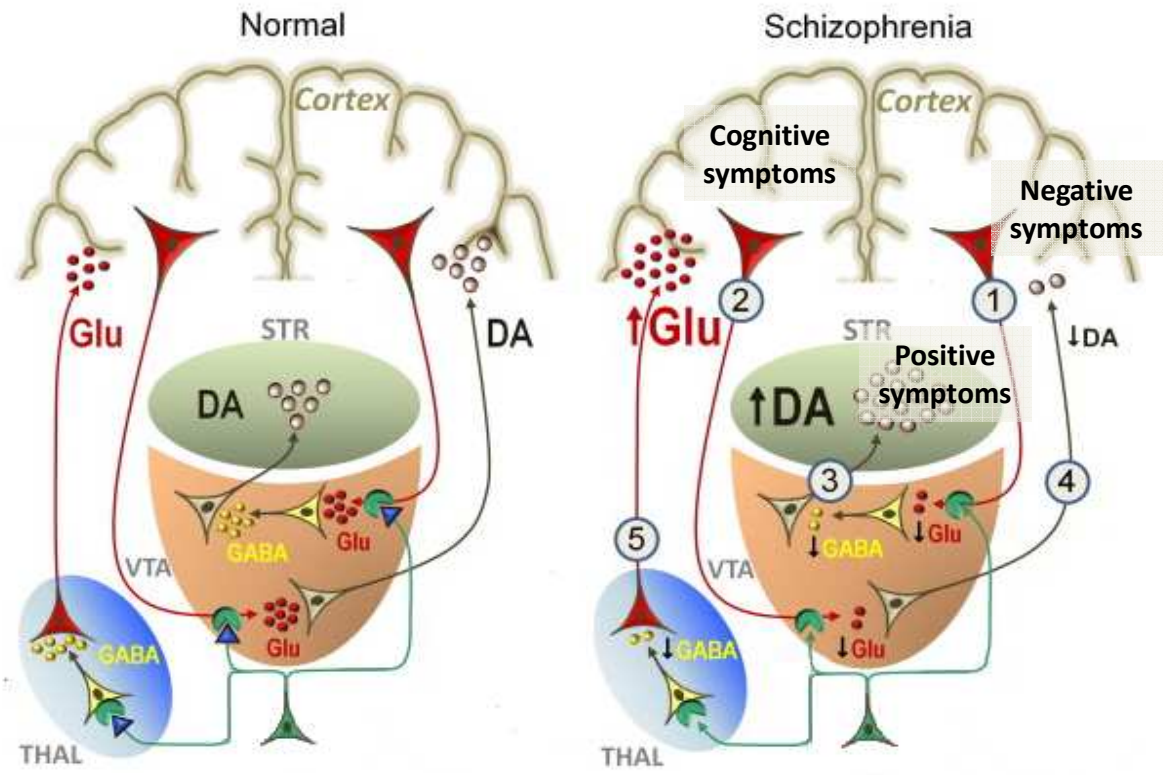


Fig. 13 - Interactions of dopaminergic and glutamatergic pathways that underlie deficits in schizophrenia. **Pathway (1)** (mesocortical) represents hypo-functionality of NMDA-R /glutamate (Glu) projections (red) from the cortex that synapse on GABA neurons (yellow) in ventral tegmental area (VTA), leading to decreased GABA release, disinhibition of dopamine (DA) projections (brown) to the ventral striatum (STR), and consequent hyperactivity of DA release in the STR, the latter illustrated by (nigrostriatal) **Pathway (3)**. **Pathway (2)** represents hypo-functionality of Glu neuron projections from the cortex that synapse directly on DA neurons in the VTA, leading to decreased glutamatergic stimulation of DA neurons that project from the VTA to the cortex and consequent decrease in cortical DA levels, the latter shown by (mesocortical) **pathway (4)**. **Pathway (5)** (thalamocortical) illustrates lower GABAergic tone in the thalamus (THAL), leading to disinhibition of Glu neurons projecting to the cortex and consequent increase in cortical Glu levels (Adapted from Moghaddam and Javitt, 2012).

In the context of the spread altered glutamatergic transmission, several reports have depicted a modified astrocytic function in schizophrenia (Kondziella et al., 2007; Schnieder et al., 2011; Schwarz and Myint, 2011; Takahashi et al., 2013). Particularly, a hyperactive glutamate uptake dysfunction has emerged as a main mechanism responsible to the decrease of the efficacy of glutamatergic synapses observed in the schizophrenic brain (Schneider et al., 1998; Nanitsos et al., 2005; Matute et al., 2005; Rao et al., 2012). Notably, an up-regulation of GLT-I mRNA, protein and function has been consistently shown in the cortex of schizophrenic patients (Simpson et al., 1998; Smith et al., 2001; Matute et al., 2005; Rao et al., 2012). This is in notable agreement with the identification of a susceptibility locus for schizophrenia within or near the GLT-I gene (Deng et al, 2004), which is deregulated in schizophrenic patients (Shao and Vawter, 2008; Spangaro et al., 2012). Also, psychotomimetics, such as phencyclidine, increase GLT-I levels and activity (Fattorini et al., 2008) whereas the anti-psychotic drug clozapine induces the reverse (Melone et al., 2003; Vallejo-Illarramendi et al., 2005). Therefore, in sharp contrast to the observation of a decrease in glutamate uptake activity in neurodegenerative diseases paving the way to increased extracellular glutamate levels and excitotoxicity (see section 2.5.1), in schizophrenia the GluT activity seems highly increased ("hyperactive"), pointing to a reduced extracellular glutamate levels and ensuing decrease on glutamatergic synaptic efficacy (Nanitsos et al., 2005).

3. Adenosine Receptors

3.1. Purinergic signaling - overview

Purinergic signaling, which corresponds to the intercellular communication mediated by adenosine triphosphate (ATP) and its breakdown products (e.g. adenosine diphosphate, ADP; adenosine monophosphate, AMP; adenosine; guanosine, inosine, urate) is, arguably, the main extracellular signalling system that integrates neuronal-astrocytes and astrocytes-astrocytes circuits in the nervous system (Inoue et al., 2010). This is because, in contrast to neurotransmitters, growth factors and ion fluxes, all cells share mechanisms for releasing ATP and membrane receptors for detecting it or its breakdown products. This enables all major types of glia to communicate via purinergic signaling and to communicate with neurons, vascular, and immune system cells. ATP provides for multiple signalling pathways within the astrocyte syncytium, being responsible for propagating Ca^{2+} waves and for long-distance astroglial communication (Hannson and Rönnbäck, 2003*;* Franke et al., 2012). Furthermore, the purinergic signalling system is intimately involved in neuropathology by mediating reactive astrogliosis, providing for glioprotection in stress conditions and assuming the main responsibility for cell proliferation, growth, and development (Inoue et al., 2010). The extended family of purinoreceptors, universally expressed in astrocytes, is coupled to numerous signalling cascades governing astrocytes physiological and pathological responses (for review see, Verkhrasky et al., 2009; Inoue et al., 2010; Franke et al., 2012).

ATP can be released from both neurons and astrocytes but recent findings suggest that the complete catabolism of ATP into the final purine metabolite adenosine originates from an astrocytic ATP source in what has been designated the “ATP-adenosine cycle” (Pascual et al., 2005; Martin et al., 2007; Halassa et al., 2009; Inoue et al., 2010) (see **Fig. 14**). In these studies it was documented that - under physiological conditions - the major source of synaptic endogenous adenosine is actually derived from astrocytic release of ATP by not yet fully

characterized pathways, which may include exocytosis, or diffusion through maxi-pore forming channels (such as hemichannels, pannexins, volume sensitive anion channels or P2X7 receptors) (Malarkey and Parpura, 2009). Afterward, ATP is extracellularly degraded to adenosine by the activity of a cascade of ectonucleotidases with a final concluding step by the action of ecto-5'-nucleotidase/CD73, a key GPI-anchored glycoprotein with a great astrocytic localization in the adult brain (Kreutzberg et al., 1978; Zimmermann and Braun, 1996; Augusto et al., 2013).

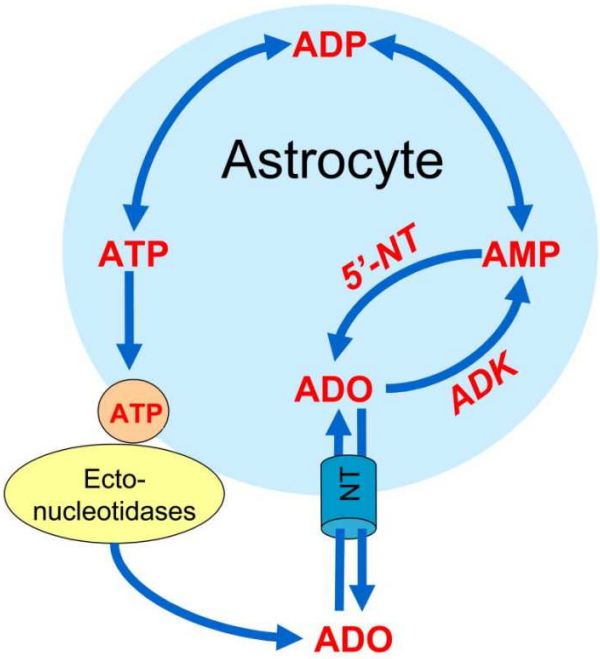


Fig.14. **- The adenosine cycle.** The regulation of extracellular levels of adenosine is largely dependent on the astrocyte-based adenosine cycle. A major source of synaptic adenosine is vesicular release of ATP followed by its extracellular degradation to adenosine (ADO) via ectonucleotidases. Nucleoside transporters (NT) equilibrate extra- and intracellular levels of adenosine. Intracellular metabolism of adenosine depends on the activity of ADK, which, together with 5′-nucleotidase (CD73/5′-NT), forms a substrate cycle between AMP and adenosine (Taken from Boison, 2008).

Adenosine is then captured by astrocytes via bi-directional nucleoside transporters (Hertz, 1978; Peng et al., 2005) and rapidly converted to AMP (and then back to ATP) via adenosine kinase (ADK), another key enzyme of the adenosine metabolism, which is also predominantly

expressed in astrocytes in the adult brain (Boison et al., 2009). In addition to the catalytic mechanisms described above, astrocytes can directly release adenosine, especially in response to hypoxic stimulation (Martin et al., 2007; Björklund et al., 2008), even though release of adenosine *per se* is more typical of neurons (Parkinson et al., 2002). In that case the release depends on export of adenosine via the equilibrative nucleoside transporters (ENTs)-e.g., the ubiquitously expressed ENT1 and ENT2 (see **Fig.14**).

Purinergic receptors are broadly divided into two categories: P_1 and P_2, representing receptors preferentially activated by adenosine and ATP, respectively (Burnstock, 2006; Fields and Burnstock, 2006). Metabotropic P_1 adenosine receptors and ionotropic P_{2X}Rs and metabotropic P_{2Y}Rs are broadly distributed in neurons and astrocytes, eliciting a remarkable variety of physiological and pathophysiological reactions (for review see Verkhrasky et al., 2009). Astrocytes respond to purinergic stimuli with increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) mainly mediated through G_q protein-coupled receptors, including those of the P_{2Y} type, with subsequent release of Ca^{2+} from intracellular stores through inositol 1,4,5-trisphosphate (IP_3) receptors (Franke et al., 2012). Propagation of Ca^{2+} waves results from the intercellular diffusion of $InsP_3$ through gap junctions or from the release of ATP in a single astrocytes, this release then producing $[Ca^{2+}]_i$ transients in the neighbouring cell by activation of P2YR/IP_3 signaling systems.

Besides being a primary signal for the astrocyte Ca^{2+} wave propagation by the activation of P_{2Y}Rs, ATP or ensuing adenosine can act pre- or post-synaptically on P_1 and P_{2X}Rs to influence excitatory synaptic activity (see section 1.4.3), facilitating glutamatergic synaptic transmission through A_{2A}R (Panatier et al., 2011), P_{2X}R (Bardoni et al., 1997; Gordon et al., 2005) or P_{2Y}Rs (Kawamura et al., 2004), or inhibiting glutamatergic neurotransmission through activation of A_1Rs (Manzoni et al. 1994; Zhang et al., 2003; Pascual et al., 2005; Serrano et al. 2006) (see **Fig. 15**). Recently it was also shown that increased astrocytic ATP release can facilitate

excitatory transmission through a reduction on GABA release induced by ATP-derived adenosine, demonstrating that ATP released from astrocytes and ensuing adenosine acts in a bidirectional fashion to regulate neuronal excitability depending on concentration (Lee et al., 2013). Finally, Halassa and colleagues demonstrated a role for adenosine hydrolyzed from ATP released by astrocytes in sleep homoeostasis by using a loss-of-function of astrocytic exocytosis model (Halassa et al., 2009). The authors further found that astrocytic signaling to adenosine A_1 receptors (A_1R) was required for the robust reduction of depressive-like behaviors following 12 hours of sleep deprivation (Hynes et al., 2013). This is the first report describing the *in vivo* significance of reduced ATP gliotransmission and point to astrocyte adenosine signaling as a possible target for a novel class of antidepressants (Hynes et al., 2013).

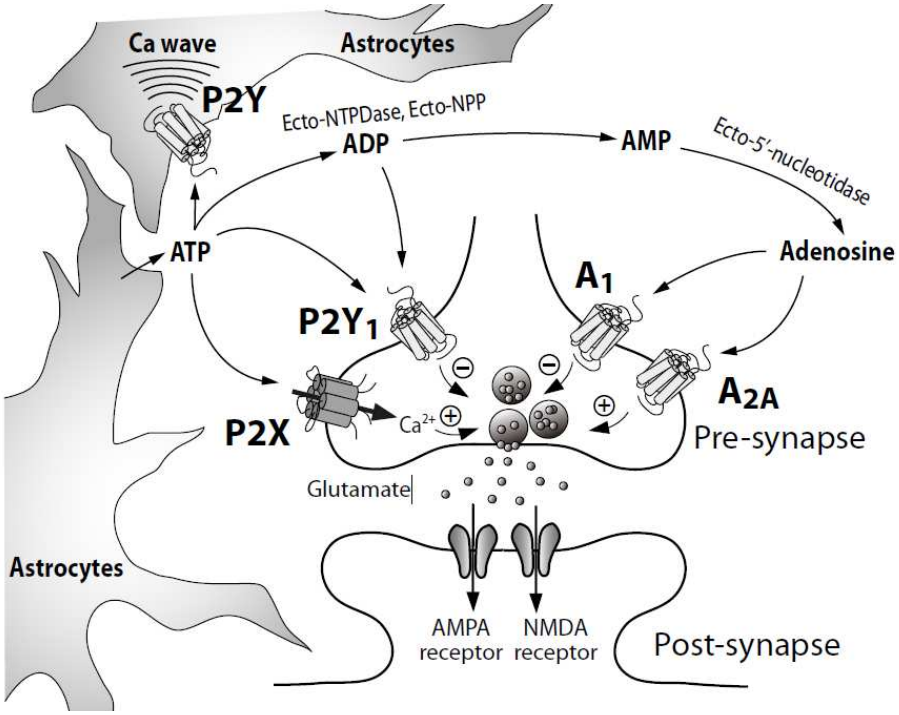


Fig.15 - Schematic illustration of astrocytic purinergic modulation of synaptic transmission. Suppression of glutamatergic transmission by adenosine from an astrocyte-ATP origin occurs through the activation of presynaptic $P_{2Y}Rs$ in cultured hippocampal neuron-glia co-cultures and the activation of presynaptic A_1Rs in acute slices (Pascual et al., 2005). In contrast, activation of $A_{2A}Rs$ rather potentiates excitatory transmission (Panatier et al., 2011). Additionally, in a large variety of central structures, the stimulation of presynaptic $P_{2X}Rs$ by ATP facilitates glutamatergic synaptic transmission *via* direct Ca^{2+} entry. (Adapted from Inoue et al., 2010)

3.2. Adenosine receptors

Adenosine is a ubiquitous neuromodulator upstream regulator of diverse brain functions uniquely positioned to integrate excitatory and inhibitory neurotransmission (Boison, 2008). In the brain, adenosine is an endogenous distress signal accumulating rapidly as an acute response to stress conditions, e.g. under oxygen deprivation during stroke or under conditions of excessive energy consumption during seizures. Adenosine modulates many neuronal and glial functions in physiological and pathophysiological conditions, such as seizure susceptibility, tissue damage and repair, and immune functions of the brain (Cunha, 2008). In addition, adenosine has direct impacts on other neurotransmitter systems and is an important upstream regulator to integrate and fine-tune glutamatergic and dopaminergic neurotransmission. The downstream effects of adenosine are mediated via activation of high-affinity A_1 or A_{2A}, low-affinity A_{2B}, or low-abundance A_3 G protein-coupled adenosine receptors (ARs) (Fredholm et al., 2005) (see **Table 2**).

The opposing activities of A_1 and A_{2A}Rs indicated in previous sections (see 1.4.3 and 3.1) imply that adenosine can exert both inhibitory and excitatory functions within the brain. A_1Rs mediate inhibitory neuromodulation by coupling to inhibitory G_i or G_o containing G proteins. Accordingly, in the hippocampus adenosine exerts a tonic inhibitory control over NMDA-R function via stimulation of A_1Rs, thus attenuating NMDA-R -mediated currents and inhibiting NMDA-R -dependent neuroplastic events including long-term potentiation (LTP) and depression (LTD) (de Mendonça and Ribeiro, 2000; Rebola et al., 2008). Conversely, activation of NMDA-R can inhibit the actions of A_1R agonists on presynaptic terminals, thus providing an additional layer of feedback control (Nikbakht and Stone, 2001). A_1R are widespread in the brain, with the highest levels in in the hippocampus, cerebellum, and cerebral cortex, being mainly located in synapses (Rebola et al., 2003), in particular in glutamatergic synapses (Rebola et al., 2005a). At synapses, A_1R inhibit glutamate release (as well as other neurotransmitters) and post-synaptically they inhibit Ca^{2+} influx through voltage-sensitive Ca^{2+} channels and NMDA-R and

also inhibit K^+ currents, leading to membrane hyperpolarization (reviewed in Fredholm et al., 2005).

In contrast to the inhibitory activity of A_1Rs, the excitatory functions of adenosine are largely mediated by activation of A_{2A}Rs (Fredholm et al., 2005; Chen et al., 2007). A_{2A}Rs mediate facilitatory neuromodulation by coupling to stimulatory G_s proteins. Thus, in contrast to inhibitory A_1R, activation of hippocampal A_{2A}Rs is required for the induction of NMDA-dependent LTP (Rebola et al., 2008), whereas A_{2A}R activation in hippocampal CA1 induces a form of NMDA-R - independent LTP (Kessey and Mogul, 1997). Thus, adenosine exerts a critical role in the fine-tuning of hippocampal synaptic plasticity which is widely believed to underlie certain forms of learning and memory (Rebola et al., 2008, Yu et al., 2009). The highest expression of A_{2A}Rs is found at the postsynaptic densities of enkephalin-enriched medium-sized spiny neurons (MSN's) in the striatum, which are involved in the control of motor function (Chen et al., 2007). A_{2A}Rs are also present at low density in different brain areas, namely in cortical and hippocampal areas where they have a predominant presynaptic localization (Rebola et al., 2005b) and in astrocytes (Boison et al., 2009). Although A_{2A}Rs have been shown to enhance the release of different neurotransmitters such as glutamate, their physiological function in the control of brain circuits is not entirely clear (Fredholm et al., 2005).

In the last decade, A_{2A}Rs have received more attention because their blockade affords a robust neuroprotection in different chronic noxious brain conditions by mechanisms still to be resolved (reviewed in Chen et al., 2007; Gomes et al, 2011). A_{2A}Rs can form heteromers with other receptors, e.g. A_1/A_{2A} heteromers, or A_{2A}/D_2 heteromers and thus can modulate adenosinergic and dopaminergic neuromodulation by direct receptor interactions. Due to these interactions with other transmitter systems and due to its action on receptors with opposing activity (A_1 *versus* A_2), adenosine is uniquely positioned as an upstream regulator to integrate and fine tune excitatory and inhibitory functions within the CNS (Cunha, 2005).

Table 2 - Characteristics of adenosine receptors. Data collected from Dunwiddie et al., 2001; Hettinger et al., 2001; Fredholm et al., 2005; Fields et al., 2006; Boison, 2008; Boison et al., 2009; Cunha, 2008; Verkhratsky et al., 2009; Wei and Chen, 2011

Adenosine receptor subtype	Adenosine affinity	Major localization in the CNS	Cellular and subcellular localization	Transduction	Specific mechanisms	Physiological properties
A_1	~70 nM	Widespread (Highly expressed in cortex, cerebellum and hippocampus; Intermediate levels in other areas)	Pre-, post- and extra-synaptically in glutamatergic neurons and striatonigral GABAergic neurons; astrocytes, oligodendrocytes, microglia	$G_{i/o}$ ↓ cAMP	Inhibits Ca^{2+} influx and decrease synaptic transmission - **inhibitory;** Inhibits the release of Glutamate, GABA and other neurotransmitters; lead to membrane hyperpolarization; decreases astrocyte proliferation	Inhbits the direct pathway of the basal ganglia, resulting in a decrease on psychomotor activity; Seizure suppression; neuroprotection; spinal analgesia; can induce sleep and wakefulness; antidepressant
A_{2A}	~150 nM	Restricted (Highly expressed in striatum, nucleus accumbens, olfactory tubercle; Low levels in other areas)	High levels post-synaptically in striatopallidal GABAergic neurons; low levels in hippocampal pre-synaptic nerve terminals, astrocytes, oligodendrocytes, microglia	$G_{i/s}$ ↑ cAMP	Stimulates Ca^{2+} influx and potentiate synaptic transmission - **facilitatory;** stimulates the uptake and release of Glutamate, GABA and other neurotransmitters; Induce astrogliosis and astrocyte proliferation	Elicits the indirect pathway of the basal ganglia, resulting in a decrease on psychomotor activity; proinflmammatory; inactivation improves learning and memory processes; induce vasodilation; promotes sleep
A_{2B}	~5100 nM	Widespread (Low levels in all areas)	Residual levels in most brain cells	$G_{i/s}$ ↑ cAMP	Stimulates Ca^{2+} influx	Pro-inflammatory activity
A_3	~6500 nM	Widespread (Intermediate levels in cerebellum and hippocampus and low levels in other areas)	Residual levels in most brain cells	$G_{i/o}G_{q/11}$ ↓ cAMP	Inhibits Ca^{2+} influx and synaptic activity during hypoxia; uncouples A_1 and mGlu receptors	Anti-inflammatory activity; Can elicit survival or apoptosis pathways

There are two other adenosine receptors (A_{2B} and A_3 receptors), but their density in the brain is lower than that of A_1R or $A_{2A}R$ and the available data are more imprecise due to the unavailability of drugs and antibodies selective for these receptors across different species (Fredholm et al., 2005). $A_{2B}R$ can couple to two different classes of G proteins, G_q and G_s. G_q regulates intracellular calcium and vesicular release, whereas G_s affects a plethora of cAMP

dependent signaling pathways. $A_{2B}R$ have been shown to induce and potentiate Ca^{2+} signaling in astrocytes (Peakman and Hill, 1994; Pilitsis and Kimelberg 1998) in addition to eliciting interleukin-6 (IL-6) (Schwaninger et al., 1997), leukemia inhibitory factor (LIF) (Moidunnny et al., 2012) and glial cell line-derived neurotrophic factor (GDNF) (Yamagata et al., 2007) release from astrocytes and thus, having a role in neuronal survival. In turn, A_3R appear to regulate apoptosis and chemokine release (Di Lorio et al., 2002) in addition to reduce ischemic brain injury in rodents (Chen et al., 2006) by a process involving modulation of synaptic AMPA-R s in CA3 pyramidal neurons (Dennis et al., 2011). Activation of A_3R by endogenous adenosine has also been shown to protect astrocytes from cell death induced, e.g., by hypoxia (Bjorklund et al., 2008) (see **Table 2**).

3.3. Adenosine receptors and motor function

Among the many functions modulated by adenosine receptors, the modulation and integration of basal ganglia's associated functions has received particular therapeutic magnitude (Chen et al., 2013). Adenosine receptors are highly enriched in the basal ganglia system, a richly interconnected neural network involved in adaptive control of behavior through interactions with sensorimotor, motivational and cognitive brain areas (Schiffmann et al., 2007).

There are two main pathways within the basal ganglia that contribute opposite influence on their targeted nuclei: the direct and the indirect pathways (see **Fig.16**). Fine-tune control of behavior (motor learning, motivation and reward) requires interplay between these two pathways (Fredholm et al., 2005):

A) The direct pathway, also known as the nigrostriatal pathway, facilitates movement through tonic input of the thalamus, thereby increasing activation of the cortex and facilitating movement (Schiffmann et al., 2007). Adenosine A_1R and dopamine D1 receptors (D_1R) are distributed throughout this pathway. The direct pathway starts with glutamatergic inputs from the cortex and/or dopaminergic inputs from the substantia nigra pars compacta (SNc) to the

dorsal striatum (caudate putamen - CPu) innervating both GABAergic neurons and cholinergic interneurons (Schiffmann et al., 2007). Higher dopamine levels in this region leads to activation of dynorphin and substance P-containing GABAergic neurons which project and inhibit GABAergic neurons in the globus pallidus interna (GPi), which normally inhibit thalamocortical activity (Schwarzschild et al., 2006). Since the GPi is being inhibited, the thalamic nuclei will be excited, sending excitatory glutamatergic inputs to different areas of the cortex, allowing movement. A_1R are antagonistically co-localized with D_1R on GABAergic striatonigral neurons, eliciting an inhibitory effect on the release of GABA on the GPi (in opposition to the facilitatory effect of D_1R), and thus eliciting a brake on the activity of the direct pathway (Fredholm et al., 2005; Schiffmann et al., 2007).

B) The indirect, or striatopallidal pathway normally yields an inhibitory influence on motor activity (Schiffmann et al., 2007). The indirect pathway contains a small number of both D_1R and A_1R, but mostly consists of dopamine D2 (D_2R) and adenosine $A_{2A}R$. Unlike D_1R and A_1R, the D_2R and $A_{2A}R$ are mainly co-localized on postsynaptic densities of the striatopallidal enkephalin-containing GABAergic neurons. They are also co-localized on cholinergic interneurons and regulate acetylcholine (ACh) release in the striatum (Kurokawa et al., 1996; Tozzi et al., 2011). The indirect pathway is considered to be the negative loop within the striatum (Schwarzschild et al., 2006). The indirect pathway starts the same way as the direct pathway, with glutamatergic inputs from the cortex and/or dopaminergic inputs from the SNc to the CPu (Fredholm et al., 2005). However, lower dopamine levels in this region lead, instead, to the activation of enkephalin-containing GABAergic neurons which project and inhibit GABAergic neurons in the globus pallidus externa (GPe), which normally inhibit the subthalamic nucleus (STN) (Schwarzschild et al., 2006). The more active STN in turn sends glutamatergic excitatory input to the GABAergic neurons of the GPi. Thus, in opposition to the direct pathway, with the activation of the GPi, the thalamic nuclei will be inhibited, decreasing the excitatory glutamatergic inputs to the cortex, and consequently reduce motor function

(Schiffmann et al., 2007). $A_{2A}R$ are antagonistically co-localized with D_2R on GABAergic striatopallidal neurons, eliciting a facilitatory effect on the release of GABA on the GPe (in opposition to the inhibitory effect of D_2R), and thus eliciting an increase on the activity of the indirect pathway, thus decreasing movement (Schwarzschild et al., 2006). In addition, it is believed that $A_{2A}Rs$ are involved in regulating this inhibition by blocking D_2R activation from the SNc or the smaller dopaminergic signal from the ventral tegmental area (VTA) to the ventral striatum. Activation of D2 receptors results in a disinhibition of the indirect pathway, which facilitates movement by decreasing inhibition to the thalamus (Schwarzschild et al., 2006).

The interplay between the direct and indirect pathway is central to the basal ganglia control of movement, motor learning, motivation and reward (Schwarzschild et al., 2006) (see **Fig.16**). Therefore, $A_{2A}R$ are a leading nondopaminergic therapeutic target in Parkinson´s disease (PD), a debilitating neurodegenerative disorder with incapacitating motor complications (Morelli et al., 2012). In PD, as a result of the loss of dopamine projections to the striatum, the direct pathway exhibits diminished activity and the indirect pathway exhibits excessive activity - a condition leading to diminished voluntary movements and the presence of involuntary movement (Dyskinesia) (Chen and Chern, 2011). Blockade of $A_{2A}R$, by decreasing the activity of the indirect pathway, has been consistently shown to restore some balance between the direct and the indirect pathways resulting in motor stimulation, without a worsening of dyskinesia (for review see, Xu et al., 2005; Schwarzschild et al., 2006; Morelli et al., 2010; Chen and Chern, 2011).

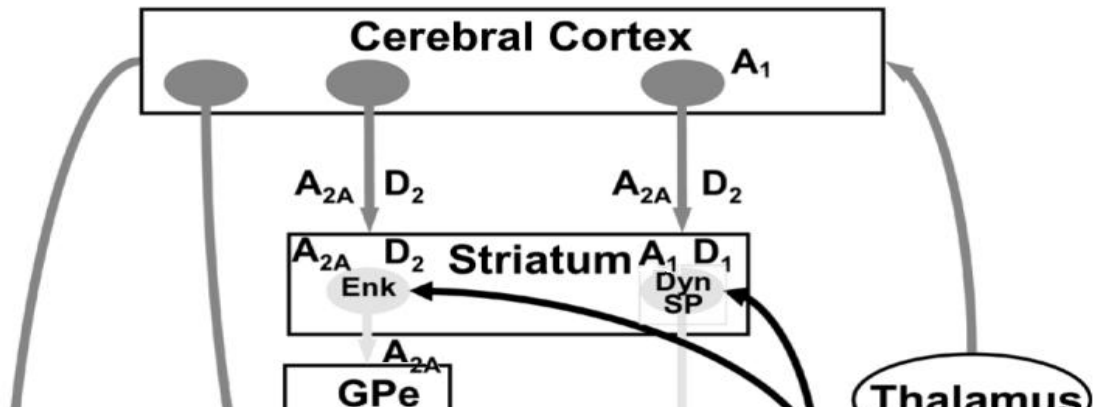


Fig.16 - Schematic representation of the basal ganglia circuitry including the major connections of the system used by the different populations of striatal GABAergic efferent neurons, together with the main localization of adenosine and dopamine receptors. Arrows represent the synaptic connections between the different structures; excitatory, inhibitory and dopaminergic connections are represented by dark grey, light grey and black arrows, respectively. Dyn, dynorphin; Enk, enkephalin; Gpe, external segment of the globus pallidus; Gpi, internal segment of the globus pallidus; SNc, substantia nigra *pars compacta;* SNe, substantia nigra *pars reticulata;* SP, substance P; STN, subthalamic nucleus. (Adapted from Schiffmann et al., 2007).

3.4. Adenosine receptors in astrocytes

Previously (see chapter 1.4.3) it was described the role of astrocyte-derived adenosine in inducing and inhibiting excitatory transmission via activation of facilitatory $A_{2A}R$ (Panatier et al., 2011) and inhibitory A_1R (Manzoni et al. 1994; Zhang et al., 2003; Pascual et al., 2005; Serrano et al. 2006; Todd et al., 2010), respectively. The importance of these mechanisms is underscored by recent findings demonstrating its involvement in sleep regulation (Halassa et al., 2009) and cognitive impairment (Stone et al., 2009).

In addition to the crucial role of astrocyte-derived adenosine in modulating neuronal ARs and synaptic transmission (see sections 1.4.3 and 3.1), adenosine can also activate astrocytic

ARs and modulate several important functions (see **Fig.17**). Astrocytes are endowed with all the known subtypes of ARs which control the release and uptake of gliotransmitters and neurotransmitters (e.g. Glutamate, GABA, ATP), metabolism of carbohydrates, astrogliosis and the release of several neuroactive substances (for review see Ciccarelli et al., 2001; Daré et al., 2007; Boison et al., 2009). It should be noted that the expression of ARs in astrocytes *in vivo* is usually low under physiological conditions and frequently below the detection limit by common histological methods (i.e. immunohistochemistry, autoradiography, or in situ hybridization) (Boison et al., 2009), although ultrastructural radioligand binding and electron microscopic studies have clearly shown the presence of all subtypes of ARs *in vivo* astrocytes (Hösli and Hösli, 1988; Hettinger et al., 2001; Schaddelee et al., 2003; Ponzio et al., 2006; Pickel et al., 2006).

As in many other types of cells, activation of A_1R not only decreases cAMP accumulation (Murphy et al., 1991; Peakman et al. 1996) but also stimulates phospholipase C (Biber et al., 1997) and induces Ca^{2+} elevations in astrocytes (Ogata et al., 1994; Porter et al., 1995; Toms et al., 1999; Cormier et al., 2001; Alloisio et al., 2004). A_1R have been also shown to protect astrocytes from hypoxic/ischemic damage (Ciccarelli et al., 2007; Björklund et al., 2008) and apoptosis (D'Alimonte et al., 2007) in addition to reduce their proliferation rate in culture (Ciccarelli et al., 1994; Abbracchio et al., 1998), partly via activation of PI3K and Erk 1/2 phosphorylation. Finally, A_1R seem involved in controlling GABA uptake (Cristóvão-Ferreira et al., 2013), ATP release (Ciccarelli et al., 1992), inducing GLT-I expression (Wu et al., 2011) and stimulating the release of neurotrophic factors (Ciccarelli et al., 1999).

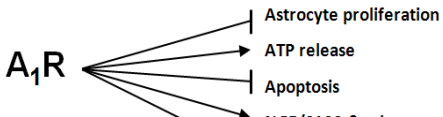
$A_{2B}R$ have been shown to induce and potentiate Ca^{2+} signaling in astrocytes (Peakman and Hill, 1994; Pilitsis and Kimelberg 1998) in addition to eliciting IL-6 (Schwaninger et al., 1997), leukemia inhibitory factor (LIF) (Moidunnny et al., 2012) and glial cell line-derived neurotrophic factor (GDNF) (Yamagata et al., 2007) release from astrocytes and thus, having a role in neuronal survival. Importantly, $A_{2B}R$ activation was demonstrated to induce glucose-6-

phosphate dehydrogenase activity and therefore glycogen synthesis (Allaman et al., 2003), a mechanism that may play a significant role in the control of brain energy metabolism,

Activation of A_3R by endogenous adenosine has been shown to protect astrocytes from cell death induced, e.g., by hypoxia (Bjorklund et al., 2008) and induce astrogliosis (Abbracchio et al., 1998).

From the various subtypes of ARs, comparatively more attention has been given to the roles performed by $A_{2A}R$ in astrocytes (Daré et al., 2007; Boison et al., 2009). Ultrastructural analysis of sections single-labeled for $A_{2A}R$ revealed that, from the total of $A_{2A}R$-postive elements in the striatum, ~6% labeled for astrocytes (with assumingly higher percentage in other brain regions such as cortex and hippocampus) (Hettinger et al., 2001). Many functional measurements (such as cAMP levels and cytokine release) coupled with pharmacological tools have clearly demonstrated the presence and active function of $A_{2A}Rs$ in astrocytes. Activation of $A_{2A}R$ in astrocytes has been shown to increase cAMP levels (van Calker et al., 1979; Rathbone et al., 1991; Murphy et al., 1991) and Ca^{2+} induction (Porter and McCarthy, 1995; Ogata et al., 1996; Alloisio et al., 1994; Doengi et al., 2008; Kanno and Nishizaki, 2012). Importantly, the expression of $A_{2A}Rs$ in astrocytes is normally induced following brain insults (Bura et al., 2008; Yu et al., 2008; Pugliese et al, 2009) and shown to elicit astrogliosis (Hindley et al., 1994; Brambilla et al., 2003; Ke et al., 2009). The induction of $A_{2A}Rs$ in astrocytes by brain insults and inflammatory signals, coupled with a local increase in adenosine and pro-inflammatory cytokines (such as IL-1β, which further induces $A_{2A}R$ expression), may serve as part of an important feed-forward mechanism to locally control neuroinflammatory responses in the brain (Boison et al., 2009).

$A_{2A}Rs$ in astrocytes have been particularly associated with the glutamatergic activity, either by inducing Ca^{2+}-dependent vesicular glutamate release (Popoli et al., 1995; Li et al., 2001; Nishizaki et al., 2002; Nishizaki 2004; Kanno and Nishizaki, 2012) or by inhibiting glutamate transport by unclear mechanisms (Nishizaki et al., 2002; Pintor et al., 2004), with recognized



consequences on the regulation of neuroinflammation, synaptic transmission and traumatic brain injury (Castillo et al., 2010; Dai et al., 2010).

In addition to the array of functions described, A_{2A}Rs have been shown to inhibit the expression of iNOS and the production of NO in astrocytes (Brodie, 1998), regulate astrocyte differentiation during development (Desfrere et al., 2007), regulate GABA transport in ass[illegible]ction (Ke[illegible]llary endothelium play a key key role in balancing brain blood flow (through astrocyte swelling and A_{2A}R-induced release of vasodilators) with neuronal activity and metabolism (Phillis, 2004) (see summary of functions in **Fig. 17**).

Fig.17 - Adenosine modulates a variety of astrocyte functions, thus indirectly affecting neurons. Arrows in the end of each thread represent "stimulation" whereas vertical lines represent "inhibition". See text for references.

3.5. Adenosine receptors and cognitive function

The notion that adenosine potentially modulates cognition probably arises from the general belief that consumption of caffeine, the most widely used psychoactive compound and a non-selective adenosine antagonist, improves cognitive performance in humans (Ribeiro and Sebastião, 2010). A growing body of pharmacological and genetic evidence now suggests that brain A_1R and $A_{2A}R$ activity may contribute to modulation of learning and memory and other cognitive processes (for review see Boison et al., 2012). Cognitive effects of caffeine are mostly due to its ability to antagonize adenosine A_1Rs in the hippocampus and cortex and $A_{2A}Rs$ in the striatum, brain areas involved in different forms of memory and learning (Squire and Schacter, 2002; Batalha et al., 2013). For example, activation of adenosine receptors impairs memory retrieval by local injection of $A_{2A}R$ agonists into the posterior cingulated cortex (Pereira et al., 2005) and impair social recognition memory by intraperitoneal injection of adenosine, which is reversed by caffeine and selective $A_{2A}R$ antagonists (Prediger et al, 2005; Wang et al., 2006). Consistent with this, transgenic animals over-expressing $A_{2A}Rs$ in the cortex display impaired spatial working memory in radial maze tests, in repeated trials of the Morris water maze and in objective recognition tests (Zhou et al.,2009). More recently it was shown that genetic and pharmacological abrogation of $A_{2A}Rs$ selectively enhanced working memory performance in different behavioral paradigms (Giménez-Llort et al., 2007; Wei et al., 2011; Batalha et al., 2013). Notably, these precognitive phenotypes of $A_{2A}R$ gene deletion are selective, with spatial reference memory, motor function, and anxiety-like behavior left intact. Thus, findings from genetic studies generally support the notion that suppression of $A_{2A}R$ activity is pro-cognitive and raise the possibility that the $A_{2A}Rs$ may represent a target for improving cognitive function under physiological conditions (for a review see Wei and Chen, 2011). $A_{2A}Rs$ in striatum are also critically involved in the mechanisms of learning and habit formation behaviors, where genetic inactivation of striatal $A_{2A}R$ was shown to lead to weaker habit formation (Yu et al., 2009). Finally, as already depicted in section 1.5, in particular relevance to the novel field of astrocyte and adenosinergic signaling, novel findings place astrocyte-derived adenosine as

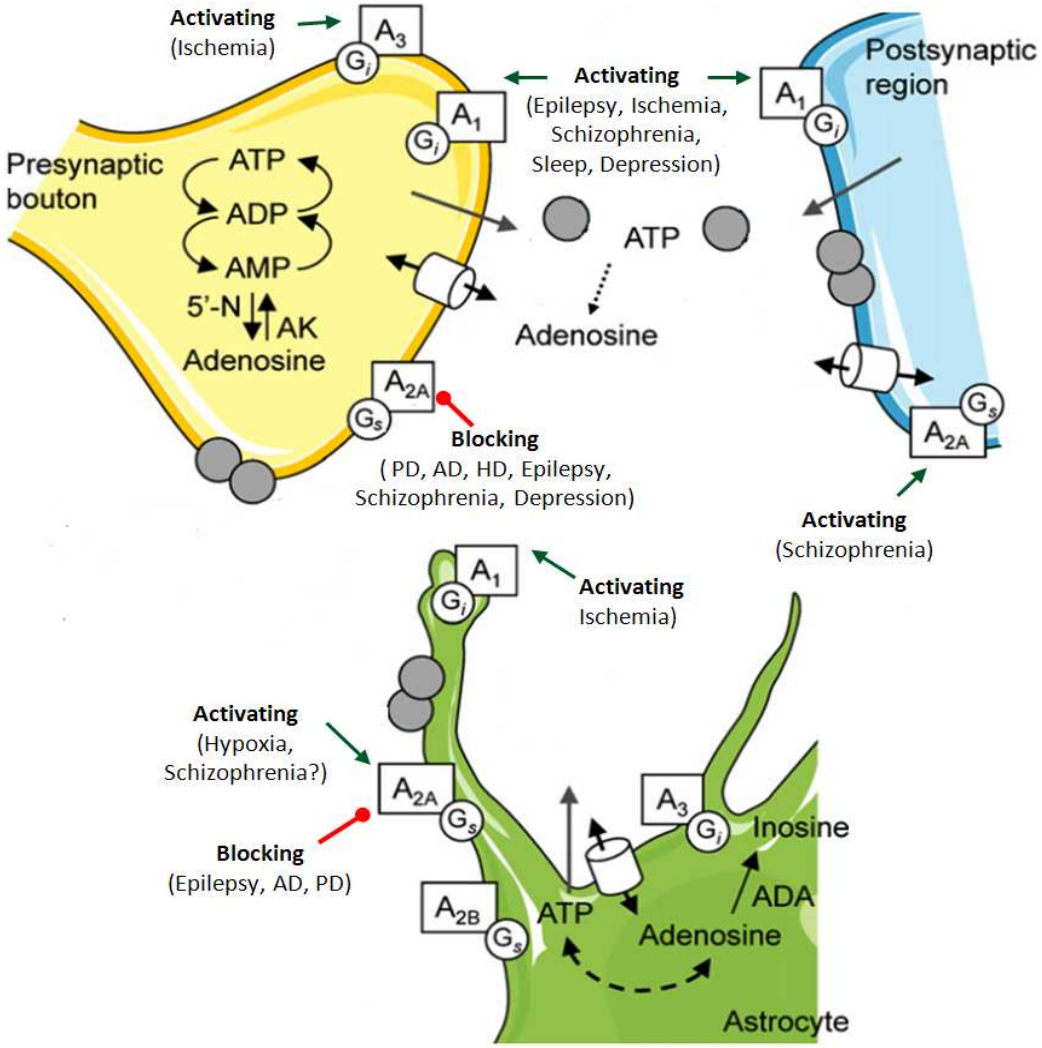
essential to the control of neuronal A_1R signaling in the modulation of sleep homeostasis and in mediating the cognitive consequences of sleep deprivation (for review see Halassa et al., 2009).

3.6. Involvement of adenosine receptors in neurologic diseases

More than a decade of research has suggested that manipulating adenosine neurotransmission might offer a valuable strategy to afford neuroprotection not only in PD (see chapter 3.3), but also in a vast array of acute and neurodegenerative conditions such as epilepsy (Boison, 2012), stroke (Chen and Chern, 2011), chronic pain (Zylka et al., 2011), HD (Chen and Chern, 2011), AD (Cunha and Agostinho, 2010) and neuropsychiatric conditions such as schizophrenia (Boison et al., 2012), depression and bipolar disorders (Lara, 2010), anxiety and panic disorders (Sebastião and Ribeiro, 2009), sleep (Halassa et al., 2009) and drug addiction (Lopes et al., 2011). Therefore, increasing attention is now given to the role of adenosine in human brain function and its potential benefit for clinical applications. This interest was bolstered by recurrent observations showing that the extracellular levels of adenosine were modified upon brain damage (Fredholm et al., 2005; Gomes et al., 2011). Thus, albeit the extracellular levels of adenosine increase with neuronal activity, they increase to considerable higher levels when brain damage occurs. This probably results from the increased use of ATP, which leads to a disproportionally higher formation of adenosine (reviewed in Cunha, 2008).

The first studies investigating adenosine and neuroprotection were conducted in models of ischemic and excitotoxic brain injury (reviewed in Fredholm et al., 2005). Under these conditions, increased extracellular adenosine in response to brain injury has been shown to act as a neuroprotectant (Morelli et al., 2010). However, a pro-neurotoxic role of adenosine has also been demonstrated, suggesting that blockade of ARs may confer neuroprotection across a range of neurodegenerative disorders (reviewed in Gomes et al., 2011). This apparent paradox reflects the complexity of adenosine transmission, with several receptor subtypes engaged in the control of different parallel processes. In addition, the same adenosine receptor subtype

expressed in different cell types (e.g. neurons or astrocytes) may mediate opposing effects in response to different neurotoxic insults (see **Fig.18**).



3.6.1. Involvement in neurodegenerative diseases - Alzheimer's disease

AD affects 20-30 million people worldwide and is characterized by progressive memory deficits, cognitive impairment and personality changes. As previously described in section 2.5.1, the main cause of AD is generally attributed to the increased production and extracellular

Fig.18 - Sites of adenosine receptors whose activation or blockade may afford protection from different neurological disorders. Green arrows indicate "stimulation", whereas red lines indicate "inhibition", representing mechanisms affording neuroprotection in the mentioned CNS disorders bellow. See text for references. PD, Parkinson's disease; AD, Alzheimer's disease; HD, Huntington disease (Adapted from Landolt, 2008).

accumulation of amyloid-beta (Aβ), in association with intracellular neurofibrillary tangle (NFT) formation (see chapter 2.5.1 for details).

The specific capacity of AR's, particularly, $A_{2A}R$ to modulate mechanisms involved in synaptic degeneration and subsequent neuronal death suggests that $A_{2A}R$ antagonists might control the apparently reversible synaptic dysfunction that occurs in early AD pathogenesis (for review see, Cunha and Agostinho, 2010; Gomes et al., 2011). Some of the strongest evidence relies on epidemiological studies where caffeine (a non-selective adenosine receptor antagonist) consumption inversely correlates with the incidence of AD (Maia & de Mendonca, 2002; Santos et al., 2010) and prevents memory impairment in animal models of AD (Dall'Igna et al., 2003; Arendash et al., 2006; Cunha et al., 2008), an effect mimicked by selective $A_{2A}R$ antagonists (Dall'Igna et al., 2007). Furthermore, $A_{2A}R$ blockade is protective in preventing Aβ-induced neurotoxicity at the synaptic terminals (Canas et al., 2009), controlling neuroinflammation (Angulo et al.,2003), avoiding the disruption of the blood-brain barrier (Chen et al., 2008) and in the control of Aβ production (Arendash et al., 2006). These data further consolidate the idea that $A_{2A}R$ blockade might afford a prophylactic benefit on memory performance, receptors which were notably found up-regulated in cortical regions of both AD animals models (Arendash et al., 2006) as well as in AD patients (Albasanz et al.,2008, Angulo et al., 2003). Although only scantily investigated, recent data also suggests that $A_{2A}R$ might modulate glutamate release ((Popoli et al., 1995; Li et al., 2001; Nishizaki et al., 2002; Nishizaki 2004; Kanno and Nishizaki, 2012) and uptake in astrocytes (Nishizaki et al., 2002), thus projecting a virtually unexplored mechanism for abrogation of the previously observed decrease in glutamate uptake in AD conditions (see section 2.5.1 for details).

3.6.2. Involvement in neuropsychiatric disorders - Schizophrenia

As previously described (see chapter 2.5.2), schizophrenia is best explained by alterations in dopaminergic and glutamatergic circuits. In addition, adenosine, as a prototypical

endogenous neuromodulator able to control both DA and Glu signaling is currently proposed to play a key integrative of these two hypotheses role in schizophrenia (for review see Boison et al., 2012). Specifically, blockade of A_1Rs (Schiffmann et al., 2007), activation of striatal A_{2A}R and blockade of extra-striatal A_{2A}Rs (Rimondini et al., 1997; Shen et al., 2008 ; Yu et al., 2008) have been suggested to confer antipsychotic action against DA hyperfunction or NMDA-R hypofunction.

A_{2A}R activity on striatal medium-sized spiny neurons (MSNs) has been shown to critically modulate LTP at cortico-accumbens glutamatergic synapses onto striatopallidal MSNs, a process thought to underlie learning and memory (Lopes et al., 2002; Shen et al., 2008). A hypofunction of the glutamatergic cortico-striatal pathway, i.e., an inhibition of the inhibitory GABA neurons by hyperactivated dopaminergic receptors as well as reduction of the glutamatergic input, is associated with opening of the thalamic filter, which leads to an uncontrolled flow of sensory information to the cortex and to psychotic symptoms (Boison et al., 2012). Furthermore, striatal A_{2A}Rs interact antagonistically with D_2Rs and NMDA-R function, which partly explain the efficacy of A_{2A}R agonists against the psychostimulant effects of NMDA-R antagonists in schizophrenia (Shen et al., 2008). On the other hand, blockade of extra-striatal A_{2A}Rs might confer antipsychotic action against dopamine hyperfunction and NMDA-R hypofunction, an observation that points to regionally distinct functions of A_{2A}Rs in modulating psychomotor activity associated with schizophrenia (Chen et al., 2000; Shen et al., 2008; Wei et al., 2011). Additionally, since A_{2A}R regulate glutamate release (Li et al., 2001; Nishizaki et al., 2002; Popoli et al., 2003; Kanno and Nishizaki, 2012) and glutamate uptake in astrocytes (Nishizaki et al., 2002), additionally they may be critical mediators for the glutamatergic dysregulated levels underlying schizophrenia.

4. Objectives and outline

As described in the previous sections, glutamate is the primary excitatory neurotransmitter in the CNS and therefore its levels must be precisely regulated in order to avoid overstimulation and excitotoxicity or low synaptic efficacy and consequent synaptic dysfunction, underlying mechanisms of many neurodegenerative and neuropsychiatric disorders (Sattler and Rothstein, 2006) (see section 2.4 and 2.5). Therefore, efforts have been made to understand the regulation of GluTs, Na^+-dependent proteins present largely in the plasma membrane of astrocytes, which regulate the basal levels of glutamate during synaptic transmission (Huang and Bergles 2004; Tzingounis and Wadiche 2007) and thus accomplish important roles in synaptic plasticity and memory formation (Maleszka et al. 2000; Yang et al. 2005; Filosa et al., 2009; Bechtholt-Gompf et al., 2010; John et al., 2012). Adenosine, a ubiquitous neuron and astrocyte function modulator (see chapter 3.4), particularly through the activation of high-affinity $A_{2A}R$ which are intimately associated with the glutamatergic activity in astrocytes (Popoli et al., 1995; Li et al., 2001; Nishizaki et al., 2002; Kanno and Nishizaki, 2012), may accomplish an important role on the regulation of glutamate uptake activity.

As a whole, the work we present here will be focused on probing the role of adenosine receptors, particularly $A_{2A}R$, on the regulation of glutamate transport in astrocytes. First, the specific mechanistic processes of glutamate uptake regulation by $A_{2A}R$ will be investigated with the aid of different pharmacological and biological tools. Secondly the importance of this process will be weighed on experimental models of brain disorders. Accordingly, the beneficial and detrimental consequences of abrogating $A_{2A}R$ activity on the control of glutamate uptake will be analyzed through a paradigm of Alzheimer's disease (AD) and in another of schizophrenia, respectively. It will be divided as follows:

In **chapter 2**, we examine the interplay between adenosine and glutamate transport on an *in vitro* and an *ex vivo (*e.g. gliosomes*)* preparation of astrocytes. In addition to a pharmacological analysis, the glutamate uptake activity of astrocytes collected from $A_{2A}R$ deficient (-/-) mice ($A_{2A}R$-GKO) was also evaluated. The identification of a functional interaction

between A_{2A}Rs and GluTs in astrocytes in this study may provide a novel mechanism to understand the ability of A_{2A}R to control synaptic function and excitotoxicity, two conditions tightly associated with the control of extracellular glutamate levels by GluTs.

In **chapter 3**, the mechanism of A_{2A}R regulation of glutamate uptake in astrocytes is investigated in more detail. Here we propose that A_{2A}R is physically and functionally associated with a Na^+/K^+-ATPase (NKA) isoform -α_2 in astrocytes in order to rapidly regulate glutamate transport. In addition to co-localization experiments and pharmacological analysis of the interactions between A_{2A}R, NKA-α_2 and GTs, we further demonstrate that the selective deletion of A_{2A}R in astrocytes (in Gfa2-A_{2A}R KO mice) leads to a concurrent increase of both glutamate uptake and NKA-α_2 levels and activity. This evidence for A_{2A}R coupling and regulation of glutamate transport through modulation of NKA-α_2 activity provides a novel mechanism for modulation of glutamatergic activity and ion homeostasis, processes intricately associated with the aetiology of several brain diseases.

In **chapter 4,** we explore the significance of A_{2A}R regulation of glutamate uptake in a model of AD, a neurodegenerative condition associated with a the contribution of a deficient glutamate uptake and resultant excitotoxicity (Li et al., 1997; Masliah et al., 2000; Scott et al., 2002). Therefore, the present study tested the ability of A_{2A}R blockade to prevent the previously observed $A\beta_{1-42}$-induced decrease in glutamate uptake (Matos et al., 2008) in different brain cortical astrocytic preparations. Results show that $A\beta_{1-42}$ leads to an augment in A_{2A}R mRNA expression and protein levels, and the blockade or genetic excision of these receptors prevents the decline in glutamate uptake in cultured astrocytes treated with $A\beta_{1-42}$ and in glial plasmalemmal vesicles (gliosomes) from $A\beta_{1-42}$-icv injected rats. Together, these results show that astroglial A_{2A}R play a crucial role in the development of Aβ-induced glutamate uptake impairment leading to glutamateric synaptic dysfunction and excitotoxicity and provide an additional justification for the promising therapeutic benefits of selective blockade of these receptors in the early stages of AD.

Finally, in **chapter 5**, we examine the behavioral consequences of an exacerbated/hyperactive glutamate uptake caused by selective deletion of $A_{2A}R$ in astrocytes (in Gfa2-$A_{2A}R$ KO mice). Here we show that Gfa2-$A_{2A}R$-KO mice exhibit an array of endophenotypes characteristic of schizophrenia, namely a decrease in working memory (WM) performance and an enhanced psychomotor response to MK-801, which were both reverted by GLT-I selective inhibition. In addition, the present results also suggest that neuronal and astrocytic $A_{2A}R$ may have opposite neuromodulation effects, since the suppression of neuronal $A_{2A}R$ activity was shown to be pro-cognitive, while the selective deletion of astrocytic $A_{2A}R$ potentiated working memory deficits. Finally, we demonstrated that the glutamatergic dysfunction observed in Gfa2-$A_{2A}R$-KO mice was associated with an alteration of NMDA-R 2B-mediated AMPA-R GluR1/GluR2 subunit membrane endocytosis, suggesting that genetic deletion of astrocytic $A_{2A}R$ may lead to drastic and extensive modifications in the glutamatergic network that confer susceptibility to a schizophrenia-type of traits. Finally, this study reveals a novel mechanistic view of the fundamental role of the neuron-astrocytic communication in cognitive processes affected in this brain pathology.

CHAPTER 2

ADENOSINE A_{2A} RECEPTORS MODULATE GLUTAMATE UPTAKE IN CULTURED ASTROCYTES AND GLIOSOMES

Marco Matos [1,2,3]*, Elisabete Augusto [1,2,3], Alexandre dos Santos-Rodrigues [1,4], Michael A. Schwarzschild [5], Jiang-Fan Chen [3], Rodrigo A. Cunha[1,2] and Paula Agostinho [1,2]

1. Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
2. Faculty of Medicine, University of Coimbra, Coimbra, Portugal
3. Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA
4. Laboratory of Cellular Neurobiology, Department of Neurobiology, Institute of Biology, Federal Fluminense University, Niterói, RJ, Brazil.

GLIA 60: 702-716 (2012)

* Marco Matos performed the experiments presented in Fig. 1-10

1. Abstract

Glutamate is the primary excitatory neurotransmitter in the CNS, where its toxic build-up leads to synaptic dysfunction and excitotoxic cell death that underlies many neurodegenerative diseases. Therefore, efforts have been made to understand the regulation of glutamate transporters (GluT), which are responsible for the clearance of extracellular glutamate. We now report that adenosine A_{2A} receptors ($A_{2A}R$) control the uptake of D-aspartate in primary cultured astrocytes as well as in an ex vivo preparation enriched in glial plasmalemmal vesicles (gliosomes) from adult rats, whereas A_1R and A_3R were devoid of effects. Thus, the acute exposure to the $A_{2A}R$ agonist, CGS 21680, inhibited glutamate uptake, an effect prevented by the $A_{2A}R$ antagonist, SCH 58261, and abrogated in cultured astrocytes from $A_{2A}R$ knockout mice. Furthermore, the prolonged activation of $A_{2A}R$ lead to a cAMP/protein kinase A (cAMP-PKA)-dependent reduction of GLT-I and GLAST mRNA and protein levels, which lead to a sustained decrease of glutamate uptake. This dual mechanism of inhibition of GluTs by astrocytic $A_{2A}R$ provides a novel candidate mechanism to understand the ability of $A_{2A}R$ to control synaptic plasticity and neurodegeneration, two conditions tightly associated with the control of extracellular glutamate levels by GluTs.

2. Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian CNS but its excessive extracellular accumulation seems to contribute for the evolution of most neurodegenerative disorders (Lipton and Rosenberg, 1994; Benarroch et al., 2010). Since glutamate is not metabolized in the extracellular environment, the maintenance of normal glutamatergic neurotransmission and the prevention of excitotoxicity depends on the re-uptake of glutamate, mainly through the astrocytic glutamate transporters, GLAST (glutamate–aspartate transporter/human homologue EAAT1) and GLT-I (glutamate transporter-1/ human homologue EAAT2) as well as neuronal and astrocytic EAAC-1 (excitatory amino acid carrier 1/human

homologue EAAT3) and cerebellar EAAT4 and retinal EAAT5 (reviewed in Dunlop, 2006 and Swanson, 2005). The importance and relevance of the activity of GluTs is underscored by the impact of modifying their activity in the control of synaptic plasticity as well as in the demise of neurodegeneration (reviewed in Tzingounis and Wadiche, 2007).

Adenosine is a classical modulator of synaptic transmission in the CNS, exerting its effects via four types of G-protein-coupled receptors: A_1, A_{2A}, A_{2B} and A_3 (Fredholm et al., 2005). Adenosine mainly controls excitatory transmission through a coordinated action of inhibitory A_1 receptors and facilitatory A_{2A} receptors (A_1R, $A_{2A}R$) (Fredholm et al., 2005). These two adenosine receptors have also been actively pursued as possible drug targets to manage neurodegeneration (reviewed in Gomes et al., 2011). Accordingly, bolstering A_1R activation of inhibiting $A_{2A}R$ function are two purported strategies to control neurodegenerative processes (Cunha, 2005; Schwarzschild, 2007). Albeit the physiological and pathological role of adenosine receptors has mainly been assumed to result from their direct action on neurons, it has also been shown that adenosine receptors are also present in astrocytes where they were shown to control the metabolism of glucose, astrogliosis, cell proliferation, cell volume changes, cell death and release of neurotrophic factors and interleukins (for review see, Daré et al., 2007; Boison et al., 2009). Given the importance of the activity of GluTs in pathological conditions, the ability of $A_{2A}R$ to afford a sustained control of GluTs could emerge as a novel candidate mechanism to understand the neuroprotection afforded by blocking $A_{2A}R$ blockade upon different brain insults (Gomes et al., 2011; Lopes et al., 2011). This hypothesis is prompted by previous observations that the control by $A_{2A}R$ of the extracellular levels of glutamate was affected by inhibitors of GluTs (Pintor et al., 2004) and that astrocytic $A_{2A}R$ activation leads to a transporter-independent and Ca^{2+}-dependent glutamate release and an inhibition of GLT-I transporter in cultured hippocampal astrocytes (Li et al., 2001; Nishizaki et al., 2002 and 2004). As pre-requisite to consider this hypothesis that $A_{2A}R$-associated neuroprotection might involve the control of GluTs, we combined pharmacological and genetic tools to explore the short as well as the long-

term control of GluTs mainly by $A_{2A}R$, but also by other adenosine receptors both in astrocytic cultures as well as in gliosomes from adult rats. The results showed a prominent effect of $A_{2A}R$ in the short-term control of both GLT-I and GLAST and revealed an ability of $A_{2A}R$ to control the expression and density levels of GLT-I and GLAST, strengthening the tight association between the adenosinergic and glutamatergic systems.

3. Material and Methods

3.1. Materials

D-Aspartate and 8-{4-(2-aminoethyl) amino] carbonylmethyloxyphenyl} xanthine (XAC) were acquired from Sigma-Aldrich (St. Louis, MO, USA), D-[^{3}H] aspartate (specific activity of 15-50 Ci/mmol) was bought from PerkinElmer (MA, USA), ZM 241385[^{3}H] (specific activity of 50 Ci/mmol) was from American Radiolabelled Chemicals (MO, USA) and Aquasafe 500 Plus liquid scintillation cocktail from Zinsser Analytic (Frankfurt, Germany). Dihydrokainate (DHK), DL-threo-β-benzoyloxyaspartate (TBOA), 4-[2-[[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride (CGS 21680), 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261), N-cyclopentyladenosine (CPA), 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (2-Cl-IBMECA), H89 dihydrochloride (H89) and dibutyryl-cAMP (dbcAMP) were all acquired from Tocris (Bristol, UK). Adenosine deaminase (ADA) was from Calbiochem (San Diego, CA, USA). Magna Pure Compact RNA isolation, Transcriptor first strand cDNA synthesis and Lightcycler FastStart DNA MasterPLUS SYBR Green I kits and all primers were from Roche Diagnostics (Amadora, Portugal) whereas the SmartCycler reaction tubes were from Cepheid (Izasa, Portugal). Reagents used in immunoblotting experiments were purchased from Bio-Rad Laboratories, except PVDF membranes that were from Millipore (Bedford, MA, USA). All cell medium components, L-leucine methyl ester (LME) and N-methyl-D-glucamine (NMG) were purchased from Sigma-

Aldrich (Saint Louis, MO, USA). Polyclonal primary antibodies rabbit anti-GLT-I/EAAT2 (C-terminus), rabbit anti-GLAST/EAAT1 (C-terminus) and Cy5.5-labelled goat anti-mouse secondary antibody conjugate were from Abcam (Cambridge, UK), Guinea Pig monoclonal anti-VGLUT-I (vesicular glutamate transporter 1) was from Synaptic Systems (Goettingen, Germany), rabbit anti-EAAC1/EAAT3 was from Alpha Diagnostics (Texas, USA) and rabbit anti-A_1R was from Affinity Bioreagents (CO, USA). Monoclonal primary antibodies rabbit anti-GFAP (Glial fibrillary acidic protein) was from Dakocytomation (Glostrup, Denmark), mouse anti-synaptophysin and anti-SNAP-25 (Synaptosomal-associated protein 25) were from Signa-Aldrich (Missouri, USA) and mouse anti-A_{2A}R (clone 7F6-G5-A2) was from Millipore (Bedford, MA).

3.2. Animals

Wistar rats (8-10 week males) and C57Bl/6 mice (4–5 day postnatal) were purchased from Charles River. Global A_{2A}R knock out (A_{2A}R-GKO) and forebrain A_{2A}R conditional knock out (Fb-A_{2A}R-KO) mice (4-5 day postnatal and 8-10 weeks) with C57Bl/6 genetic background were generously provided by Jiang-Fan Chen (Boston University School of Medicine, Boston, MA). The generation and characterization of Fb-A_{2A}R has been described previously (Bastia et al., 2005; Wei et al., 2011; Yu et al., 2008). GFAP gene promoter-driven A_{2A}R conditional knock out (Gfa2-A_{2A}R-KO) mice in a congenic C57Bl/6 background were generated as detailed in Xu et al. (in preparation). Briefly, both transgenic *Gfa2-cre* mice (Bajenaru et al., 2002) and separately ''floxed'' A_{2A}R gene ($A_{2A}R^{flox/flox}$) mice (Bastia et al., 2005) were backcrossed for 10-12 generations to C57Bl/6 mice (Charles River; Wilmington, MA). *GFAP-cre*, $A_{2A}R^{flox/flox}$ mice were then crossed with nontransgenic (no cre) $A_{2A}R^{flox/flox}$ mice to generate the Gfa2-A_{2A}R-KO mice and littermate, nontransgenic controls studied here. Animals were maintained under controlled environment (23 ± 2℃; 12 h light/dark cycle; ad li bitum access to food and water) and handled according to European Union guidelines (86/609/EEC).

3.3. Preparation of primary astrocytes cultures

Primary astrocytes cultures were prepared from cerebral cortices of 4-5-days postnatal CB57-Bl6 mice according to a previous described procedure (Matos et al., 2008), but with some additional modifications to restrain microglial contamination (Saura, 2007). After preparation of the mixed glial cultures, the microglial cells were separated from the astrocytic monolayer by shaking at 200 rpm in an orbital shaker for 4-6 h at 37 ℃. Then, the astrocytes that remained attached in the flasks (mainly type-I) were washed with HBSS buffer containing EDTA (1 mM) and further detached by a mild trypsinization procedure using HBSS with 0.1% trypsin. The cells were reseeded with fresh astrocytes culture medium on culture cell plates, at a low density ($5x10^4$ cells/cm^2) and maintained in culture until confluence (usually, 2 days). In order to further reduce the enduring microglial content, two to three days after confluence, the astrocytes were treated with 5 mM LME lysosomotropic agent (Hambry et al., 2006) which was added to astrocytes cultures for 4-5 h (or 50 mM for 1 h, in the case of high-density cultures) before washing and replacement with fresh culture medium. After 1-2 days of cell readjustment, the astrocytes were ready for the experiments and could be maintained in culture for 1-2 weeks. Our cultures displayed a high percentage of astrocytes (≥95%), as determined by immunostaining against the astrocyte selective protein GFAP and the microglial selective marker CD11b/OX-42.

3.4. Preparation of glial plasmalemmal vesicles (gliosomes)

Brain cortical gliosomes were obtained from wild-type rats, and A_{2A}R-GKO, Fb-A_{2A}R-KO and Gfa2-A_{2A}R-KO mice through a discontinuous Percoll gradient, as previously described (Nakamura et al., 1993; Stigliani et al. 2006), but with modifications gathered from existing protocols of synaptosomal preparation (Rodrigues et al. 2005; Dunkley et al. 2008). Animals were killed and the brain cortex was quickly removed. The tissue was homogenized in a medium containing 0.25 M sucrose and 10 mM HEPES (pH 7.4), using a glass-Teflon tissue

grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 1000 *g* at 4℃) to remove nuclei and debris, and the supernatant (S1) gently stratified on a discontinuous Percoll gradient (2, 6, 15 and 23% v/v in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4) and centrifuged at 31,000 *g* for 5 min with braking speed set down to zero after reaching 1500 *g* (Dunkley et al. 2008). The layers between 2 and 6% Percoll (gliosomal fraction) and between 15 and 23% Percoll (purified presynaptic nerve terminal - synaptosomal - fraction) were collected, washed in 10 mL of HEPES buffered medium (HBM: 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) and further centrifuged at 22,000 *g* for 15 min at 4℃ in order to remove myelin components and post-synaptic material from the gliosomal and synaptosomal fractions, respectively. Both fractions were then removed and re-suspended in HBM for immunocytochemical studies. In the case of Western blot experiments, the gliosomal layer was re-suspended in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) with protease inhibitor cocktail (CLAPS) (10 µg/ml chymostatin, leupeptin, antipain and pepstatin A (Sigma) in DMSO) and the protein content was measured with BCA method (Pierce Technology); for the functional uptake experiments, the gliosomal pellets were re-suspended in Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, 1 $CaCl_2$, pH 7.4) or NMG buffer (where NaCl is isosmotically replaced by N-methylglucamine-NMG) and were viable for experiments between 4 and 6 h.

3.5. Drug treatments

Non-transportable glutamate transport inhibitors TBOA and DHK were incubated in the uptake buffer at different concentrations. TBOA is a non-selective glutamate transport inhibitor with IC_{50} values of 70 µM for GLAST/EAAT1 and 6 µM for GLT-I/EAAT2 and EAAC1/EAAT1; the prototypical non-transportable and selective GLT-I/EAAT2 inhibitor - DHK - has an IC_{50} value of 23 µM for GLT-I and 3000 µM for GLAST/EAAT1 and EAAC1/EAAT1 (IC_{50} values

available in Tocris data sheets). The $A_{2A}R$ agonist, CGS 21680, and antagonist, SCH 58261, the A_1R agonist, CPA, and the A_3R agonist, 2-CI-IB-MECA, were added to the astrocyte cultures for 10 min or 24 h before the beginning of the uptake experiments. Adenosine deaminase (ADA; specific activity of 225 units/mg protein) was prepared in Na^+-free N-Methyl-D-glucamine (NMG) buffer and added to astrocytic cultures or gliosomal preparations to achieve a final concentration of 2 U/ml and always 15 min before the addition of adenosine agonists and/or antagonists. The PKA inhibitor, H89, or the cAMP analogue, dbcAMP, were added for 24 h to the astrocytic cultures and in, the case of uptake studies, these drugs were also present during the uptake assay.

3.6. D-[^{3}H] aspartate uptake

D-Aspartate was used in the uptake studies because it is an excellent substrate for the high-affinity glutamate carriers and is little metabolized inside the cells (Bender et al., 1997; Furness et al., 2008). The analysis of D-[^{3}H] aspartate uptake in cultured astrocytes was carried out as previously described (Matos et al., 2008). The cultured astrocytes were incubated with Krebs buffer containing D-[^{3}H] aspartate (0.1 µCi/ml) and 50 µM D-aspartate for 10 min at 37℃, except for the saturation kinetics assays where the total D-aspartate concentrations ranged from 5 to 800 µM. After this period, the medium was removed, and the cultured cells were placed on ice and washed twice with cold NMG buffer to terminate the uptake. Then, the cells were lysed with 0.5 M NaOH and transferred to a scintillation vial to be mixed with Aquasafe 500 Plus liquid scintillation cocktail. The radioactivity content (disintegrations per minute) was determined using a TRICARB® 2900TR liquid scintillation analyzer. The remaining cell suspension was assayed for protein content. Some experiments were performed with NMG medium at 4℃ to determine the nonspecific uptake. The uptake rates for the cells, in each well, were expressed as the uptake per minute and per milligram of protein. The kinetic constants (i.e., V_{max} and K_M) were determined by nonlinear regression (curve fit) analysis using a one-site binding (hyperbola)

equation provided by the GraphPad Prism software. The analysis of D-[^{3}H] aspartate uptake into gliosomes was performed according to previous procedures (Köfalvi et al., 2005; Suchak et al., 2003) but with some modifications. Briefly, the gliosomal fractions were diluted in Krebs or NMG buffer and left to equilibrate at 37℃ for 10 min. Afterwards, the fractions were added to previously prepared reaction tubes with loading medium containing D-[^{3}H] aspartate (0.1 µCi/ml), D-aspartate (concentrations ranging from 5 to 100 µM in kinetic studies) with or without inhibitors of glutamate transport, TBOA and DHK. The A_{2A}R agonist CGS 21680 was added to the samples 20 min before the uptake assay. The mixtures were incubated for 10 min at 37℃ and the reaction terminated by rapid layering the mixture onto glass microfiber filters (Whatman GF/C, GE Healthcare). Then the filters were washed, dried and the radioactivity measured using a liquid scintillation cocktail on a scintillation analyzer. The rate of D-aspartate uptake was calculated as described above.

3.7. A_{2A}R binding to gliosomal and synaptosomal membranes

Gliosomal and synaptosomal membranes were prepared as previously described (Rebola et al., 2003) from the cerebral cortices of either Cre/loxP positive or negative A_{2A}R-GKO, Fb-A_{2A}R-KO and Gfa2-A_{2A}R-KO adult mice (9 weeks). The gliosomal or synaptosomal membranes were re-suspended in a pre-incubation solution (containing 50 mM Tris, 1 mM EDTA and 2 mM EGTA, pH 7.4), and a sample was collected for determining protein concentration using the BCA assay. The membranes were then incubated with 2 U/ml of ADA for 30 min at 37℃, to remove endogenous adenosine. The mixtures were centrifuged at 25,000 *g* for 20 min at 4℃ and the pelleted gliosomal or synaptosomal membranes were re-suspended in Tris-Mg solution (containing 50 mM Tris and 10 mM $MgCl_2$, pH 7.4) with 4 U/ml of ADA. Binding with 3 nM of the selective A_{2A}R antagonist, [^{3}H] ZM 241385 was performed for 1 h at room temperature with ~50-100 µg of gliosomal or synaptosomal total membranes, with constant shaking. The binding reactions were stopped by addition of 4 ml of ice-cold Tris-Mg solution filtration of the solution

through Whatman GF/C glass microfiber filters (GE Healthcare). The radioactivity was measured as described above. Specific binding was estimated by subtraction of non-specific binding, which was measured in the presence of 12 μM of XAC (Sigma-Aldrich), a mixed adenosine $A_1R/A_{2A}R$ antagonist, and [^{3}H] ZM 241385 specific binding was expressed as fmol/mg protein. All binding assays were performed in duplicate.

3.8. Western blotting

Western blotting of cultured cortical astrocytes and gliosomes extracts was performed as previously described by our group (Canas et al., 2009; Matos et al., 2008; Rodrigues et al., 2005). Incubation with the primary antibodies, namely anti-GFAP (1:2000), anti-GLAST/EAAT1 (1:1000), anti-GLT-I/EAAT2 (1:1000), anti-EAAC1 (1:500), anti-synaptophysin (1:1000), anti-SNAP-25 (1:1000), anti-VGLUT-I (1:1000), anti-A_1R (1:1000) or anti-$A_{2A}R$ (1:500), all diluted in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) with 0.1% Tween (TBS-T) and 3% BSA (fatty acid free) or 5% non-fat dry milk (for $A_{2A}R$), was carried out overnight at 4 ℃. After washing with TBS-T, the membranes were incubated with appropriate IgG secondary antibodies for 2 h at room temperature. After washing, the membranes were revealed using an ECF kit (Amersham ECL*)* and visualized under a fluorescence imaging system (VersaDoc 3000, Bio-Rad). The membranes were then stripped of the antibodies and reprobed for α-tubulin immunoreactivity using a mice anti-α-tubulin antibody (1:40,000). The densiometric analysis of protein bands was performed using a VersaDoc precision digitizer equipped with the Quantity One software version 4.4.1 (Bio-Rad).

3.9. Immunocytochemical analysis

The immunocytochemistry assays were adapted from our previous experience with primary cell cultures and purified subcellular fractions (Canas et al., 2009; Garção et al., 2006; Rodrigues et al., 2005). Thus, astrocytic cultures or gliosomes were fixed in poly-L-lysine-coated coverslips, permeabilized and blocked with 3% BSA in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO_4, 1.47 mM KH_2PO_4, pH 7.4). These preparations were then incubated with the

primary antibodies, namely rabbit anti-GLAST/EAAT1 (1:500) or rabbit anti-GLT-I/EAAT2 (1:500), and guinea pig anti-$A_{2A}R$ (1:250) or mouse anti-GFAP (1:1000). The astrocytes or gliosomes were then washed with PBS with 3% BSA and incubated for 1 h at room temperature with AlexaFluor-594-labelled anti-rabbit IgG (1:200), AlexaFluor-488-labelled anti-mouse IgG (1:200) or cyanine 5.5-labelled anti-mouse IgG antibodies (1:500). After washing and mounting onto slides with Prolong Antifade (Invitrogen), the preparations were visualized by transmission and fluorescence microscopy (Zeiss Axiovert 200, with Axiovision software 4.6) under a 100 x 10 magnification for both astrocytes and gliosomes. For co-localization counting purposes in gliosomes, the fluorescence in each coverslip (3-4 per experiment) was analyzed by counting 5-7 different fields using Image J software (Windows version; National Institutes of Health), classifying gliosomes as particles between 4 and 25 pixel resolution in a RGB 256 color scale. Only pixel intensities above background (measured in structures labelled only with secondary antibodies) were considered (see Rodrigues et al., 2008).

3.10. Quantitative PCR

Total RNA was isolated from cultured astrocytes using the Magna Pure Compact RNA isolation kit. Each sample (10 ng/μL of RNA) was subsequently synthesized to complementary DNA (cDNA) by reverse transcription using Transcriptor first strand cDNA synthesis kit in a GeneAmp PCR system 2400 thermal cycler (Perkin Elmer, Norwalk. CT, USA). The real-time PCR was performed using Lightcycler FastStart DNA MasterPLUS SYBR Green I kit customized for amplification of the target cDNA's in a SmartCycler system (Cepheid, Izasa Portugal). Primers used for real-time PCR for GLAST/EAAT1 and GLT-I/EAAT2 cDNA amplification, were synthesized with 100% homology to the mouse (*Mus musculus*) sequence by homology search through the NCBI BLAST program. Forward 5' (5'-CCTTCGTTCTGCTCACGGTC-3') and reverse 3' (5'-TTCACCTCCCGGTAGCTCAT-3') GLAST primers (accession number NM_148938) produced a 90 bp amplicon. Forward 5' (5'-GTGCAAGCCTGTTTCCAGC-3') and reverse 3' (5'-GCCTTGGTGGTATTGGCCT-3') GLT-I

primers (accession number NM_001077514) produced an 85 bp amplicon. The mRNA expression of both GLAST and GLT-I genes were normalized using histone H1 (H1) as an internal control gene after examination of the absence of variation in the experimental conditions. This was done by calculating the fold change in the internal control (H1), comparing the CGS 21680-treated and untreated samples, which was estimated to be around 1 in replicate samples (Schmittgen and Livak, 2008). Forward 5' (5'-CTTGGCTTTGGGCTTCACGGGTTTT-3') and reverse 3' (5'-CCACGGACCACCCCAAGTATTCAG-3') H1 primers (accession number NM_012578.2) produced a 487 bp amplicon. Samples, together with SYBR Green I reaction mix, were run for 45 cycles of amplification, each composed by 10 min at 95 ℃; denaturation at 95 ℃ for 10 s; primer annealing for 5 s at 58 ℃ (GLAST), at 55 ℃ (GLT-I) or at 71 ℃ (H1); and extension at 72 ℃ for 6 s (for GLAST and GLT-I) an d 20 s (for H1). All studies were performed after checking primer specificity in 2% agarose gels and further calculation of the primer efficiencies after setting a series of dilutions of the samples to generate a standard curve with the log of dilution factor plotted against the Ct value obtained during amplification of each dilution (Schmittgen and Livak, 2008). All primers had a good linearity (R^2 > 0.980) and calculated efficiencies of 1.9/90% for GLAST, 1.88/88% for GLT-I and 1.91/91% for H1b with consistency across triplicate reactions. Relative quantification was performed using the Ct/Livak method ($R=2^{-\Delta\Delta Ct}$) for GLAST and GLT-I and expressed as fold change in arbitrary values (Wong and Medrano, 2005; Schmittgen and Livak, 2008).

3.11. Statistical analysis

Data were expressed as arbitrary values or percentages of values obtained in control conditions or conditions mentioned in the figures legends, and were presented as means ± S.E.M. for the number of experiments indicated in the figure captions. Statistical significance was determined using F test for one-variable analysis of variance (one-way ANOVA) followed by Dunnett and Tukey and multiple comparison *post hoc* tests.

2

4. Results

4.1. $A_{2A}R$ decreases D-Aspartate uptake in cultured astrocytes and gliosomes

To determine the role of adenosine receptors in the control of the activity of GluTs in astrocytes, we tested the effect of selective agonists of A_1R (CPA), $A_{2A}R$ (CGS 21680) and A_3R (CI-IB-MECA) on D-[^{3}H] aspartate uptake by cultured astrocytes from neocortex of wild-type (C57Bl/6J) mice. Figure 1 shows that only the $A_{2A}R$ selective agonist, CGS 21680, significantly affected D-aspartate uptake by astrocytes. Indeed, CGS 21680 significantly ($p < 0.05$) decreased D-[^{3}H] aspartate uptake by 25.35 ± 4.30% (n=4, at 50 nM) and by 39.00 ± 7.00% (n=4 at 100 nM), compared to control cells. A_1R and A_3R selective agonists, CPA (50 and 100 nM) or 2-CI-IB-MECA (50 and 100 nM) had no impact on the D-aspartate uptake (**Fig. 1**).

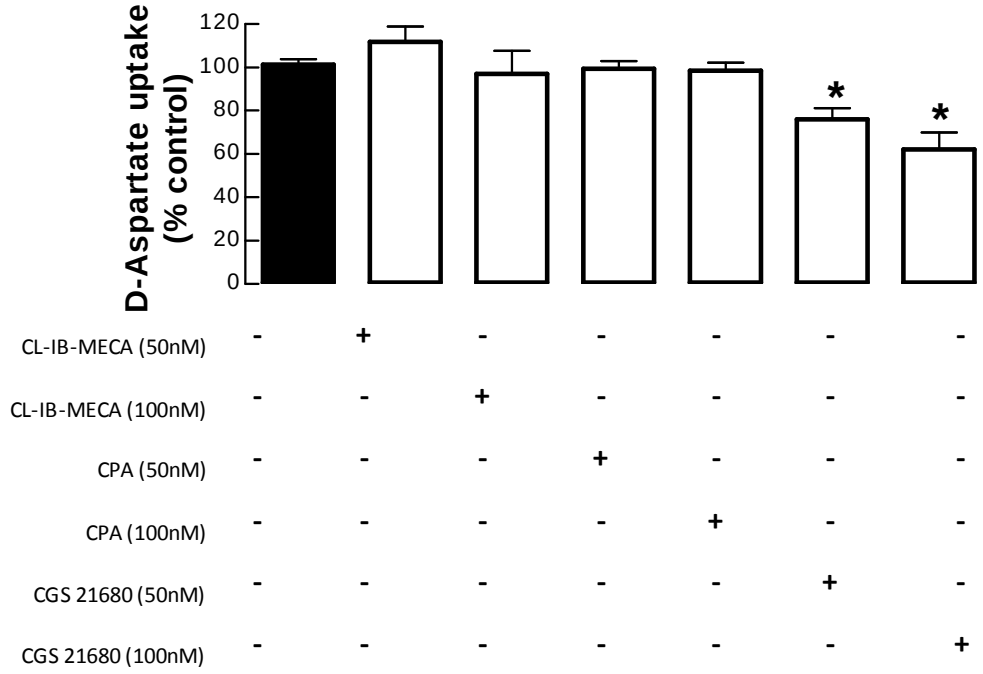
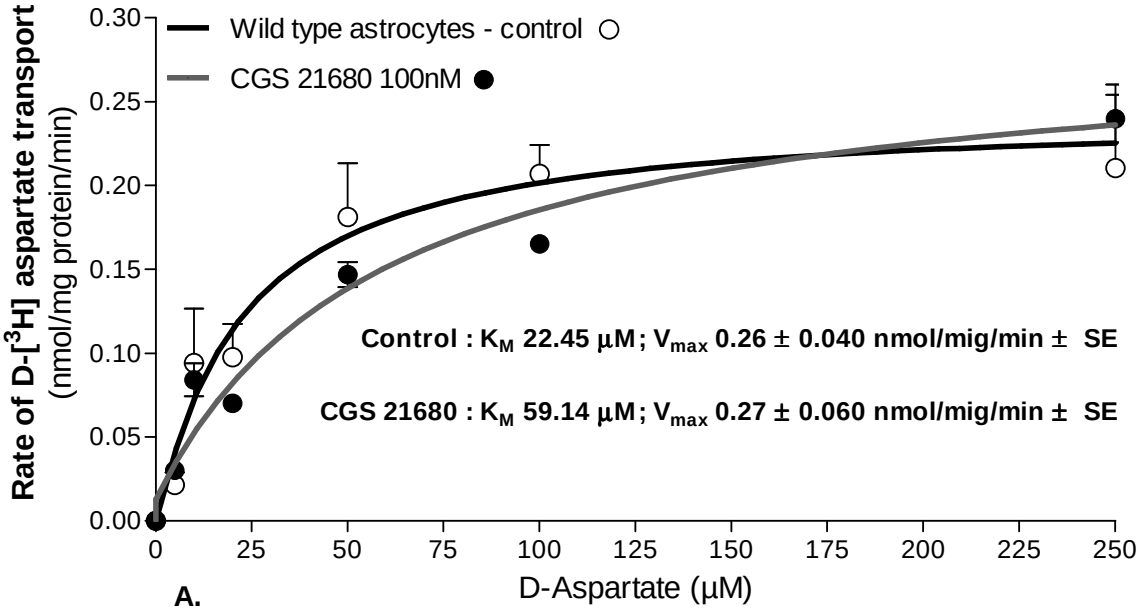
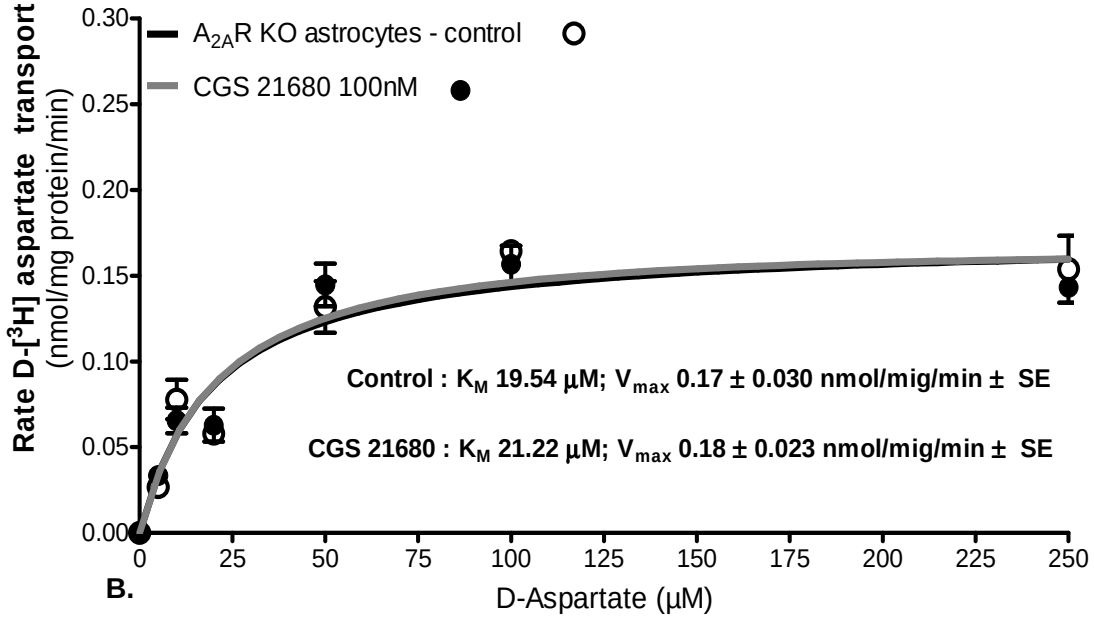


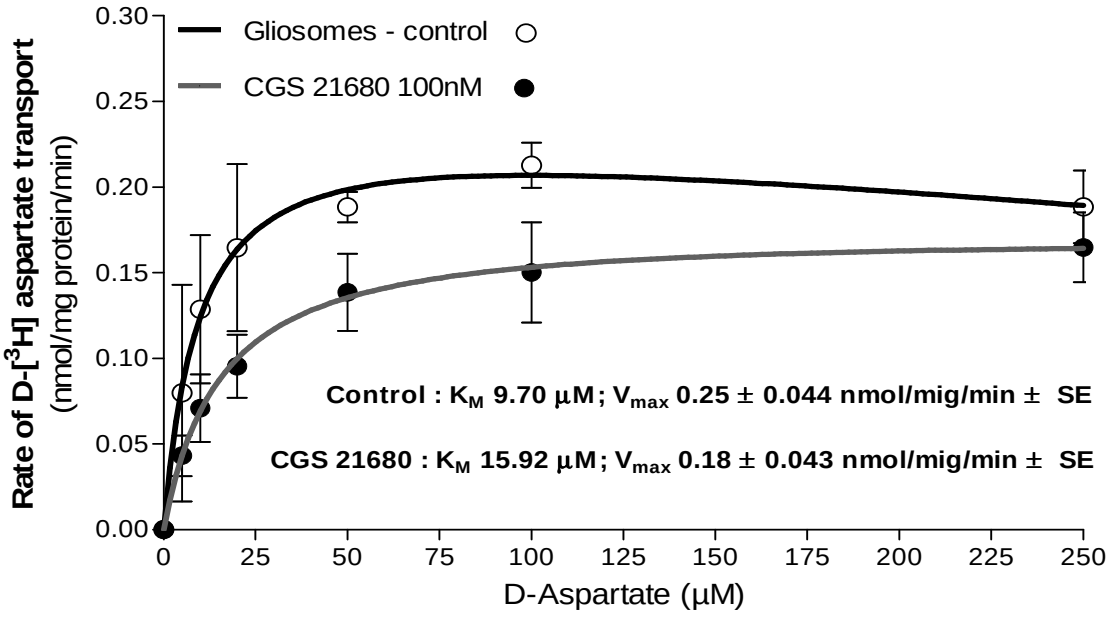
Fig. 1 - Acute (30 min) activation of $A_{2A}R$, but not of A_1R or A_3R, inhibits D-aspartate uptake in cultured cortical astrocytes. Primary cultures of astrocytes with 15 DIV, obtained from the neocortex of post-natal (P4-5) wild-type (C57Bl/6J) mice, were firstly pre-incubated for 10 min with adenosine deaminase (2 U/ml) to remove all endogenous adenosine and then acutely exposed or not (control) to A_1R, $A_{2A}R$ or A_3R selective agonists, CPA, CGS 21680 and CI-IB-MECA, respectively, for 30 min in Krebs medium at 37℃. The D-[^{3}H] aspartate uptake assay was then carried out for 10 min. Data are expressed as the percentage of D-aspartate transport in control conditions obtained in the absence of drugs (filled bar), and are the means ± SEM of at least 4 independent experiments done in triplicate. *$p < 0.05$ compared to control (filled bar).

In the second set of experiments, we explored the effect of CGS 21680 on D-[^{3}H] aspartate uptake in different astrocytic preparations, namely in astrocytic cultures from A_{2A}R-GKO mice and in cortical gliosomes, an *ex vivo* heterogeneous astrocytic preparation composed by plasma membrane vesicles, which has been described as a useful model to assess gliotransmitter uptake and release (Milanese et al., 2009). As shown in Figure 2, the analysis of the saturation kinetic curves of D-aspartate uptake in these different astrocytic preparations shows that acute (30 min) activation of A_{2A}R with CGS 21680 decreased D-aspartate uptake in astrocytic cultures from wild type mice as well as in gliosomes, but not in astrocytic cultures from A_{2A}R-KO mice. It is interesting to note that, in spite of the expected variability of kinetic assays, there was a remarkable coincidence in the kinetic parameters and in the effect of CGS 21680 in gliosomes and in cultured astrocytes from wild type mice (**Fig. 2**). Furthermore, it was observed that there was a decreased capacity of D-aspartate uptake in astrocytes from A_{2A}R-GKO mice when compared with their wild-type counterparts (**Fig. 2**).





B.



C.

Fig. 2 - The acute (30 min) activation of $A_{2A}R$ decreases D-aspartate uptake in cultured astrocytes from wild-type (A) but not $A_{2A}R$-GKO mice (B), as well as in rat cortical gliosomes (C). The different preparations were pre-incubated with adenosine deaminase (2 U/ml) for 10 min, subsequently incubated with or without CGS 21680 for 30 min and further incubated for 10 min with 0-250 µM of D-aspartate (and 0.1 µCi/ml D-[^{3}H] aspartate) in Na^+- buffer (total uptake) or in NMG (Na^+-free) buffer with TBOA (non-specific uptake), at 37℃. The saturation kinetic curves of specific glutamate transport were obtained by subtracting the non-specific from the total uptake and the kinetic constants K_M and V_{max} (shown beneath each plot) were determined by nonlinear regression and fitting of the data points with a rectangular hyperbola. Each data point represents the mean ± SEM of at least 3 separate experiments, measured in triplicate.

In addition to this genetic proof, we also attempted to pharmacologically define the involvement of $A_{2A}R$ in the control of glutamate uptake. As shown in Figure 3, the selective $A_{2A}R$ antagonist, SCH 58261 (100 nM, pre-incubated for 30 min before the uptake assays) prevented ($p < 0.05$) the inhibitory effect of CGS 21680 on D-aspartate uptake in cultured astrocytes (**Fig. 3A**) and in gliosomes (**Fig. 3B**), while SCH 58261 was devoid of effects alone (**Fig. 3**).

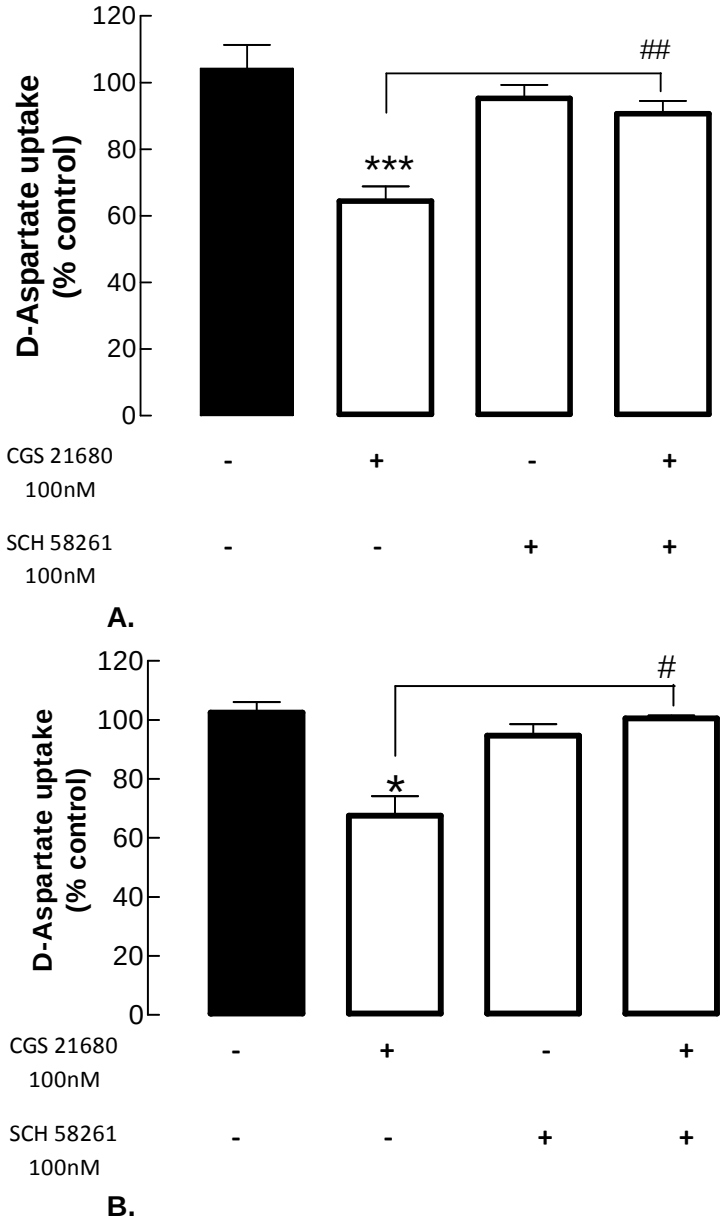


Fig.3- Pharmacological demonstration of the involvement of $A_{2A}R$ in the control of D-aspartate uptake in cultured astrocytes (A) and in gliosomes (B). Cultured astrocytes or gliosomes were pre-incubated with adenosine deaminase (2 U/ml) for 10 min and then treated with $A_{2A}R$ antagonist SCH 58261 for 15 min before incubation with $A_{2A}R$ selective agonist CGS 21680 (30 min). The D-[^{3}H] aspartate uptake assay was then carried out for 10 min. Data are expressed as the percentage of control transport obtained in the absence of drugs treatment (control) and are the means ± SEM of at least 3 independent experiments done in triplicate. ***$p < 0.001$ compared to control (no added drugs, filled bar); $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ between indicated bars.

4.2. Impact of $A_{2A}R$ on the relative activities of GLT-I, GLAST and EAAC1

The close similarity between the kinetic parameters and the amplitude of $A_{2A}R$-mediated inhibition of D-aspartate uptake (30-35% decrease) in astrocytes and gliosomes (**Fig. 2**), prompted us to test if the same GluTs were affected in the different astrocytic models. Due to the current unavailability of selective inhibitors of the main GluTs (e.g. GLAST and EAAC1), we used TBOA (non-selective glutamate transport inhibitor) or DHK (selective GLT-I inhibitor) at different concentrations and in combination with buffer containing Na^+ or not (NMG) in order to pharmacologically discriminate and estimate the contributions of each glutamate transporter to the total glutamate uptake in the different astrocytic models, as explain in detail in Tables I, II and III.

TABLE I - Short-Term Inhibitory Effect of the $A_{2A}R$ agonist CGS 21680 on Glutamate Uptake Components in Cultured Astrocytes Obtained from Wild-Type Mice

Astrocytes-Wild type (uptake compartment)	Control (nmol/mg/min)	CGS 21680 (100 nM) (nmol/mg/min)	Control (% of control)	CGS 21680 (100 nM) (% of control)	*P*
Total	0.23 ± 0.035	0.20 ± 0.018	100.00 ± 7.066	74.07 ± 7.055	<0.05
GLAST	0.08 ± 0.002	0.02 ± 0.001	103.76 ± 13.431	22.30 ± 3.502	<0.01
GLT-I	0.15 ± 0.023	0.16 ± 0.002	100.00 ± 15.053	105.20 ± 13.400	–

Pharmacological discrimination of glutamate transporters inhibited upon acute (30 min) activation of $A_{2A}R$ in cultured astrocytes from wild-type mice.

Table I shows that GLT-I and GLAST were responsible, respectively, for 54% (0.15 ± 0.02 nmol/mg/min, n=5) and 29% (0.080 ± 0.002 nmol/mg/min, n=5) of D-aspartate uptake in cultured astrocytes from wild type mice (the remaining 17% of the specific uptake might either be due to simple diffusion, glutamate-cystine exchangers, functional hemichannels or “low affinity” GluTs; see Danbolt, 2001). CGS 21680 selectively reduced the GLAST uptake contribution (≈86% reduction, 103.76 ± 13.43% in control astrocytes *versus* 22.30± 3.50% in CGS 21680-treated astrocytes, $p < 0.01$, n=5), whereas GLT-I activity seemed unaffected.

TABLE II - Short-Term Inhibitory Effect of the A_{2A}R agonist CGS 21680 on Glutamate Uptake Components in Cultured Astrocytes Obtained from A_{2A}R-GKO Mice

Astrocytes-A_{2A}R-GKO (uptake compartment)	Control (nmol/mg/min)	CGS 21680 (100 nM) (nmol/mg/min)	Control (% of control)	CGS 21680 (100 nM) (% of control)	*P*
Total	0.14 ± 0.021	0.12 ± 0.070	101.52 ± 17.023	94.38 ± 4.154	–
GLAST	0.05 ± 0.003	0.04 ± 0.014	100.01 ± 16.018	91.02 ± 27.020	–
GLT-I	0.07 ± 0.021	0.06 ± 0.013	109.61 ± 15.053	95.74 ± 15.165	–

Pharmacological discrimination of the glutamate transporters inhibited upon acute (30 min) activation of A_{2A}R in cultured astrocytes from A_{2A}R-GKO mice.

Astrocytes cultured from A_{2A}R-GKO mice (where CGS 21680 is devoid of effects), display a major reduction on both GLT-I and GLAST transporting activities when compared to cultured astrocytes from wild-type mice (**Table II**). Thus, GLT-I activity is diminished by 53% (0.15 ± 0.02 nmol/mg/min in control astrocytes *versus* 0.07 ± 0.02 nmol/mg/min in astrocytes from A_{2A}R-GKO mice, n=5) and GLAST activity is decreased by 38% (0.080 ± 0.002 nmol/mg/min in control astrocytes *versus* 0.050 ± 0.003 nmol/mg/min in astrocytes from A_{2A}R-GKO mice, n=5) (**Table I** and **II**).

TABLE III - Short-Term Inhibitory Effect of the A_{2A}R agonist CGS 21680 on Glutamate Uptake Components from Rat Cortical Gliosomes

Gliosomes (uptake compartment)	Control (nmol/mg/min)	CGS 21680 (100 nM (nmol/mg/min)	Control (% of control)	CGS 21680 (100 nM) (% of control)	*P*
Total	0.22 ± 0.014	0.14 ± 0.022	102.65 ± 6.309	61.81 ± 10.020	<0.01
GLAST	0.04 ± 0.003	0.03 ± 0.005	102.13 ± 2.794	72.50 ± 12.37	<0.05
GLT-I	0.10 ± 0.015	0.06 ± 0.009	100.86 ± 3.726	54.10 ± 3.992	<0.01
EAAC1	0.03 ± 0.005	0.02 ± 0.003	109.02 ± 6.252	76.0 ± 10.03	<0.05

Pharmacological discrimination of the glutamate transporters inhibited upon acute (30 min) activation of A_{2A}R in gliosomes.

Tables I-III - The preparations were preincubated with ADA (2 U/mL) for 10 min and then incubated for 30 min with different glutamate transport inhibitors, with or without the A_{2A}R agonist CGS 21680 (100 nM), before the initiation of D-[^{3}H] aspartate uptake assays. The separation of the contribution of each glutamate transporter was based on the use of different experimental conditions: (1) Krebs buffer (with no drugs) to determine total glutamate uptake; (2) NMG buffer + TBOA (300 μM, inhibits all glutamate transporters) to determine the nonspecific binding/uptake; (3) Krebs buffer + TBOA (20 μM, inhibits GLT-I and EAAT3) to determine GLAST contribution (4) Krebs buffer + DHK (100 μM selectively inhibits GLT-I) to determine the contribution of GLAST and EAAC1 (in gliosomes). The data expressed as nmol/min/mg protein and as % of control represent the contribution of each glutamate transporter to the total uptake and the transport mediated by each uptake components, respectively. The values are means 6 SEM of 5 independent experiments measured in triplicate; $P < 0.05$ and $P < 0.01$ comparing the uptake values in the absence and presence of CGS 21680.

Owing to the additional presence of EAAC1 transporters in gliosomes (but not in our cultured astrocytes), as detected by Western blot analysis (see **Fig. 5**), and in accordance with a previous report (Suchak et al., 2003), further combinations had to be made to pharmacologically estimate the relative contribution of this transporter in gliosomes (**Table III**). Table III shows that GLT-I, GLAST and EAAC1 were responsible, respectively, for 45% (0.10 ± 0.01 nmol/mg/min, n=5), 18% (0.040 ± 0.008 nmol/mg/min, n=5) and 13% (0.030 ± 0.005 nmol/mg/min, n=5) of the whole D-aspartate uptake in adult cortical brain gliosomes. The inhibitory effect of CGS 21680 on D-aspartate uptake in this preparation is likely due to a decrease of the activity of all GluTs; however, GLT-I seems more affected (≈ 46% reduction, 100.86 ± 3.73% in control gliosomes *versus* 54.10 ± 4.00% in CGS 21680-treated gliosomes, $p < 0.01$, n=5) than GLAST (≈28% reduction, 102.13 ± 3.00% in control gliosomes *versus* 72.50 ± 12.40 % in CGS 21680-treated gliosomes, $p < 0.01$, n=5) and EAAC1 (≈30% reduction, 109.02 ± 6.30% in control gliosomes *versus* 76.00 ± 10.03% in CGS 21680-treated gliosomes, $p < 0.01$, n=5).

Overall, the results indicate that the acute activation of $A_{2A}R$ leads to a preferential and selective decrease of GLAST capacity in cultured astrocytes (**Table I**) and a major decrease of GLT-I but also of GLAST and EAAC1 capacity in gliosomes (**Table III**).

4.3. Co-localization between $A_{2A}R$ and glutamate transporters

The ability of $A_{2A}R$ to control the activity of GluTs implies a co-localization of $A_{2A}R$ together with GLT-I or GLAST. We first confirmed by Western blot analysis, by using total membranes from the striatum as a positive control (Fredholm et al., 2005), that $A_{2A}R$ was present in cultured astrocytes but not present in astrocytes collected from $A_{2A}R$-GKO mice (**Fig. 4A**). Furthermore, $A_{2A}R$ immunoreactivity is present in GFAP-labeled elements and is co-localized with GLT-I (**Fig.4B**) or GLAST (**Fig.4C**) immunoreactivity in cultured astrocytes from wild-type mice. It was also interesting to observe that $A_{2A}R$ are also present in GFAP-negative but vimentin- and

GLAST-positive immature astrocytes, which may underlie possible roles of the adenosinergic system during astrocytic development and maturation (data not shown).

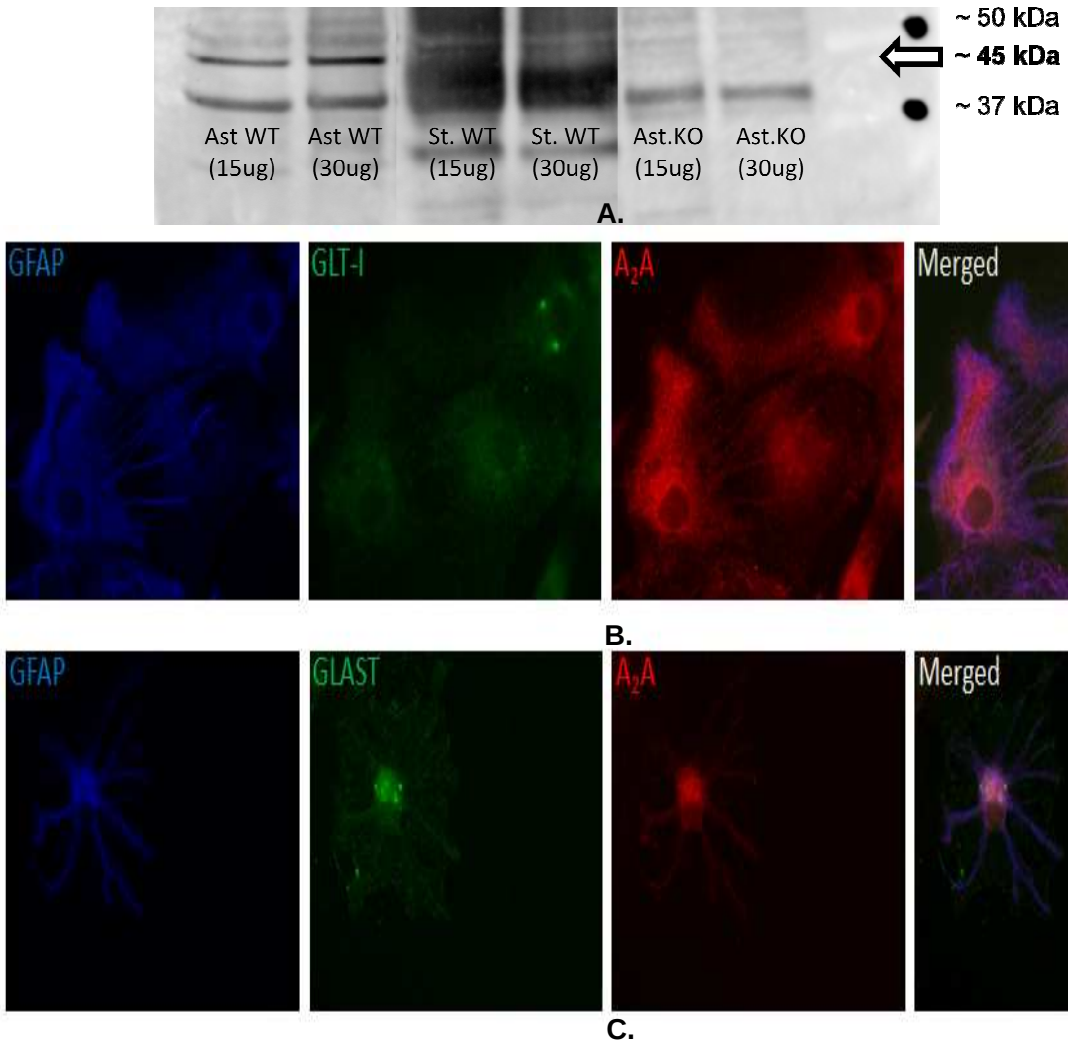


Fig. 4 - A_{2A} receptors are present in cultured astrocytes and are co-located with GFAP and GLT-I (B) or GLAST (C) in cultured astrocytes. Panel (**A)** shows that A_{2A}R are present in cultured astrocytes from wild type mice (Ast WT), similarly to total striatal membranes (St WT), and are absent in cultured astrocytes from A_{2A}R-GKO mice (Ast KO). The representative Western blot shows that two different amonts of protein from Ast WT or St WT yield increasing immunoreactivity at 45 kDa, which is absent in Ast KO. Panels (**B)** and **(C)** are representative images of immunocytochemical labeling of cultured astrocytes with GFAP (first column, blue color), and GLT-I (in A) or GLAST (in B) (third column, green color), A_{2A}R (second column, red color) and finally the merged images (far right column), which shows the co-localization of A_{2A}R with GLT-I or GLAST in GFAP-positive elements. The Western blot and the images are representative of 4 independent experiments.

Before attempting to document the co-localization of $A_{2A}R$ and GluTs in gliosomes, we had to carry out a careful characterization of the gliosomal preparation, a task somehow complicated by the evidence that astrocytic membranes have a plethora of presynaptic proteins identical to those of presynaptic nerve terminals (Maienschein et al., 1999; Montana et al., 2006). Thus, we decided to use a combination of different markers of gliosomes and synaptosomes (purified nerve terminals) to conclude on the purity (and degree of contamination) of both fractions. The characterization of the gliosomal preparation displayed in Figure 5, shows that it contains higher levels of GFAP, GLT-Iα, and GLAST ($p < 0.001$) compared to synaptosomes, as previously described (Milanese et al., 2009); in contrast, synaptosomes had significantly ($p < 0.001$) higher levels of the synaptic vesicle markers synaptophysin and VGLUT-I (**Fig. 5**), whereas similar amounts of the SNARE complex protein SNAP-25 were present in both fractions; finally, low post-synaptic contaminations were present in both fractions, as gauged by the low levels of the post synaptic density protein of 95 kDa (PSD-95), as previously described (Maienschein et al., 1999; Wilhelm et al., 2004).

After defining the gliosomes as a heterogenous but mainly astrocytic preparation, we tested the presence of $A_{2A}R$ immunoreactivity, which was found to be lower ($p < 0.05$) than that found in synaptosomes (**Fig. 5**). Accordingly, receptor-binding studies confirmed that the selective $A_{2A}R$ antagonist, [^{3}H] ZM 241385 bound with a greater density (36.00 ± 7.00 fmol/mg protein, n=5) to synaptosomal rather that gliosomal cortical membranes (20.00 ± 3.00 fmol/mg protein, n=5). The availability of transgenic mice with global deletion of $A_{2A}R$ ($A_{2A}R$-GKO) and Cre/loxP system selective deletions of $A_{2A}R$ in astrocytes (Gfa2-A_{2A}KO, GFAP promoter-driven deletion) and in forebrain neurons (Fb-A_{2A}KO - CAMK-IIα promoter-driven deletion, see Yu et al., 2008; Wei et al., 2011) further allows re-enforcing our contention that gliosomes are indeed a purified astrocytic preparation suited to probe properties of astrocytes from the adult brain. As expected, the number of [^{3}H] ZM 241385 binding sites was significantly ($p < 0.001$) decreased in both synaptosomes and gliosomes prepared from $A_{2A}R$-GKO mice (**Fig. 6**). Furthermore, in Gfa2

promoter-driven A_{2A}R-KO mice, the density of [^{3}H] ZM 241385 binding sites were significantly ($p < 0.01$) decreased in gliosomes (**Fig. 6A**), but not in synaptosomes (**Fig. 6B**); conversely in CAMK-II-driven A_{2A}R-KO mice, the density of [^{3}H] ZM 241385 binding sites was significantly ($p < 0.001$) decreased in synaptosomes (**Fig.6A**), but not in gliosomes (**Fig. 6B**).

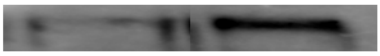
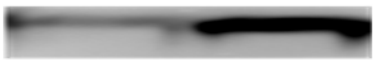
Proteins	Gliosomes/Synaptosomes	Gliosomes (control)	Synaptosomes (% of control)	P
GFAP (~ 50 kDa)		100%	29,2 ± 10,8 %	< 0,001
GLT-I (~ 62 kDa)		100%	21,4 ± 6,0 %	< 0,001
GLAST (~ 51 kDa)		100%	26,0 ± 7,0 %	< 0,001
EAAC1 (~ 69 kDa)		100%	57,0 ± 6,0 %	< 0,001
VGLUT-1 (~ 50 kDa)		100%	225,0 ± 20,2 %	< 0,01
Synaptophysin (~ 38 kDa)		100%	306,0 ± 21,4%	< 0,001
SNAP-25 (~ 25 kDa)		100%	109,2 ± 4,40 %	-
PSD-95 (~ 95 kDa)		100%	131,0 ± 24,8 %	-
A_{2A}R (~ 45 kDa)		100%	155,7± 18,8 %	< 0,05

Fig. 5 - Differential presence of A_{2A}R in cortical gliosomes and synaptosomes. Comparison by Western blot analysis of the density of A_{2A}R and of different putative markers (GFAP, GLT-I, GLAST, EAAC1, VGLUT-I, synaptophysin, SNAP-25, and PSD-95) in gliosomes and synaptosomes purified from the rat cerebal cortex. Values were expressed as percentage of the density values determined for cortical gliosomes and are presented as mean ± SEM of at least four independent experiments. Note that markers of nerve terminals (synaptophysin and VGLUT-I) are more abundant in synaptosomes, whereas putative markers of astrocytes (GFAP and all the glutamate transporters tested) are more abundant in gliosomes, which display A_{2A}R immunoreactivity with a density lower than that found in synaptosomes. *P < 0.05, **P < 0.01 and ***P < 0.001, compared with correspondent gliosomal control.

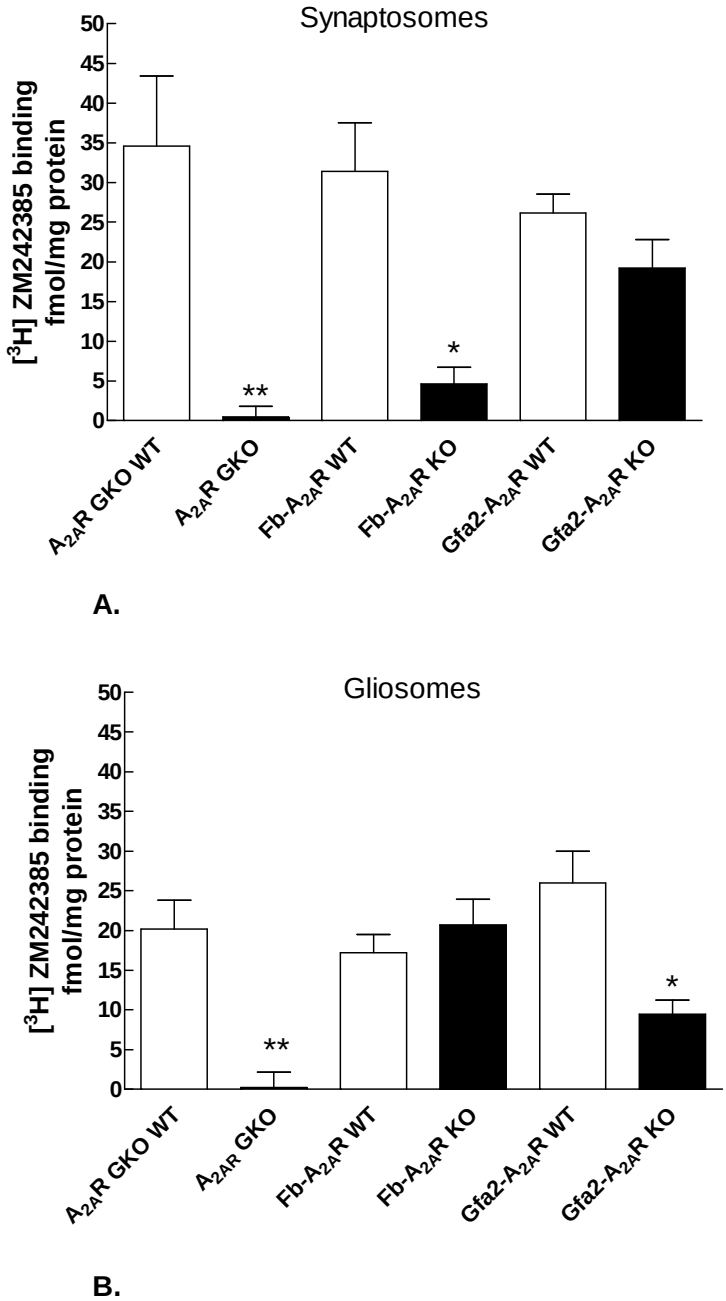


Fig. 6 - Differential $A_{2A}R$ binding to cortical gliosomal and synaptosomal membranes. Figures A and B show the density of $A_{2A}R$ in cortical synaptosomal **(A)** and gliosomal **(B)** membranes from transgenic mice with general deletion of $A_{2A}R$ ($A_{2A}R$ GKO) or selective deletions of $A_{2A}R$ in forebrain neurons (Fb-$A_{2A}R$ KO) or in astrocytes (Gfa2-$A_{2A}R$ KO) compared with their strain-matched wild-type counterparts. $A_{2A}R$ density was evaluated as the specific binding of the selective $A_{2A}R$ antagonist [^{3}H] ZM 241385 (3 nM), which was incubated for 1 h with ~50–100 µg of gliosomal or synaptosomal total membranes, after their treatment with adenosine deaminase (4 U/mL). Specific binding was performed by subtraction of nonspecific binding, which was measured in the presence of 12 µM of XAC, a mixed adenosine A_1R/ $A_{2A}R$ antagonist. Values are mean 6 SEM of duplicates of five independent samples and are expressed as fmol/mg/ prot. *P < 0.01 and **P < 0.001, compared with the corresponding wild-type mice.

This enabled us to evaluate with greater confidence the co-localization of $A_{2A}R$ and glutamate transporters using gliosomes as a model preparation of astrocytes from the adult brain. As shown in Figure 7, it was observed that $A_{2A}R$ were co-localized with GLT-I and GLAST immunoreactivities. Thus, in spite of their low-density levels in cortical gliosomes, $A_{2A}R$ are well

positioned for cross-talking with glutamate transporters, supporting the role of adenosine as a gliomodulator of glutamatergic transmission through $A_{2A}R$ (**Fig. 7**).

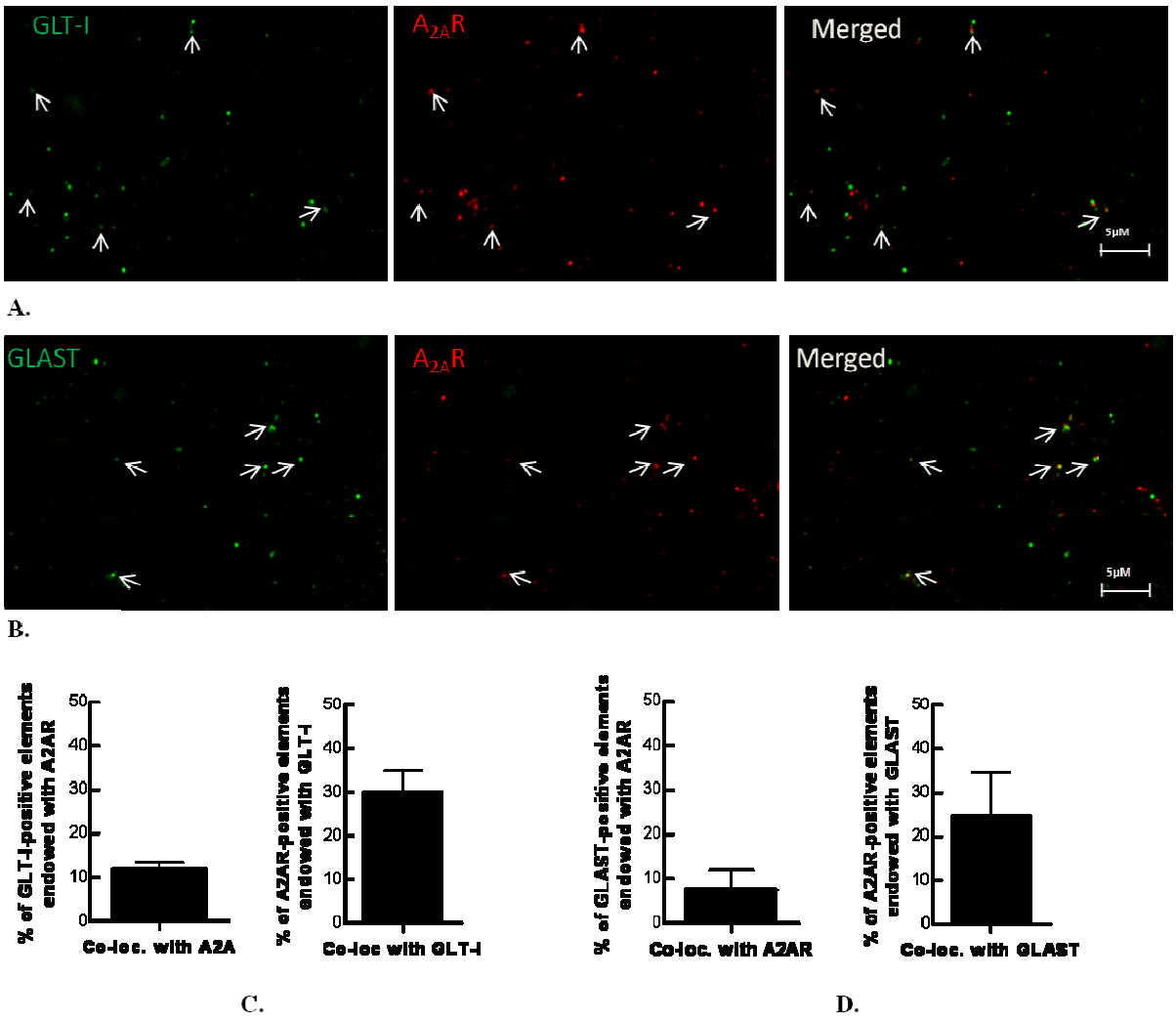


Fig. 7 - Co-localization of $A_{2A}R$ and glutamate transporters GLT-I (A and C) or GLAST (B and D) in rat cortical gliosomes. (A) Immunocytochemical detection of GLT-I (left image, in green), of $A_{2A}R$ (centre image, in red) and merging of the two images (right image) showing the co-localization of both membrane proteins in gliosomes from the rat cerebral cortex. **(B)** Immunocytochemical detection of GLAST (left image, in green), of $A_{2A}R$ (centre image, in red) and merging of the two images (right image) showing the co-localization of both membrane proteins in gliosomes from the rat cerebral cortex. **(C)** Quantification of the co-localization $A_{2A}R$ and GLT-I immunoreactivities in the total gliosomal population. **(D)** Quantification of the co-localization of $A_{2A}R$ and GLAST immunoreactivities in the total gliosomal population. The images were obtained by fluorescence microscopy with a total magnification of 1,000X and are representative of five different fields per coverslip. The data in the bar graphs are mean ± SEM of three to four independent experiments carried out in gliosomal preparations from different animals.

4.4. $A_{2A}R$ decreases D-Aspartate uptake through an increase on cAMP/PKA

Since we and others have previously shown that $A_{2A}R$ are constitutively and continuously activated to control neurodegeneration associated with abnormal glutamatergic transmission (reviewed in Cunha, 2005), we next explored the effect of long-term (24 h) activation of $A_{2A}R$ on astrocytic D-[^{3}H] aspartate uptake capacity. As shown in Figure 8, the prolonged activation of $A_{2A}R$ with its selective agonist CGS 21680 for 24 h still significantly inhibited the D-[^{3}H] aspartate uptake in cultured astrocytes. Indeed, the treatment for 24 h with 100 nM CGS 21680 significantly ($p < 0.01$) decreased D-[^{3}H] aspartate uptake by 30.28 ± 6.53% (n=5), compared to control (**Fig. 8**). Similar to the acute activation of $A_{2A}R$ (**Fig. 3**), the pre-incubation with SCH 58261 significantly ($p < 0.05$) prevented the decrease of D-aspartate uptake caused by the prolonged (24 h) exposure to CGS 21680.

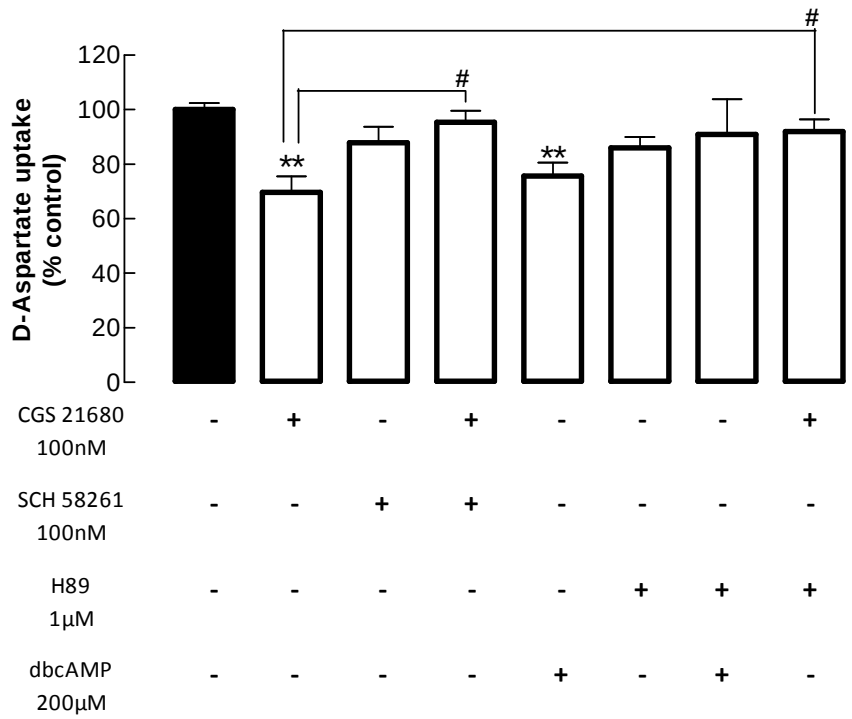


Fig. 8 The long term activation of $A_{2A}R$ attenuates D-aspartate uptake through a PKA-signaling pathway in cultured astrocytes. Astrocytes were treated with the $A_{2A}R$ antagonist SCH 58261 or with the PKA inhibitor H89 for 15 min before incubation with the $A_{2A}R$ selective agonist CGS 21680 or with the cAMP analogue dbcAMP for 24 h. The D-[^{3}H] aspartate uptake assay was then carried out for 10 min. Data are expressed as % of the transport determined in control astrocytes (filled bar) and are the means ± SEM of at least 3 independent experiments performed in triplicate. **p < 0.01 compared to control astrocytes (filled bar); $^{\#}p < 0.05$ between indicated bars.

In order to identify the signalling pathway recruited by $A_{2A}R$ to inhibit glutamate uptake, we tested the effect of the PKA inhibitor H89 and of the cyclic adenosine monophosphate (cAMP) analogue Dibutyryl-cAMP (dbcAMP). As shown in Figure 8, dbcAMP (200 µM) mimicked the inhibitory effect of CGS 21680 on D-aspartate uptake (24.27 ± 5.34% reduction, $p < 0.01$, compared to untreated cells, n=3). Furthermore, the inhibition of PKA with H89 (1 µM) significantly ($p < 0.05$) prevented the decrease of D-aspartate uptake induced by either CGS 21680 or dbcAMP (**Fig. 8**).

4.5. $A_{2A}R$ activation decreases GLT-I and GLAST expression

We next tested if $A_{2A}R$ activation decreased the activity of GluTs only through an inhibition of their activity or also through a control of their expression and density. Quantitative PCR assays showed that the expression of both GLT-I and GLAST were generally decreased between the acute (30 min) and the prolonged (12-24 h) activation of $A_{2A}R$ (**Fig.9A** and **B**). Thus, immediately after 30 min of CGS 21680 exposure, there was a decrease of GLT-I mRNA levels (-2.50 ± 0.31 fold change, n=5), reaching its maximum at 3 h of incubation (-6.20 ± 3.88 fold change, $p < 0.001$, n=5), maintaining the decrease after 12 h of incubation (-4.60 ± 2.60 fold change, $p < 0.01$, n=5) and finally recovering to control levels after 24 h of treatment (**Fig. 9A**). Similarly but even more steadily, right after 30 min of CGS 21680 exposure, there was a significant decrease in GLAST mRNA levels (-3.00 ± 0.72 fold change, $p < 0.01$), which became larger after 3 h of incubation (-5.00 ± 2.28 fold change, $p < 0.01$, n=5), reaching its maximum after 12 h of incubation (-6.50 ± 3.06 fold change, $p < 0.001$, n=3) and attenuated but still present after 24 h (-1.50 ± 0.48 fold change, n=3) (**Fig. 9B**). The $A_{2A}R$-induced decrease of GLAST expression was confirmed at the protein level, as testified by the Western blot analysis showing lower levels of GLAST protein after 24 h of exposure to CGS 21680 (**Fig. 10**). This analysis could not be carried out for GLT-I due to the poor signal of the antibody in astrocytic cultures.

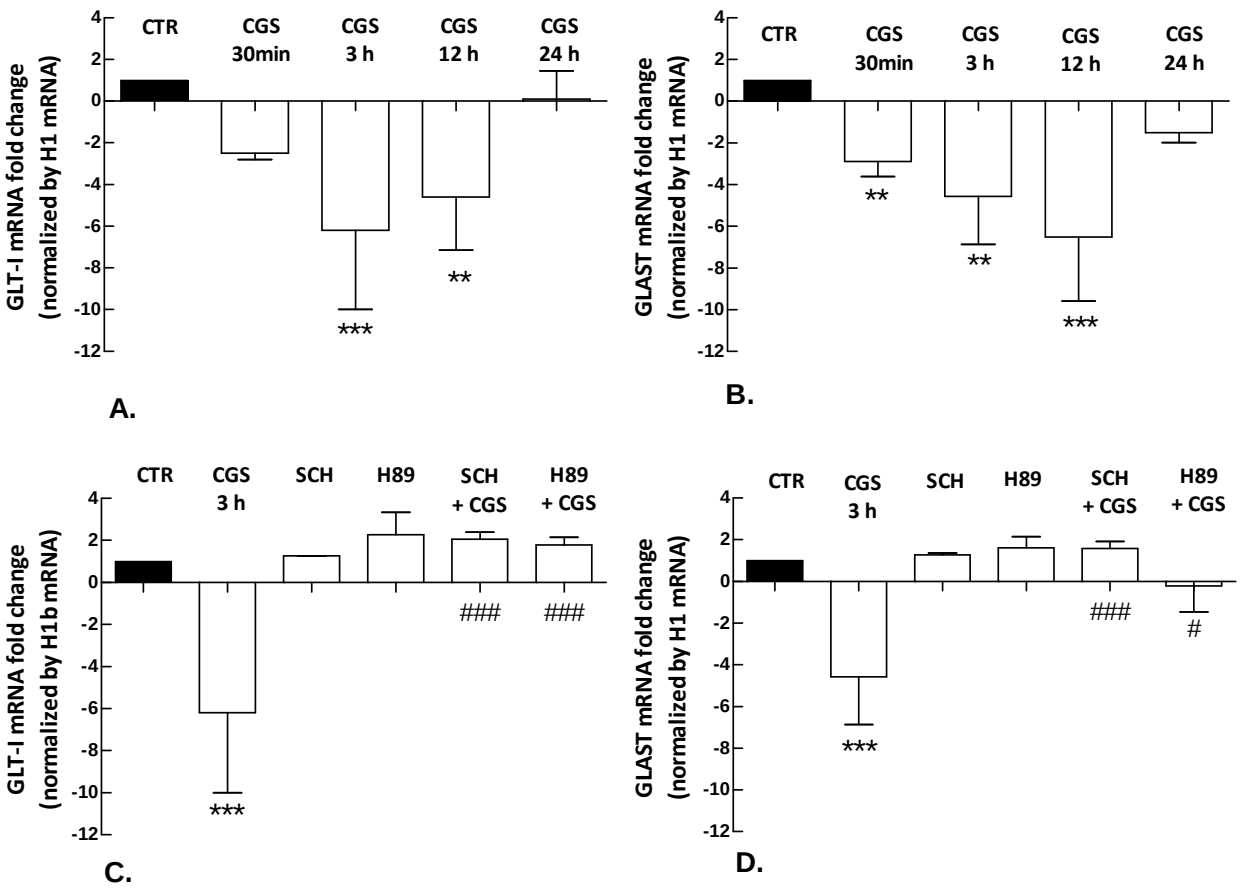


Fig. 9 - Prolonged activation of $A_{2A}R$ decreases the expression of GLT-I and GLAST mRNA in cultured astrocytes from wild type mice, through a cAMP-PKA signaling cascade. **(A, B)** Cultured astrocytes from wild-type mice were challenged with CGS 21680 (CGS) (100 nM) during the period indicated above each bar and the levels of GLT-I **(A)** and GLAST **(B)** mRNA were quantified by real-time PCR. The agonist of $A_{2A}R$ decreases the levels of GLT-I **(A)** and GLAST **(B)** mRNA in a time-dependent manner (30 min- 24h). **(C, D)** The $A_{2A}R$ antagonist SCH 58261 (SCH) (100 nM) and the protein kinase A inhibitor H89 (1 µM) prevented the decrease of GLT-I **(C)** and GLAST **(D)** mRNA levels caused by exposure for 3 h to CGS 21680 (100 nM). In all panels, the mRNA expression of both GLAST and GLT-I genes was normalized by using histone H1 (H1) mRNA as an internal control gene after examination of the absence of variation in the experimental conditions. Relative quantifications were performed using the C_t /Livak method ($R=2^{-\Delta\Delta Ct}$) for the GLAST and GLT-I genes of interest and expressed as fold change in arbitrary values. The data are mean ± SEM of at least three independent PCR experiments. **$p < 0.01$ and ***$p < 0.001$ compared to control (no added drugs; filled bar); $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to the effect of CGS 21680 (second bar from the left).

Next, we attempted to probe the involvement of the cAMP-PKA signalling pathway on the observed $A_{2A}R$-induced decrease of GLT-I and GLAST expression. As shown in Figure 9C, the blockade $A_{2A}R$ with SCH 58261 and the inhibition of PKA with H89 significantly ($p < 0.001$)

prevented the CGS 21680-induced decrease of GLT-I mRNA levels (**Fig. 9C**). Similarly, both SCH 58261 and H89 significantly ($p < 0.001$ and $p < 0.05$, respectively) prevented the CGS 21680-induced decrease of GLAST expression (**Fig. 9D**).

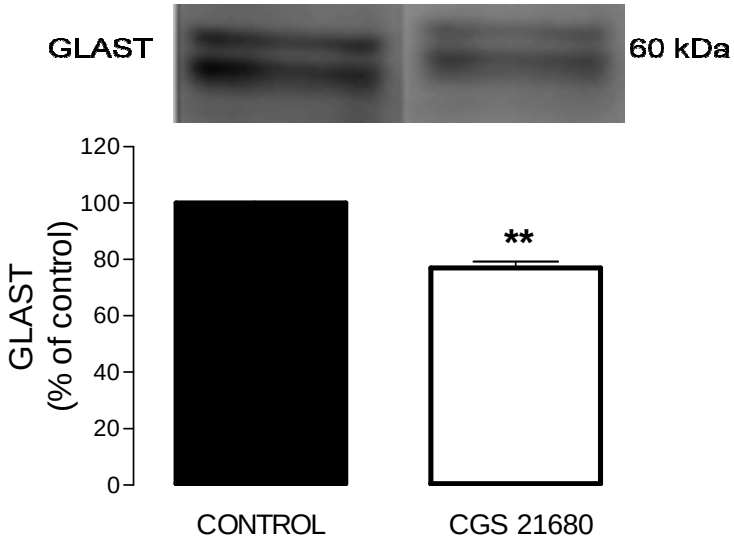


Fig. 10 - Astrocyte GLAST protein levels are decreased by the activation of A_{2A}R. A_{2A}R activation through CGS 21680 long treatment (24 hr) leads to a decrease of 20% in GLAST levels in mice wild-type cultured cortical astrocytes. Cell lysates were examined by immunoblotting with an anti-GLAST polyclonal antibody at non-saturating protein quantities. Immunoreactive bands were visualized by scanning on a Versadoc Image system, and the levels of proteins were quantified in the Quantity One program. Bars represent the relative levels of GLAST and were expressed as percentage of control of untreated astrocytes. Data are the means ± SEM of at least three independent experiments. **p < 0.001, significantly different from control cells.

5. Discussion

The present study provides the first direct evidence that the activation of astrocytic A_{2A} receptors (A_{2A}R) decreases the uptake of glutamate by inhibiting their activity as well as their expression. Furthermore, we also show that A_{2A}R are the main adenosine receptor controlling glutamate clearance since none of the other adenosine receptors tested (A_1R and A_3R) modified glutamate uptake. Thus, the present identification of the presence of A_{2A}R in different astrocytic preparations, the co-localization of A_{2A}R with different GluTs and their ability to control the

activity and the expression of astrocytic GluTs prompts a potential novel role of astrocytic $A_{2A}R$ in the control of physio-pathological processes known to be influenced by the activity of GluTs.

One major aim was to identify if $A_{2A}R$ modulated any particular glutamate transporter subtype. This question is not straightforward since it is known that different preparations from different brain regions and from different species display a different relative activity and availability of the different GluTs (Danbolt et al., 2001). In astrocytic cultures from both neonatal or adult brain, GLT-I transporter proteins were scarcely detectable (Kondo et al., 1995; Swanson et al., 1997; Gegelashvili et al., 2000) or even absent (Abe and Saito, 2000), arguably because of the lack of soluble inducible neuronal factors that increase GLT-I expression and activity (Gegelashvili et al., 1997; Swanson et al. 1997); therefore GLAST is considered by some as the major transporter in primary astrocytes cultures. However, many other studies show the presence of GLT-I in postnatal rat brain astrocytes cultures (Miralles et al., 2001; Plachez et al., 2004; Vallejo-Illarramendi et al., 2005; Matos et al., 2008) and occasionally even identify GLT-I as the sole transporter in these cultures (Nishizaki et al., 2002). In our cultures of neocortical astrocytes obtained from post natal (P4-5) mice, both GLT-I and GLAST were detected, and GLT-I was shown to be the most important glutamate uptake carrier, accounting for 54% of the Na^+-dependent glutamate uptake (where GLAST accounts for ≈30% and no activity was detected for EAAC1) (**Table I**). These conclusions were drawn from our pharmacological dissection using the GLT-I selective inhibitor DHK, since the Western blot analysis of astrocytes extracts showed very low and unworkable levels of the GLT-I protein (even though GLT-I mRNA was detected). An aspect that might not be captured by cultured astrocytes but may be present in gliosomes is the heterogeneity of the distribution of GluTs. Thus, the targeting of the transporters to particular parts of the astrocytic plasma membrane is non-uniform and apparently directed to the locations where they are most needed (Reichenbach, 2010; Danbolt et al., 2001). We observed that gliosomes displayed some heterogeneity with varying distribution and co-localizations of both membrane GLT-I and GLAST

but also of other common astrocytic markers, such as GFAP (**Fig.5**). In our gliosomes prepared from the adult rat cortex, GLT-I emerges as the most important glutamate carrier, responsible for 45% of the Na^+-dependent glutamate uptake, whereas GLAST accounts for 18% of total uptake and EAAC1 for 13% (**Table III**). Thus, albeit there are differences in the relative contribution of different transporters in different preparations, GLT-I is the predominant transporter in both preparations used. Interestingly, the acute activation of $A_{2A}R$ seems to predominantly affect the activity of GLAST in astrocytes cultures (**Table I**), whereas it decreases the activity of all GluTs in gliosomes (**Table III**). The mechanism by which the acute activation of $A_{2A}R$ affects GluTs in cultured astrocytes may involve a direct regulation of the activity of GLAST transporter proteins, since CGS 21680 increases the K_M with no change of V_{max}. A similar mechanism may occur in gliosomes, albeit the observation that CGS 21680 affects both V_{max} and K_M further suggests that a possible $A_{2A}R$-mediated control of the internalization of GluTs (see Sattler and Rothstein, 2006). This likely involves an $A_{2A}R$-mediated phosphorylation of GluTs (Guillet et al., 2005; Gehring et al., 2009; Wu et al., 2010), albeit surprisingly little is known about receptor-mediated control of the activity of GluTs (reviewed in Robinson, 2006).

The present study also provides the first evidence showing that the long-term (24 h) activation of $A_{2A}R$ decreases the expression of GLT-I and GLAST in cultured astrocytes (**Fig. 9** and **10**). The present study allowed defining the involvement of the cAMP-protein kinase A transducing pathway in this $A_{2A}R$-mediated control of the expression of GluTs, but the mechanisms underlying this control still remain to be unraveled mainly because too little is currently known about the control of the expression of GluTs and several conflicting results have been reported. For instance, neurotrophic factors were shown to bolster the astrocytic glutamate uptake activity through the activation of protein kinase A (Gegelashvili et al., 1997, 2000). Accordingly, different studies have shown that the cAMP/ protein kinase A transducing pathway can either enhance (Gegelashvili et al., 1997; Figiel et al., 2003), fail to affect (Gegelashvili et al., 1997; Danbolt, 2001) or decrease the expression or activity of GLT-I and GLAST (Nishizaki

et al., 2002; Lim and Kim, 2003; Adolph et al., 2007; Rath et al., 2008) in astrocytic cultures. Thus, it seems that protein kinase A can potentially recruit different mechanisms with opposite effects on the expression of glutamate receptors and it may be the particular membrane receptors triggering this transduction pathway that will define its impact on the expression of GluTs.

This dual ability of $A_{2A}R$ to rapidly control the activity of GluTs, and to cause a long-term down regulation of their expression is strikingly related to the dual role of $A_{2A}R$ in the control of physio-pathological events that are dependent on the control of the extracellular levels of glutamate. Thus, $A_{2A}R$ are well known to control synaptic plasticity phenomena (D'Alcantara et al., 2001; Rebola et al., 2008; Costenla et al., 2011), an effect that has largely been assumed to result from the action of synaptically-enriched $A_{2A}R$ (Rebola et al., 2005). Notably, it has also been shown that the control of the activity of GluTs can influence synaptic plasticity (Tsvetkov et al., 2004; Filosa et al., 2009; Omrani et al., 2009; Goubard et al., 2011). Therefore, the presently observed ability of $A_{2A}R$ to control the activity of GluTs is a strong argument to propose that the control of synaptic plasticity by $A_{2A}R$ might also involve the action of astrocytic $A_{2A}R$.

The blockade of $A_{2A}R$ is also known to afford a robust neuroprotection against different brain insults (Cunha, 2005; Chen et al., 2007; Gomes et al., 2011) and the excessive extracellular glutamate accumulation seems to contribute for the evolution of most neurodegenerative disorders (Lipton and Rosenberg, 1994). Interestingly, $A_{2A}R$-mediated neuroprotection has been proposed to result from the action of both non-neuronal (Yu et al., 2008; Dai et al., 2010; Rebola et al., 2011) and neuronal $A_{2A}R$ (Carta et al., 2009; Canas et al., 2009) through mechanisms that are still unclear. Since the modulation of GluTs is also known to affect the susceptibility and/or extent of neuronal damage (Harada et al., 2007; Sattler and Rothstein, 2006; Domercq et al., 2005; Tanaka et al., 1997), our present conclusion that $A_{2A}R$ control GluTs offers a likely mechanism that could explain the neuroprotective effects of $A_{2A}R$ antagonists.

In summary, the present results provide additional evidence for the presence of $A_{2A}R$ in astrocytes and demonstrate their crucial role in modulating perhaps the most important role of astrocytes, glutamate uptake. Indeed, the primary role of astrocytic $A_{2A}R$ could be to sense synaptic transmission in order to adjust the extracellular level of glutamate, either by modulation of the transporter activity (shown in these studies) or by exocytotic glutamate release (Li et al., 2001; Nishizaki et al., 2002), therefore playing a potentially key role in the control of synaptic plasticity and neurodegeneration.

6. Acknowledgements

The authors thank Livia Bajenaru and David Gutmann at the University of Washington for providing transgenic Gfa2-cre mice, and Yuehang Xu for expert technical assistance.

CHAPTER 3

ANTAGONISTIC INTERACTION BETWEEN ADENOSINE A_{2A} RECEPTORS AND Na^+/K^+-ATPase-α2 CONTROLLING GLUTAMATE UPTAKE IN ASTROCYTES

Marco Matos [1,2,3]*, Elisabete Augusto [1,2,3], Paula Agostinho [2,3], Rodrigo A. Cunha [2,3] and Jiang-Fan Chen [1]

1. Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA
2. Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
3. Faculty of Medicine, University of Coimbra, Coimbra, Portugal

J NEUROSCI. (2013) (accepted)

* Marco Matos performed the experiments presented in Fig. 1-6

1. Abstract

Astrocytic glutamate transporter-1 (GLT-I) is critical to control the bulk of glutamate uptake and, thus, to regulate synaptic plasticity and excitotoxicity. GLT-I glutamate uptake is driven by the sodium gradient implemented by Na^+/K^+-ATPases (NKA) and the α_2 subunit of NKA (NKA-α2) is actually linked to GLT-I to regulate astrocytic glutamate transport. We recently found that adenosine A_{2A} receptors (A_{2A}R), which control synaptic plasticity and neurodegeneration, regulate glutamate uptake through unknown mechanisms. Here we report that A_{2A}R activation decreases NKA activity selectively in astrocytes to inhibit glutamate uptake. This results from a physical association of A_{2A}R with NKA-α2 in astrocytes, as gauged by co-immunoprecipitation and *in situ* proximity ligation assays, in the cerebral cortex and striatum, two brain regions where A_{2A}R inhibit the astrocytic glutamate uptake. Moreover, the selective deletion of A_{2A}R in astrocytes (using Gfa2-A_{2A}R-KO mice) leads to a concurrent increase of both astrocytic glutamate uptake and NKA-α_2 levels and activity in the striatum and cortex. This coupling of astrocytic A_{2A}R to the regulation of glutamate transport through modulation of NKA-α_2 activity provides a novel mechanism linking neuronal activity to ion homeostasis controlling glutamatergic activity, all of which are processes intricately associated with the etiology of several brain diseases.

2. Introduction

Glutamate is the most abundant neurotransmitter, mediating nearly 80% of the synaptic transmission in the brain (Benarroch et al., 2010). Due to its rapid extracellular buildup and deleterious consequences when over-stimulating glutamate receptors, an efficient transport system dynamically regulates the extracellular glutamate levels to prevent glutamate accumulation and “spillover” between neighboring synapses (Dunlop, 2006). The astroglial specific glutamate transporter-I subtype (GLT-I) is the dominant glutamate transporter in the

brain, which importance is underscored by the impact of modifying GLT-I activity on synaptic plasticity as well as on neurodegeneration (Sattler and Rothstein, 2006). GLT-I are Na^+-dependent transporters, relying on the Na^+ electrochemical gradient generated by Na^+/K^+-ATPases (NKA) to drive glutamate uptake (Anderson and Swanson 2000). NKA are a class of ubiquitous plasma membrane enzymes responsible for maintaining the membrane potential of cells using the energy of adenosine triphosphate (ATP) hydrolysis (Reinhard et al., 2013). A functional NKA consists of a large catalytic α-subunit harboring the ATP-binding sites and a smaller β-subunit required for full enzymatic activity and also functioning as an anchoring protein (Aperia et al., 2007). In the brain, three different α-subunits isoforms are present in a cell-specific manner: the low-affinity α_1 is present in all cell types, the high-affinity α_2 isoform is restricted to astrocytes and the high-affinity α_3 isoform is expressed exclusively in neurons (Benarroch et al., 2011). It is thus not surprising that NKA activity and specifically the α_2-isoform has emerged as a robust modulator of glutamate uptake in astrocytes as heralded by the observations that: i) ATP depletion leads to a reversal of glutamate uptake (Longuemare et al., 1999); ii) inhibitors of NKA such as ouabain impair glutamate transporter activity (Pellerin and Magistretti, 1997; Rose et al., 2009; Genda et al., 2011) and lead to glutamate transporter clustering and redistribution (Nakagawa et al., 2008; Nguyen et al., 2010); iii) the α_2 subunit of the NKA co-localizes and physically associates in the same protein complex with glutamate transporters (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011).

We have previously shown that adenosine, a classical and ubiquitous modulator of synaptic transmission (Fredholm et al., 2005), by activating astrocytic adenosine A_{2A} receptors ($A_{2A}R$), controls the uptake of glutamate through a dual-mechanism (Matos et al., 2012b): a long term activation of $A_{2A}R$ triggers a cAMP/protein kinase A-dependent decrease of the expression of GLT-I and GLAST prior to the reduction of the levels and activity of both transporters (Matos et al., 2012b), whereas the acute short-term activation of astrocytic $A_{2A}R$ decreases the activity of glutamate transporters through an unknown mechanism that might depend on the physical

proximity of A_{2A}R and GLT-I (Matos et al., 2012b). We have now tackled the mechanism of A_{2A}R-mediated inhibition of the astrocytic glutamate transport, which was found to depend on a physical association and modulation by A_{2A}R of NKA-α_2 in astrocytes. This provides the first demonstration that A_{2A}R control ion homeostasis, paving the way to understand the broad neuroprotective impact of A_{2A}R antagonists in different brain disorders (Gomes et al., 2011).

3. Material and Methods

3.1. Animals

Initial experiments were carried out using adult (2-3 months old) male c57Bl6 mice. We also used GFAP gene promoter-driven A_{2A}R conditional knock out (Gfa2-A_{2A}R-KO) mice, which were generated using the Cre/loxP system, as previously described (Matos et al., 2012b). The Gfa2-Cre line was obtained from David Gutmann (Dept. Neurology, Washington University School of Medicine, St. Louis, Missouri) using the gfa2 transgene construct (Bajenaru et al., 2002). The transgene construct consists of the 2.2-kb fragment of the human glial fibrillary acidic protein (GFAP) promoter (Gfa2) (obtained from M. Brenner, National Institute of Neurological Disorders and Stroke) coupled to the encephalomyocarditis virus IRES and to a cDNA encoding the nucleus-targeted Cre recombinase (for details see Lee et al., 2006 and 2008). The 55 bp segment of the gfa2 promoter, spanning bp 21488 to 21434 with respect to the RNA start site, has been shown to contain a 45 bp sequence spanning bp 21443 to 21399 required for silencing expression in neurons. Thus, the specific Gfa2 promoter, in opposition to other GFAP promoter constructs, has been elegantly shown as astrocyte specific in all CNS regions (Lee et al., 2008). Briefly, both transgenic *Gfa2-cre* mice (Bajenaru et al., 2002) and mice carrying the ''floxed'' A_{2A}R gene ($A_{2A}^{flox/flox}$) (Bastia et al., 2005) were back-crossed for 10-12 generations to C57Bl/6 mice (Charles River; Wilmington, MA). Gfa2-*cre* mice were then crossed with non-transgenic (no *cre*) $A_{2A}^{flox/flox}$ mice to generate Gfa2-A_{2A}R-KO and Gfa2-A_{2A}R-WT mice. Animals were maintained under controlled environment (23 ± 2℃; 12 h light/dark cycle; *ad libitum*

access to food and water) and handled according to the Animal Care and Use Committee at Boston University School of Medicine and the NIH Guide for the Care and Use of Laboratory Animals (1982).

3.2. Preparation of total membranes

Mice were euthanized by decapitation after deep anesthesia with isoflurane and cortical and striatal brain tissue was collected and homogenized in sucrose (0.32 M) solution [containing 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, USA), pH 7.4] at 4 ºC. The homogenates were centrifuged at 3,000 *x g* for 10 min at 4 ℃ and the resulting supernatants were centrifuged again at 14,000 *x g* for 10 min at 4 ℃. The pellets were washed in Krebs-HEPES-Ringer (KHR) solution (140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl, 5 mM glucose and pH 7.4) at 4 ºC and further centrifuged at 14,000 x *g* for 10 min at 4 ºC. The pellets were resuspended either in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) with protease inhibitor cocktail (CLAPS, composed by 10 µg/mL of chymostatin, leupeptin, antipain and pepstatin A; from Sigma-Aldrich, USA) and the protein content measured with the bicinchoninic acid (BCA) assay (Thermo Scientific, USA).

3.3. Preparation of gliosomes and synaptosomes

After the homogenization of the brain tissue (cortex or striatum), purified synaptosomes and gliosomes were obtained using a discontinuous Percoll gradient (2, 6, 15, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4), as previously described (Matos et al., 2012b). The layers between 2 and 6% of Percoll (gliosomal fraction) and between 15 and 23% of Percoll (purified presynaptic nerve terminals - synaptosomal fraction) were collected, washed in 10 mL of HEPES buffered medium (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES and pH 7.4) and further centrifuged at 22,000 x *g* for 15 min at 4 ºC to remove myelin components and

postsynaptic material from the gliosomal and synaptosomal fractions, respectively. Crude synaptosomes were prepared after consecutive differential centrifugations of the brain homogenate in sucrose solution and in a 45% Percoll solution at 4 ºC (Canas et al., 2009). The fractions were resuspended in Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, 1 $CaCl_2$, pH 7.4) or NMG buffer (where NaCl is isosmotically replaced by N-methylglucamine-NMG).

3.4. Na^+/K^+-ATPase (NKA) activity assay

NKA activity in synaptosomes and gliosomes was measured using a high sensitivity colorimetric ATPase assay kit following the manufacturer's instructions (Innova Biosciences, UK). Gliosomes or synaptosomes (20 µg) were incubated with the reaction buffer containing 100 mM Tris, 1 mM ATP and 5 mM $MgCl_2$ at pH 7.4, in the absence or in the presence of ouabain (0.01 µM to 2 mM), CGS 21680 (30-100 nM) and/or SCH 58261 (50 nM) for 30 min, at 37 ℃. The amount of inorganic phosphate (Pi) relea sed was quantified colorimetrically at 630 nm, as previously described (Sarkar et al., 2002; Nguyen et al., 2010) and the protein content measured with the BCA assay. The specific activity of NKA was calculated by subtracting the ouabain-insensitive activity from the overall activity (in the absence of ouabain) and expressed as µmol Pi liberated from ATP by 1 µg of protein (µmol Pi/µg protein).

3.5. D-[^{3}H]aspartate uptake

The uptake of the non-metabolizable glutamate analogue D-[^{3}H]aspartate is a validated readout of the activity of GluTs (Anderson and Swanson, 2000) and was carried out as previously described (Matos et al., 2012a and b). Briefly, the gliosomal or synaptosomal fractions were diluted in Krebs or NMG buffer and equilibrated at 37℃ for 10 min. Triplicates (150 µL) of each fractions were added to 150 µL of Krebs or NMG medium containing a final concentration of 50 nM D-[^{3}H] aspartate (11.3 ci/mmol; PerkinElmer, USA). The mixtures were incubated for 10 min at 37 ℃ and the reaction term inated by rapid vacuum filtration over glass

microfibre filters Whatman GF/C (GE Healthcare, USA) and further washed 3 times with ice cold NMG buffer. Filters were dried overnight, drenched in 2 mL of liquid scintillation cocktail (PerkinElmer, USA) and counted on a LKB Wallac 1219 Liquid Scintillation Counter (Wallac, Finland). The specific uptake of D-[^{3}H]aspartate was calculated by subtraction from the total uptake of the non-specific uptake measured in a Na^*-free medium (NMG).

3.6. Drug treatments

The selective $A_{2A}R$ agonist [[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2 yl]amino]ethyl]benzene propanoic acid hydrochloride (CGS 21680, Tocris, USA), the $A_{2A}R$ antagonist 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261, Tocris, USA) and the NKA inhibitor ouabain octahydrate (Tocris, USA) were added to synaptosomes and gliosomes to reach final concentrations of 100 nM, 50 nM and 1 mM (or other when specified), respectively, at 30 min before the D-[^{3}H]aspartate uptake and the NKA activity assays, as previously described (Matos et al., 2012a, b).

3.7. Co-immunoprecipitation (Co-IP)

Co-IP was performed as previously described (Ciruela et al., 2006). Briefly, total membranes from the cortex or striatum were prepared as described above and washed in PBS (140 mM NaCl, 3 mM KCl, 20 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH7.4) before centrifugation at 14,000 *g* for 10 min at 4 ºC. The pellets were resuspended in the immunoprecipitation buffer (IPB; containing 20 mM Tris, pH 7.0, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 1 µM okadaic acid, 0.1 mM PMSF and 1:1000 protease inhibitor cocktail) with 1% Triton X-100, sonicated for 30 sec on ice and further spun down for 10 min to remove insoluble materials. A sample was collected for determining protein concentration using the BCA assay, another was stored at -20 ºC as input (positive control) and the rest was processed for IP at a dilution of 0.5 mg/mL. Protein A sepharose beads were incubated with the sample for 1 h at 4 ºC under rotation to pre-absorb any protein that non-specifically bound to the

protein A sepharose beads. The supernatant was recovered by centrifugation and 3 µg of anti-A_{2A}R antibody (Millipore, USA) or irrelevant IgG (for negative control) were added and incubated for 3 h at 4 ºC under rotation. To pool-down the immune complexes, the samples were incubated with protein A sepharose beads for 2 h at 4 ºC and centrifuged. The pellets were washed twice in IPB with 1% Triton X-100, 3 times in IPB with 1% Triton X-100 and 500 mM NaCl and twice in IPB. The immunoprecipitates were resolved by SDS-PAGE buffer, and Western blots were performed with anti-NKA-α2 isoform or anti-GLT-I/EAAT2 antibodies (see Western blotting).

3.8. Western blotting

Western blotting of gliosomal or synaptosomal extracts was performed as previously described (Canas et al., 2009; Matos et al., 2012a). Incubation with the primary antibodies, namely anti-A_{2A}R (1:200; Millipore), anti-GLT-I/EAAT2 (1:1000; Millipore), anti-NKA-α2 isoform (1:200; Millipore) and anti-β-actin (1:5000, Sigma-Aldrich, USA), all diluted in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) with 0.1% Tween (TBS-T) and 3% BSA (fatty acid free), was carried out overnight at 4 ℃. After washing, the membranes were revealed using an ECF kit (Amersham*, USA)* and visualized under a fluorescence LAS-4000 digital imaging system (Fujifilm, USA). The densiometric analysis of protein bands was performed using Quantity One software version 4.4.1 (Bio-Rad, USA).

3.9. Immunohistochemistry

Immunohistochemistry in brain slices was performed as described previously (Canas et al., 2009). After a transcardiac perfusion, the brains were post-fixed overnight in PBS with 4% paraformaldehyde and cryopreserved in PBS containing 25% sucrose. The frozen brains were sectioned (30 µm coronal slices) with a Leica CM3050S cryostat (Leica Microsystems). The sections corresponding to cortex and striatum were permeabilized, blocked and incubated overnight at room temperature in the presence of goat polyclonal anti-NKA-α2 isoform antibody

(1:500) and mouse monoclonal anti-GLT-I/EAAT2 (1:1000) antibody. The sections were subsequently incubated with donkey anti-mouse and anti-goat secondary antibody conjugated with a fluorophore (Alexa Fluor 488 or Alexa Fluor 555, 1:200, Invitrogen) for 2 h at room temperature. After rinsing, the sections were mounted on slides and allowed to dry. Vectashield mounting medium with DAPI (Vector Laboratories) was applied as well as the cover glass. All sections were examined under a fluorescence Nikon eclipse E600 microscope, with SPOT software 4.7 (Diagnostic Instruments, Inc).

3.10. *In situ* Proximity Ligation Assay (PLA)

The PLA was performed as previously described (Soderberg et al., 2006; Augusto et al., 2013) in brain sections from Gfa2-A_{2A}R-KO and WT littermates prepared as described for immunohistochemistry. The sections were rinsed in TBS (0.1 M Tris, pH.7.4, and 0.9% w/v NaCl) and blocked with TBS with 10% fetal bovine serum and 0.5% Triton X-100 for 2 h at room temperature. Subsequently, the slices were incubated with goat polyclonal anti-NKA-α2 isoform antibody (1:500) and rabbit polyclonal anti-A_{2A}R antibody (1:500) overnight at room temperature. After washing in TBS with 0.2% Triton X-100, the slices were incubated for 2 h at 37 ℃ with the PLA secondary probes anti-rabbit PLUS and anti-goat MINUS (1:5; Olink Bioscience) under gentle agitation. Afterward, the slices were washed twice with Duolink II Wash Buffer A (Olink Bioscience) and incubated with the ligation-ligase solution (Olink Bioscience) for 30 min at 37 ℃. After a new rinse, the slices were incubated with DNA polymerase (1:40; Olink Bioscience) in the amplification solution (Olink Bioscience) for 100 min at 37 ºC. After several washes in consecutive decreasing concentrations of SSC buffers (Olink Bioscience), the slices were mounted on slides, allowed to dry and the coverslips applied with Duolink Mounting Medium (Olink Bioscience). Fluorescence images were acquired on an Axiovert 200M inverted confocal microscope (Carl Zeiss Microscopy) using a 40× N.A objective. The images were then analyzed and the PLA puncta signals quantified with ImageJ software. A

threshold was selected manually to discriminate PLA puncta from background fluorescence. The built in macro "Analyze Particles" was then used to count all objects in the thresholded image. Objects larger than 5 μm^2 were rejected, thereby effectively removing nuclei. The remaining objects were counted as $A_{2A}R$- NKA-α2 PLA positive puncta.

3.11. Statistical analysis

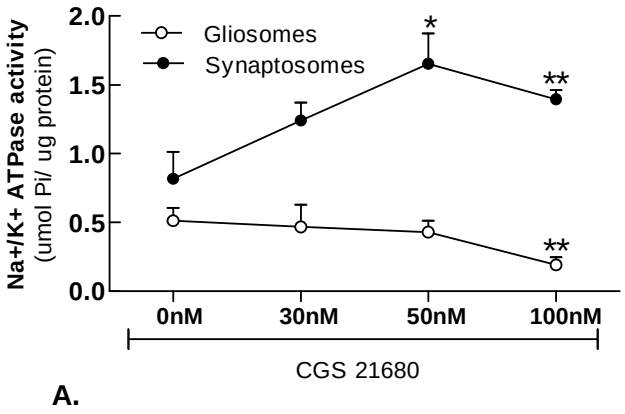
Data are expressed as absolute or arbitrary values or percentages of values obtained in control conditions or conditions mentioned in the figures legends, and are presented as means ± S.E.M.. Parametric analysis of variance (ANOVA) was used to determine statistically significant differences, with the indicated pos-hoc test. All data were analyzed using GraphPad Prism software (Version 5.0, GraphPad, USA).

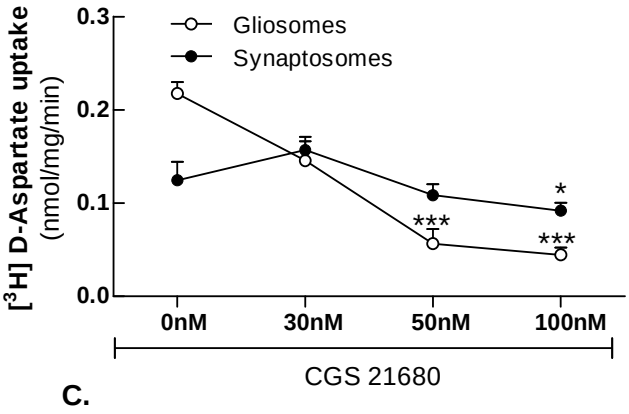
4. Results

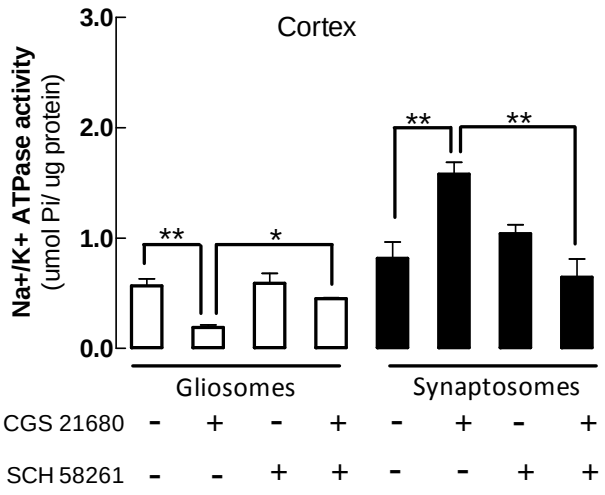
4.1. Activation of $A_{2A}R$ decreases Na^+/K^+-ATPase (NKA) activity in gliosomes

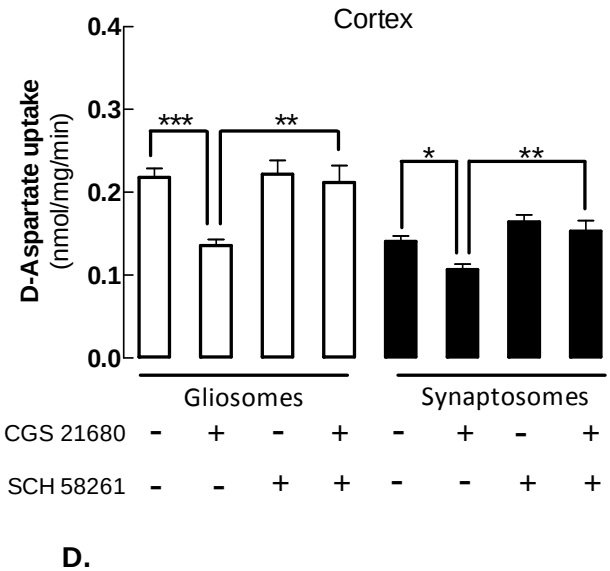
Since $A_{2A}R$ control the uptake of glutamate by the astrocytic GluTs GLT-I (Matos et al., 2012b) and the efficiency of GluTs depend on the sodium gradient ensured by the activity of Na^+/K^+-ATPase (NKA) (Benarroch, 2011), we tested the impact of $A_{2A}R$ activation on the activity of NKA in astrocytes and neurons. We first prepared gliosomes (astrocyte-enriched plasmalemmal vesicles) and synaptosomes (enriched nerve terminals) from the cerebral cortex of adult mice and challenged them with the selective $A_{2A}R$ agonist CGS 21680 and/or the $A_{2A}R$ antagonist SCH 58261 before determination of NKA activity, assessed as the ouabain-sensitive ATP hydrolysis (**Fig.1**). Activation of $A_{2A}R$ in cortical gliosomes by CGS 21680 (at 100 nM, but not at lower concentrations of 30-50 nM), led to a 66.0±4.0% decrease (n=4, $p<0.01$) of NKA activity in comparison with non-treated gliosomes (**Fig. 1A**); this effect was prevented (n=4, $p<0.05$) by the pre-administration of SCH 58261 (50 nM) (**Fig. 1B**). In contrast, CGS 21680 (100 nM) induced a 93.0±13.0% increase (n=4, $p<0.01$) of the NKA activity in synaptosomes, which was prevented by SCH 58261 (n=4, $p<0.01$; **Fig. 1A, B**). A similar trend was observed in

the striatum (**Fig. 1E**), another brain area where the $A_{2A}R$ modulation of glutamate uptake in astrocytes has been documented (Pintor et al., 2004). Thus, in striatal gliosomes, CGS 26180 (100 nM) decreased NKA activity by 36.0±8.4% (n=3, p<0.05), an effect prevented by SCH 58261 (50 nM; n=3, p<0.05); in contrast 100 nM CGS 26180 tended to increase (57.0±27.0%, n=3; p=0.05) NKA activity in striatal synaptosomes (**Fig. 1E**).









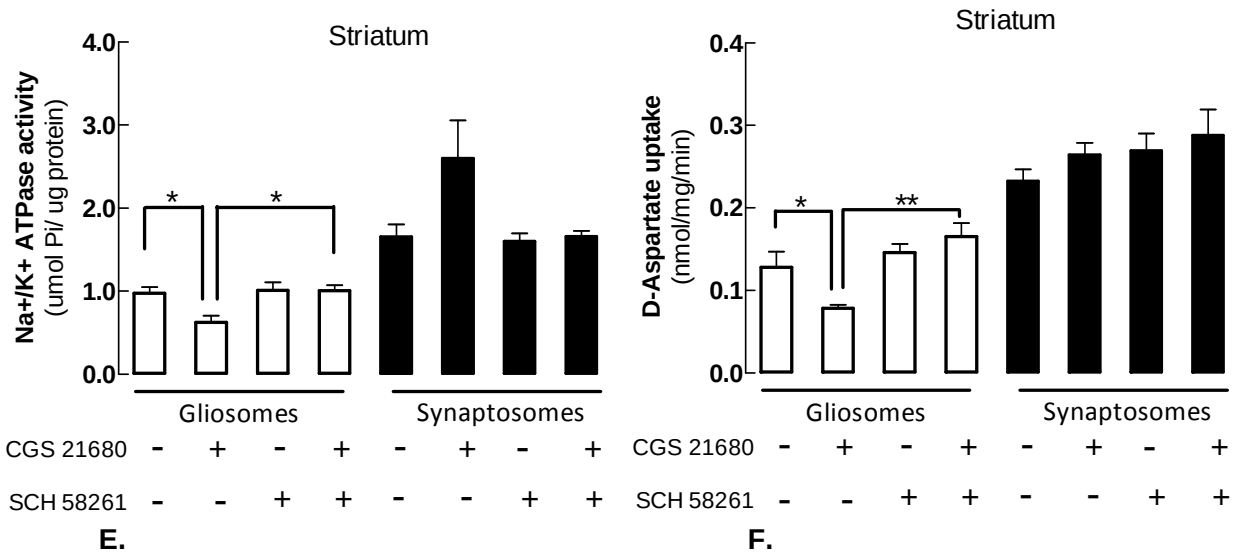


Fig.1. Activation of A_{2A}R leads to a selective decrease of the activities of both NKA and GluTs in gliosomes but not in synaptosomes from either the cerebral cortex or striatum. Gliosomes and synaptosomes from brain cortex or striatum were incubated without or with the A_{2A}R selective agonist CGS 21680 (30-100 nM) and/or antagonist SCH 58261 (50 nM). **(A)** The activation of A_{2A}R by CGS 21680 in cortical gliosomes (open symbols) reduces NKA activity, whereas it increases NKA activity in synaptosomes (closed symbols). **(B)** These opposite effects of CGS 21680 (100 nM) on NKA activity in cortical gliosomes and synaptosomes were prevented by SCH 58261, the same occurring in striatal gliosomes **(E)**. **(C)** The activation of A_{2A}R with CGS21680 (30-100 nM) caused an inhibition of D-aspartate uptake both in cortical gliosomes and in synaptosomes and SCH 58261 prevented this effect of CGS 21680 (100 nM) **(D)**. **F)** A_{2A}R activation by CGS 21680 (100 nM) also inhibited D-aspartate uptake in striatal gliosomes, whereas no significant effects were observed in striatal synaptosomes. NKA activity was determined by subtracting the total ATPase activity from the ATPase activity in the presence of membrane ATPase inhibitor ouabain and was expressed as µmol Pi liberated from ATP by 1 µg of protein (µmol Pi/µg protein), whereas the specific uptake of [^{3}H]D-aspartate was calculated by subtracting the uptake activity from the uptake activity in the presence of Na^+-free buffer NMG and was expressed as nmol of D-aspartate retained per mg of gliosome protein per minute. Data are mean ± SEM of at least 3 independent experiments done in triplicate. Statistical differences were gauged using the Tukey's post-hoc test applied after One-Way ANOVA with *$p < 0.05$ and **$p < 0.01$, when compared with non-treated conditions.

4.2. Comparison of the effect of A_{2A}R on NKA activity and on D-aspartate uptake

In order to explore a possible link between NKA activity and glutamate uptake, we begun by comparing the impact of CGS 21680 and of SCH 58261 on NKA activity and on D-aspartate uptake in gliosomes and synaptosomes from either the cerebral cortex or of the striatum. As shown in Figure 1C, CGS 21680 (50-100 nM) inhibited D-[^{3}H]aspartate uptake both in cortical

gliosomes (79.2±3.2% at 100 nM, n=4; p<0.001) as well as in cortical synaptosomes (26.4±7.2% at 100 nM, n=4; p<0.05). This CGS 21680-induced inhibition was prevented by SCH 58261 in both cortical gliosomes (n=4; p<0.01) and cortical synaptosomes (n=4; p<0.01) (**Fig. 1D**). A similar profile of A_{2A} receptor-mediated inhibition of D-aspartate uptake was observed in gliosomes from the striatum (**Fig. 1F**).

Overall, these results show a parallel effect of A_{2A}R controlling NKA activity and the uptake of D-aspartate in gliosomes, whereas there is a qualitative dissociation between the impact of A_{2A}R on the activity of NKA and on glutamate uptake in synaptosomes.

4.3. Low concentrations of NKA-inhibitor ouabain blunt the A_{2A}R-mediated inhibition of D-aspartate uptake in astrocytes

In order to strengthen the link between NKA activity and glutamate uptake in astrocytes, we next analyzed the concentration-dependent effect of the NKA inhibitor ouabain both on NKA activity (**Fig.2A**) and on [^{3}H]D-aspartate uptake (**Fig.2B**) in gliosomes from the cerebral cortex of adult mice, where the uptake of D-aspartate had been found to be nearly twice greater than in striatal gliosomes (Figs. 1D and 1F) and where NKA and D-aspartate uptake were similarly modulated by A_{2A}R (Figs 1A and 1C). Ouabain caused a bimodal but parallel impact on the activities of both NKA (**Fig.2A**) and of GluTs (**Fig.2B**) in cortical gliosomes. Thus, a low ouabain concentration (0.1 μM) induced a 40.0±5.0% increase (n=4, p<0.05) of NKA activity compared with non-treated gliosomes, in agreement with previous reports (Lichstein et al., 1985; Gao et al., 2002; Antolović et al., 2006); a low/moderate concentration of ouabain (1 μM) had no effect on NKA activity, whereas moderate/higher concentrations (10-100 μM) inhibited (n=4, p<0.05) and a higher concentration (2 mM) of ouabain caused a 73.0±11.2% inhibition (n=4, p<0.01) of NKA activity (Fig. 2A). In accordance with the key NKA-mediated control of GLT-I activity, a low ouabain concentration (0.1 μM) increased [^{3}H]D-aspartate uptake by 26.1±4.1% (n=4, p<0.05), a low/moderate concentration (1 μM) had no effect, a moderate/higher concentration (10 μM)

inhibited (n=4, p<0.05), and a higher concentration (2 mM) inhibited glutamate uptake by 75.0±9.0% (n=4, p<0.001) (**Fig. 2B**), as previously observed (Pellerin and Magistretti et al., 1997; Rose et al., 2009).

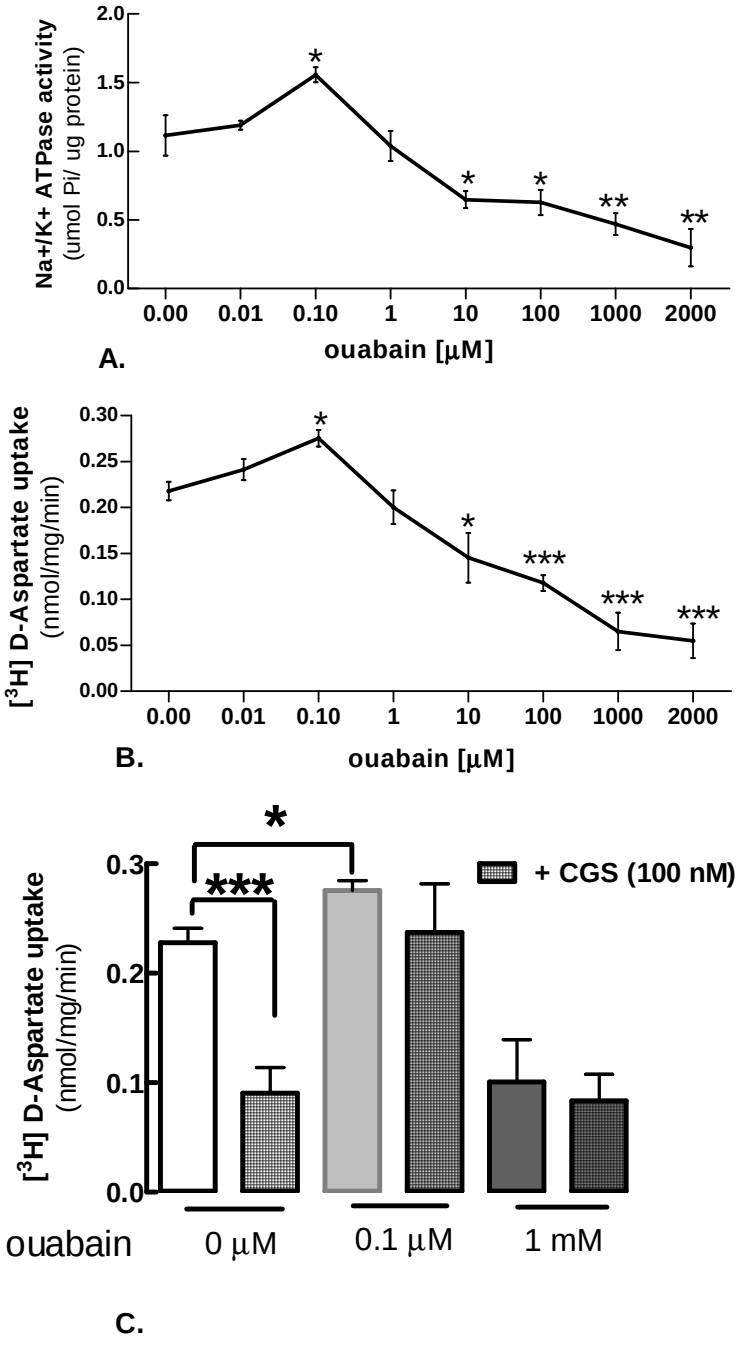


Fig.2. The NKA-inhibitor ouabain prevents has a parallel impact on the activities of NKA and of glutamate transport and blunt the impact of $A_{2A}R$ on D-aspartate uptake in cortical gliosomes. (A) Concentration-dependent inhibition of NKA activity by ouabain in cerebral cortical gliosomes from WT mice. Ouabain at 0.1 µM enhanced NKA activity, in opposition with concentrations above 10 µM. NKA activity was expressed as µmol Pi liberated from ATP by 1 µg of protein (µmol Pi/µg protein). **(B)** Concentration-dependent inhibition of [^{3}H]D-aspartate uptake in cerebral cortical gliosomes from WT mice. Ouabain at 0.1 µM enhanced [^{3}H]D-aspartate uptake, in opposition with concentrations above 100 µM. The specific uptake of [^{3}H]D-aspartate was expressed as nmol of D-aspartate retained per mg of gliosome protein per minute. **(C)** Acute (30 min) incubation of cerebral cortical gliosomes with the $A_{2A}R$ selective agonist CGS 21680 (100 nM) decreased [^{3}H]D-aspartate uptake, an effect no longer observed upon perturbation of the activity of NKA by pre-incubation with either a low (0.1 µM) or a high concentration of ouabain (1 mM). Data are the mean ± SEM of 5 independent experiments done in triplicate. Statistical difference was assessed using a Two-way ANOVA analysis.*p < 0.05, **p < 0.01, ***p < 0.001, comparison with control-non-treated condition.

We next analyzed how a low and high concentration of ouabain affected the $A_{2A}R$-induced inhibition of the astrocytic glutamate uptake. As depicted in Figure 2C, activation of $A_{2A}R$ in cortical gliosomes with 100 nM CGS 21680 decreased glutamate uptake by 61.0±1.1% (n=5, p<0.001), and this effect of CGS 21680 was blunted in the presence of either a low (0.1 μM) or a high (1 mM) concentration of ouabain concentration. In fact, in the presence of 0.1 μM ouabain, the effect of CGS 21680 on glutamate uptake was no longer significant, the same occurring in the presence of 1 mM ouabain (Fig. 2C). These data show that the perturbation of NKA activity blunts the ability of $A_{2A}R$ to control glutamate uptake, indicating that astrocytic $A_{2A}R$ may be coupled to NKA in order to rapidly modulate glutamate uptake.

4.4. NKA activity is increased selectively in astrocytes from Gfa2-A_{2A}R-KO mice

To further re-enforce the association between $A_{2A}R$ and NKA to control astrocytic glutamate uptake, we next investigated how the selective deletion of $A_{2A}R$ in astrocytes, using Gfa2-A_{2A}R-KO mice (see Matos et al., 2012b), also affected NKA activity in astrocytes and neurons. As portrayed in Figure 3, gliosomes collected from the cortex (**Fig. 3A**) or striatum (**Fig. 3B**) of Gfa2-A_{2A}R-KO mice displayed a significantly higher NKA activity than gliosomes collected from WT littermates (58.1±9.0%, n=4, p<0.05 in the cortex; 33.1±6.0%, n=4, p<0.05 in the striatum). In contrast, NKA activity was not significantly different in cortical (n=4, p=0.94) or striatal (n=4, p=0.24) synaptosomes from Gfa2-A_{2A}R-KO or Gfa2-A_{2A}R-WT mice. A similar analysis of the activity of GluTs revealed that D-[^{3}H]aspartate uptake was significantly increased (62.0±7.2%, n=4, p<0.001) in cerebral cortical gliosomes, but not in synaptosomes (n=4, p>0.05), from Gfa2-A_{2A}R-KO mice compared to WT littermates (**Fig. 3C**). Similarly, D-[^{3}H] aspartate uptake was also selectively increased (44.0±9.0%, n=4, p<0.01) in striatal gliosomes, but not synaptosomes (n=4, p>0.05), from Gfa2-A_{2A}R-KO mice compared to WT littermates (**Fig. 3D**). Albeit the modification of glutamate uptake in astrocytes upon selective elimination of $A_{2A}R$ from astrocytes may result from a short- and/or long-term regulation (see Matos et al., 2012b), the

observed parallel modification of NKA and glutamate uptake activities selectively in gliosomes of Gfa2-A_{2A}R-KO mice is further suggestive of the view of an astrocyte-selective coupling between A_{2A}R and NKA to regulate glutamate uptake.

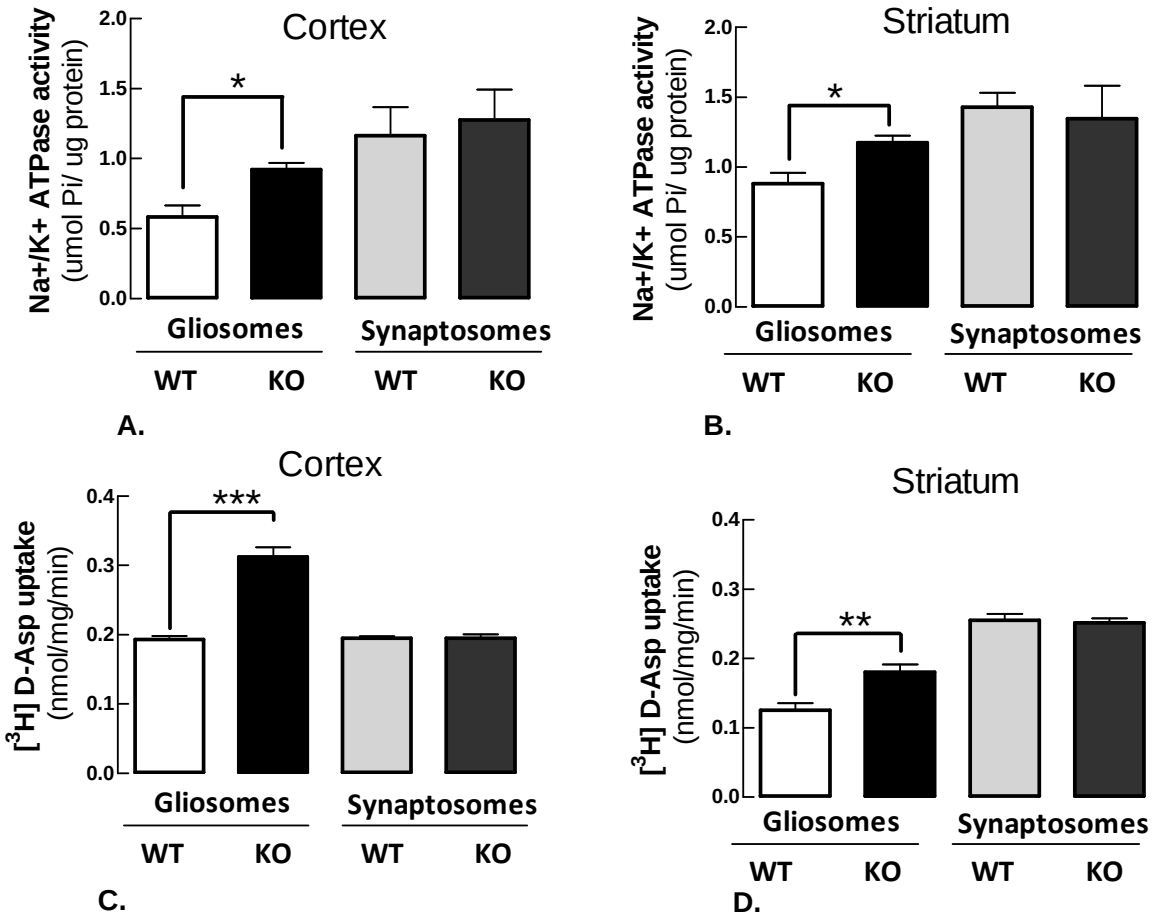
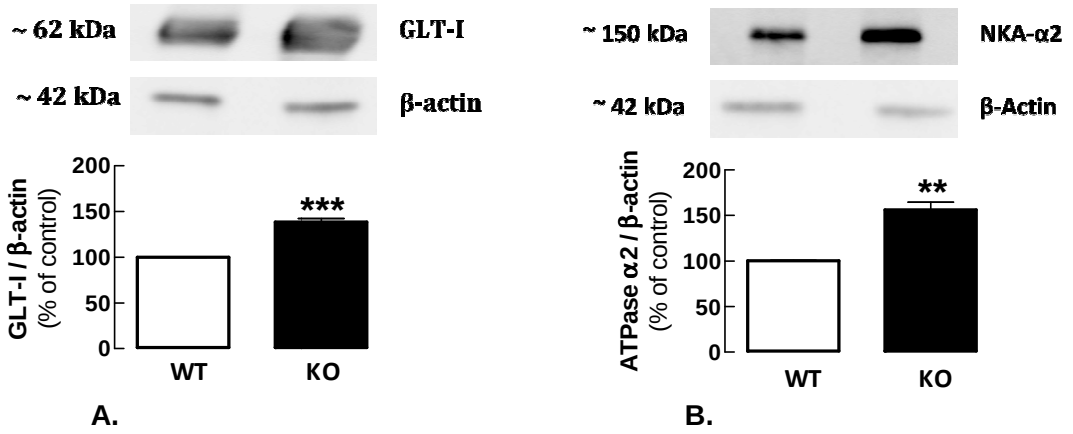
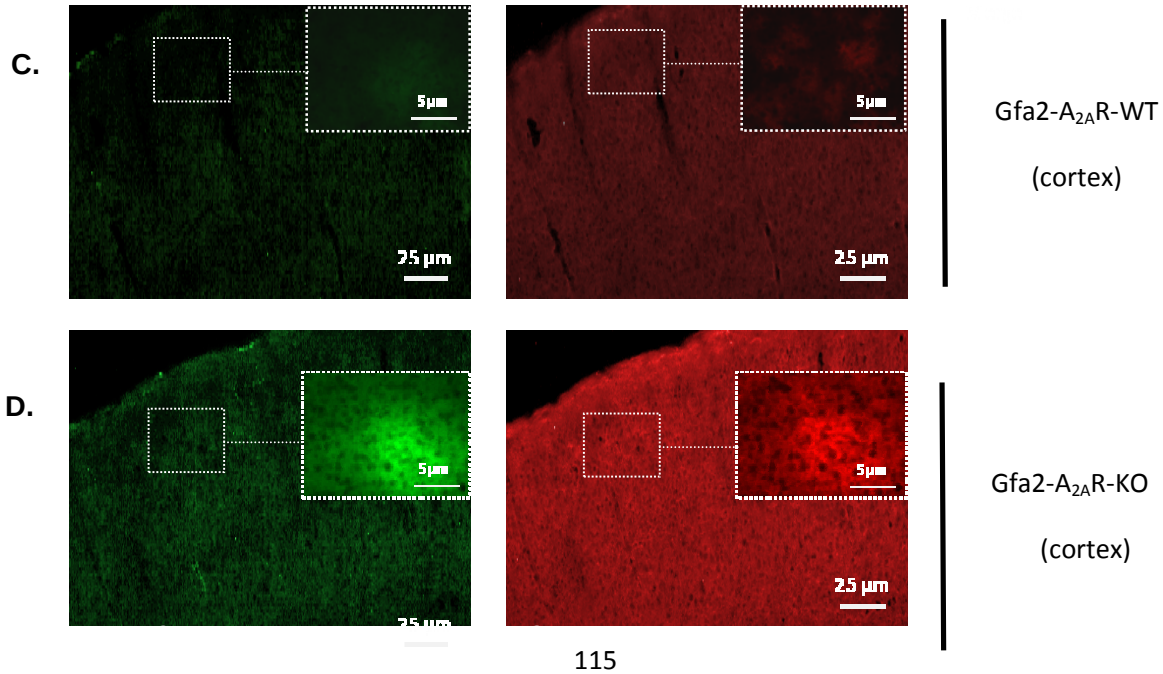


Fig.3. NKA activity and glutamate uptake are increased in parallel selectively in gliosomes from the cortex or striatum of Gfa2-A_{2A}R-KO mice. Gliosomes and synaptosomes from Gfa2-A_{2A}R-KO mice and from corresponding WT littermates were prepared before the NKA activity **(A, B)** and the [^{3}H]D-aspartate uptake (C, D) assays. The increased NKA activity was restricted to gliosomes from GFAP-A_{2A}R-KO mice (black columns), particularly in the cortex **(A)** but also in the striatum **(B)**, compared to WT mice (white columns). [^{3}H]D-aspartate uptake was also selectively increased in gliosomes from the cortex **(C)** and striatum **(D)**. Data are mean ± SEM of at least 4 independent experiments. Statistical differences were gauged using the Tukey's post-hoc test applied after One-Way ANOVA with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

4.5. GLT-I and NKA-α2 immunoreactivities are increased in Gfa2-A_{2A}R-KO mice

As a first step toward testing the hypothesis that A_{2A}R, NKA-α2 and GluTs might be physically associated in astrocytes, we compared the density and distribution of GLT-I and NKA-α2 in the cerebral cortex and striatum from Gfa2-A_{2A}R-KO mice and WT littermates (**Fig. 4**). Western blot analysis showed that the density of GLT-I was significantly increased in the cortex (138.1±4.4%; n=6, p<0.001) and striatum (121.1±2.0%; n=6, p<0.01) of Gfa2-A_{2A}R-KO compared to Gfa2-A_{2A}R-WT mice (**Fig. 4 A**, **E**). Notably, the density of NKA-α2 was also significantly increased in the cortex (156.0±9.0%; n=6, p<0.001) and striatum (124.0±7.0%; n=6, p<0.05) of Gfa2-A_{2A}R-KO compared to WT mice (Fig. **4 B**, F).





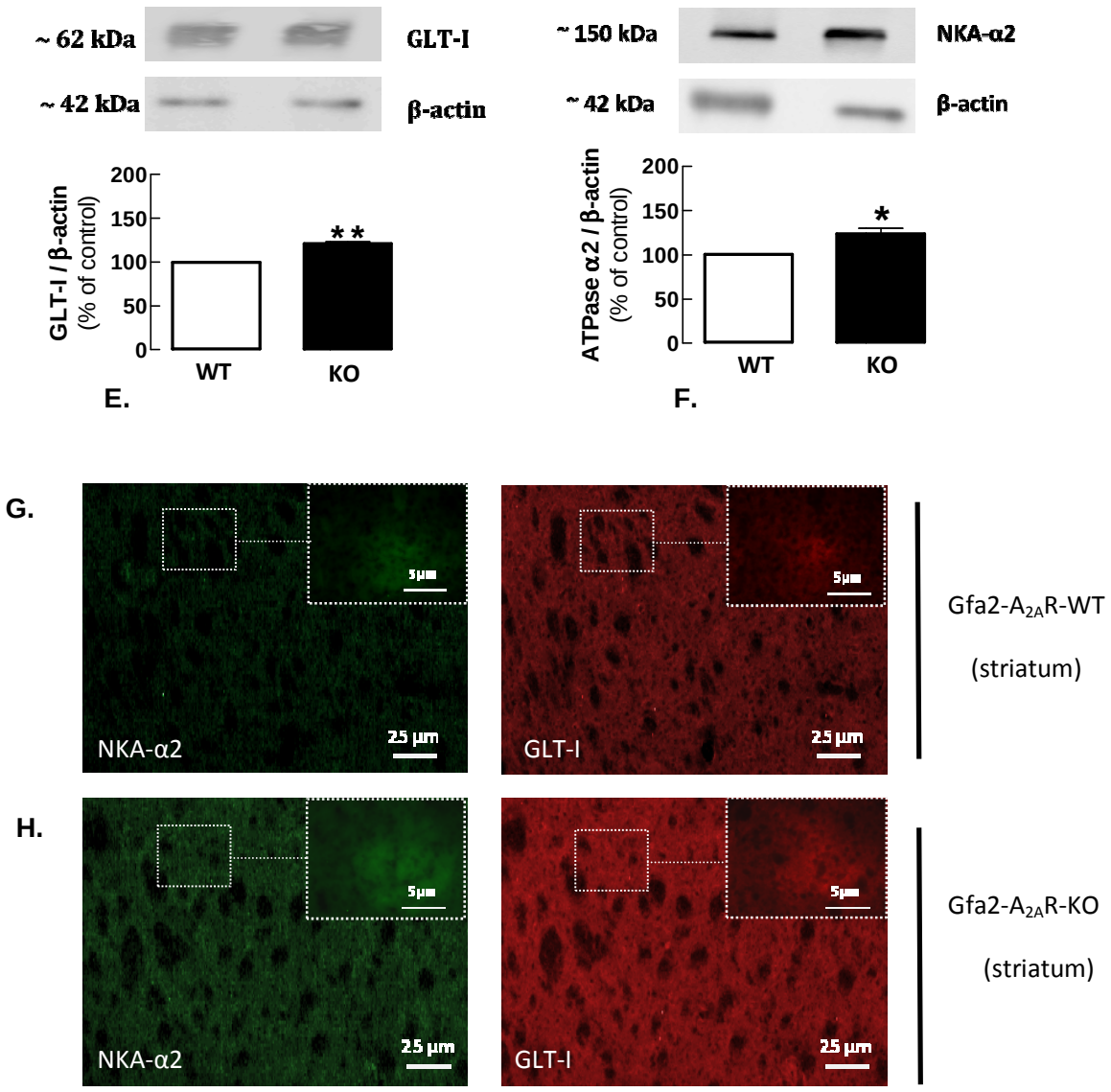


Fig.4. GLT-I and NKA-α2 immunoreactivities are increased in Gfa2-A_{2A}R-KO mice. Western blot analysis of total membranes showed that the density of GLT-I **(A, E)** and of NKA-α2 **(B, F)** were significantly increased in the cortex (**A,B)** and striatum **(E, F)** of Gfa2-A_{2A}R-KO *vs* Gfa2-A_{2A}R-WT mice. The bars **(A, B, E,** and **F)** represent the relative immunoreactivity obtained with each primary antibody normalized with anti-β actin (reference) immunoreactivity and were expressed as percentage of WT littermates. The immunohistochemical data **(C, D, G, H)** shows the immunoreactivity of GLT-I and NKA-α2 in the cortex **(A-D)** and in the striatum **(E-H)** of Gfa2-A_{2A}R-KO **(D, H)** and Gfa2-A_{2A}R-WT littermates **(C, G)** with correspondent higher amplifications in the right-up corner of each image. Data are mean ± SEM of at least 6 independent experiments. Statistical differences were gauged using the Tukey's post-hoc test applied after One-Way ANOVA with *p < 0.05, ***p*<0.01 and ****p*<0.001, comparison with naive WT littermates. The calibration bar in each row (25 µm/ 5 µm) is valid for all pictures in the row.

Immunohistochemical analysis confirmed the Western blot results, showing an increased immunoreactivity of both GLT-I and NKA-α2 in the frontal cortex (**Fig.4 C**, **D**) and dorsal striatum (**Fig.4 G**, **H**) of Gfa2-A_{2A}R-KO compared to Gfa2-A_{2A}R-WT mice (n=6). These observations are in agreement with the reported superimposable ultrastructural distribution of the α_2 subunit of NKA and GLT-I (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011; Bauer et al, 2012) and further suggest that astrocytic A_{2A}R is a key modulator of this coupling.

4.6. A_{2A}R is physically associated with NKA-α2

Previous co-immunoprecipitation studies revealed a closed association between GLT-I and NKA-α2 (Rose et al., 2009; Genda et al., 2011; Bauer et al, 2012) forming a protein complex at the plasma membrane of astrocytes to ensure the maintenance of the electrochemical Na^+ gradient required for glutamate uptake during neuronal activity. Since we have also shown a close association between A_{2A}R and GluTs (Matos et al., 2012b), we next sough to test if A_{2A}R and NKA-α2 might also co-purify in the cerebral cortex or striatum. The pull-down of A_{2A}R from cortical and striatal homogenates was followed by a Western blot analysis of the A_{2A}R-immunoprecipate with the anti-NKA-α2 antibody (IP) (or with an anti-IgG antibody as a negative control (CTR -)), while confirming the presence of NKA-α2 in the input sample in non-immunopreciptated membranes (CTR +) and the presence of A_{2A}R in the input and pull-down samples (upper lanes - WB). As depicted in Figure 5, we observed a close association between NKA-α2 and A_{2A}R in the brain extracts from Gfa2-A_{2A}R-WT mice (n=3) (lower lanes - IP), which was highly decreased in both cortical (Fig. 5A) or striatal extracts (Fig. 5B) from Gfa2-A_{2A}R-KO mice (n=3), in comparison with the WT littermates. These data provide strong evidence of a close association between A_{2A}R and NKA-α2 in astrocytes, which is absent in Gfa2-A_{2A}R-KO mice. We next attempted to confirm the existence of A_{2A}R and NKA-α2 complexes in cortical and striatal brain slices of Gfa2-A_{2A}R-KO or Gfa2-A_{2A}R-WT littermates, using an *in situ* proximity ligation assay (PLA) (Soderberg et al., 2006; Augusto et al., 2013) (**Fig.6**).

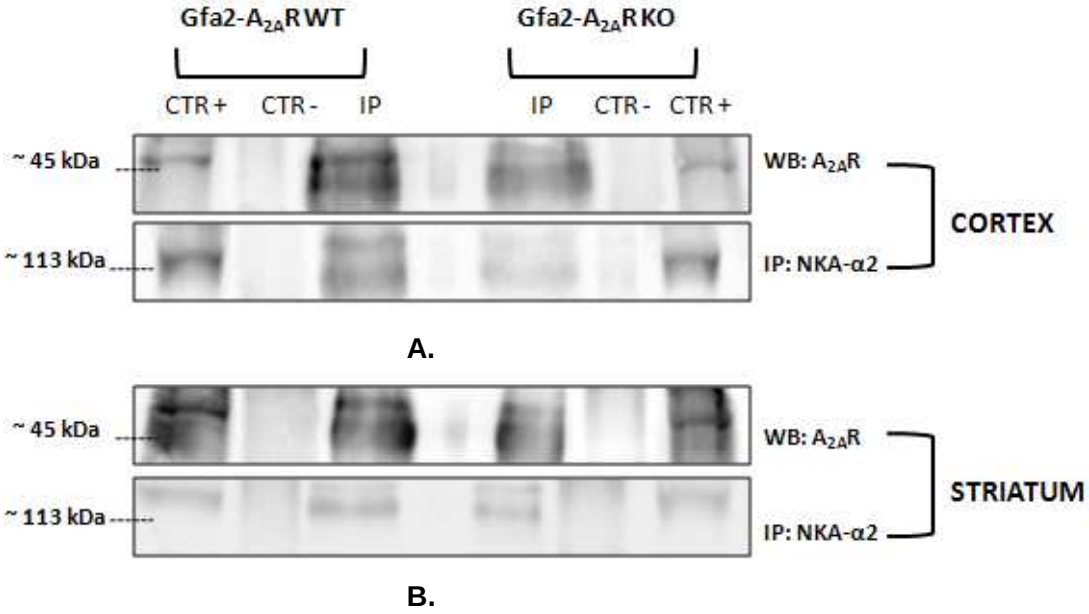


Fig.5 - $A_{2A}R$ is physically associated with NKA-α2 and this coupling is abrogated in Gfa2-$A_{2A}R$-KO mice. **(A-B)** Immunoprecipitation of $A_{2A}R$ from cerebral cortical (A) or striatal (B) total membranes from Gfa2-$A_{2A}R$-KO mice and Gfa2-$A_{2A}R$-WT littermates with anti-$A_{2A}R$ antibody (IP) or lack of $A_{2A}R$ pull-down with IgG (CTR -), followed by Western blot analysis with anti-NKA-α2 antibody, revealed an association between NKA-α2 and $A_{2A}R$ in the WT immunoprecipitate (IP), which was absent in Gfa2-$A_{2A}R$-KO mice. The presence of NKA-α2 in the input sample was confirmed in non-co-immunoprecipitated membranes (CTR +) in the lower (IP) lanes. The presence of $A_{2A}R$ was confirmed by Western blot analysis in the upper lanes (WB).

PLA is an antibody-based method in which the $A_{2A}R$ and NKA-α2 proteins were first immunolabeled with primary antibodies and then with secondary antibodies conjugated to complementary oligonucleotides, which can only ligate and be amplified if the $A_{2A}R$ and NKA-α2 antibody molecules are in close proximity (below 16 nm) to be identified as fluorescent $A_{2A}R$-NKA-α2 puncta (Trifilieff et al., 2011). Figure 6A illustrates the existence of $A_{2A}R$-NKA-α2 positive signals in both the cerebral cortex and striatum with a higher $A_{2A}R$-NKA-α2 cross-linking signal in the cortex than in the striatum (35.0±10.0% of cortical positive signals, n=3), possibly reflecting the different density of astrocytes in the two brain areas (Kalman and Hajos, 1989; Taft et al., 2005) or an eventual different density of $A_{2A}R$ in astrocytes in these two brain regions. The specific association between $A_{2A}R$ and NKA-α2 in astrocytes is further re-enforced by the sharp and significant decrease of the $A_{2A}R$-NKA-α2 positive signals in the cortex

(93.0±3.0%, n=3, p<0.001) and in the striatum (82.3±27.0% decrease, n=3, p<0.01) of Gfa2-A_{2A}R-KO mice compared with WT littermates (**Fig 6A**, **B**).

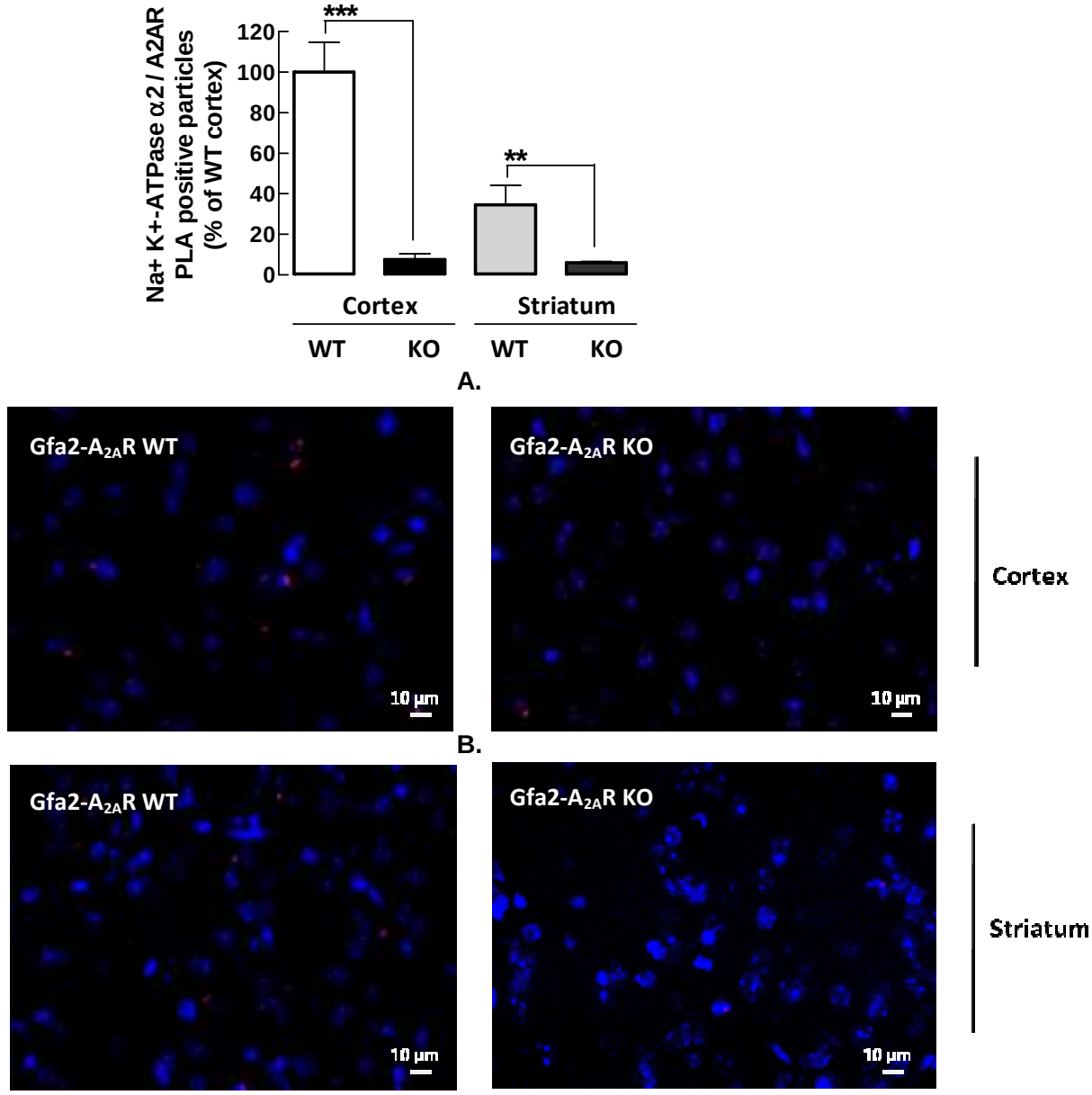


Fig.6. A_{2A}R is closely associated with NKA-α2 and this coupling is abrogated in Gfa2-A_{2A}R-KO mice. **(A)** The PLA assay corroborated the closed proximity (≤ 16 nm) between astrocyte A_{2A}R and NKA-α2 in the cortex and striatum from Gfa2-A_{2A}R-WT mice, which was blunted in Gfa2-A_{2A}R-KO mice. **(B)** Representative confocal images of the PLA assay showing distinct bright red spots in the cortex and striatum from WT mice, corresponding to the amplification products between DNA probes linked to the anti-A_{2A}R and anti-NKA-α2 antibodies. Data are mean ± SEM of at least 3 independent experiments. Statistical differences were gauged using the Tukey's post-hoc test applied after One-Way ANOVA with ***p*<0.01 and ****p*<0.001. The calibration bar is 10 µm.

5. Discussion

The present results provide the first direct evidence of the co-localization and functional interaction between A_{2A}R and Na^+/K^+-ATPase (NKA-α2) specifically in astrocytes in the mouse adult brain. Additionally, we show that this A_{2A}R-mediated control of NKA activity provides a novel mechanism by which A_{2A}R directly regulate glutamate uptake by astrocytes. This was concluded based on a combination of parallel neurochemical assays of NKA activity and D-[^{3}H]aspartate uptake, coupled to pharmacological manipulations of A_{2A}R and NKA activity and further confirmed by co-immunoprecipitation and PLA assays, all validated though the comparative study of Gfa2-A_{2A}R-KO and WT mice (see **Fig.7**).

The key role of NKA in the control of astrocytic glutamate transport is well established, as heralded by the ability of the NKA inhibitor ouabain to impair glutamate uptake (Pellerin et al., 1997; Cholet et al., 2002; Rose et al., 2009; Nguyen et al, 2010). Notably, this NKA-mediated modulation of GluTs involves a physical association between NKA-α_2 and GLT-I and GLAST, as evidenced by their co-localization, co-purification and co-immunoprecipitation (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011; Bauer et al., 2012) and by the reversed ability of GluTs to modulate NKA activity (Gegelashvili et al., 2007). In parallel, we had previously documented the co-localization and functional interaction between A_{2A}R and GLT-I in astrocytes (Matos et al., 2012a, b). The present demonstration that A_{2A}R physically associate with NKA-α_2 suggests the existence of a macromolecular complex encompassing A_{2A}R, NKA-α_2 and GLT-I in astrocytic membranes, in accordance with the role of NKA as a docking station of molecular signaling hubs (Reinhard et al., 2013) and the versatility of A_{2A}R to interact with different neurotransmitters receptors, enzymes and anchoring proteins (Burgueño et al., 2003; Ferré et al., 2007; Zezula and Freissmuth, 2008; Navarro et al., 2009). From the functional point of view, this ability of A_{2A}R to control NKA-α_2 provides a novel mechanism to understand how the acute A_{2A}R manipulation decreases glutamate uptake by astrocytes (see **Fig.7** for resume). Thus,

$A_{2A}R$ activation not only triggers a cAMP-PKA-dependent pathway to decrease the expression of astrocytic GluTs, but also triggers a rapid inhibition of astrocytic glutamate transport (Matos et al., 2012b). The molecular mechanism operated by $A_{2A}R$ to control NKA may involve a direct conformational control of NKA (Arystarkhova and Sweadner, 2005) as a result of the observed physical association between $A_{2A}R$ and NKA-α2, which would allow understanding the opposite impact of $A_{2A}R$ on astrocytic NKA-α2 activity (inhibition) and neuronal NKA-α3 activity (stimulation). It is worth noting that the mechanism by which $A_{2A}R$ control neuronal (putatively NKA-α3) activity is still unresolved, although it seems unrelated to the control of glutamate clearance. In fact, in contrast to gliosomes, neuronal $A_{2A}R$ modulate in an opposite manner NKA (facilitation) and glutamate uptake (inhibition), indicating that the $A_{2A}R$-mediated control of neuronal NKA activity is not directly coupled to the control of glutamate clearance in synaptosomes. This is in agreement with the predominant role of astrocytes rather than neurons in the clearance of extracellular glutamate (Danbolt, 2001; Sattler and Rothstein, 2006).

The presently identified selective interaction and co-localization of NKA-α2 with $A_{2A}R$ to mediate the fast control of glutamate uptake provides new insights into our understanding of important neurobiological processes including synaptic plasticity, cognition and neurodegeneration that are influenced by the abnormal functioning of either GluTs (Dunlop et al., 2006; Benarroch et al., 2010) or NKA-α2 (Moseley et al., 2007; Benarroch et al., 2011) and which are known to be controlled by $A_{2A}R$ (Chen et al., 2007; Gomes et al., 2011)**.** For example, modification of glutamate uptake biases synaptic plasticity and affects cognition (Huang and Bergles, 2004; Tzingounis and Wadiche, 2007; Bechtholt-Gompf et al., 2010) and other mood-related disturbances related to schizophrenia-like endophenotypes (Schwarz and Myint, 2011); similarly, mutations of the NKA-α2 gene have been associated with impaired spatial learning, spontaneous epileptic seizures and anxiety (Lingrel et al., 2007; Moseley et al., 2007; Benarroch, 2011). Our finding of the direct interaction between $A_{2A}R$ and NKA-α2 controlling GLT-I activity provides the tentative explanation that the $A_{2A}R$-mediated control of synaptic

plasticity (Costenla et al., 2011), working memory (Zhou et al. 2009; Wei et al. 2011) and memory impairment in animal models of Alzheimer's disease (Canas et al., 2009; Cunha and Agostinho, 2010) may involve an $A_{2A}R$-mediated control of glutamate uptake by astrocytes (Matos et al., 2012a). Likewise, the ability of $A_{2A}R$ to modulate mood disorders (Cunha et al., 2008; Shen et al., 2012) might also result from this ability of astrocytic $A_{2A}R$ to regulate NKA-α2 and GLT-I activities. This corresponds to a shift from neurons to astrocytes as an additional important cellular site of action of $A_{2A}R$ to control different brain pathologies (Chen et al., 2007; Gomes et al., 2011). In fact, the predominant localization of $A_{2A}R$ in medium spiny neurons (Schiffmann et al., 2007) and in synapses throughout the brain (Rebola et al., 2005) has prompted neuronal-based mechanisms as responsible for $A_{2A}R$-mediated neuroprotection (Chen et al., 2007; Gomes et al., 2011), whereas the role of $A_{2A}R$ in astrocytes (reviewed in Boison et al., 2009) has received less attention. This presently reported ability of $A_{2A}R$ to control astrocytic NKA activity implies a tight regulation by $A_{2A}R$ of ionic homeostasis (see below) in astrocytes (Türközkan et al, 1996; Leite et al., 2011) indirectly controlling glutamatergic neurotransmission, which may provide the explanation for the broad spectrum of neuroprotection of $A_{2A}R$ antagonists in diverse brain regions against a variety of brain insults (Chen et al., 2007; Gomes et al., 2011). This contention is in agreement with our reported coupling of $A_{2A}R$, NKA-α2 and GluTs both in the striatum and cortex. However, we also noted that the activities of NKA and of glutamate uptake, as well as the number of $A_{2A}R$/NKA-α2 PLA puncta were different between the striatum and cortex. This suggests a general qualitatively similar control of NKA-α2 and GLT-I by $A_{2A}R$ in different brain regions, but also indicates quantitative differences between different brain regions, probably related to different expression of astrocytic $A_{2A}R$ and/or the different astrocyte-neuron interplay in controlling the extracellular glutamate levels in different brain regions.

It is worth noting that the impact of $A_{2A}R$-mediated control of NKA activity in astrocytes may actually override the importance of the control of glutamate uptake. In fact, the brain has one of

the highest concentrations of NKA in the body (Reinhard et al, 2013), and NKA-α2 has a prime role in maintaining the Na^+ and K^+ gradients, which provide the driving force for multiple and vital cellular functions such as regulation of cell volume, pH, energization of the resting membrane potential and Na^+-coupled secondary transport of H^+, Ca^{2+} and glucose across the astrocytic plasma-membrane (Aperia, 2007; Kirischuk et al., 2012). Thus the regulation of astrocytic NKA-α2 by $A_{2A}R$ suggests a potential ability of $A_{2A}R$ to impact on each of these astrocytic processes to affect a variety of neurobiological processes. For instance, NKA-α2 activity controls the extracellular K^+ homeostasis to regulate neuronal depolarization, synaptic fidelity and the signal-to-noise ratio of synaptic transmission (Wang et al., 2012), which may well underlie the ability of $A_{2A}R$ to control synaptic plasticity and the salience of information encoding in neuronal networks (reviewed in Cunha, 2008). Also, the control of extracellular K^+ and pH by astrocytic NKA-α2 (Obara et al., 2008; Benarroch, 2011) may provide novel mechanistic insights for the ability of $A_{2A}R$ to control abnormal excitability characteristic of animal models of epilepsy (El Yacoubi et al., 2008). Additionally, the control by $A_{2A}R$ of astrocytic ion homeostasis may also be involved in the control of glucose and lactate metabolism, in accordance with the impact of caffeine (an adenosine receptor antagonist) and $A_{2A}R$ on brain metabolism (Hammer et al., 2001; Duarte et al., 2009).

Notably, our novel key observation that $A_{2A}R$ physically associate with and inhibit NKA-α2 also prompts a novel mechanism to link metabolic control with ion homeostasis. Thus, NKA activity is the chief controller of ion homeostasis at the cost of considerable energetic support. As NKA activity consumes ATP, it generates adenosine, and this local metabolic imbalance then feeds back to curtail excessive activity of NKA-α2 and control ion homeostasis through the activation of $A_{2A}R$. Thus, this novel observation that $A_{2A}R$ regulates NKA-α2 activity unveils the hitherto unrecognized possibility that the impact of $A_{2A}R$ and of caffeine consumption on brain dysfunction may involve a primary target on astrocytic ion homeostasis indirectly impacting on synaptic function and viability. Interestingly, we observed an opposite $A_{2A}R$ modulation of NKA

activity in gliosomes and synaptosomes, which suggest a complex and potential "fine-tuning" modulation of NKA activity in astrocytes and neurons to impact on cognition, mood and neurodegeneration processes (see **Fig.7**).

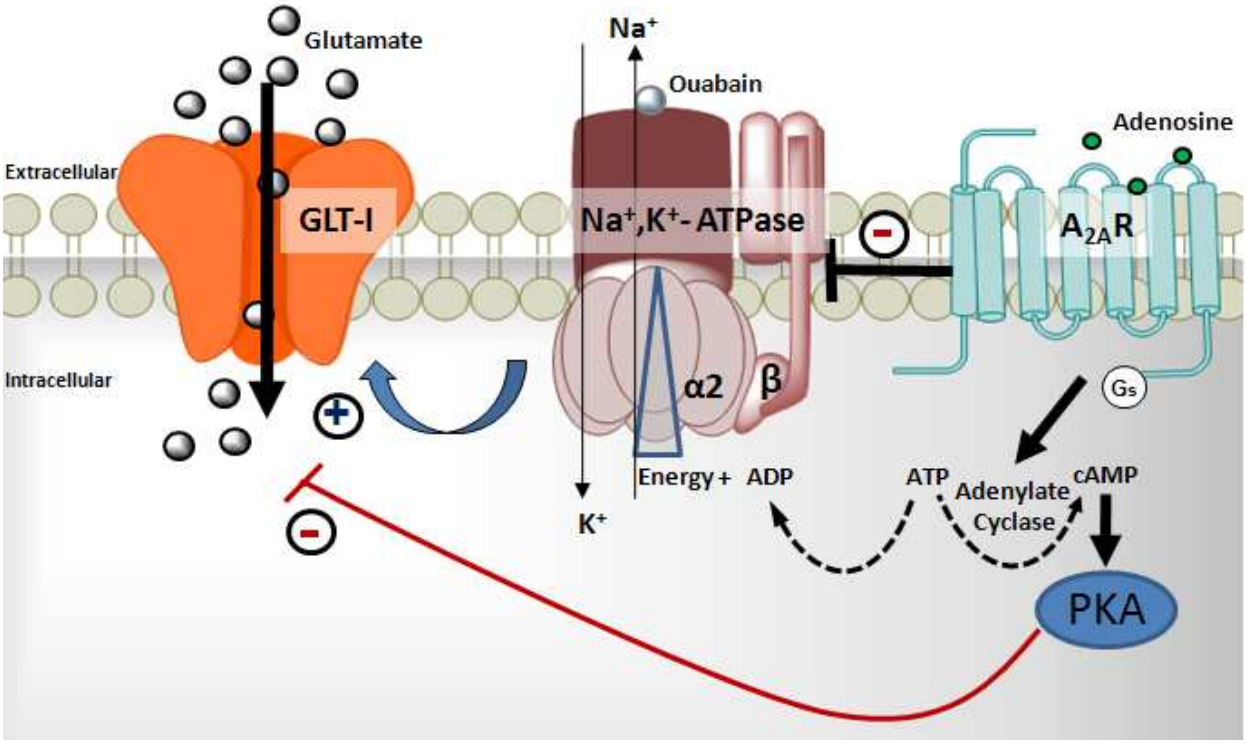


Fig. 7 - Proposed mechanism for the GLT-I-NKA-α2-$A_{2A}R$ interactions in the astrocyte plasma membrane. The binding of adenosine to $A_{2A}R$ elicits the activation of stimulatory Gs protein to activate adenylate cyclase, which, in turn, induces the synthesis of intracellular cAMP from ATP and the consequent activation of the cAMP-PKA signaling pathway. The physical interaction $A_{2A}R$ with NKA-α2 and the decrease in the amount of local ATP available to maintain Na^+ and K^+ pump activity may lead to a fast inhibition of the NKA-α2 activity and an ensuing decline in the GLT-I uptake activity (Cholet et al., 2002; Nakagawa et al., 2008; Rose et al., 2009; Genda et al., 2011). The long-term activation of the PKA pathway leads to a reduction in the expression of mRNAs for GLT-1 and GLAST glutamate transporters and a sustained decrease of glutamate uptake (see chapter 2 or Matos et al., 2012b).

In conclusion, we provide molecular and functional evidences showing the physical association of $A_{2A}R$ and NKA-α2 and the ability of $A_{2A}R$ to decrease NKA-α2 activity. This was shown to constitute the mechanism by which the acute manipulation of $A_{2A}R$ controls the transport of glutamate by astrocytes as an example of the possible importance of this novel

A_{2A}R-NKA-α2 molecular hub to understand the neuroprotective impact of caffeine and A_{2A}R antagonists on diverse neurological conditions.

6. Acknowledgements

The authors thank Michael A. Schwarzschild at the Molecular Neurobiology Laboratory, Department of Neurology, Massachusetts General Hospital, for providing the transgenic Gfa2-A_{2A}R-KO mice.

CHAPTER 4

ASTROCYTIC ADENOSINE A_{2A} RECEPTORS CONTROL THE β-AMYLOID PEPTIDE-INDUCED DECREASE OF GLUTAMATE UPTAKE

Marco Matos [(1,2)*], Elisabete Augusto [(1,2)], Nuno Machado [(1,2)], Alexandre dos Santos-Rodrigues [(1,3)],Rodrigo A. Cunha [(1,2)] and Paula Agostinho [(1,2)]

1. Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal
2. Faculty of Medicine, University of Coimbra, Coimbra, Portugal
3. Laboratory of Cellular Neurobiology, Department of Neurobiology, Institute of Biology, Federal Fluminense University, Niterói, RJ, Brazil.

J.ALZHEIMERS DIS. 31: 555-567 (2012)

* Marco Matos performed the experiments presented in Fig. 1-6

1. Abstract

Alzheimer's disease (AD) is characterized by a progressive cognitive impairment tightly correlated with the accumulation of β-amyloid peptides (Aβ, mainly $Aβ_{1-42}$). There is a precocious disruption of glutamatergic synapses in AD, in line with an ability of Aβ to decrease astrocytic glutamate uptake. Accumulating evidence indicates that caffeine prevents the burden of AD, likely through the antagonism of A_{2A} receptors ($A_{2A}R$) which prevent Aβ-induced memory impairment and synaptotoxicity. Since $A_{2A}R$ also modulate astrocytic glutamate uptake, we now tested if $A_{2A}R$ blockade could prevent the decrease of astrocytic glutamate uptake caused by Aβ. In cultured astrocytes, $Aβ_{1-42}$ (1 µM for 24 hours) caused an astrogliosis typified by an increased density of GFAP, which was mimicked by the $A_{2A}R$ agonist, CGS 26180 (30 nM), and prevented by the $A_{2A}R$ antagonist, SCH 58261 (100 nM). $Aβ_{1-42}$ also decreased D-aspartate uptake by 28±4%, an effect abrogated upon genetic inactivation or pharmacological blockade of $A_{2A}R$. In accordance with the long term control of glutamate transporter expression by $A_{2A}R$, $Aβ_{1-42}$ enhanced the expression and density of astrocytic $A_{2A}R$ and decreased GLAST and GLT-I expression in astrocytes from wild type, but not from $A_{2A}R$ knockout mice. This impact of $Aβ_{1-42}$ on GluTs and uptake dependent on $A_{2A}R$ function was also confirmed in an *ex vivo* astrocyte preparation (gliosomes) from rats intracerebroventricularly (icv) injected with $Aβ_{1-42}$. These results provide the first demonstration for a direct key role of astrocytic $A_{2A}R$ in the ability of Aβ-induced impairment of glutamate uptake, which may underlie glutamatergic synaptic dysfunction and excitotoxicity in AD.

2. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized symptomatically by a progressive cognitive and memory loss (for review see Querfurth and LaFerla, 2007). Albeit the ethiology of AD is still debated, evidence has accumulated to nail the excessive production of β-amyloid peptide (Aβ), especially $Aβ_{1-42}$, as a candidate causative mechanism

(Hardy and Higgins, 1992). This may involve an initial dysfunction and loss of cortical synapses (Selkoe, 2002; Coleman et al., 2004), which is already observed in patients with Mild Cognitive Impairment (MCI) (Scheff et al., 2006). Notably, it seems that glutamatergic synapses are at particularly risk in AD (Bell et al., 2006; Kirvell et al., 2006; Kashani et al., 2008; D'Amelio et al., 2011). This heralds the hypothesis that glutamate excitotoxicity, a prominent executioner in most brain diseases (Lipton and Rosenberg, 1994), may play a role in the Aβ-induced neurodegeneration associated with AD (Hynd et al., 2004).

The elucidation of the ethiology of AD has also evolved based on the exploration of the molecular mechanisms of different neuroprotective strategies effective in AD. Recent years have witnessed a remarkable convergence of epidemiological and animal studies defining the ability of caffeine to prevent AD (Maia and de Mendonça, 2002). Thus, epidemiological studies have shown that the chronic consumption of caffeine attenuates dementia and its associated neuropathology (Maia and de Mendonça, 2002; Eskelinen et al., 2002; Smith, 2009; Santos et al., 2010; Gelber et al., 2011). Animal studies have confirmed these findings (Dall'Igna et al., 2003 and 2007; Chen et al., 2008; Arendash et al., 2009) and allowed proposing the putative molecular target of caffeine, adenosine A_{2A} receptors ($A_{2A}R$), as a main neuroprotective system in AD (Dall'Igna et al., 2007; Canas et al., 2009). The observation that caffeine and $A_{2A}R$ blockade prevent memory impairment triggered by different noxious stimuli (reviewed in Takahashi et al., 2008; Cunha and Agostinho, 2010) suggests that $A_{2A}R$ may control fundamental mechanisms governing neurodegeneration (Gomes et al., 2011) and glutamatergic transmission emerges as a prominent mechanism in view of the enrichment (Rebola et al., 2005) and key role of $A_{2A}R$ in controlling the plasticity of cortical glutamatergic synapses (Rebola et al., 2005; Costenla et al., 2010).

In addition, we have previously shown that glial $A_{2A}R$ can play a prominent role of in the control of neurodegeneration (Yu et al., 2008). This may result from the tight ability of astrocytes to control glutamatergic synapses, which has led to the emergence of the concept of

the tripartite synapse (Halassa et al., 2007; Perea et al., 2009). In particular, the definition of the duration of a synaptic glutamatergic signal is defined by the ability of astrocytes to remove extracellular glutamate (Tzingounis and Wadiche, 2007). This is achieved through the activity of the mainly astrocytic high-affinity glutamate transporters GLAST (glutamate-aspartate transporter) and GLT-I (glutamate transporter-I) (Danbolt et al., 2001), which function has been shown to be important to define both physiological processing of glutamatergic synapses (Tsvetkov et al., 2004; Arnth-Jensen et al., 2002) as well as neuronal damage (Rothstein et al., 1996; Tanaka et al., 1997). Notably, the brain of AD patients displays a decrease of GluTs (Hardy et al., 1982; Westphalen et al., 2003; Jacob et al., 2007; Schallier et al., 2011; Scott et al, 2011) and Aβ impairs glutamate uptake by astrocytes (Harris et al., 1996; Parpura-Gill et al., 1997; Lauderback et al., 1999; Harkany et al., 2000; Matos et al., 2000), which could lead to glutamate overload, dysfunction and damage of glutamatergic synapses. Additionally, we recently reported that A_{2A}R also control glutamate uptake by astrocytes (see chapter 2 or Matos et al., 2012b), paving the way to consider the control of astrocytic glutamate uptake as a key mechanism associated with glutamatergic dysfunction and damage in AD amenable to A_{2A}R-mediated neuroprotection. To begin exploring this hypothesis, we now tested if the pharmacological and genetic blockade of A_{2A}R might control Aβ -induced impairment of glutamate uptake by astrocytes.

3. Material and Methods

3.1. Materials

D-Aspartate and 8-{4-(2-aminoethyl) amino] carbonylmethyloxyphenyl} xanthine (XAC) were acquired from Sigma-Aldrich (St. Louis, MO, USA), D-[^{3}H] aspartate (specific activity of 15-50 Ci/mmol) was bought from PerkinElmer (MA, USA), ZM241385[^{3}H] (specific activity of 50 Ci/mmol) was from American Radiolabelled Chemicals (MO, USA) and Aquasafe 500 Plus liquid scintillation cocktail from Zinsser Analytic (Frankfurt, Germany). Dihydrokainate (DHK),

DL-threo-β-benzoyloxyaspartate (TBOA), 4-[2-[[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-yl]amino]ethyl]benzene propanoic acid hydrochloride (CGS21680), 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH58261), N-cyclopentyladenosine (CPA), 1-[2-chloro-6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide (2-Cl-IBMECA), H89 dihydrochloride (H89) and dibutyryl-cAMP (dbcAMP) were all acquired from Tocris (Bristol, UK). Adenosine deaminase (ADA) was from Calbiochem (San Diego, CA, USA). Magna Pure Compact RNA isolation, Transcriptor first strand cDNA synthesis and Lightcycler FastStart DNA MasterPLUS SYBR Green I kits and all primers were from Roche Diagnostics (Amadora, Portugal) whereas the SmartCycler reaction tubes were from Cepheid (Izasa, Portugal). Reagents used in immunoblotting experiments were purchased from Bio-Rad Laboratories, except PVDF membranes that were from Millipore (Bedford, MA, USA). All cell medium components, L-leucine methyl ester (LME) and N-methyl-D-glucamine (NMG) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Polyclonal primary antibodies rabbit anti-GLT-I/EAAT2 (C-terminus), rabbit anti-GLAST/EAAT1 (C-terminus) and Cy5.5-labelled goat anti-mouse secondary antibody conjugate were from Abcam (Cambridge, UK), Guinea Pig monoclonal anti-VGLUT-I (vesicular glutamate transporter 1) was from Synaptic Systems (Goettingen, Germany), rabbit anti-EAAC1/EAAT3 was from Alpha Diagnostics (Texas, USA) and rabbit anti-A_1R was from Affinity Bioreagents (CO, USA). Monoclonal primary antibodies rabbit anti-GFAP (Glial fibrillary acidic protein) was from Dakocytomation (Glostrup, Denmark), mouse anti-synaptophysin and anti-SNAP-25 (Synaptosomal-associated protein 25) were from Signa-Aldrich (Missouri, USA) and mouse anti-A_{2A}R (clone 7F6-G5-A2) was from Millipore (Bedford, MA).

3.2. Animals

Wistar rats (8-10 week males) and C57Bl/6 mice (4-5 day postnatal) were from Charles River and A_{2A}R global knockout mice with C57Bl/6 genetic background were generated by Jiang-Fan Chen (Boston University School of Medicine, MA). Animals were maintained under controlled environment (23 ± 2℃; 12 h light/dark cycle; *ad libitum* access to food and water) and handled according to European Union guidelines (86/609/EEC).

3.3. D-[^{3}H] aspartate uptake

Primary astrocytes cultures were prepared from cerebral cortices of 4-5 days postnatal C57-Bl6 mice, as previously described (Matos et al., 2008; Matos et al, 2012b). The astrocytes, free of microglia (see Matos et al, 2012b), were platted at low density ($5x10^4$ cells/cm^2) to analyze glutamate uptake, as described previously (Matos et al., 2008; Matos et al, 2012b) using D-aspartate because it is an excellent substrate for the high-affinity glutamate carriers and is little metabolized inside cells (Bender et al., 1997; Furness et al., 2008). Briefly, astrocytes were incubated with Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 1 $CaCl_2$, 6 glucose, 10 HEPES, pH 7.4) containing D-[^{3}H] aspartate (0.1 µCi/ml; from Perkin-Elmer, MA, USA) and 50 µM D-aspartate (Sigma, Sintra, Portugal) for 10 min at 37 ℃ before terminating the uptake assay by placing cells on ice and washing twice with cold N-methyl-glucamine (NMG) buffer (a Krebs buffer where NaCl is isosmotically replaced by NMG). Then, the cells were lyzed with 0.5 M NaOH, an aliquot used to determine the radioactivity content (disintegrations per minute) and the remaining used to measure the protein content with the BCA method (Pierce Technology, Rockford, USA).

$Aβ_{1-42}$ (Bachem, Bubendorf, Switzerland) was dissolved in a 0.1% NH_3 solution (pH>9) and stored at -20℃ until use. This solution, mainly containing soluble monomers (see Canas et al., 2009), was directly apllied to astrocytes to achieve a final concentration of 1 µM incubated during 24 hours before the uptake assays. When testing the effect of A_{2A}R ligands, either the selective agonist 4-[2-[[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2-

yl]amino]ethyl]-benzene propanoic acid (CGS 21680, 30 nM) or the selective antagonist 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 58261, 100 nM), they were added for 30 min before incubation with $A\beta_{1-42}$.

3.4. Quantitative PCR

The extraction of total RNA from cultured astrocytes, its reverse transcription into cDNA and the real-time PCR was performed using Lightcycler FastStart DNA Master^{PLUS} SYBR Green I kit customized for amplification of the target cDNA's in a SmartCycler system *(*Cepheid*,* Izasa Portugal). qPCR analysis were essentially carried out as previously described [48] using forward *(5'*-GCCTGCTTTGTCCTGGTCC-3') and reverse (*5'*- GGAGTGGAATTCGGATGGC-3') $A_{2A}R$ primers, forward *(5'*-CCTTCGTTCTGCTCACGGTC-3') and reverse (*5'*-TTCACCTCCCGGTAGCTCAT-3') GLAST primers, forward *(5'*- GTGCAAGCCTGTTTCCAGC-3') and reverse (*5'*-GCCTTGGTGGTATTGGCCT -3') *GLT*-I primers, and forward *(5'*-CTTAGAGGGACAAGTGGCG -3') and reverse (*5'*- GGACATCTAAGGGCATCACA -3') *18S* primers used as internal control for normalization of the fold changes of gene expression. All primers had a good linearity *(*R^2 > 0.980*)* and calculated efficiencies of 2.1/107% for $A_{2A}R$, 1.9/90% for GLAST, 1.88/88% for GLT-I and 1.9/95% for 18S (internal control) with consistency across triplicate reactions (Matos et al., 2012b)*.* Briefly*, s*amples together with SYBR Green I reaction mix, Fast start Taq DNA polymerase, reaction buffer, 10 mM $MgCl_2$, dNTP mix and activated SYBR green I dye, were run in *a* SmartCycler thermal cycler for 45 cycles composed by activation of Taq polymerase (95℃ for 10 min), denaturation (95℃ for 10 s), annealing (5 s at 56℃ for $A_{2A}R$, at 58℃ for GLAST, at 55℃ for 18 s for GLT-I) and extension (72℃ for 6 s)**.** Relative quantification was performed using the C_t /Livak method ($R=2^{-\Delta\Delta Ct}$) for the $A_{2A}R$, GLAST and GLT-I genes and expressed as fold change in arbitrary values *(*Wong and Medrano, 2005; Schmittgen and Livak, 2008). Briefly, to determine the relative expression of the GLT-I, GLAST and $A_{2A}R$ target genes in the test samples (Abeta-treated astrocytes) and calibrator samples (non-treated astrocytes) using reference gene 18s as the normalizer, the expression

levels of both the target and the reference genes were determined using RT-qPCR. After the CT values were measured, the expression level of the target genes in Aβ-treated astrocytes relative to non-treated astrocytes was performed using the Livak comparative method, also known as the $2^{-\Delta\Delta Ct}$ method, after determining the efficiencies of the target and internal control genes (Wong and Medrano, 2005; Schmittgen and Livak, 2008).

3.5. Western blotting

Western blot analysis was performed as previously described (Matos et al, 2012b). Briefly, the primary antibodies, namely anti-GFAP (1:2,000; from Dakocytomation, Glostrup, Denmark), anti-GLAST/EAAT1 (1:1,000; from Abcam, Cambridge, UK), anti-GLT-I/EAAT2 (1:1,000; from Abcam) or anti-A_{2A}R (1:500; from Millipore, Billerica, USA), all diluted in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) with 0.1% Tween (TBS-T) and 3% bovine serum albumin (fatty acid free; Sigma) or 5% non-fat dry milk (for A_{2A}R), were incubated overnight with the membranes at 4 ℃. After washing with TBS-T, the membranes were incubated with appropriate alkaline-phosphatase-conjugated IgG secondary antibodies (1:10,000; Molecular Probes, Leiden, The Netherlands) for 2 h at room temperature. After washing, the membranes were revealed using ECF (Amersham, GE Healthcare, Carnaxide, Portugal) and visualized under a fluorescence imaging system (VersaDoc 3000; Bio-Rad, Amadora, Portugal) to determine the intensity of the bands with the Quantity One software version 4.4.1 (Bio-Rad).The membranes were then stripped of the antibodies and reprobed for α-tubulin immunoreactivity using an anti-α-tubulin antibody (1:40,000; from Sigma).

3.6. A_{2A}R binding to primary astrocyte membranes

Total membranes from mice cortical primary astrocytes cultures were prepared as previously described for synaptosomal membranes (Lopes et al., 2004) and re-suspended in an incubation solution (50 mM Tris and 10 mM $MgCl_2$, pH 7.4) to estimate the density of A_{2A}R, as previously described (Lopes et al., 2004). This was achieved by incubating the astrocytic

membranes (circa 100 µg of protein) with a supra-maximal concentration (6 nM) of the selective $A_{2A}R$ antagonist, [^{3}H]SCH 58261 (specific activity of 77 Ci/mmol; prepared by Amersham and offered by Dr. E.Ongini, Shering-Plough, Italy) for 1 hour at room temperature (23-25 ℃) in the incubation solution containing 2 U/ml adenosine deaminase (Sigma). Specific binding was determined by subtraction of the nonspecific binding, which was measured in the presence of 12 µM of 8-{4-(2-aminoethyl)amino]-carbonylmethyloxyphenyl}xanthine (XAC; Sigma), a mixed A_1R/A_2R antagonist and specific binding was expressed as fmol/mg protein. Binding assays were performed in duplicate and the protein concentration was determined as described above.

3.7. *Ex vivo* analysis of gliosomes from $A\beta_{1-42}$-icv injected rats

Rats were injected intra-cerebroventricularly (icv) with vehicle (0.1% NH_3) or with $A\beta_{1-42}$ (single injection of 2 nmol in 4 µl), which we have previously shown to trigger memory impairment after 15 days (Canas et al., 2009; Cunha et al., 2008). After measuring spontaneous locomotion in an open filed arena (see Canas et al., 2009; Cunha et al., 2008), memory performance was evaluated now using a modified Y-maze test, which allows estimating spatial memory (Dellu et al., 1997). This consisted of two trials separated by an inter-trial interval of 2 hours; the first trial (training) had an 8 min duration and rats were allowed to explore only two arms with the third (novel) arm closed; this was open during the second trial (8 min) to determine the total time spent in each arm (see Cognato et al., 2010).

Vehicle-treated and Aβ-treated rats with memory impairment were then used to prepare gliosomes, a heterogeneous preparation that we and others have defined to be derived from astrocytes (Nakamura et al., 1993; Stigliani et al., 2006; Matos et al., 2012b). Briefly, after killing a rat, homogenising the cerebral cortex and removing nuclear debris, the purification of gliosomes was achieved through a discontinuous Percoll gradient (2, 6, 15 and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4) and a centrifugation at 31,000 *g* for 5 min; as previously described, gliosomes were collected between 2 and 6% of

Percoll, and further centrifuged at 22,000 *g* for 15 min at 4℃ to remove myelin contaminants (see Matos et al., 2012b).

For Western blot analysis, the gliosomes were re-suspended in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) with protease inhibitor cocktail (CLAPS, composed by 10 µg/ml chymostatin, leupeptin, antipain and pepstatin A; from Sigma). Instead, for the functional uptake experiments, the gliosomal pellets were re-suspended in Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, 1 $CaCl_2$, pH 7.4) or NMG buffer (where NaCl is isosmotically replaced by NMG) and were viable for experiments between 4-6 h. The uptake of D-[^{3}H] aspartate into cortical gliosomes was carried out as previously described [48]. After equilibrating at 37℃ for 10 min, gliosomes were in cubated for 10 min at 37 ºC with Krebs medium containing D-[^{3}H]aspartate (0.1 µCi/ml) and D-aspartate (50 µM) and the reaction was terminated by filtration (Whatman GF/C, GE Healthcare, Kent, USA) for determination of the tritium retained by the gliosomes in the filters, as described above.

3.8. Statistical analysis

Data were expressed as arbitrary values or percentages of values obtained in control conditions or conditions mentioned, and were presented as mean ± S.E.M.. Statistical significance was determined using a Student unpaired *t* test for comparison between 2 groups or a one-way ANOVA followed by Dunnett or Tukey *post hoc* tests for mean comparisons with the control group or between multiple groups, respectively.

4. Results

4.1. $A_{2A}R$ blockade prevents $A\beta_{1-42}$-induced astrogliosis and reduced D-aspartate uptake

Figure 1 shows that the incubation for 24 hours of cultured astrocytes with $A\beta_{1-42}$ (1 µM) decreased D-[^{3}H]aspartate uptake by 28±4% (n=8, p<0.01). This was completely prevented (p<0.001) by pre-incubation with the selective $A_{2A}R$ antagonist SCH 58261 (100 nM) (**Fig. 1A**). To confirm this key role of $A_{2A}R$ in the Aβ-induced inhibition of glutamate uptake, we tested the impact of Aβ on the uptake of D-[^{3}H] aspartate in astrocytes cultured from $A_{2A}R$ global KO mice. The selective $A_{2A}R$ agonist, CGS 21680 (100 nM), which decreased D-aspartate uptake in astrocytes from wild type mice (Matos et al., 2008), was devoid of effects in astrocytes derived from $A_{2A}R$ global KO mice. Likewise, $A\beta_{1-42}$ (1 µM, 24 hours) also failed to affect D-aspartate uptake in astrocytes from $A_{2A}R$ global KO mice (**Fig.1B**).

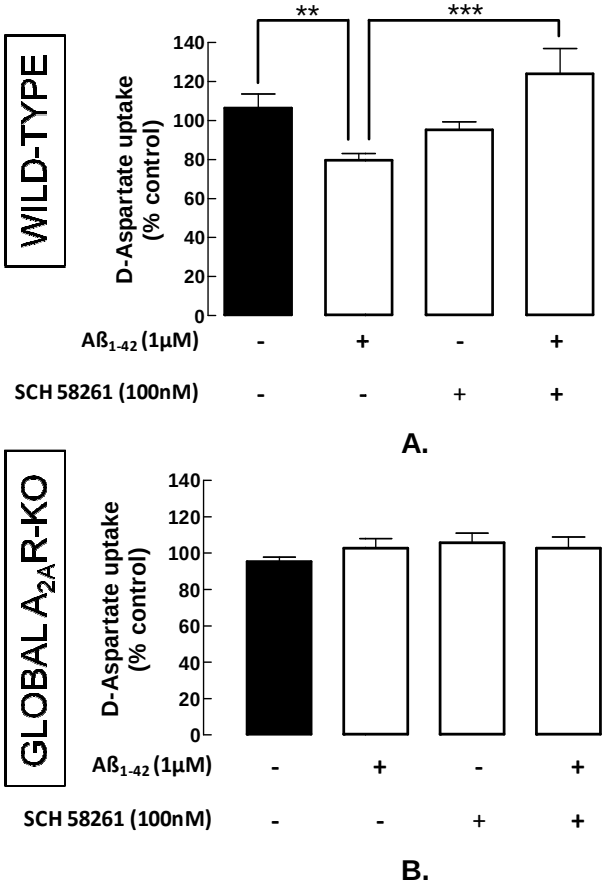


Fig. 1 - $A_{2A}R$ blockade (A) or genetic inactivation (B) prevents $A\beta_{1-42}$-induced decrease in D-aspartate uptake. Primary cultured astrocytes from the neocortex of wild-type **(A)** or global $A_{2A}R$ KO mice **(B)** pups (P2) were maintained in culture for 15 days. The cells were treated or not (control) with the $A_{2A}R$ antagonist SCH 58261 (100 nM) or with the $A_{2A}R$ agonist CGS 21680 (100 nM) for 30 min at 37℃. The cells were then incubated with $A\beta_{1-42}$ (1 µM) or with vehicle for 24 h before the D-[^{3}H] aspartate uptake assay, which was carried out for 10 min. Data are expressed as the percentage of control transport obtained in the absence of drug treatment and are the mean ± SEM of at least eight independent experiments, each done in triplicate. Statistical differences detected using the Tukey test applied after ANOVA. **p < 0.01, significantly different from non-treated astrocytes; $^{###}$p < 0.001, significantly different from $A\beta_{1-42}$ treated astrocytes.

As an additional measure of the impact of Aβ on astrocytes, we report that $Aβ_{1-42}$ (1 µM, 24 hours) increased by 21±3% (n=6, p<0.01) the density of GFAP (**Fig. 2**), a marker of astrogliosis (reviewed in Middeldorp and Hol, 2011). This was mimicked by exposure to CGS 21680 (100 nM, n=6) and SCH 21680 (100 nM, n=6) prevented (p<0.01) the effects of either CGS 26180 or $Aβ_{1-42}$ (**Fig. 2**). This confirms the mandatory role of A_{2A}R in the Aβ-induced impact of astrocytes, namely on the decrease of astrocytic glutamate uptake.

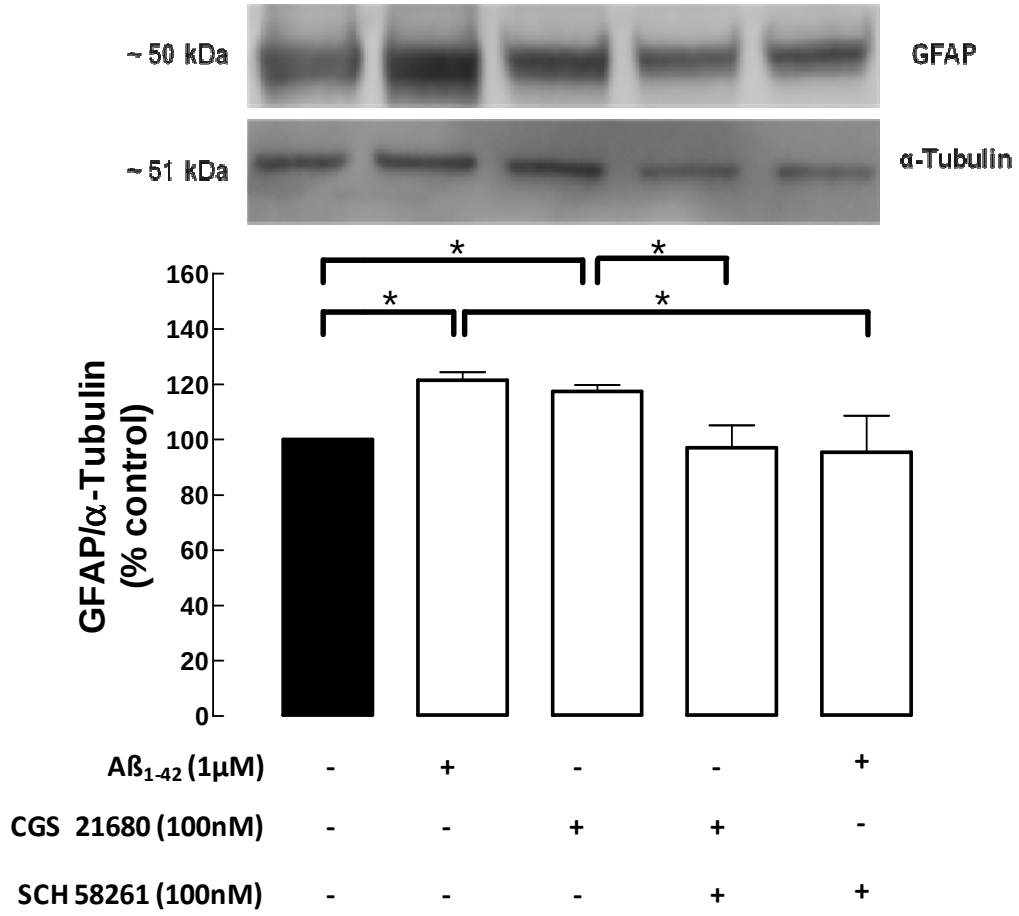


Fig. 2 - A_{2A}R blockade prevents $Aβ_{1-42}$-induced astrogliosis. Primary cultured astrocytes from the neocortex of wild-type mice pups (P2) were maintained in culture for 15 days before treatment for 24 h with $Aβ_{1-42}$ (1 µM) with or without previous incubations with CGS 21680 (100 nM) and/or SCH 58261 (100 nM), an A_{2A}R agonist and antagonist, respectively. Cell lysates were examined by immunoblotting with an anti-GFAP monoclonal antibody at non-saturating protein quantities. Bars represent the relative levels of GFAP to α-Tubulin (reference) immunoreactivity and were expressed as percentage of control astrocytes obtained in the absence of drug treatment. Data are mean ± SEM of at least four independent experiments. Statistical differences detected using the Dunnett's test applied after ANOVA. *p < 0.01, significantly different from non-treated astrocytes.

4

4.2. $A_{2A}R$ blockade prevents $A\beta_{1-42}$-induced downregulation of glutamate transporters

We have previously shown that $A_{2A}R$ control glutamate uptake by astrocytes in a dual manner: through a short term modulation of the activity of the transporters and through a longer term reduction of the expression of GluTs. Since the impact of Aβ on the activity of GluTs required a prolonged incubation, we reasoned that this should involve an $A_{2A}R$-mediated control of the expression of the main GluTs, GLT-I and GLAST. Accordingly, we found that the exposure of cultured astrocytes to $A\beta_{1-42}$ (1 µM, 24 hours) significantly ($p<0.001$) decreased the expression of both GLT-I (-3.0±0.33 fold change, n=6) and of GLAST (-4.0±0.35 fold change, n=6) (**Fig. 3**).

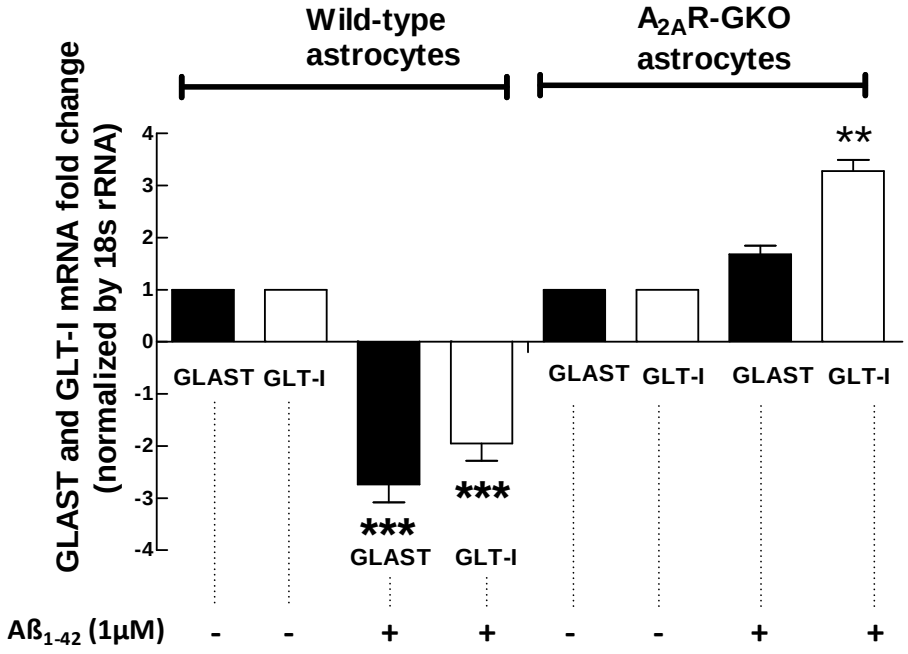
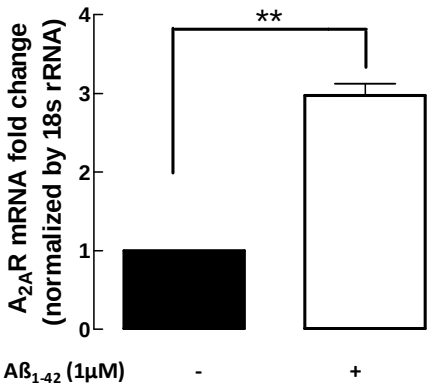


Fig. 3 - $A\beta_{1-42}$ peptide decreases the astrocytic GLT-I and GLAST mRNA levels. The mRNA expression of GLAST (open bars) and GLT-I (filled bars) genes was assayed by real-time PCR and normalized using 18s rRNA as an internal control gene. Relative quantifications were performed using the C_t /Livak method for the GLAST and GLT-I genes and expressed as fold change in arbitrary values. Data are mean ± SEM of at least four independent PCR experiments, each with 8 different samples. Since the comparative Ct ($2^{-\Delta\Delta Ct}$)) method was chosen the comparison of each aimed gene expression (GLAST or GLT-I) was made for each sample (WT astrocytes or $A_{2A}R$-KO astrocytes) between Aβ treated and non-treated astrocytes, in direct correlation with the internal control gene variation. Statistical differences were detected using the Tukey *post hoc* test applied after ANOVA for each gene mRNA. **$p < 0.01$ and ***$p < 0.001$, significantly different from non-treated astrocytes.

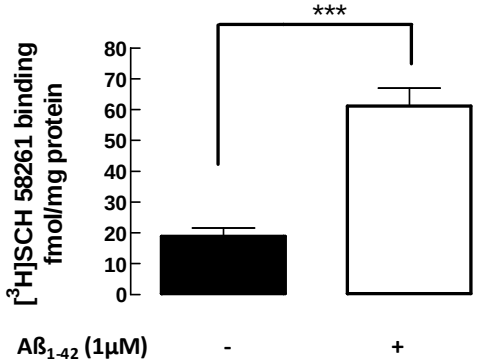
To gauge the role of A_{2A}R in the Aβ-induced modulation of the expression of GluTs, we tested the effect of $A\beta_{1-42}$ on GLT-I and GLAST expression in cultured astrocytes obtained from A_{2A}R global KO mice. Figure 3 shows that the ability of $A\beta_{1-42}$ to decrease the expression of GLT-I and GLAST observed in wild-type cultured astrocytes was totally abrogated in astrocytes prepared from A_{2A}R global KO mice; actually, GLT-I expression was even increased (2.4±0.23 fold change, n=6; $p<0.01$) upon $A\beta_{1-42}$ exposure. These results further re-enforce that $A\beta_{1-42}$ acts through A_{2A}R activation to trigger a decrease in GluTs expression and function.

4.3. $A\beta_{1-42}$ increases the expression and density of astrocytic A_{2A}R

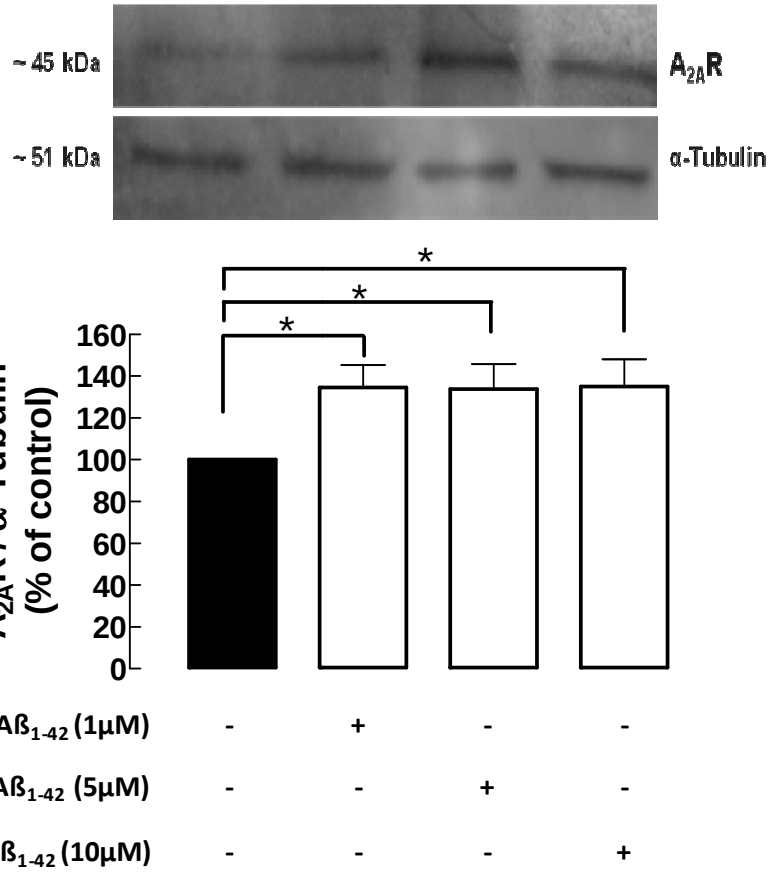
The qPCR analysis also revealed that A_{2A}R mRNA levels were significantly increased (2.0±0.40 fold change, n=6; $p<0.01$) after treatment of cultured astrocytes with $A\beta_{1-42}$ (1 μM, 24 hours) (**Fig. 4A**). This was translated into an increased density of A_{2A}R proteins since the exposure of cultured astrocytes to different concentrations of $A\beta_{1-42}$ (1-10 μM, 24 hours) increased ($p<0.05$, n=6) the density of A_{2A}R, as gauged by Western blot analysis (**Fig. 4C**). This was confirmed using a binding assay (**Fig. 4B**), which showed that the selective A_{2A}R antagonist [^{3}H]SCH 58261 (see Lopes et al., 2004) bound to membranes from cultured astrocytes with a density of 18.9±2.6 fmol/mg (n=6) and exposure to $A\beta_{1-42}$ (1 μM, 24 hours) caused a 3.3-fold increase ($p<0.001$) of this binding density (61.2±6.0 fmol/mg, n=6). These data suggest that Aβ triggers an up-regulation of A_{2A}R, compatible with their key role in mediating the effects of Aβ on astrocytes.



A.



B.



C.

Fig. 4 - $A\beta_{1-42}$ increases the expression and density of astrocytic $A_{2A}R$. (A) The exposure to $A\beta_{1-42}$ increased the astrocytic $A_{2A}R$ mRNA levels, as assayed by real-time PCR, using 18s rRNA as an internal control gene. (**B)** This enhanced levels of $A_{2A}R$ mRNA in astrocytes upon exposure to $A\beta_{1-42}$ (1 µM), was confirmed with a binding assay using astrocytic total membranes (1 mg/mL) incubated with 5 nM [^{3}H]SCH 58261 for 1 h.(**C)** The exposure to $A\beta_{1-42}$ (1 µM) also increased $A_{2A}R$ protein levels, as examined by immunoblotting with an anti-$A_{2A}R$ monoclonal antibody at non-saturating protein quantities. Bars represent the relative levels of $A_{2A}R$ to α-tubulin (reference) immunoreactivity and were expressed as percentage of control astrocytes obtained in the absence of $A\beta_{1-42}$ treatment. Data are mean ± SEM of at least six independent experiments. *p < 0.05, significantly different from non-treated astrocytes, using the Dunnett's post hoc test applied after ANOVA. **p < 0.01 and ***p < 0.001, significantly different from non-treated astrocytes, using the Student's t test.

4.4. $Aβ_{1-42}$-ICV injection causes memory deficits, astrogliosis and decreased D-aspartate uptake - role of A_{2A}R

We have previously shown that a single icv injection of Aβ caused, after 15 days, a synaptotoxicity and cognitive impairment in rodents, which was prevented by A_{2A}R pharmacological or genetic blockade (Dellu et al., 1997; Dall'Igna et al., 2007; Canas et al., 2009). Using a different memory test designed to selectively probe hippocampal-dependent memory (modified Y maze), we observed that $Aβ_{1-42}$-injected rats explored the novel arm during less time than control rats ($p<0.05$, n=9; see **Fig. 5A**), without modification of spontaneous locomotion in an open field arena (data not shown; see Cunha et al., 2008; Canas et al., 2009). This indicates that spatial memory was indeed impaired two weeks after $Aβ_{1-42}$ injection.

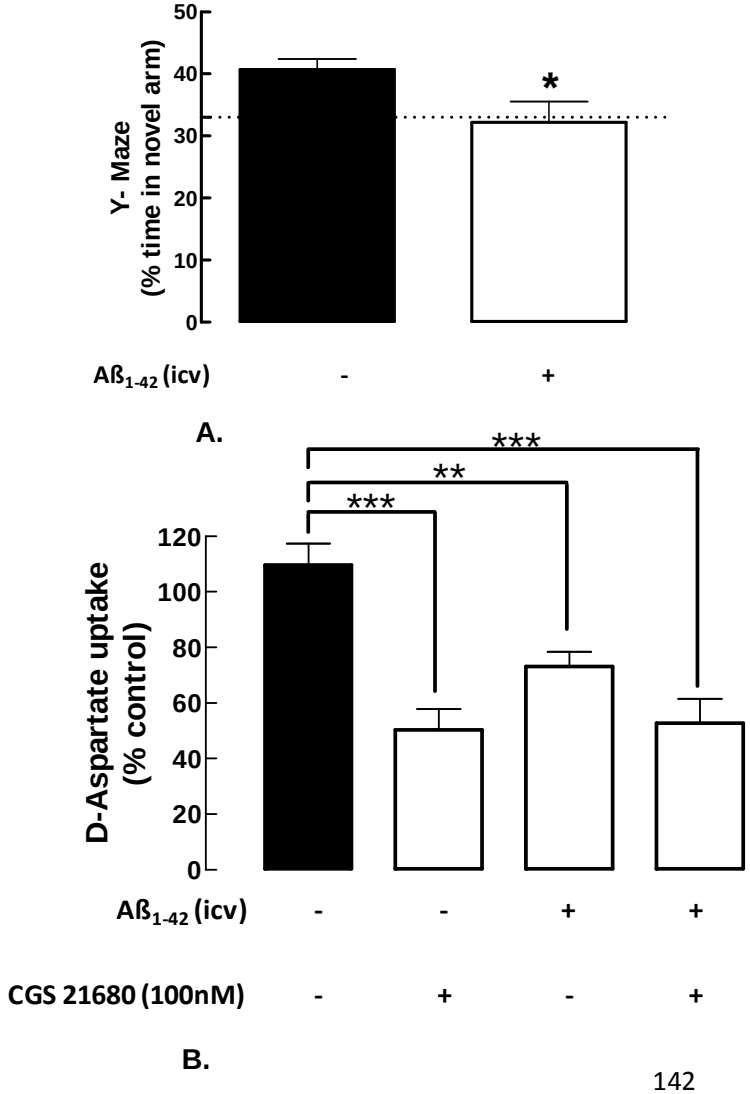


Fig. 5 - $Aβ_{1-42}$ *in vivo* administration causes memory deficits and decreases D-aspartate uptake. **(A)** Two weeks after icv injection of $Aβ_{1-42}$ (2 nmol), adult Wistar rats displayed a memory deficit in a modified (two-trial recognition) Y-maze test. Data are expressed as the percentage of time expended in exploration in the novel arm and are mean±SEM of nine rats analyzed *per* group ($Aβ_{1-42}$ - or vehicle- icv injected rats). **(B)** Cortical brain gliosomes prepared from vehicle or $Aβ_{1-42}$ -icv injected rats displayed a reduced D-aspartate uptake capacity. Gliosomes were obtained from $Aβ_{1-42}$ -icv and vehicle-icv (control) injected rats 20 days after $Aβ_{1-42}$-icv injection. Gliosomes were then treated 30 min with the A_{2A}R agonist CGS 21680 (100 nM) before the D-[^{3}H]aspartate uptake assay, which was carried out for 10 min. Data are expressed as the % of control transport obtained from control group and are mean±SEM of at least 6 independent experiments, each done in triplicate. Statistical differences were gauged using the Tukey's test applied after ANOVA with $^{**}p < 0.01$ and $^{***}p < 0.001$.

We next used these memory impaired rats to prepare gliosomes (an *ex vivo* preparation enriched in glial plasmalemmal vesicles) from their cerebral cortices. Figure 5B shows that cortical gliosomes from $A\beta_{1-42}$-injected animals displayed a significantly lower D-aspartate uptake (36.5±5.3% inhibition, n=6; $p<0.01$) compared to vehicle injected rats. Further arguing for a enhanced long term effect of $A_{2A}R$ on the impact of Aβ on GluTs, we found that the acute (30 minutes) activation of $A_{2A}R$ with CGS 21680 (100 nM) inhibited D-aspartate uptake by 59.3±7.5% (n=6, $p<0.001$) in cortical gliosomes from vehicle-injected rats, whereas CGS 21680 failed to further affect glutamate uptake in cortical gliosomes from $A\beta_{1-42}$-injected rats (**Fig. 5B**).

We next attempt confirming if gliosomes from Aβ-treated rats displayed the cardinal modifications which allowed concluding in cultured astrocytes that the $A_{2A}R$-mediated decrease of the expression of GluTs was the mechanism by which Aβ inhibited glutamate uptake. Thus, we compared the density of GluTs (GLAST and GLT-I), of the main marker of astrogliosis (GFAP) and of $A_{2A}R$ in cortical gliosomes from vehicle and $A\beta_{1-42}$-injected rats. Figure 6A shows that $A_{2A}R$ density was increased in gliosomes obtained from $A\beta_{1-42}$-icv injected rats ($p<0.01$, n=5) concomitant to an increased astrogliosis, as suggested by the higher GFAP levels ($p<0.05$, n=4; **Fig. 6B**). By contrast, GLT-I levels were significantly reduced in gliosomes ($p<0.05$, n=5; **Fig. 6D**), whereas the GLAST levels were only slightly decreased (7.0±3.0%, n=5; **Fig. 6C**). These results are in accordance with these found in cultured astrocytes, thus supporting that Aβ depresses astrocytic glutamate uptake through an up-regulation of $A_{2A}R$ which leads to a down-regulation of the expression of the main GluTs and a long-term reduction of their density.

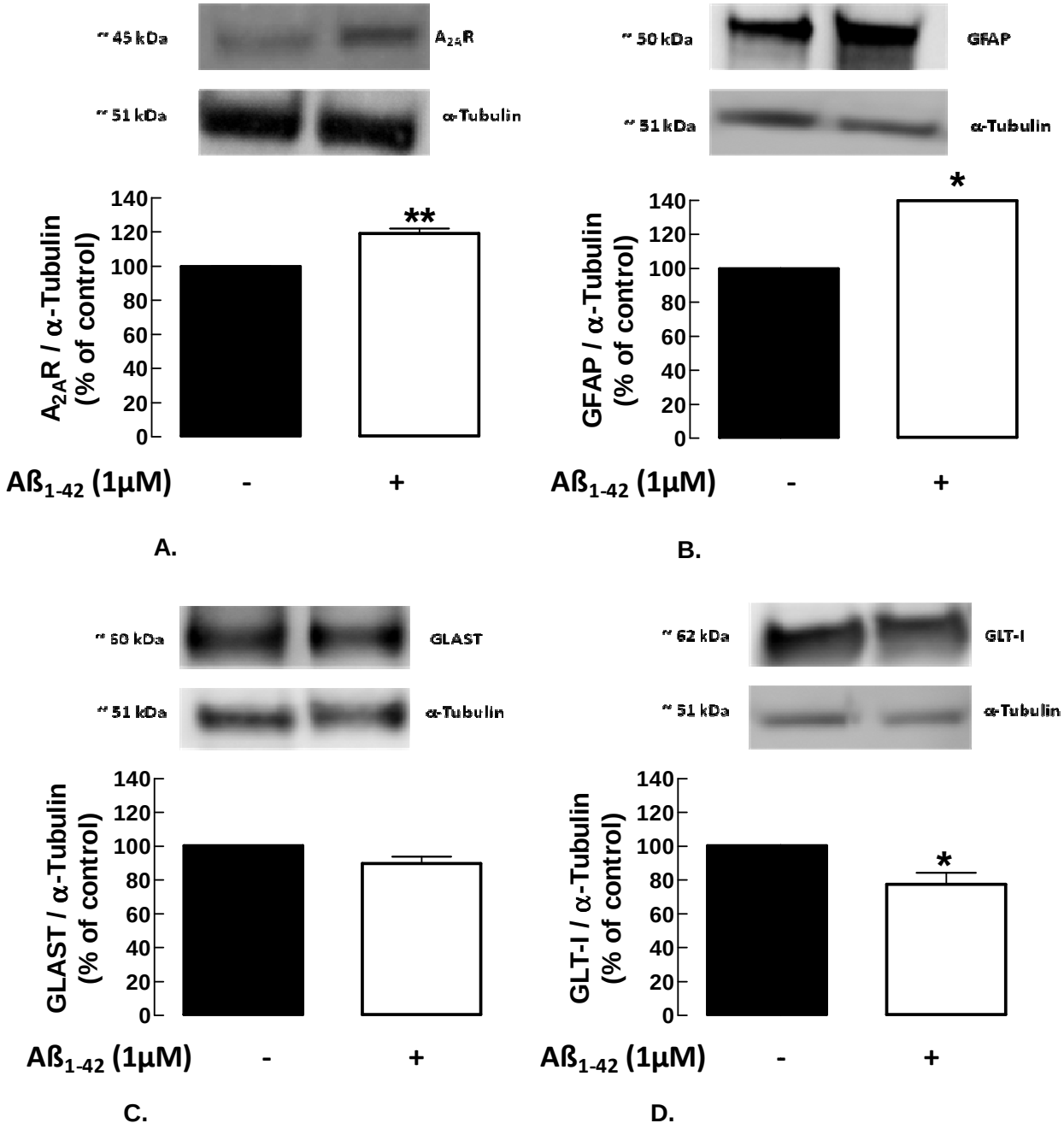


Fig. 6 - Reduction in GLAST and GLT-I levels and increase in GFAP and A_{2A}R levels in gliosomes from $Aβ_{1-42}$-icv injected rats. Gliosomes were prepared from the cortex $Aβ_{1-42}$-icv and vehicle-icv (control) injected adult Wistar rats, 20 days after $Aβ_{1-42}$ injection, to evaluate A_{2A}R **(A)**, GFAP **(B)**, GLAST **(C)** and GLT-I **(D)** immunoreactivity by Western blotting at non-saturating protein quantities. Values are expressed as percentage of control (gliosomes prepared from vehicle-icv injected rats without drug treatment). Data are mean ± SEM of at least 3 independent experiments. *p < 0.05 * and **p < 0.01, significantly different from control gliosomes, using the Student's t test.

5. Discussion

The present results provide the first demonstration that adenosine A_{2A} receptors play a key role in the mechanism by which $A\beta_{1-42}$ affects the expression of astrocytic GluTs, to such an extent that the blockade of adenosine A_{2A} receptors abrogates the deleterious impact of $A\beta_{1-42}$ on glutamate uptake.

This general conclusion is in agreement with the previously documented modification of glutamate transporters both in the brain of AD patients (Hardy and Higgins, 1992; Westphalen et al., 2003; Jacob et al., 2007; Schallier et al., 2011; Scott et al., 2011), as well as in animal models of AD (Masliah et al., 2000), as well as in *in vitro* (Harris et al., 1996; Parpura-Gill et al., 1997; Lauderback et al., 1999; Matos et al., 2008) and *in vivo* assays (Harkany et al., 2000) showing a direct ability of β-amyloid peptides to decrease glutamate uptake by astrocytes. Further strengthening a causal role for the decreased density of GluTs in memory impairment associated with AD, it was recently described that the partial loss of the glutamate transporters GLT-I [GLT-I(+/-) mice crossed to transgenic mice expressing mutations of the amyloid-β protein precursor and presenilin-1, AβPPswe/PS1ΔE9] unmasked spatial memory deficits at 6-months of age, at a time when memory deficits are not yet observable in AβPPswe/PS1ΔE9 (Mookherjee et al., 2011). The expected consequence of a decreased ability of astrocytes to clear glutamate is the enhancement of the extracellular levels of glutamate and promotion of glutamate excitotoxicity, which has been argued to be a prominent executor of damage in different neurodegenerative diseases (Lipton and Rosenberg, 1994). This scenario predicts that glutamatergic synapses should be at greater risk given it is the localization where glutamate receptors are most abundant and functionally more relevant (Tzingounis and Wadiche, 2007). Accordingly, there is robust evidence showing that the loss of cortical synapses is the best morphological correlate of memory impairment in MCI and early AD (reviewed in Selkoe, 2002; Coleman et al., 2004; Cunha and Agostinho, 2010) and it is interesting to note that indeed glutamatergic synapses seem to be the most affected, as

gauged from the few studies addressing this issue (Bell et al., 2006; Kirvell et al., 2006; Kashani et al., 2008; D'Amelio et al., 2011). What is not so far clarified is the mechanism underlying the decrease of the expression, density and overall capacity of GluTs in AD. And the present study provides evidence supporting a novel and pioneering hypothesis, which pinpoints the enhanced density and responsiveness of adenosine A_{2A} receptors as a key step mediating the decreased expression of glutamate receptors in the presence of Aβ.

The involvement of adenosine A_{2A} receptors (A_{2A}R) in neurodegeneration and control of memory impairment in AD has consistently been observed in cellular and animal models of AD (Dall'Igna et al., 2003; Dall'Igna et al., 2007; Canas et al., 2009 and Cunha et al., 2008). In fact, A_{2A}R are recognised as the main target of chronic caffeine consumption (Chen et al., 2007; Ferré, 2008) and there is a notable agreement between the neuroprotective and memory sparing properties of caffeine and of pharmacological or genetic blockade of A_{2A}R (reviewed in Takahashi et al., 2008 and Cunha and Agostinho, 2010). Thus, it is anticipated that the ability of caffeinated coffee or caffeine to reduce the incidence of memory impairment (Smith, 2009; Ritchie et al., 2007), AD (Maia and de Mendonça, 2002; Eskelinen et al., 2009; Smith, 2009; Santos et al., 2010; de Mendonça and Cunha, 2010) and neuropathological findings compatible with AD (Gelber et al., 2011), may also involve the antagonism of A_{2A}R. Since A_{2A}R are most abundantly located in synapses, namely in glutamatergic synapses in cortical regions (Rebola et al., 2005) and that they control the release of glutamate (Lopes et al., 2002) and the activation of NMDA receptors (Rebola et al., 2008), it has been proposed that the ability of A_{2A}R to control neurodegeneration and memory impairment was associated with a control of glutamatergic synapses (d'Alcantara et al., 2001; Chen et al., 2007; Cunha and Agostinho, 2010; Gomes et al., 2011). This would be in agreement with the ability of caffeine and A_{2A}R blockade to control abnormal synaptic plasticity (d'Alcantara et al., 2001; Flajolet et al., 2008; Rebola et al., 2008; Costenla et al., 2010) as well as the consequent synaptotoxicity (Silva et al., 2007; Canas et al., 2009; Cognato et al., 2010; Rebola et al., 2011). The present study

opens a novel mechanistic insight into the ability of $A_{2A}R$ to prevent neurodegeneration, i.e. through the control of the expression of GluTs. This still maintains glutamatergic excitotoxicity and the glutamatergic synapse in the central stage of early modifications in AD, but shift the main role of $A_{2A}R$ from nerve terminals to astrocytes, in accordance with a previous suggestion that glial rather neuronal $A_{2A}R$ played the leading role in the control of neurodegeneration in an animal model of Parkinson's disease (Yu et al., 2008). However, it is most likely that it is a synergism of different actions controlled by $A_{2A}R$ that defines their robust ability to control neurodegeneration and memory impairment (Cunha and Agostinho, 2010; Gomes et al., 2011). Thus, albeit the presently reported ability of $A_{2A}R$ to control the Aβ-induced decrease of the glutamate uptake is fully compatible with the known involvement of GluTs deficits in AD and with the neuroprotective properties of $A_{2A}R$ antagonists, this should not exclude that other mechanisms such as the control of neuroinflammation (Rebola et al., 2011), of the blood-brain barrier permeability (Chen et al., 2008), of the production of granulocyte-colony stimulating factor (GCSF) (Cao et al., 2011) or of the production/clearance of Aβ (Arendash et al., 2009) could also contribute for $A_{2A}R$-mediated control of AD.

This gain of function of $A_{2A}R$ at the onset of neurodegenerative diseases (review in Cunha, 2005) is due to two concurrent factors: 1) an enhanced release of adenosine, which occurs also after exposure to Aβ (Canas et al., 2009) as it does following numerous noxious stimuli (Latini and Pedata, 2001); and, 2) an up-regulation of $A_{2A}R$, which we now showed to also occur in astrocytes after exposure to Aβ. A modest increase of $A_{2A}R$ density was also observed in dystrophic neurites of senile plaques in necropsic cortical tissue of AD patients (Angulo et al., 2003; Albasanz et al., 2008), as well as in the hippocampus of a transgenic (APP Swedish mutation) mouse model of AD (Arendash et al., 2006). However, this is the first study showing that both $A_{2A}R$ expression and density are increased upon exposure to Aβ and in particular in astrocytes.

In summary, the present study provides the first evidence that the impact of Aβ on GluTs is indirectly mediated by an up-regulation of A_{2A}R and its ability to trigger a decreased expression of the main astrocytic GluTs. Conversely, the study also shows that the pharmacological or genetic blockade of A_{2A}R abrogates the ability of Aβ to decrease glutamate uptake by astrocytes, a modification consistently found in AD models and patients and which may be at the onset of AD-associated neurodegeneration.

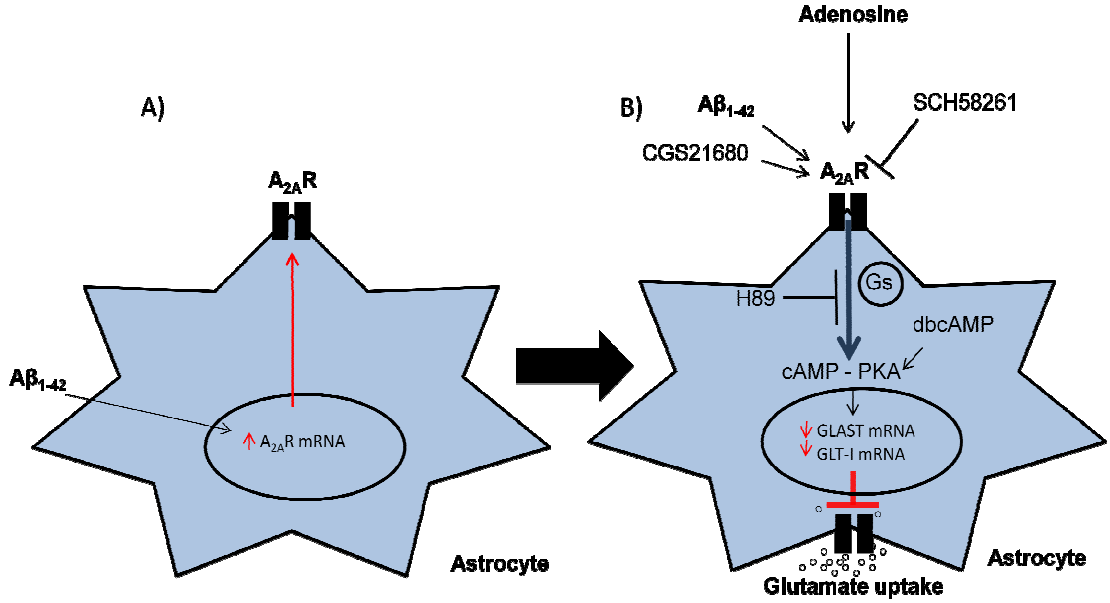


Fig. 7 - Proposed scheme for the $A\beta_{1-42}$-A_{2A}R mediated decrease in glutamate uptake. **(A)** $A\beta_{1-42}$ leads to an augment in A_{2A}R expression and levels by unclear mechanisms. This augment and persistent A_{2A}R activation in turn, **(B)** leads to a decrease in GLAST and GLT-I expression and levels and uptake capacity, an event which may underlie glutamatergic dysfunction and excitotoxicity in AD.

6. Acknowledgements

The authors thank Prof. Teresa Gonçalves and Marta Mota (Department of Microbiology, Faculty of Medicine, University of Coimbra), for allowing the use of the SmartCycler system and for their technical assistance in the PCR studies.

CHAPTER 5

DELETION OF ADENOSINE A_{2A} RECEPTORS IN ASTROCYTES ALTERS GLUTAMATERGIC SYNAPSES AND TRIGGERS PSYCHOMOTOR AND COGNITIVE DYSFUNCTIONS CHARACTERISTIC OF SCHIZOPHRENIA

Marco Matos [1,2,3] *, Elisabete Augusto [1,2,3], Catherine J. Wei [1], Michael A. Schwarzschild [4], Yu Tian Wang [5], Paula Agostinho [2,3], Rodrigo A. Cunha [2,3] and Jiang-Fan Chen [1]

1. Department of Neurology, Boston University School of Medicine, Boston, Massachusetts, USA.
2. Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal.
3. Faculty of Medicine, University of Coimbra, Coimbra, Portugal.
4. Molecular Neurobiology Laboratory, Massachusetts General Hospital, Boston, USA.
5. Department of Medicine & Brain Research Centre, Vancouver Hospital & Health Sciences Centre, University of British Columbia, Vancouver, Canada

SUBMITTED MANUSCRIPT

* Marco Matos performed the experiments presented in Fig. 1-9

1. Abstract

Schizophrenia is a severe mental disorder characterized by impaired cognitive and emotional processes. Adenosine, an endogenous neuromodulator controlling dopaminergic and glutamatergic signaling, is proposed to play a key integrative role in schizophrenia. We here show that the selective deletion of $A_{2A}R$ in astrocytes (in Gfa2-$A_{2A}R$ KO mice) triggers endophenotypes characteristic of schizophrenia, namely enhanced psychomotor activity to the psychotomimetic MK-801 and decreased working memory. The underlying mechanism involved an astrocyte-to-neuron adaptive process, beginning with an upregulation of the astrocytic glutamate transporter GLT-I, associated with increased presynaptic release of glutamate and NMDA-NR2B up-regulation, and culminating with an internalization of AMPA receptors. Accordingly, both GLT-I selective inhibition and prevention of GluR1/2 endocytosis with a brain-penetrating peptide, prevented both the working memory impairment and the MK-801-induced hyperlocomotion in Gfa2-$A_{2A}R$ KO mice. These results indicate that astrocytic $A_{2A}R$, by controlling GLT-I activity and additional glutamatergic mechanisms, may be critical mediators of the glutamatergic dysfunction underlying schizophrenia.

2. Introduction

Schizophrenia is an incurable mental disorder with a presumed neuro-developmental origin (Ross et al., 2006). The spectrum of symptoms includes (i) positive symptoms, such as delusions, hallucinations, and disorganized thinking, (ii) negative symptoms, such as anhedonia and social withdrawal, and (iii) cognitive symptoms, characterized by working memory and executive deterioration (van Os and Kapur, 2009). Existing pharmacotherapy and conventional models have long emphasized dopaminergic (DA) dysfunction as the prime neurochemical basis of schizophrenia, building upon the pharmacological evidence that striatal DA hyperfunction seems associated with the positive symptoms (Snyder, 1976). However, the limitations of the DA model to account for negative and cognitive symptoms led to the

emergence of glutamatergic models, based on the observation that psychotomimetic agents such as phencyclidine (PCP) and dizocilpine (MK-801) induce psychotic and cognitive disturbances in human and animals similar to those observed in schizophrenic patients by blocking N-methyl-D-aspartate receptors (NMDA-R) (Moghaddam and Javitt, 2012). In fact, cortical glutamatergic hypofunction is well positioned to explain not only the positive and negative symptoms, but also the cognitive decline that is at the premorbid stage of schizophrenia (Ranganath et al., 2008). This seems to result from the ability of NMDA-R to control the impaired interactions between multiple brain regions, which are a hallmark of the schizophrenic brain (Lisman et al., 2008; Field et al., 2011), rather than a particular locus of dysfunction. A modified astrocytic function emerges as an attractive mechanism to bridge several findings related to schizophrenia, since astrocytes are modified in the schizophrenic brains (Takahashi and Sakurai, 2013; Schnieder and Dwork, 2011), they control the glutamatergic system through the uptake of glutamate (Anderson and Swanson, 2000) and they control integrated brain responses (Halassa and Haydon, 2010). Notably, an up-regulation of glutamate transporter 1 (GLT-I) mRNA, protein and function has been consistently shown in the cortex of schizophrenic patients (Simpson et al., 1998; Smith et al., 2001; Matute et al., 2005; Rao et al., 2012). Also, psychotomimetics, such as PCP, increase GLT-I levels and activity (Fattorini et al., 2008) whereas the anti-psychotic drug clozapine induces the reverse (Melone et al., 2003; Vallejo-Illarramendi et al., 2005).

Adenosine is an endogenous neuromodulator able to control both DA and glutamate signaling and exerts potent inhibitory or stimulatory influences on synaptic activity through activation of adenosine A_1 or A_{2A} receptors ($A_{2A}R$), respectively (Fredholm et al., 2005). Adenosine is proposed to play a key integrative role in schizophrenia (Boison et al., 2012; Shen et al., 2012). Thus, striatal $A_{2A}R$ activation and extra-striatal $A_{2A}R$ blockade are proposed to confer antipsychotic action against DA hyperfunction or NMDA-R hypofunction (Rimondini et al., 1997; Shen et al., 2008; Shen et al. 2012). In addition, astrocytic $A_{2A}R$ tightly regulate GLT-I

activity (see chapter 2 or Matos et al., 2012b) unraveling another potential link to the pathogenesis of schizophrenia. Indeed, we now present a novel mechanism linking $A_{2A}R$ in astrocytes and its control of GLT-I activity with an array of altered pathological hallmarks characteristic of schizophrenia, namely a decrease of working memory performance and an enhanced psychomotor response to MK-801; we also report that the observed glutamatergic dysfunction in GFAP-$A_{2A}R$-KO mice was associated with an alteration of NMDA-NR2B-mediated GluR1/GluR2 subunit membrane trafficking. Thus the genetic deletion of astrocytic $A_{2A}R$ causes drastic modifications of the glutamatergic system leading to the emergence of schizophrenia endophenotypes. This brings to the center stage the critical role of the astrocytes-to-neuron communication in cognitive processes affected upon schizophrenia.

3. Material and Methods

3.1. Animals

CaMK2-α gene promoter-driven forebrain $A_{2A}R$ knockout (Fb-$A_{2A}R$-KO) mice were previously characterized (Bastia et al., 2005; Yu et al., 2008; Wei et al., 2011). GFAP gene promoter-driven $A_{2A}R$ knock out (Gfa2-$A_{2A}R$-KO) mice were generously provided by Michael A. Schwarzschild (Massachusetts General Hospital Charlestown, Massachusetts, USA) and generated using the Cre/loxP system as previously described (see chapter 2 or Matos et al., 2012b). The GFAP-Cre line was obtained from David Gutmann (Department of Neurology, Washington University School of Medicine, St. Louis, Missouri, Bajenaru et al., 2002) using the gfa2 transgene construct. The transgene construct consists of the 2.2-kb fragment of the human glial fibrillary acidic protein (GFAP) promoter (Gfa2) (obtained from M. Brenner, National Institute of Neurological Disorders and Stroke) and the encephalomyocarditis virus IRES, the cDNA encoding the nucleus-targeted Cre recombinase (Lee et al., 2006). The 55 bp segment of the gfa2 promoter, spanning bp 21488 to 21434 with respect to the RNA start site has been shown to contain a 45 bp sequence spanning bp 21443 to 21399 required for silencing

expression in neurons. Thus, the specific Gfa2 promoter, in opposition to other GFAP promoter constructs, has been elegantly shown as astrocyte specific in all CNS regions (for characterization see Lee et al., 2008). Briefly, both transgenic *Gfa2-cre* mice (Bajenaru et al., 2002) and mice carrying the ''floxed'' $A_{2A}R$ gene ($A_{2A}^{flox/flox}$) (Bastia et al., 2005) were back-crossed for 10-12 generations to C57Bl/6 mice (Charles River; Wilmington, MA). *Gfa2-cre* mice were then crossed with non-transgenic (no *cre*) $A_{2A}^{flox/flox}$ mice to generate Gfa2-$A_{2A}R$-KO mice and Gfa2-$A_{2A}R$-WT littermates. Mice were maintained under controlled environment (23 ± 2℃; 12 h light/dark cycle with *ad libitum* access to food and water) and handled according to the Animal Care and Use Committee at Boston University School of Medicine and the NIH Guide for the Care and Use of Laboratory Animals (1982).

3.2. Preparation of total membranes

Mice were euthanized by decapitation after deep anesthesia with isoflurane and cortical brain tissue was collected and homogenized in sucrose (0.32 M) solution [containing 1 mM EDTA, 10 mM HEPES, 1 mg/mL bovine serum albumin (BSA; Sigma-Aldrich, USA), pH 7.4] at 4 ºC. The homogenates were centrifuged at 3,000 *x g* for 10 min at 4 ℃ and the resulting supernatants were centrifuged again at 14,000 *x g* for 10 min at 4 ℃. The pellets were washed in Krebs-HEPES-Ringer (KHR) solution containing 140 mM NaCl, 1 mM EDTA, 10 mM HEPES, 5 mM KCl, 5 mM glucose and pH 7.4 at 4 ºC and further centrifuged at 14,000 *g* for 10 min at 4 ºC. Depending on the experimental aim, the pellets were resuspended either in the specific incubation buffer or in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) with protease inhibitor cocktail (CLAPS, composed by 10 µg/mL of chymostatin, leupeptin, antipain and pepstatin A; from Sigma-Aldrich, USA) and the protein content measured with the BCA assay (Pierce Technology, USA).

3.3. Preparation of gliosomes and synaptosomes

After the homogenization of the cortical brain tissue, purified synaptosomes and gliosomes were obtained using a discontinuous Percoll gradient (2, 6, 15, and 23% v/v of Percoll in a medium containing 0.32 M sucrose and 1 mM EDTA, pH 7.4), as previously described (see chapter 2 or Matos et al., 2012b). The layers between 2 and 6% of Percoll (gliosomal fraction) and between 15 and 23% of Percoll (purified presynaptic nerve terminal - synaptosomal fraction) were collected, washed in 10 mL of HEPES-buffered medium containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES and pH 7.4, and further centrifuged at 22,000 *g* for 15 min at 4 ºC to remove myelin components and postsynaptic material from the gliosomal and synaptosomal fractions, respectively. Crude synaptosomes were prepared after several differential centrifugations of the brain homogenate in a 45% Percoll solution at 4 ºC, as previously described (Rodrigues et al., 2005). The fractions were resuspended in RIPA buffer for Western blot analysis, whereas for functional glutamate uptake and release experiments, the fractions were re-suspended in Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 6 glucose, 10 HEPES, 1 $CaCl_2$, pH 7.4) or NMG buffer (where NaCl is isosmotically replaced by N-methylglucamine-NMG).

3.4. D-[^{3}H] aspartate uptake

The uptake analysis of the non-metabolizable glutamate analogue D-[^{3}H]aspartate in gliosomes and synaptosomes was carried out as previously described (Matos et al., 2012a and b). Briefly, the gliosomal and synaptosomal fractions were diluted in Krebs or NMG buffer (with excess for BCA protein determination) and left to equilibrate at 37 ℃ for 10 min. Afterwards, 150 µL triplicates of both fractions were added to 150 µL of loading Krebs or NMG medium containing a final concentration of 50 nM D-[^{3}H] aspartate (specifc activity: 11.3 ci/mmol; PerkinElmer, USA). The mixtures were incubated for 10 min at 37 ℃ and the reaction terminated by rapid layering of the mixture on onto glass microfibre filters Whatman GF/C (GE

Healthcare, USA) placed on a vacuum pump filtration system and further washed 3 times with ice-cold NMG buffer. Filters were dried overnight, drenched in 2 mL of liquid scintillation cocktail (PerkinElmer, USA) and counted on a LKB Wallac 1219 Liquid Scintillation Counter (Wallac, Finland). The specific D-[^{3}H]aspartate uptake transport was calculated by subtraction from the total uptake of the non-specific uptake measured in a Na^+-free medium (NMG) in the presence of 100 nM of the glutamate transporter inhibitor dihydrokainate (DHK; Tocris, UK).

3.5. L-[^{3}H] glutamate release

L-[^{3}H] glutamate release from synaptosomes and gliosomes was measured as previously described (Milanese et al., 2009), with modifications. Synaptosomal and gliosomal preparations were incubated in a non-depolarizing medium (low K^+) consisting of KHR (HEPES 10 mM, NaCl 135 mM, KCl 5 mM, EDTA 1 mM, glucose 5 mM) with 200 nM L-[^{3}H]glutamate (specifc activity: 49 ci/mmol; PerkinElmer, USA) for 15 min**,** at 37 ℃. One aliquot was taken for measuring total L-[^{3}H]glutamate in the beginning of the experiment. Labeled gliosomes and synaptosomes were then centrifuged at 13,000 × *g* for 1 min**,** at 4 ℃. The supernatants were discarded and the pelleted gliosomal or synaptosomal membranes resuspended again in 300 µL of two types of buffer for 90 s, at 37 ℃:

i) for basal L-[^{3}H]glutamate release a physiological K^+ and Ca^{2+} buffer (HEPES 10 mM, NaCl 135 mM, KCl 5 mM, $CaCl_2$ 0.1 mM, glucose 5 mM) was used; and

ii) for evoked L-[^{3}H]glutamate release a high K^+ (NaCl decreased accordingly) and Ca^{2+} buffer (HEPES 10 mM, NaCl 100 mM, KCl 40 mM, $CaCl_2$ 1.2 mM, glucose 5 mM) was used, in the absence (control) or presence of the $A_{2A}R$ agonist CGS21680 (100 nM; Tocris, USA).

The mixtures were then immediately centrifuged at 16,000 *x g* for 1 min at 4℃ and the radioactivity present in supernatants was measured in a liquid scintillation analyzer. The released L-[^{3}H]glutamate was calculated as a percentage of the total amount of radiolabel in the gliosomal and synaptosomal preparations at the start of the incubation period.

3.6. Biotinylation assay of surface receptors

The density of GluR1/GluR2 subunits of AMPA-R present at the surface of the plasma membrane of synaptosomes was estimated using a biotinylation assay, as previously described (Schenk et al., 2003; Kim et al., 2007). Membranes from crude cortical synaptosomes were prepared as previously described (Rodrigues et al., 2005). After three washes with ice-cold KHR, 10% of the synaptosomal sample was stored for total protein quantification and the remaining was incubated with 1 mg/mL EZ-Link Sulfo-NHS-LC-Biotin (Thermo-Scientific, USA) for 30 min, at 4 ℃. To remove and inactivate biotin residues, the synaptosomes were centrifuged (14000 rpm for 5 min) and washed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO_4, 1.47 mM KH_2PO_4, pH 7.4) containing 50 mM glycine. The biotinylated proteins in the pellet were then extracted using a RIPA buffer supplemented with protease inhibitors (CLAPS) for 1 h. Triton X-100-insoluble material was removed by centrifugation at 700 *x g* for 3 min. The isolation of biotinylated proteins was then performed with 60 µL of Ultra-link immobilized Streptavidin Resin beads (Pierce, USA) on a rotator overnight at 4 ℃. Beads were washed three times and bound biotinylated proteins eluted with a SDS sample buffer. The samples were then boiled at 95-100 ℃ for 5 min (to denature the proteins and separate them from the streptavidin beads) and then centrifuged at 2.500 rpm for 5 min. The supernatants with biotinylated surface proteins and the total protein stored at the beginning of the experiment were then analyzed by Western blotting.

3.7. Western blotting

Western blotting of gliosomal, synaptosomal and total membranes extracts was performed as previously described (Rodrigues et al., 2005; Matos et al., 2012b). Incubation with the primary antibodies, namely anti-GFAP (1:1000; Santa Cruz Biotechnology, USA), anti-GLAST/EAAT1 (1:1000; Abcam, USA), anti-GLT-I/EAAT2 (1:1000; Millipore, USA), anti-NMDA-NR1 (NR1) (1:1000; Millipore, USA), anti-NMDA-NR2A (NR2A) (1:500; Millipore, USA), anti-

NMDA-R2B (NR2B) (1:1000; Abcam, USA), anti-AMPA-GluR1 (1:1000; Abcam, USA), AMPA-GluR2 (1:1000; Millipore, USA) and anti-β-actin (1:5000, Sigma-Aldrich, USA), all diluted in Tris-buffered saline (137 mM NaCl, 20 mM Tris–HCl, pH 7.6) with 0.1% Tween (TBS-T) and 3% BSA (fatty acid free), was carried out overnight at 4 ℃. After washing with TBS-T, the membranes were incubated with appropriate IgG secondary antibodies for 2 h at room temperature. After washing, the membranes were revealed using an ECF kit (*ECL,* Amersham*, USA)* and visualized under a fluorescence LAS-4000 digital imaging system (Fujifilm, USA). The densiometric analysis of protein bands was performed using Quantity One software version 4.4.1 (Bio-Rad, USA).

3.8. Working memory analysis

The working memory of Gfa2-A_{2A}R-KO mice was assessed using two approaches: the Y-maze spontaneous alternation test and the 8 baited-arms radial arm maze (RAM) test.

The Y-maze spontaneous alternation test was performed as previously described before (Duarte et al., 2012). Individual mice were placed at the end of one arm and freely explored the maze for 5 min. The sequence of entrance in each arm was recorded and the number of alternations (entrance in the 3 different arms sequentially) was quantified. The percentage of spontaneous alternation consists in the percentage of alternations in the total possible alternations (total number of arms' changes minus 2).

The 8 baited RAM test was carried out in mice were maintained on a food deprivation regime, which was gradually introduced with a progressive reduction in the daily available food, until the animals reached a stable weight of not less than 85% of the *ad libitum* weight. The RAM had 8 identical and equally spaced arms (56 cm long, 12 cm wide) radiating from a central octagonal platform (side-length = 12 cm). The mice were exposed to the maze for 5 min each day with a food reward at the end of each arm. The habituation was performed until the animals were able to finish the task within 5 min. Afterwards, 6 test trials divided by 6 days were

performed in which the 8 arms were set with a food reward and the mice were able to freely explore the maze until they ate the 8 food rewards, with a maximal time limit of 10 min. Working memory errors were defined as re-entries into previously visited arms and the global performance indexed by the ‘‘number of correct choices until the first error’’, as previously described (Singer et al., 2012).

3.9. Reference memory analysis

Spatial reference memory was assessed using the “forced-trial” in a modified Y-maze, as previously described (Cognato et al., 2010; Singer et al., 2012) to measure the innate tendency of mice to recognize spatial novelty, aided by spatial cues. The test consisted of two phases separated by a variable time interval (delay). Each animal was assigned two arms (start arm and familiar arm) to which they were exposed during the acquisition phase. The remaining third arm constituted the novel arm to be used in the second phase (retrieval test phase). Numerous visual cues were placed on the walls of the testing room and were kept constant throughout the behavioral test. The assay consisted of two 5-min trials separated by a 30 min interval. In the first trial (acquisition), one arm of the Y-maze was closed with a door and the mice were released from the end of the start arm facing the centre of the maze. After entering the familiar arm the animal was allowed to freely explore both the start and familiar arms for 5 min for 5 min. During the second trial (retrieval), animals had free access to the three arms and were allowed to explore the maze for another 5 min. The time and number of entrances in each arm was recorded and the percentage of time and entrances in the “novel arm” quantified.

3.10. Locomotion in home cage

Horizontal locomotor activity was assessed in polypropylene cages that were placed into adjustable frames equipped with seven infrared photocell beams, recorded, and analyzed on a computer (San Diego Instruments, San Diego, CA). Ambulation was quantified as the number of sequential breaks in adjacent beams. All mice were habituated to the test cages for at least 120

min before recording basal locomotion for 60 min in 5-min bins. To assess the motor-stimulatory effect of NMDA-R antagonist dizocilipine (MK-801; Sigma-Aldrich, USA) the animals were injected intraperitoneally at an efficient dose (0.5 mg/kg), and the motor activity recorded for the next 3 hours (see Chen et al., 2000).

3.11. Drug administration

The prototypical non-transportable and selective GLT-I/EAAT2 inhibitor dihydrokainic acid (DHK; 10 mg/kg, i.p) was administrated 30 min before the injection of MK-801 or the performance of memory tasks, and compared with vehicle (i.p., 75% saline, 15% DMSO, 10% castor oil) injected mice. The dose of DHK was derived from previously published behavioral data (Namura et al., 2002; Chu et al., 2007; John et al., 2012).

The selective $A_{2A}R$ antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-*e*]-1,2,4-triazolo [1,5-*c*]pyrimidine (SCH58261; Tocris, UK) was injected intraperitoneally at an efficacious dose (0.1 mg/kg) (Canas et al., 2009), in vehicle solution and applied 30 min before killing the mice to prepare gliosomes and synaptosomes. The $A_{2A}R$ agonist [[6-amino-9-(N-ethyl-β-D-ribofuranuronamidosyl)-9H-purin-2yl]amino]ethyl]benzene propanoic acid hydrochloride (CGS 21680, Tocris, USA) was added to synaptosomes and gliosomes to reach a final concentration of 50 nM 30 min before the L-[^{3}H]glutamate release studies, as previously described (Matos et al., 2012b).

The synthetic peptide (Tat-Glur2_{3Y}) (generously provided by Yu Tian Wang, University of British Columbia, Vancouver, Canada) contains tyrosine residues that block the phosphorylation-mediated endocytosis of AMPA-R. Previous studies showed that Tat-Glur2_{3Y} blocks the regulated AMPA-GluR2 endocytosis without affecting constitutive AMPA-R endocytosis (Wong et al., 2007; Ge et al., 2010). Since Tat-GluR2_{3Y} reaches peak concentrations in the brain 90 min after injection, Tat-GluR2_{3Y} or the scrambled control peptide,

Tat-GluR2$_{3S}$, dissolved in 0.9% NaCl, were injected in mice (3 µmol/kg, i.p.) 60 min before performance of memory task and 90 min before the administration of MK-801.

3.12. Statistical analysis

Data are mean ± S.E.M. of the number of experiments indicated in the figure captions. Parametric analysis of variance (ANOVA) was used to determine statistically significant differences. To further delineate the nature of significant outcomes, we conducted *post hoc* analyses to subsets of the data included in the overall ANOVA, or pair-wise comparisons based on the associated error terms taken from the overall ANOVA. All data were analyzed using GraphPad Prism software (Version 5.0, GraphPad, USA).

4. Results

4.1. Opposite modulation of MK-801-induced psychomotor activity by astrocyte *versus* forebrain neuronal A_{2A}R

We first aimed to characterize the impact on psychomotor activity of A_{2A}R deletion in astrocytes using astrocyte-specific Gfa2-A_{2A}R KO mice, which was contrasted with the known impact of A_{2A}R deletion in neurons, using the previously characterized forebrain neuronal-specific Fb-A_{2A}R-KO mice (Bastia et al., 2005; Shen et al., 2008). To produce a selective deletion of A_{2A}Rs in astrocytes, the GFAP-Cre line was obtained using the gfa2 transgene containing the 2.2-kb fragment of the human glial fibrillary acidic protein (GFAP) promoter (Gfa2) (Lee et al., 2006 and 2008), which encompasses a 45 bp sequence spanning 21443 to 21399 required for silencing expression in neurons (see experimental procedures for details). After habituation to the activity cages, Gfa2-A_{2A}R KO, Fb-A_{2A}R-KO and their corresponding WT littermate mice received a saline injection (control) and their motor activity was recorded for 1 hour; motor activity was then recorded for 3 additional hours after MK-801 administration, which has been consistently used to model schizophrenic traits in animal models, based on the notion

that the enhanced motor activity triggered in rodents is an faithful indicator of the propensity of a drug to elicit or exacerbate psychosis in humans (Moghaddam and Javitt, 2012).

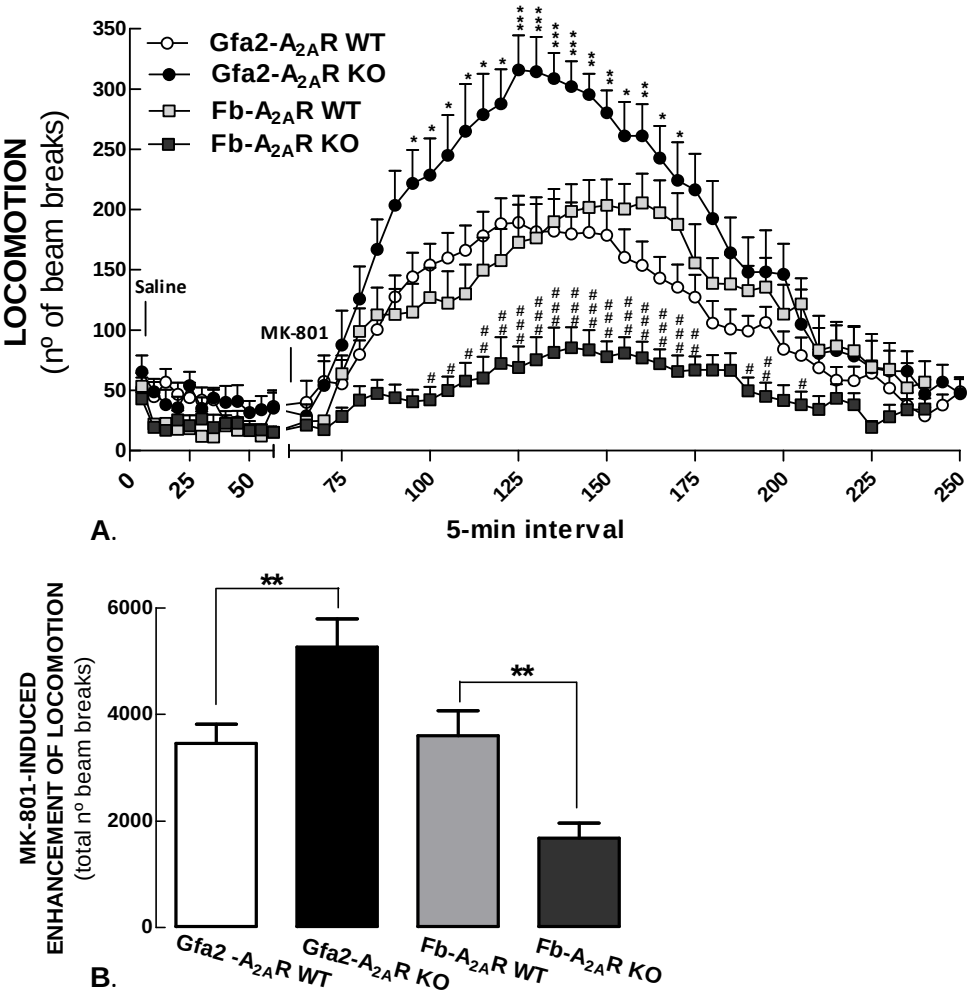


Fig.1. - **MK-801-induced psychomotor activity is increased in Gfa2-A_{2A}R-KO but attenuated in Fb-A_{2A}R-KO mice**. All mice were habituated to the activity cages for at least 2 h (data not shown). **(A)** The data show the 4-h period of recording of locomotor activity. Mice first received a saline injection (i.p.) and activity recorded for 1 h. Then mice received an injection of MK-801 (0.5 mg/kg, i.p.) and activity was recorded for another 3 h. MK-801 administration produced a marked increase of locomotor activity compared to saline injection alone in all mice. Notably, Gfa2-A_{2A}R-KO mice showed a significantly higher peak response than Gfa2-A_{2A}R-WT mice; in contrast, MK-801 caused a significantly lower increase of locomotor activity in Fb-A_{2A}R-KO than in Fb-A_{2A}R-WT mice. Data points plotted are 5-min bin interval means. **(B)** The bar graph show the grouped analysis of the total locomotor activity in the MK-801 post-injection period (75-200 min), confirming that the selective deletion of astrocytic A_{2A}Rs *versus* neuronal A_{2A}Rs produced opposite effects on the MK-801-induced hyperlocomotor response. Values represent mean ± SEM and statistical difference was assessed with a two-way ANOVA and Bonferroni post-hoc analysis with n = 12 per group. *p<0.05, **p<0.01, ***p<0.001, Gfa2-A_{2A}R-WT *vs* Gfa2-A_{2A}R-KO and $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001, Fb-A_{2A}R-WT *vs* Fb-A_{2A}R-KO.

As depicted in Figure 1A, MK-801 injection increased locomotor activity in all mouse lines whereas no differences were found between the groups after saline injection. However, the effect of MK-801 was more pronounced in Gfa2-A_{2A}R KO mice compared to Gfa2-A_{2A}R-WT mice, as confirmed by a 2 x 46 (Genotype × 5-minute Bins) Two-way ANOVA of the number of beam breaks, which yielded a significant effect of bins ($F_{(59,1086}$=31.73, p<0.0001, n=12) and its interaction with genotype ($F_{(59,1086)}$=2.53, p<0.0001, n=12). MK-801 injection also triggered hyperlocomotion in both Fb-A_{2A}R-KO and Fb-WT mice (**Fig. 1A**), with a significant effect of bins ($F_{(47,888)}$=13.56, p<0.0001, n=12). Notably, A_{2A}R deletion in neurons resulted in an opposite phenotype to A_{2A}R deletion in astrocytes, since Fb-A_{2A}R-KO mice displayed a lower MK801-induced hyperlocomotor response when compared to Fb-WT mice (interaction with genotype: $F_{(47,888)}$=3.62, p<0.0001, n=12). One-Way ANOVA of the total number of beam breaks in the MK-801 post-injection period for each group (**Fig. 1B**) corroborated this conclusion: Gfa2-A_{2A}R-KO mice showed an exacerbated response to MK-801 (p<0.01, n=12) whereas Fb-A_{2A}R-KO had lower psychomotor response to MK-801 (p<0.01, n=12), compared with their corresponding WT littermates. These data show that astrocytic and forebrain neuronal A_{2A}Rs exert an opposite control of psychomotor activity, and indicate a requirement for astrocytic A_{2A}Rs, but not of neuronal A_{2A}Rs to avoid exacerbated responses to psychosis-inducing drugs.

4.2. Spatial working memory is impaired in Gfa2-A_{2A}R-KO, in contrast to Fb-A_{2A}R-KO mice

Impairment of working memory (WM) performance is a key endophenotype of schizophrenia (Amann et al., 2010), which is more strongly related to the functional impairment of schizophrenics than positive symptoms and other neuropsychological traits (Van Snellenberg, 2009). Since A_{2A}R activity may contribute to the modulation of learning and memory and neuronal A_{2A}Rs control (inhibit) WM performance (Zhou et al., 2009; Wei et al., 2011), we next investigated if spatial WM performance was also affected upon selective deletion of astrocytic

A_{2A}Rs. To exclude the possibility that potential WM alterations might result from non-specific motor deficits or impairment of sensory perception and/or memory of spatial cues, we first evaluated the reference memory of Gfa2-A_{2A}R-KO mice. As depicted in Figure 2 (**A**, **B**), no alterations were found in the performance of reference memory between Gfa2-A_{2A}R-KO and WT mice, both spending a similar time exploring (**Fig. 2A**) and entering (**Fig. 2B**) the novel arm in a modified Y-maze test. However, when analyzing the WM score in a conventional Y-maze spontaneous alternation paradigm (**Fig. 3A**), a significant impairment was found in Gfa2-A_{2A}R-KO mice compared to their corresponding WT mice (n=20/group, $p<0.01$), with no change of locomotion (**Fig. 3B**). WM was also assessed in a more sensitive test using an 8-baited RAM (**Fig. 3E, F**). In this task, the total WM error score during the 6 trials was significantly higher ($p<0.01$, n=10) in Gfa2-A_{2A}R-KO compared to WT mice. This indicates that astrocytic A_{2A}R deletion lead to a selective impairment of WM, the opposite impact resulting from the deletion of neuronal A_{2A}Rs (Zhou et al., 2009; Wei et al., 2011).

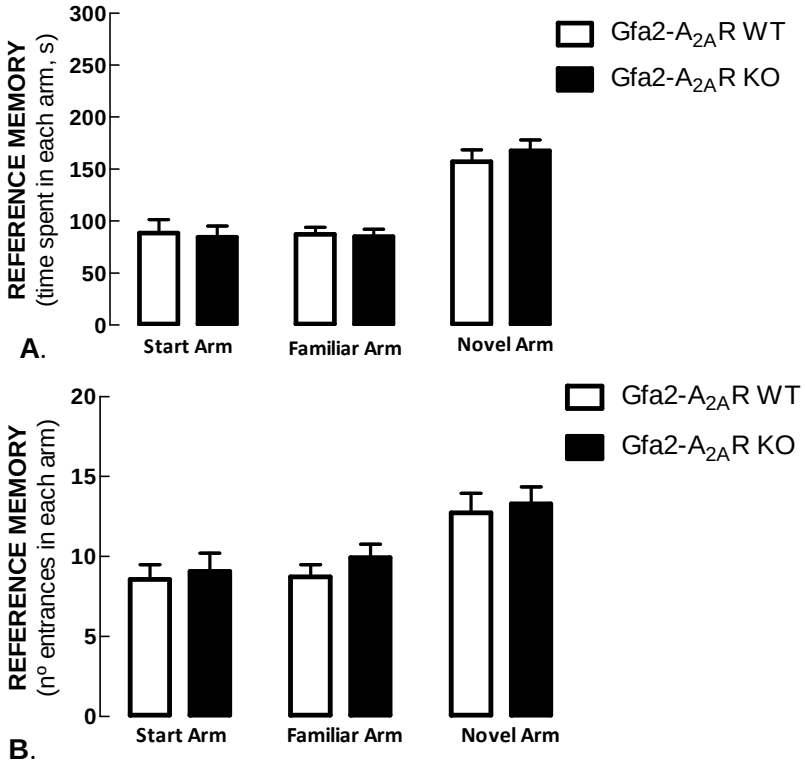


Fig.2 - **Reference memory is not affected in Gfa2-A_{2A}R-KO mice.** Reference memory performance was gauged by the time spent **(A)** or by the number of entrances **(B)** in the novel arm in a forced Y-maze paradigm, which was not modified in Gfa2-A_{2A}R-KO mice. Statistical difference was assessed using Tukey's post-hoc test applied after One-Way ANOVA and the values are the mean ± S.E.M. of experiments with n = 10-18 per group.

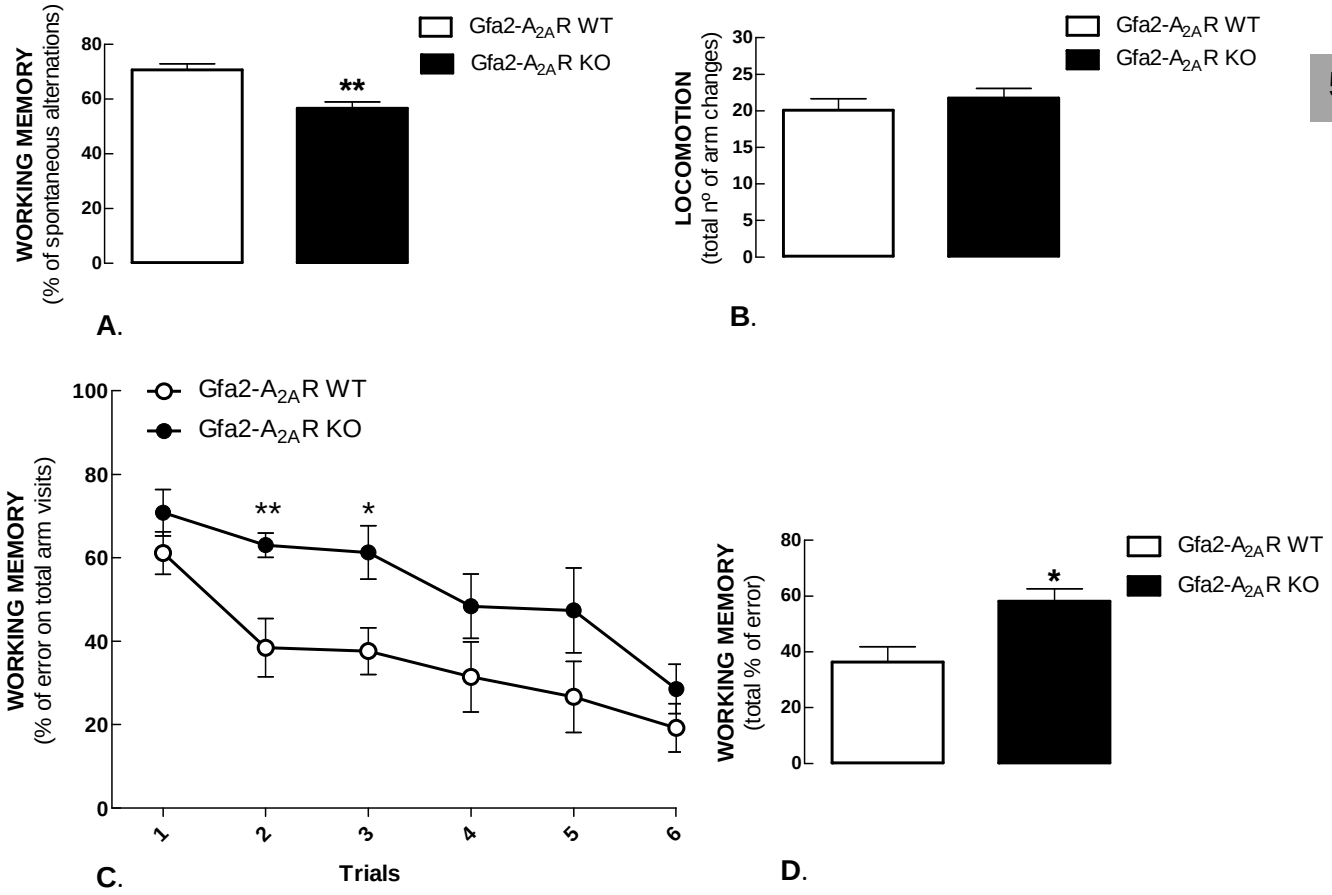


Fig.3 - **Spatial working memory performance is impaired in Gfa2-A_{2A}R-KO mice. (A)** Gfa2-A_{2A}R-KO mice display a spatial working memory deficit in a classical Y-maze paradigm. **(B)** Gfa2-A_{2A}R-KO mice show an identical basal locomotion when compared with the WT littermates, in the classical Y-maze paradigm. (**C, D)** Working memory performance was consistently impaired in Gfa2-A_{2A}R-KO compared to WT mice, in an 8-baited arms RAM test. Working memory performance in this paradigm was indexed to the number of correct choices until the first error. The data in **(C)** show the time course experiment for 6 consecutive trials and **(D)** displays the grouped analysis of the total number of errors for the entire 6-trial experiment. Statistical difference was assessed using Tukey's post-hoc test applied after One-Way ANOVA and the values are the mean ± S.E.M. of experiments with n = 10-18 per group. *p<0.05, **p<0.01, Gfa2-A_{2A}R-WT Gfa2-A_{2A}R-KO.

4.3. Glutamate uptake is increased in Gfa2-A_{2A}R-KO but not in Fb-A_{2A}R-KO mice

The modification of memory performance has been associated with alterations of glutamatergic function, which is also dependent on the astrocytic clearance of extracellular glutamate that is known to be affected in schizophrenia (*e.g*. Matute et al., 2005). Since we have previously shown that astrocytic A_{2A}Rs control both the activity and the expression of GluTs (Matos et al., 2012b), we next compared glutamate uptake capacity in Gfa2-A_{2A}R-KO and Fb-A_{2A}R-KO mice and in their corresponding WT littermates. The uptake of D-[^{3}H]aspartate was significantly increased in gliosomes (astrocyte-enriched plasmalemmal vesicles) from Gfa2-A_{2A}R-KO mice (175.00±12.00% *vs* WT mice; $p<0.001$, n=6) but not from Fb-A_{2A}R-KO (**Fig. 4A, B**) or in synaptosomes (purified nerve terminals) from both transgenic lines (**Fig. 4C**, **D**). The key role of astrocytic A_{2A}Rs in this modified glutamate uptake by gliosomes selectively observed in Gfa2-A_{2A}R-KO mice was further strengthen by the observation that the selective A_{2A}R antagonist, SCH58261 (100 nM), increased (167.70±8.83%; $p<0.05$, n=6) the uptake of glutamate by gliosomes from Fb-A_{2A}R-KO mice (**Fig. 4B**) but was devoid of effects in gliosomes from Gfa2-A_{2A}R-KO mice (**Fig. 4A**).

In accordance with these functional observations, Western blot analysis of total membranes showed that the density of GLT-I was significantly increased (138.10±4.40%; $p<0.001$, n=6) in Gfa2-A_{2A}R-KO *vs* WT mice (**Fig. 4E**), with no modifications of the densities of either glutamate-aspartate transporter (GLAST) (**Fig. 4F**) or the astrocytic marker glial fibrillary acidic protein (GFAP) (**Fig. 4G**).

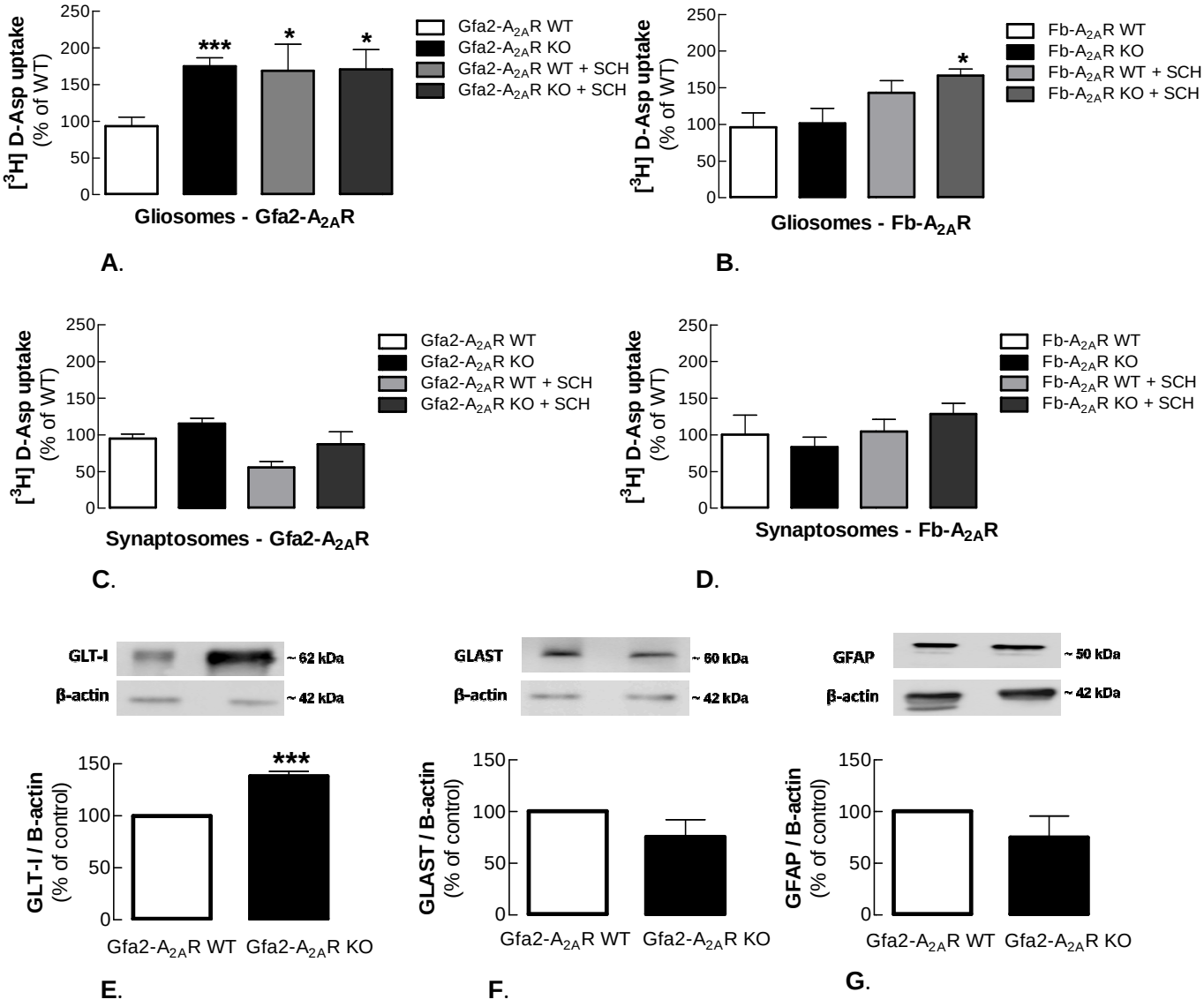


Fig.4 - **Glutamate uptake is selectively increased in cerebral cortical gliosomes from Gfa2-A_{2A}R-KO mice (A) but not from Fb-A_{2A}R KO mice (B) or in nerve terminals (C, D)**. Gliosomes and synaptosomes from Gfa2-A_{2A}R-KO, Fb-A_{2A}R-KO and corresponding WT mice were prepared after acute (30 min) administration of vehicle (i.p.) or SCH 58261. Then, glutamate uptake was estimated by assaying D-[^{3}H]aspartate uptake for 10 min. The hypothesis of an astrocytic specific mechanism of A_{2A}R regulation of glutamate uptake is corroborated by the observation that glutamate uptake was not modified in gliosomes (**B)** or in synaptosomes (**D)** from Fb-A_{2A}R-KO mice. GLT-I density **(E)** was also significantly increased in total membranes from the cerebral cortex of Gfa2-A_{2A}R-KO (black columns) compared to WT mice (white columns), with no difference in GLAST **(F)** or GFAP **(G)**. Bars **(E-G)** represent the relative levels of each primary antibody with anti-β actin immunoreactivity and were expressed as percentage of WT littermates. Data are mean ± SEM of at least 6 independent experiments. Statistical differences were gauged using the Tukey's post-hoc test applied after One-Way ANOVA with *p<0.05, **p<0.01, ***p<0.001, compared with naive WT mice.

4.4. Selective GLT-I inhibition prevents the exacerbation of MK-801-induced psychomotor activity and reverses the working memory deficits in Gfa2-A_{2A}R-KO mice

To test the role of this hyperactive glutamate uptake in the cognitive impairment observed in the Gfa2-A_{2A}R-KO, we tested the effect of dihydrokainate (DHK), a selective inhibitor of GLT-I with low affinity for ionotropic glutamate receptors (James et al., 1980; Anderson and Swanson, 2000). As previously described (see **Fig. 1**), MK-801 caused a higher locomotor activity in Gfa2-A_{2A}R-KO than in WT mice (**Fig. 5A, B**). Notably, the administration of DHK (10 mg/kg, ip) 30 min before MK-801 blunted the exacerbated psychomotor response to MK-801 in Gfa2-A_{2A}R-KO mice, which became similar to that of WT mice, whereas DHK was devoid of effects in WT mice (**Fig. 5A, B**). Indeed, ANOVA analysis (DHK treatment × 5-minute Bins) showed a significant attenuated response to MK-801 in Gfa2-A_{2A}R-KO mice treated with DHK, when compared with Gfa2-A_{2A}R-KO mice treated with vehicle (interaction with DHK treatment: $F_{(45,683)}$=2.47, p<0.0001, n=12). One-Way ANOVA of the total number of beam breaks in the MK-801 post-injection period confirmed this conclusion (**Fig. 5B**): Gfa2-A_{2A}R-KO mice displayed a significant exacerbated response to MK-801 (p<0.05, n=12) when compared to their corresponding WT littermates, whereas DHK-treated Gfa2-A_{2A}R-KO mice had a significant attenuation of the exacerbated psychomotor response to MK-801 (p<0.05, n=11). These data indicate that altered levels of extracellular glutamate resulting from GLT-I up-regulation are involved in the exacerbated response of Gfa2-A_{2A}R-KO mice to the psychosis-inducing drug MK-801.

We next tested the impact of DHK on the previously observed WM impairment found in Gfa2-A_{2A}R-KO mice (see **Fig. 3**). As shown in Figure 5C, *post hoc* pair-wise comparisons between Gfa2-A_{2A}R-KO and WT mice treated or not with DHK (10 mg/kg, ip), showed that the WM impairment observed in Gfa2-A_{2A}R-KO mice (p<0.01, n=20) was prevented by DHK (p<0.001, n=10), whereas DHK was devoid of effects in Gfa2-A_{2A}R-WT mice (p=0.41). This

further indicates that the deregulated astrocytic glutamate transport is causally associated with the cognitive impairment found in Gfa2-A_{2A}R-KO mice (**Fig.5 C**).

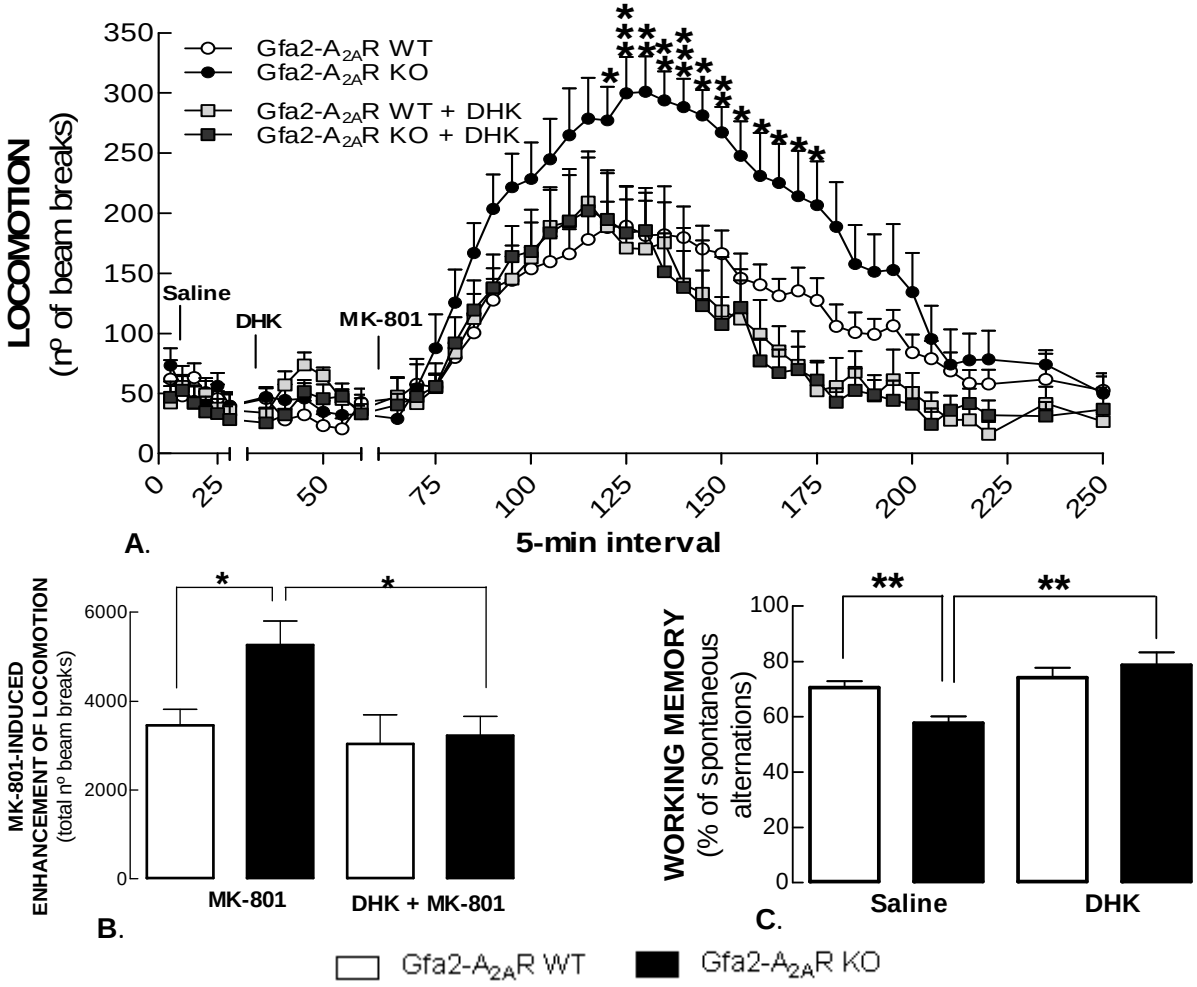


Fig.5 - **The selective inhibitor of the glutamate transporter GLT-I, DHK, prevents the exacerbation of MK-801-induced psychomotor activity and reverses the working memory impairment in Gfa2-A_{2A}R-KO mice**. **(A)** The course experiment (4 h) to assess locomotor activity, where all the mice were first habituated in the test cages for 120 min before receiving a saline injection (i.p.). Then DHK was administered 30 min before MK-801 and activity was recorded for another 3 h. As expected, MK-801 increased locomotor activity, an effect greater in Gfa2-A_{2A}R-than Gfa2-A_{2A}R-WT mice. However, DHK blunted the exacerbated response of Gfa2-A_{2A}R-KO mice, while it was devoid of effects in Gfa2-A_{2A}R-WT mice. **(B)** The bar graph displays the grouped analysis of the total locomotor activity in the MK-801 post-injection period (75-200 min), showing the complete blockade by DHK of the enhanced psychomotor response to MK-801 in Gfa2-A_{2A}R-KO mice. **(C)** The observed impairment of the working memory performance, assessed as the spontaneous alternation in a Y-maze test, that was observed in Gfa2-A_{2A}R-KO mice, was completely restored by the previous acute (30 min) administration of DHK. Data is mean ± S.E.M. of the percentage of alternations in the total possible alternations (total number of arms' changes minus 2). The statistical differences were assessed using Tukey's post-hoc test applied after One-Way ANOVA with n= 8-12 per group. *p<0.05, **p<0.01, comparing the columns indicated in the figure.

4.5. The evoked glutamate release from nerve terminals is increased in Gfa2-A_{2A}R-KO mice

5

Since the dynamics of the glutamatergic system depends on the clearance but also on the production of extracellular glutamate, we next investigated if Gfa2-A_{2A}R-KO mice displayed a modified pattern of basal- and K^+-stimulated L-[^{3}H]glutamate release from synaptosomes (*i.e.* as a neurotransmitter) and from gliosomes (*i.e*. as a gliotransmitter). The depolarization of either gliosomes (**Fig. 6A**) or synaptosomes (**Fig. 6B**) triggered an increased outflow of glutamate in accordance with the known ability of synaptosomes to sustain a vesicular release of glutamate (Nicholls et al., 2003) and gliosomes to release glutamate through vesicular release and glutamate transport reverse activity (Milanese et al., 2009).

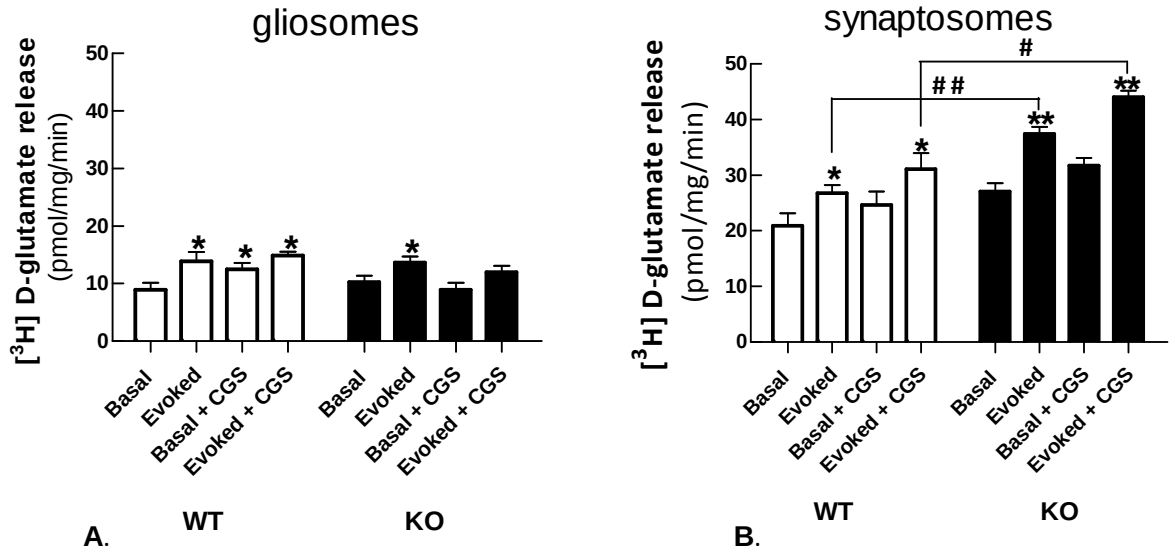


Fig.6 - **The evoked release of glutamate is increased in synaptosomes from Gfa2-A_{2A}R-KO mice and further exacerbated by the A_{2A}R agonist CGS21680**. The basal (5 mM KCl, 0.1 mM $CaCl_2$) or depolarization (higher K^+-induced) Ca^{2+}-dependent evoked (40 mM KCl, 1.2 mM $CaCl_2$, for 90-s) release of L-[^{3}H]glutamate release from cerebral cortical **(A)** gliosomes or **(B)** synaptosomes was evaluated in the absence or in the presence of the A_{2A}R agonist CGS21680 (50 nM). Depolarising conditions triggered an evoked release from both gliosomes **(A)** and synaptosomes **(B)**, which was larger in synaptosomes from Gfa2-A_{2A}R-KO mice, the only preparation where CGS21680 exacerbated the evoked release of glutamate. Statistical difference was assessed using Tukey's post-hoc test applied after One-Way ANOVA. Data are mean ± S.E.M. from six independent experiments performed in triplicates. *p<0.05, **p<0.01, comparing with corresponding basal conditions. $^{\#}$p<0.05, $^{\#\#}$p<0.01, comparing with corresponding WT conditions.

Notably, the evoked release of glutamate from synaptosomes (**Fig. 6B**), but not from gliosomes (**Fig. 6A**) was significantly larger in Gfa2-A_{2A}R-KO mice that in their WT littermates ($p<0.01$, n=6). This became even more evident in the presence of the A_{2A}R agonist CGS21680 (50 nM) ($p<0.05$, n=6; **Fig. 6B**), whereas in gliosomes (**Fig. 6A**) CGS21680 only enhanced the basal outflow of glutamate from WT mice ($p<0.05$, n=6; **Fig. 6A**) but not from Gfa2-A_{2A}R-KO mice. Thus, the deletion of astrocytic A_{2A}Rs causes a profound modification of the extracellular glutamate dynamics resulting from a parallel reduction of the extra-synaptic glutamate levels (as a consequence of the up-regulation of astrocytic glutamate uptake) together with an increase of the synaptic glutamate levels (as a consequence an enhanced evoked release from the presynaptic terminals). This further indicates that the cognitive impairment observed in the Gfa2-A_{2A}R-KO mice might be associated with an imbalance of glutamatergic transmission.

4.6. Increased NMDA-NR2B and decreased AMPA-GluR1/2 surface levels in GFAP-A_{2A}R-KO mice

Synaptic changes of glutamate levels trigger adaptive modifications of the density of AMPA and NMDA receptors (NMDA-R) in different brain pathologies, as typified by the relation between altered NMDA-R levels and schizophrenia (Kristiansen et al., 2007; Moghaddam and Javitt, 2012). Thus, we first compared the levels of NR1, NR2A and NR2B subunits of NMDA-R in total membranes from the cerebral cortex of Gfa2-A_{2A}R-KO and WT mice. Western blot analysis showed that while there were no significant changes in the levels of NR1 (**Fig. 7A**) and NR2A subunits (**Fig. 7B**), a significant increase of the density of NR2B subunits was found in Gfa2-A_{2A}R-KO mice ($p<0.01$, n=6; **Fig. 7C**). Since NR2B-containing NMDA-R control the AMPA-R trafficking, namely their endocytosis (Tigaret et al., 2006; Ge et al., 2010), we next carried out biotinylation experiments in crude cortical synaptosomes to scrutinize changes in the density of surface AMPA GluR1/GluR2 proteins in Gfa2-A_{2A}R-KO mice. As shown in Figure 7

(D, E), the surface levels of both GluR1 and GluR2 were significantly diminished ($p<0.05$, n=3) in Gfa2-A_{2A}R-KO mice.

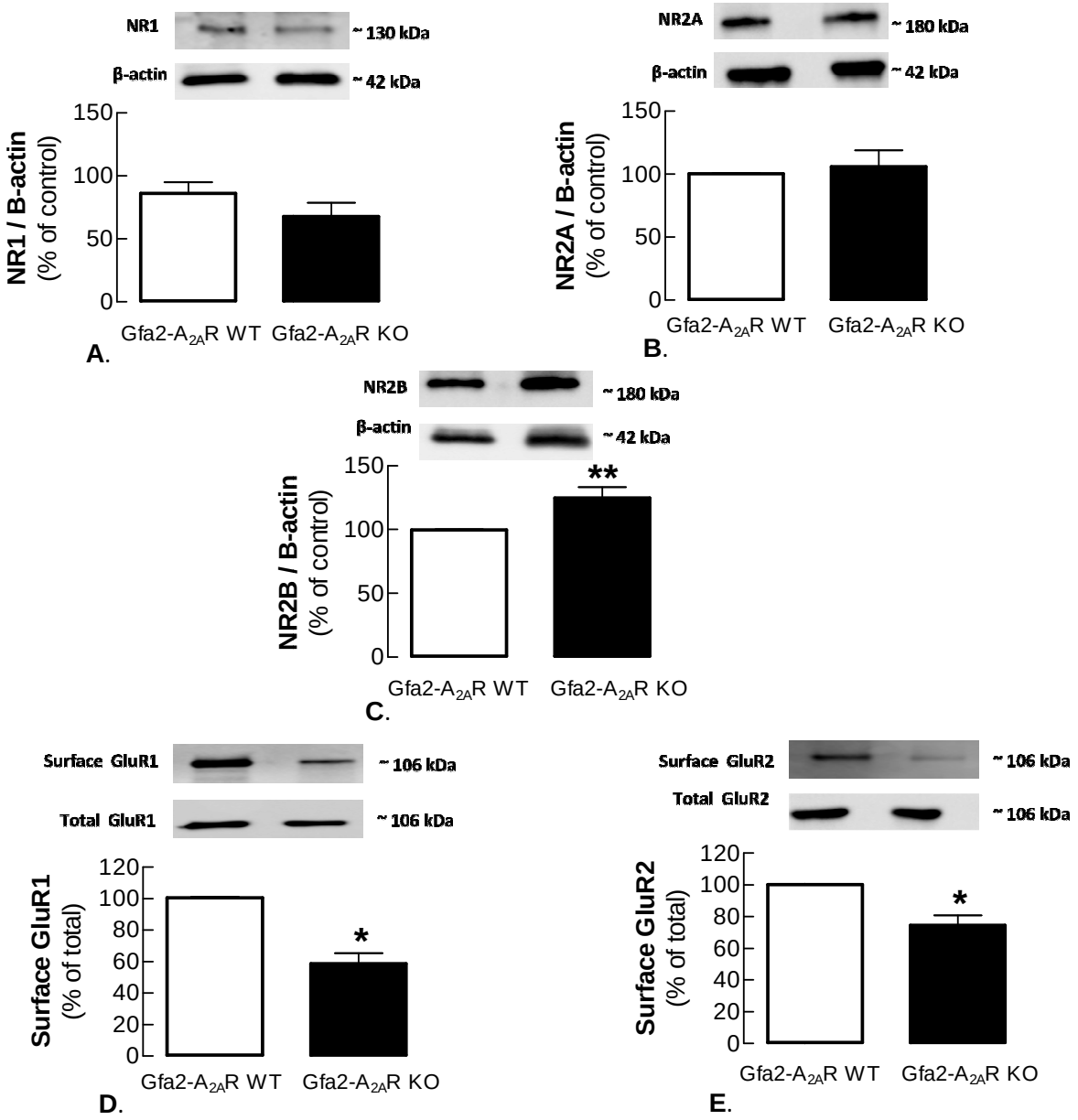


Fig.7 - **Gfa2-A_{2A}R-KO mice display an enhanced density of NR2B and decreased membrane surface levels of GluR1/GluR2 in crude synaptosomes from the cerebral cortex**. Western blot comparison of the density of NMDA-R-NR1 **(A)**, NMDA-R-NR2A **(B)** and NMDA-R-NR2B **(C)** in synaptosomal membranes, and of AMPA-R-GluR1 **(D)** and AMPA-R-GluR2 **(E)** in membranes and biotinylated extracts from synaptosomes from the cortex of GFAP-A_{2A}R-KO and WT mice. The identified protein bands were analyzed by densitometry, and receptor ratios for AMPA-R subunits were determined by dividing the surface intensity by the total intensity. Statistical difference was assessed using Tukey's post-hoc test applied after One-Way ANOVA. Values are the mean ± S.E.M. of n=3 experiments. *$p<0.05$, **$p<0.001$, comparing with WT mice.

4.7. Selective blockade of regulated AMPA-R internalization reverts the cognitive impairment in GFAP-A_{2A}R-KO mice

It has been shown that the endocytosis of GluR1/2-containing AMPA-R occurs through a controlled pathway recruited by NR2B-containing NMDA-R to implement changes of synaptic strength that are thought to underlie learning and memory processes (*e.g.* Wong et al., 2007; Han et al., 2012). This regulated GluR2 endocytosis can be selectively inhibited with the synthetic peptide Tat-Glur2_{3Y}, without affecting constitutive AMPA-R endocytosis, allowing to selectively control WM performance and the underlying changes in synaptic strength (Wong et al., 2007; Ge et al., 2010; Han et al., 2012). Thus, we now compared the impact of Tat-GluR2_{3Y} and of the scrambled control peptide, Tat-GluR2_{3S}, in the behavioral changes found in the Gfa2-A_{2A}R-KO mice to probe the involvement of the NR2B-regulated GluR1/2 endocytic process.

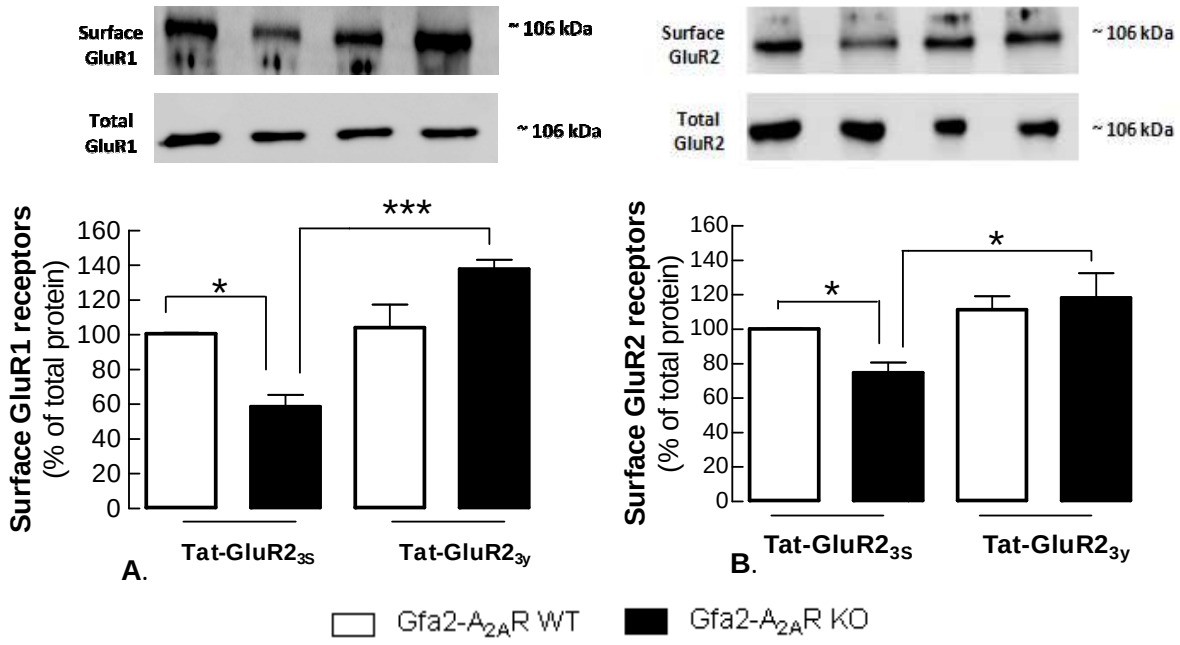


Fig.8 - **The decrease in the membrane levels of GluR1 (A) and GluR2 (B) in Gfa2-A_{2A}R-KO mice is prevented by Tat-Glur2_{3Y} peptide and unaffected by the control Tat- GluR2_{3S} peptide**. Tat-GluR2_{3Y} (3 µmol/kg, i.p.) or the scrambled control peptide, Tat- GluR2_{3S} (3 µmol/kg, i.p.), were injected in Gfa2-A_{2A}R-KO or WT mice, 60 min before their sacrifice for preparation of cerebral cortical synaptosomes, analyzed by biotinylation and Western blot. Statistical difference was assessed using Tukey's post-hoc test applied after One-Way ANOVA. Values are mean ± S.E.M. of n = 8-12 mice per group. *p<0.05, **p<0.01, comparing the indicated columns.

We first confirmed that the injection of Tat-GluR2$_{3Y}$, but not of Tat-GluR2$_{3S}$, 60 min before the sacrifice of the mice for preparation of cortical synaptosomes, significantly prevented the decrease in the levels of biotin-labeled GluR1 ($p<0.001$, n=3; **Fig. 8A**) and GluR2 ($p<0.05$, n=3; **Fig. 8B**) at the surface of synaptic terminals from Gfa2-A$_{2A}$R-KO mice, without affecting WT littermates. These results show the high efficiency of peripherally-injected Tat-GluR2$_{3Y}$ peptide to the regulated endocytosis of AMPA-R in nerve terminals of the cerebral cortex.

We next injected Gfa2-A$_{2A}$R-KO or WT mice with Tat-GluR2$_{3Y}$ or Tat-GluR2$_{3S}$ 60-90 min before testing their psychomotor response to MK-801 or their WM performance. As shown in Figure 9A, Tat-GluR2$_{3Y}$, but not Tat-GluR2$_{3S}$, attenuated the abnormally larger MK-801-induced psychomotor response in Gfa2-A$_{2A}$R-KO mice, while being devoid of effects in Gfa2-A$_{2A}$R-WT mice. Indeed, a two-way ANOVA of the number of beam breaks confirmed a significant reduction of the MK-801 response in Gfa2-A$_{2A}$R-KO mice treated with Tat-GluR2$_{3Y}$, when compared with Gfa2-A$_{2A}$R-KO mice treated with control Tat-GluR2$_{3S}$ (interaction with Tat-GluR treatment: $F_{(71,1079)}=8.02$, $p<0.001$, n=12; **Fig. 9A**). This conclusion was further supported by a one-way ANOVA of the total number of beam breaks in the MK-801 post-injection (**Fig. 9B**): Gfa2-A$_{2A}$R-KO mice treated with scrambled Tat-GluR2$_{3S}$ peptide showed a significantly exacerbated response to MK-801 ($p<0.05$, n=12) when compared to WT littermates, whereas Tat-GluR2$_{3Y}$-treated Gfa2-A$_{2A}$R-KO mice displayed a significant attenuation of the exacerbated psychomotor response to MK-801 ($p<0.001$, n=12). Likewise, as shown in Figure 9C, Tat-GluR2$_{3Y}$ pre-treatment also prevented the WM impairment of Gfa2-A$_{2A}$R-KO mice. Thus, *post hoc* comparisons between Gfa2-A$_{2A}$R-KO and WT mice treated with either Tat-GluR2$_{3Y}$ or Tat-GluR2$_{3S}$, showed that the impairment of WM in the Y maze task observed in Gfa2-A$_{2A}$R-KO mice ($p<0.001$, n=12) was significantly prevented by Tat-GluR2$_{3Y}$ ($p<0.001$, n=12) (**Fig. 9C**). Altogether, these data pinpoint the modification of the regulated endocytosis of GluR1/2-containing AMPA-R as a key process responsible for the schizophrenia-like psychomotor and cognitive dysfunctions found in Gfa2-A$_{2A}$R-KO mice.

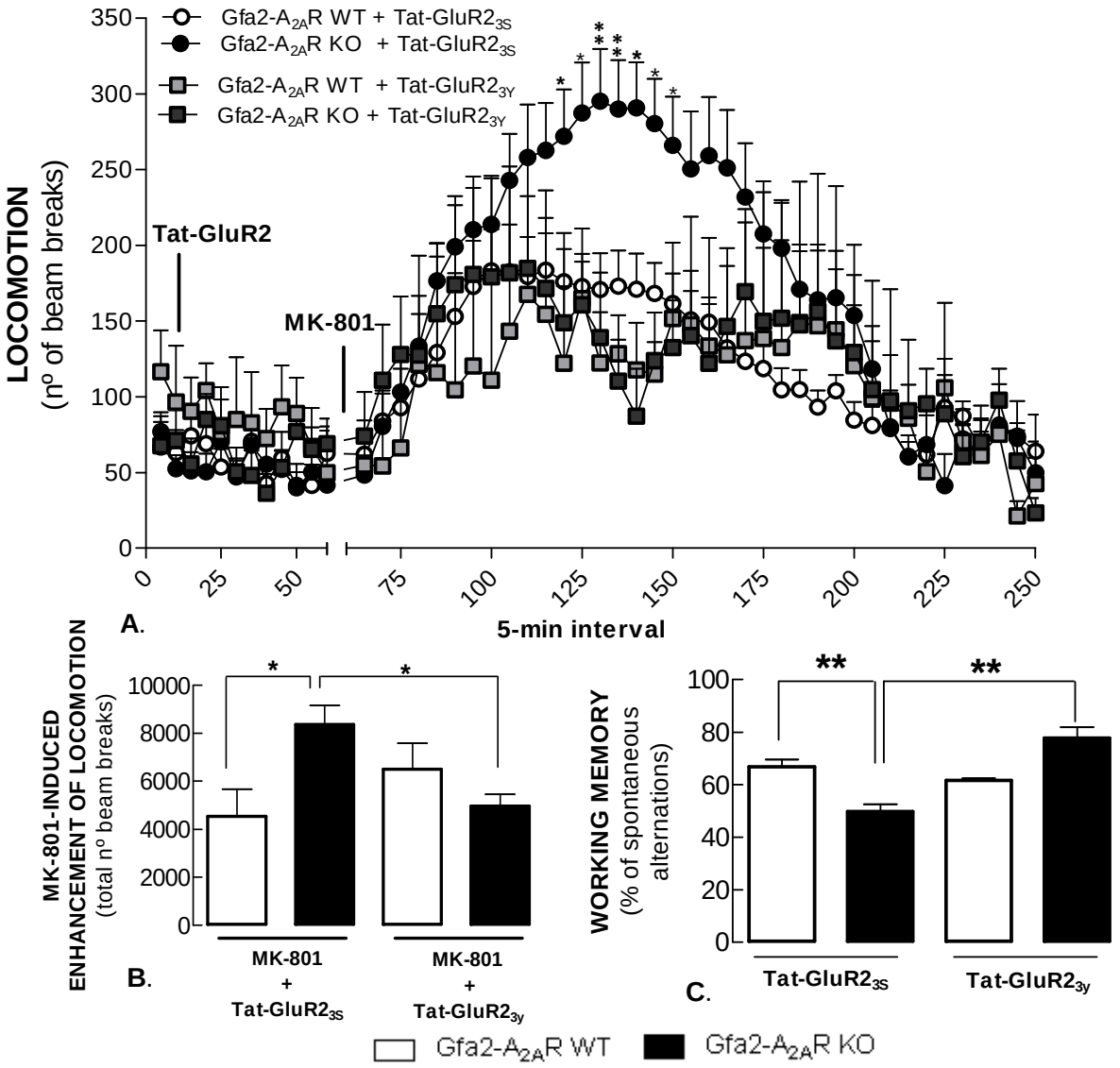


Fig.9 - **The synthetic peptide Tat-Glur2$_{3Y}$, which blocks the regulated endocytosis of AMPA-R GluR2 subunits, blunts the exacerbation of MK-801-induced psychomotor activity and reverses the working memory impairment in Gfa2-A$_{2A}$R-KO mice**. Tat-GluR2$_{3Y}$ or Tat-GluR2$_{3S}$ were injected in Gfa2-A$_{2A}$R-KO mice 90 min before injection with MK-801 or 60 min before performance of the memory task. All the mice were habituated in the test cages for at least 120 min before recording (data not shown). **(A)** Gfa2-A$_{2A}$R-KO mice treated with Tat-GluR2$_{3Y}$ did not show the significant exacerbated response to MK-801, which was present in the presence of Tat-GluR2$_{3S}$. Statistical differences were assessed using a Two-way ANOVA and Bonferroni post-hoc analysis. **(B)** The bar graph displays the grouped analysis of the total locomotor activity in the MK-801 post-injection period (70-250 min), showing the ability of Tat-Glur2$_{3Y}$, but not Tat-GluR2$_{3S}$, to prevent the enhanced MK-801 response in Gfa2-A$_{2A}$R-KO mice. Statistical differences were assessed using the Tukey's post-hoc test applied after One-Way ANOVA. **(C)** The data show that the impairment of working memory performance observed in Gfa2-A$_{2A}$R-KO mice subject to a conventional Y-maze test is prevented by the previous administration of Tat-Glur2$_{3Y}$, but not Tat-GluR2$_{3S}$. Values are mean ± S.E.M. of n = 8-12 mice per group. *p<0.05, **p<0.01, comparing the indicated columns.

5. Discussion

This present study identifies a novel molecular mechanism linking $A_{2A}R$ in astrocytes through its control of GLT-I activity and increased endocytosis of GluR1/2-containing AMPA-R, to key endophenotypic behaviors characteristic of schizophrenia, namely an exacerbation of MK-801-induced psychomotor response and a decrease of working memory. The exploration of the neurochemical features underlying these schizophrenia-like endophenotypes revealed a astrocytes-to-neuron wave of alterations (see **Fig. 10**): i) an upstream dysfunction of $A_{2A}R$-mediated control of glutamate uptake causing ii) an intermediary adaption of glutamatergic synapses with an extra-synaptic increase of the astrocytic clearance of glutamate compensated by an opposite increase of synaptic glutamate release, both leading to iii) a selective up-regulation of NR2B-containing NMDA-R triggering iv) a downstream endocytosis of GluR1/2-containing AMPA-R. This prompts a novel view of the behavioral modifications characteristic of cognitive symptoms associated with schizophrenia that involve a primary astrocytic locus ($A_{2A}R$-mediated control of GluTs) together with a mandatory astrocyte-to-neuron communication that entrains a modification of the set-up of ionotropic glutamate receptors sustaining the cognitive and psychomotor abnormalities.

The present results further illustrate the central role of astrocytes in the development of schizophrenia-like symptoms. This is in line with the increased awareness of the importance of astrocytes to control higher brain functions (Perea, 2009; Halassa and Haydon, 2010; Panatier et al., 2011), typified by the observations that altering astrocytic functions such as D-serine (Panatier et al., 2006) or ATP release (Halassa et al., 2009), glycogenolysis or lactate transport (Suzuki et al., 2011) or the density of aquaporin-4 water channels (Li et al., 2012) triggers a dysfunction of cognitive processes. We now report that the primary impact of astrocytic $A_{2A}R$ is the up-regulation of glutamate transport, namely GLT-I, in astrocytes. This is in notable agreement with the identification of a susceptibility locus for schizophrenia within or near the GLT-I gene (Deng et al, 2004), which is deregulated in schizophrenic patients (Shao and

Vawter, 2008; Spangaro et al., 2012). Accordingly, GLT-I mRNA, protein and function are consistently reported to be increased in the brain of schizophrenic patients (Simpson et al., 1998; Smith et al., 2001; Matute et al., 2005; Rao et al., 2012). Furthermore, GLT-I upregulation impairs adaptation of the startle reflex in adult rats, which was prevented by the glutamate transport inhibitor DHK (Bellesi et al., 2009). Finally, the antipsychotic clozapine specifically downregulates GLT-I expression and function (Melone et al., 2003; Vallejo-Illarramendi et al., 2005).

Notably, these astrocytic A_{2A}R-induced modifications of GLT-I function were found to cause an adaptation of glutamatergic synapses, typified by an enhanced density of NR2B subunits of NMDA-R, an increase of synaptic glutamate release and an internalization of AMPA-R (see **Fig. 10**). This is in accordance with the ability of NR2B-containing NMDA-R to control both working memory (Wang et al., 2013; von Engelhardt et al., 2008) and the response to drugs of abuse (Huang et al., 2009; Mao et al., 2009), as well as with the association of AMPA-R internalization with working memory performance (Vazdarjanova et al., 2011; Ge et al., 2010) and mood-associated behaviors (Boudreau et al., 2007; Du et al., 2010). Indeed, we now confirmed that inhibiting the regulated AMPA-R internalization blunted the abnormal working memory and response to MK-801, which mimicked by inhibition of GluTs. This provides a causal demonstration that the modification of the astrocyte glutamate uptake (A_{2A}R selectively controlled GLT-I activity in gliosomes) triggers an astrocytes-to-neuron communication leading to changes in the density of NR2B-containing NMDA-R function and impacting on AMPA-R internalization, as might also occur upon stressful conditions (Wong et al., 2007).

Collectively, these data provide a novel framework to consider a pathogenesis role of astrocytic A_{2A}R-driven astrocyte-to-neuron communication in schizophrenia-related abnormal behavioral responses, albeit the underlying signaling mechanisms still remain to be unraveled. This shift towards modified astrocytic function as primers of schizophrenic endophenotypes is in line with the increased recognition that the dysfunction of the schizophrenic brain likely results

from a widespread impaired interaction between cortical and subcortical glutamatergic networks rather than resulting from a defined modification in a particular brain area (Lisman et al., 2008; Field et al., 2011). Indeed, astrocytes are slower and long-range integrators of neuronal function (Verkhratsky and Toescu, 2006; Halassa and Haydon, 2010); they control global brain processes such as sleep (Halassa et al., 2009) and the disruption of cortical astrocytic networks is associated with pathologies affecting the whole brain, such as epilepsy (Oberheim et al., 2008). This notion that schizophrenia endophenotypes result from an astrocytic-mediated imbalance of the connectivity between different brain regions is also in line with the opposite effects resulting from a localized and more global manipulation of the activity of either GluTs, NR2B-containing NMDA-R or AMPA-R trafficking on memory performance or response to psychoactive drugs (Wang et al., 2013; von Engelhardt et al., 2008; Bechtholt-Gompf et al., 2010; Rao-Ruiz et al., 2011). This underscores the need to evaluate global than local changes when grasping the etiology of schizophrenic endophenotypes.

The present study also highlights the prominent role of astrocytic $A_{2A}R$ in the control of schizophrenia since the genetic deletion of astrocytic $A_{2A}R$ leads to severe modifications in the glutamatergic system that confer susceptibility to a schizophrenia-type of traits. This finding is of particular significance since it indicates a physiological requirement for astrocytic $A_{2A}R$ to avoid exacerbated responses to psychosis-inducing drugs such as MK-801. This is in accordance with the adenosine hypofunction of schizophrenia (Lara and Souza, 2000; Boison et al., 2012). Indeed, the enhanced production of adenosine ameliorates the positive and cognitive endophenotypes of schizophrenia (Shen et al., 2012), which is in remarkable agreement with our reported ability of astrocytic $A_{2A}R$ deletion to trigger schizophrenia endophenotypes. Thus, the impact of $A_{2A}R$ on schizophrenia-like behavioral abnormalities is unlikely to be limited to the direct ability of $A_{2A}R$ to interfere with dopamine D_2R, as previously noted (Rimondi et al., 1997; Boison et al., 2012); instead, astrocytic $A_{2A}R$ may affect the dopaminergic system indirectly since an hyperactive glutamate transport has been consistently associated with a

hypofunctionality of NMDA-R mesocortical projections that elicit the nigrostriatal and thalamocortical dopaminergic and glutamatergic imbalance underlying schizophrenia (Moghaddam and Javitt, 2012).

In addition, the present results suggest that neuronal and astrocytic $A_{2A}R$ may have opposite neuromodulation effects, since the suppression of neuronal $A_{2A}R$ activity is pro-cognitive (Zhou et al., 2009; Wei et al. 2011) while the selective deletion of astrocytic $A_{2A}R$ potentiated working memory deficits. Indeed, it appears that the still poorly appreciated role of astrocytic $A_{2A}R$ may override neuronal $A_{2A}R$ since Gfa2-$A_{2A}R$-KO mice display overt behavioral abnormalities that are not present in Fb-$A_{2A}R$-KO mice. However, our current observation that the modification of astrocytic $A_{2A}R$ lead to a glia-to-neuron wave of modifications, that also included a modified efficiency of presynaptic $A_{2A}R$ controlling the evoked release of glutamate, actually suggests that there is a coordinated function of astrocytic and neuronal $A_{2A}R$ to fine-tune information processing between neuronal networks. This joins previous reports showing that $A_{2A}R$ can modulate behavior in different ways depending on the brain area, cell type and cell compartment where they are expressed (Shen et al., 2008; Wei et al., 2011), which is probably related with different sources of adenosine fulfilling different roles as modulators and homeostatic regulators of synaptic and brain function (Cunha, 2001). In this respect, it is worth noting that a recent study also found that the selective interference with another modulation system typically associated with neuronal adaptive control, cannabinoid CB_1R, also impacts on working memory in opposite manners according to whether neuronal or astrocytic CB_1R are manipulated (Han et al., 2012). The parallel role of cannabinoids and adenosine as activity-dependent homeostatic systems and the previously described functional and physical association between $A_{2A}R$ and CB_1R (Ferré et al., 2010), prompt the hypothesis that homeostatic mechanisms sensed by astrocytes are designed to control the functional integration of the function between brain circuits that is characteristically impaired in schizophrenia.

In conclusion, we now identified a novel molecular mechanism linking $A_{2A}R$ in astrocytes through its control of GLT-I activity and endocytosis of GluR1/2-containing AMPA-R to key endophenotypic behaviours characteristic of schizophrenia. This supports a potential role of $A_{2A}R$ specifically in astrocytes for the development of schizophrenia, which seems based on an astrocytic-to-neurons wave of modifications (see **Fig. 10**).

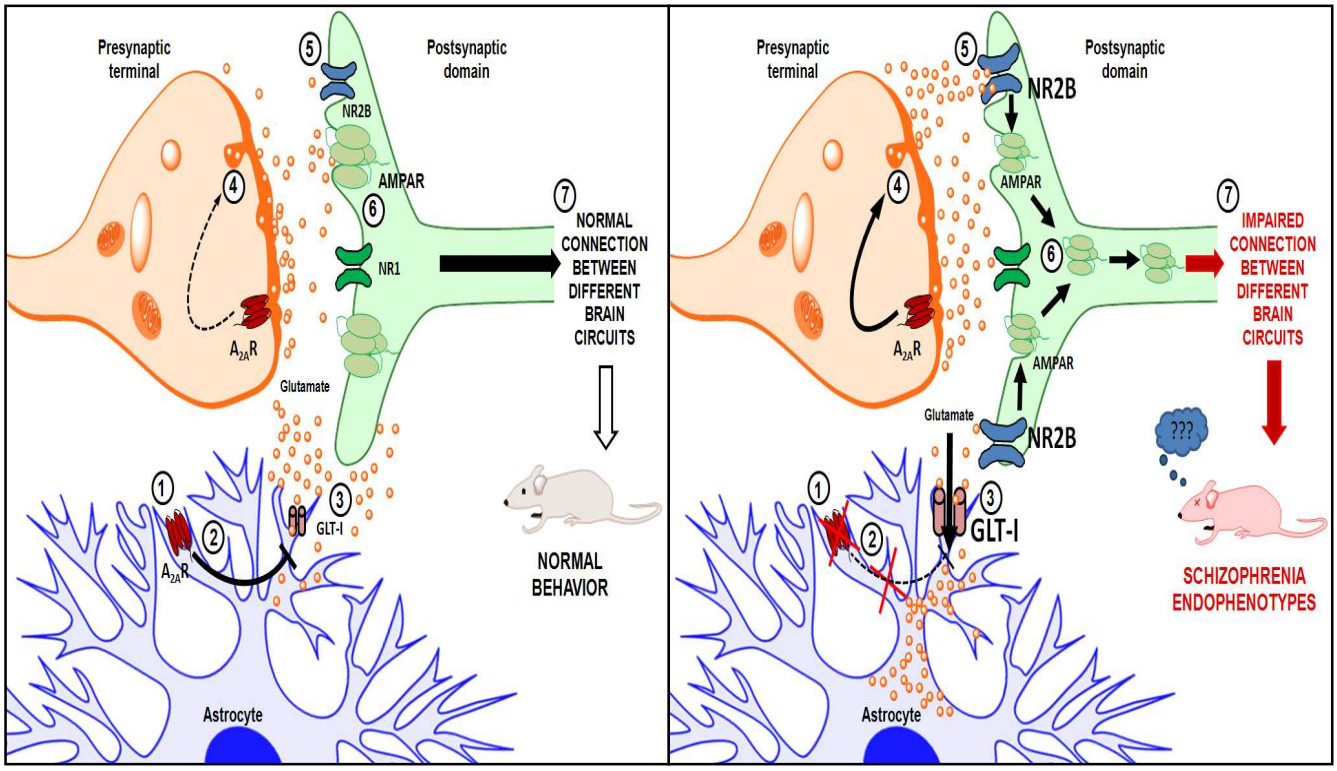


Fig.10 - **Proposed model for glutamatergic dysfunction and subsequent working memory impairment and emergence of schizophrenia-like endophenotypes in Gfa2-A_{2A}R-KO mice**. ***(A)*** In WT mice, astrocytic A_{2A}Rs play a crucial role inhibiting the astrocytic uptake of glutamate through GLT-I transporters (Matos et al., 2012b) **(1-2)**; this allows resolving an abnormal accumulation of extra-synaptic glutamate levels **(3)** and does not trigger an adaption of the neuronal glutamatergic system, with a lack of aberrant A_{2A}R-induced bolstering of synaptic glutamate levels neuronal **(4)** precluding changes in the density of NR2B-containing NMDA-R **(5)** and internalization of AMPA-R **(6)** maintaining a proper connectivity between brain circuits and a normal behavior **(7)**. **(B)** The lack of A_{2A}Rs in astrocytes of Gfa2-A_{2A}R-KO mice **(1)** removes the inhibition of GLT-I transporters **(2)**, leading to enhanced levels of GLT-I transporters and decreased extra-synaptic levels of glutamate **(3)**. Additionally, the function of presynaptic A_{2A}Rs is bolstered leading to enhanced levels of synaptic glutamate **(4)**; this causes a re-organization of post-synaptic glutamate receptors typified by an enhanced density of NR2B-containing NMDA-R **(5)** leading to the internalization of AMPA-R **(6)**; altogether, these changes lead to an impaired connectivity between brain circuits with the emergence of endophenotypes characteristic of schizophrenia such as enhanced response to psychotomimetics and decreased working memory performance. Thus, an astrocytic modification (elimination of astrocytic A_{2A}Rs) leads to a wave of astrocyte-to-neuron modifications associated with the emergence of schizophrenia-like endophenotypes.

CHAPTER 6

GENERAL CONCLUSIONS

1. Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and is critically involved in mechanisms of synaptic plasticity and memory (Benarroch, 2010). However, the excessive accumulation of this extracellular neurotransmitter leads to a neuronal enhanced excitability and cell death in addition to an altered synaptic function (Martin et al., 1997). In addition, increased levels of extracellular glutamate and deficient glutamate uptake have been implicated in neurodegenerative diseases, such as Alzheimer's disease (Benarroch et al., 2010) and neuropsychiatric disorders such as schizophrenia (Simpson et al, 1992). Because glutamate is not metabolized in the extracellular environment, the maintenance of normal glutamatergic neurotransmission or the prevention of glutamate-induced neurodegenerative disorders depends on the presence of GluTs mainly GLT-I and GLAST, which together account for more than 90% of the entire glutamate uptake in the forebrain (Anderson and Swanson, 2000).

Recent evidence suggests an intimate involvement between the glutamatergic and adenosinergic systems, mainly through the action of adenosine A_{2A}Rs. It was shown that the switch of A_{2A}R activation in glial cells and the regulation of neuroinflammation is dependent on the local glutamate level (Dai et al., 2010). Various studies have also demonstrated that A_{2A}R astrocyte activation leads to a transporter-independent and Ca^{2+}-dependent glutamate release in cultured hippocampal astrocytes (Li et al., 2001; Nishizaki et al., 2002), in striatal tissue and cultured striatal neurons (Popoli et al. 2003; Pintor et al., 2004). Therefore, to explore the possible role of A_{2A}R on the regulation of glutamate uptake we employed D-aspartate uptake radioligand assays as well as semi-quantifitative PCR and immunocytochemical approaches to examine the effect of A_{2A}R activation on the glutamate uptake in primary cultured astrocytes from wild type and A_{2A}R-global KO mice pups as well as in an *ex-vivo* biological preparation enriched in glial plasmalemmal vesicles (gliosomes) obtained from adult rats. The data we

present in **Chapter 2** are in accordance to what had also been described by Nishizaki and its colleagues (Nishizaki et al., 2004) and further suggest two mechanisms of $A_{2A}R$ modulation on glutamate uptake in astrocytes: i) one at an acute level through fast association between $A_{2A}R$ and GLT-I or GLAST and ii) another at a longer time scale through $A_{2A}R$ -mediated PKA signalling, leading to general decrease in GLT-I and GLAST mRNA and protein levels.

Collectively, the studies depicted in **Chapter 2** demonstrate the crucial role of astrocytic $A_{2A}Rs$ in modulating perhaps the most important role of astrocytes, i.e. the control of glutamate uptake; they also revealed that $A_{2A}R$ operate different mechanisms affecting all the GluTs in different astrocytic preparations (*in vitro* and *ex-vivo*). Since extracellular adneosine is produced as a function of neuronal activity (Dunwiddie and Masino, 2000), the primary role of astrocytic $A_{2A}Rs$ could indeed be to sense synaptic transmission in order to adjust the extracellular level of glutamate, either by modulation of the transport activity shown here, or by active glutamate release (Nishizaki et al., 2002). The development of novel therapies, perhaps based on the selective blockade of adenosine $A_{2A}Rs$, may therefore greatly contribute to the improvement from a vast array of neuropathologies that have glutamatergic synaptic dysfunction and excitotoxicity as central triggering processes (Volterra and Meldosi, 2005) (see **Fig 1**).

2. Antagonistic interaction between Adenosine A_{2A} receptors and Na^+/K^+-ATPase-α_2: implications for astrocyte glutamate uptake

We had previously shown in Chapter 2 that adenosine, by activating astrocytic $A_{2A}R$, controls the uptake of glutamate through a dual-mechanism. The astroglial specific glutamate transporter-I subtype (GLT-I) is the dominant glutamate transporter in the CNS, whose importance is underscored by the impact of modifying GLT-I activity in synaptic plasticity as well as in neurodegeneration (Benarroch et al., 2010). GLT-I are Na^+-dependent transporters, relying on Na^+ electrochemical gradients generated by NKA to drive glutamate uptake (Anderson and Swanson, 2000). In addition, consisting data has shown that the astrocyte-

specific NKA isoform NKA-α2 co-localizes and is a potent regulator of GluTs expression, processing and activity (Cholet et al., 2002; Rose et al., 2009; Genda et al., 2011). In **Chapter 3** we tackled the mechanism of $A_{2A}R$-mediated inhibition of the astrocytic glutamate transport, which was found to depend on a physical association and control by $A_{2A}R$ of NKA-α_2 in astrocytes. The results provide the first evidence of a co-localization and functional interaction between $A_{2A}R$ and Na^+/K^+ATPase-α2 in the plasma membrane of astrocytes of the mice adult brain, depicting an unique way employed by $A_{2A}R$ to regulate glutamate uptake. By means of coimmunoprecipitation, PLA, and assays of NKA activity and D-[^{3}H]aspartate uptake, we showed the existence of a physical and functional link between $A_{2A}R$, NKA-α2 and GLT-I at the cell surface of astrocytes. Furthermore, through a biological control where $A_{2A}R$ was specifically deleted from astrocytes, we revealed that this heterotrimeric interaction is abrogated in gliosomes from Gfa2-$A_{2A}R$ KO mice, eliciting a simultaneous deregulatory increase in the levels and activity of NKA-α2 and GLT-I. These results reinforce the view that NKA might act as molecular hubs by mediating the function of $A_{2A}R$ and GluT association (Bøttger et al., 2012). The selective interaction of NKA-α2 with $A_{2A}R$ in the fast control of glutamate uptake may provide new insights into our understanding of many cognitive processes and in the aetiology of several neuropathological conditions known to be influenced by the activity of GluTs and NKA (see **Fig 1**).

3. Astrocyte adenosine A_{2A} receptors control the Amyloid-β peptide induced decrease of glutamate uptake

Research advances have enabled a detailed understanding of the molecular pathogenesis of the main hallmark of Alzheimer's disease (AD) - i.e., the presence of high levels of beta-amyloid (Aβ) peptide, especially $Aβ_{1-42}$ (Hardy and Higgins, 1992; Blennow et al., 2006). In addition, synaptic malfunction and cognitive deterioration often appears in advance of Aβ plaque formation and neuronal cell loss and it seems to be the changes of the extracellular

levels of soluble forms of Aβ that are more closely related to the pattern of cognitive and memory decline (Shankar et al., 2008; Ondrejcak et al., 2009). Furthermore, in AD, cortical glutamatergic pathways are disrupted in a way proportional to the degree of dementia (Hynd et al*.,* 2004). One possible mechanism involved in the degenerative process is that the ability of Aβ peptide to enhance the vulnerability of neurons to glutamate overloading and excitotoxicity (Gray and Patel, 1995; Hynd et al*.,* 2004). In addition, crucial astrocytic functions seem to be compromised (for review see: Nagele et al., 2004; Rodríguez et al., 2009; Siu et al., 2007), often culminating in atrophy and apoptosis in astrocytes surrounding the amyloid deposits. These reactions develop simultaneously and may represent the underlying mechanisms for loss in synaptic connectivity and plasticity, which in turn determine the cognitive deficits. Furthermore, astrogliosis or astrocytic atrophy comes with a price, namely a reduced ability for glutamate uptake, thus increasing the neuronal vulnerability to glutamate excitotoxicity (Benarroch, 2010). Indeed, previous studies have shown a significant reduction of glutamate transporter activity in AD (Scott et al. 1995; Masliah et al. 1996; Liang et al., 2002; Matos et al., 2008; Piermartiri et al; 2010) so that overcoming the loss of GluTs function could be therapeutically relevant for AD. To this respect, adenosine $A_{2A}R$ are well positioned to fulfill this purpose, since we previously shown in Chapter 2 that astrocyte $A_{2A}R$ regulate glutamate uptake. In addition, the specific ability of $A_{2A}R$ to modulate mechanisms involved in synaptic degeneration and subsequent neuronal death opens the possibility that $A_{2A}R$ antagonists might control the apparently reversible synaptic dysfunction that occurs in early AD pathogenesis (for review see, Cunha and Agostinho, 2010; Gomes et al., 2010). Some of the strongest evidence relies on epidemiological studies where caffeine (a non-selective adenosine receptor antagonist) consumption inversely correlates with the incidence of AD (Maia & de Mendonca, 2002; Santos et al., 2010). However, it still remained undefined whether astrocytic $A_{2A}R$ could indeed control the modifications of astrocytic glutamate uptake that occur upon dementia.

In **Chapter 4** we analyzed if the astrocytic $A_{2A}R$ was able to modulate the previously observed decrease on glutamate uptake triggered by Aβ peptide (Matos et al., 2008). The exposure of primary cultures of mice cortical astrocytes to $A\beta_{1-42}$ inhibited D-aspartate uptake and this inhibitory effect of $A\beta_{1-42}$ was prevented by selective blockade of $A_{2A}R$ and completely absent in cultured astrocytes from $A_{2A}R$ global knockout (KO) mice. Furthermore, the exposure to $A\beta_{1-42}$ also induced a decrease in GLAST and GLT-I mRNA and protein levels, which was abrogated in cultured astrocytes from $A_{2A}R$ KO mice. This key role of astrocytic $A_{2A}R$ may result from an up-regulation of $A_{2A}R$, as concluded from the ability of $A\beta_{1-42}$ to enhance both the expression of $A_{2A}R$ mRNA, protein levels as well as the binding density of $A_{2A}R$ in cultured astrocytes. The control by $A_{2A}R$ of Aβ-induced inhibition of glutamate uptake was confirmed to occur on an *ex vivo* preparation of astrocytes; thus, cortical gliosomes from rats intracerebroventricularly (icv) injected with $A\beta_{1-42}$, which displayed memory deficits, displayed a lower uptake of D-aspartate and $A_{2A}R$ selective agonist CGS 21680 was no longer able to decrease D-aspartate uptake. Together, these results indicate that astroglial $A_{2A}R$ control glutamate uptake and play a key role in the Aβ-induced impairment of glutamate uptake, which may lead to glutamatergic synaptic dysfunction and excitotoxicity. This provides an additional justification for the promising therapeutic benefits of $A_{2A}R$ antagonists in early AD (see **Fig 1**).

4. Deletion of adenosine A_{2A} receptors in astrocytes alters glutamatergic synapses and triggers psychomotor and cognitive dysfunctions characteristic of schizophrenia

In an attempt to further define the impact of astrocytic $A_{2A}R$ in the control of brain function, we undertook a behavioral characterization of a novel mouse transgenic line with a specific deletion of $A_{2A}R$ in astrocytes (GFAP-$A_{2A}R$-KOs). In **Chapter 5**, we showed that GFAP-$A_{2A}R$-KOs reproduced key endophenotypic behavioral modifications characteristic of schizophrenia, namely an exacerbation of MK-801-induced psychomotor response and a decrease of working memory. Such observations matched neurochemical alterations in glutamatergic activity,

resulting from a hyperactive astrocyte glutamate uptake and a consequent increase in facilitated glutamate release from the presynaptic nerve terminals. In addition we shown that the observed glutamate dysfunction was associated with an alteration of NMDAR2B-mediated AMPAR GluR1/GluR2 subunit membrane trafficking, suggesting that the genetic deletion of astrocytic $A_{2A}R$ may lead to severe modifications in the glutamatergic system that confer susceptibility to schizophrenia-type of traits. Collectively, these data provide a novel framework to consider schizophrenia-related abnormal behavioral responses as resulting from an impaired astrocyte-neuron communication, where $A_{2A}R$ seem to play a key balancing role.

By maintaining brain extracellular glutamate concentrations below toxic levels (Rosenberg and Aizenman, 1989; Brown, 1999; Kawahara et al., 2002) the clearance of extracellular glutamate by GluTs are also responsible for the modulation of synaptic activity (Huang and Bergles, 2004). The immunolocalization of GluTs shows these transporters to be concentrated in areas that face or surround neuronal spinal processes (Chaudry et al., 1995; Lehre et al., 1995; Minelli et al., 2001; Melone et al., 2009) and excitatory synaptic activity has been shown to activate astrocyte GluTs currents (Bergles and Jahr, 1995; Pita-Almenar et al., 2006; Zhang et al., 2009). The effects of GluTs on synaptic transmission are illustrated by the fact that glutamate uptake inhibitors prolong postsynaptic glutamate-induced currents which induce long-term potentiation (LTP) and long-term depression (LTD) events (Tzingounis and Wadiche, 2007). This process limits glutamate diffusion beyond the synaptic vicinity and consequent heterosynaptic plasticity, thus contributing to synapse independence, efficacy and specificity (Huang and Bergles, 2004). Minimizing the diffusion of glutamate from the synapse is especially relevant in the primate CNS, since central glutamate-mediated synapses are believed to operate as independent entities (Tzingounis and Wadiche, 2007). However, it is still unclear if a steady increase of the function of GluTs actually benefits the the highly sensitive glutamatergic transmission (Katagiri et al, 2001). In fact, an hyperactive glutamate transport has been consistently associated with schizophrenia (Schneider et al., 1998; Nanitsos et al., 2005; Matute

et al., 2005; Rao et al., 2012). Our data gathered from the analysis of Gfa2-A_{2A}R-KO mice, which display increased glutamate transport and several schizophrenia-like deficits, is fully compatible with a view that hyperactive GluT activity is detrimental for brain function. Furthermore, our data also suggest that local disturbances in the glutamate levels between the synaptic and perisynaptic compartments may result in an altered post-synaptic NMDAR function. Synaptic NMDARs are primarily comprised of NR1/NR2A subunits, while NR1/NR2B have a preferential extrasynaptic localization (Hardingham and Bading, 2010). Interestingly, we found that an hyperactive perisynaptic (astrocytic) glutamate uptake results in compensatory increases in NR2B-NMDAR subunits which elicit AMPAR endocytosis that result in cognitive impairment. It seems that, in order to overcome the glutamate deficiency created by the exacerbated astrocytic glutamate uptake, there is an adaptive increase in pre-synaptic glutamate release bolstered by neuronal A_{2A}R activation and a parallel increase in the number of NMDAR complexes at the plasma membrane. Thus, our results imply a coordinated action of astrocytic A_{2A}R and of presynaptic A_{2A}R to fine-tune glutamatergic transmission, whereby the elimination of astrocytic A_{2A}R (leading to a decrease of the extracellular levels of glutamate) bolsters the function of presynaptic A_{2A}R (enhancing the presynaptic release of glutamate.

By controlling GLT-I activity (Matos et al., 2012b) and glutamate release (Popoli et al., 2003), astrocytic A_{2A}Rs may be critical mediators of the neuron-astrocytic glutamatergic dysfunction underlying the pathophysiology of schizophrenia. Mechanistically, the remarkable ability of a dysregulated glutamate transport caused by suppression of astrocyte A_{2A}Rs, to elicit to severe changes in the glutamatergic system and produce schizophrenia-related endophenotypes in rodents may lead to new insights into the neurochemical basis for the disorder (see **Fig 1**).

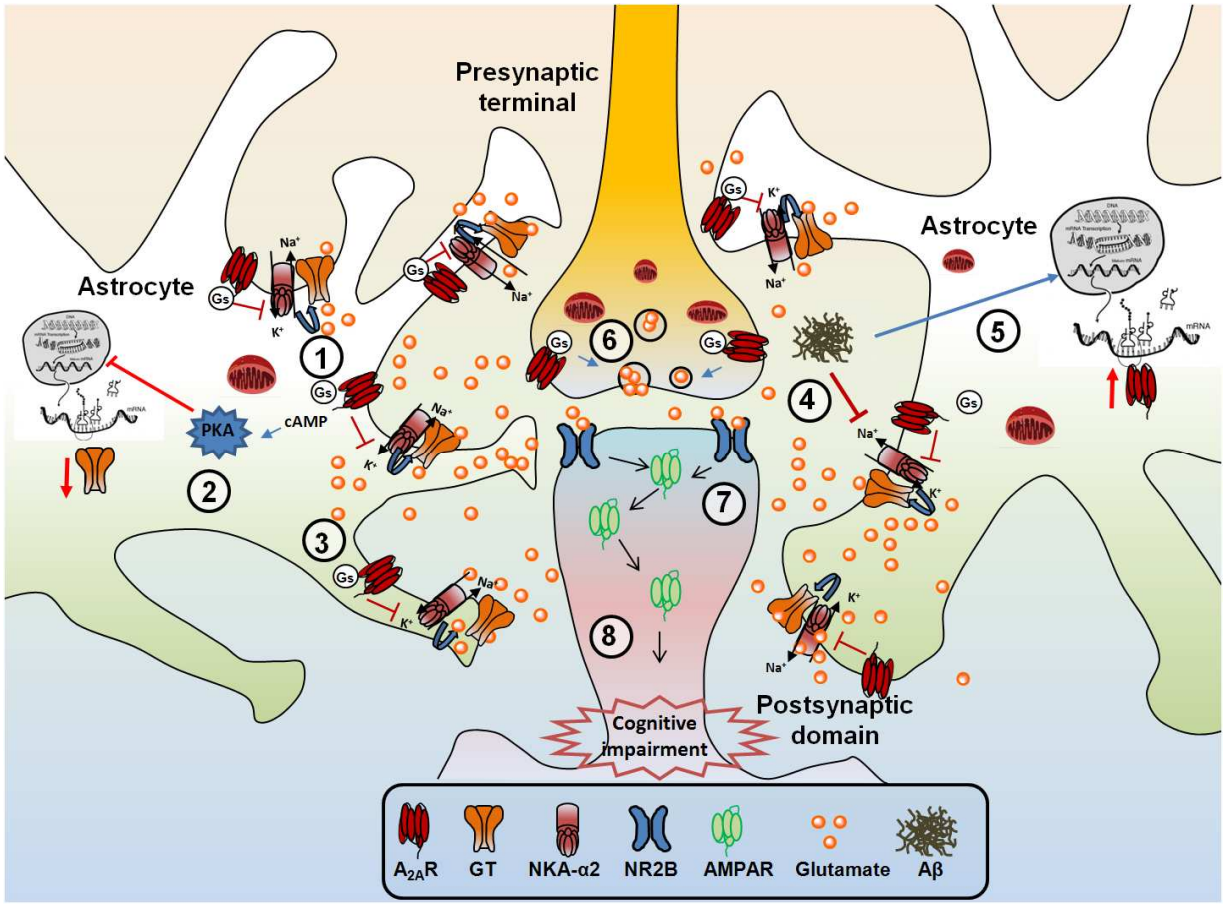


Fig.1. **Role of adenosine A_{2A}Rs in astrocytes - implications for glutamatergic activity**. A_{2A}R is a critical regulator of glutamate uptake in astrocytes (Matos et al., 2012b). **(1)** The binding of adenosine to astrocyte A_{2A}R elicits the activation of stimulatory Gs protein to activate adenylate cyclase, which, in turn, induces the activation of the cAMP-PKA signaling pathway **(2)** which leads to a long-term abrogation of GluTs expression and a sustained decrease of glutamate uptake (see **chapter 2**). **(3)** As an additional process of rapid modulation of glutamatergic activity, and owing to the close physical interaction between astrocyte A_{2A}R and NKA-α2, the activation of A_{2A}R leads to an immediate inhibition of glutamate uptake (see **chapter 3**). **(4)** In Alzheimer's disease conditions, $A\beta_{1-42}$ peptide, the candidate causative mechanism for the neurodegenerative cascade, leads to an unclear persistent A_{2A} receptor activation and increased expression **(5)** which, in turn, leads to a decrease in GLAST and GLT-I expression and levels and uptake capacity, an event which may underlie glutamatergic dysfunction and excitotoxicity in AD (see **chapter 4**). **(6)** Pre-synaptic neuronal A_{2A}R activation leads to a vesicular release of glutamate (Popoli et al., 2003), which rapidly builds up in the synaptic and perisynaptic clefts. In physiological conditions, astrocyte A_{2A}R contains the aberrant increase in glutamate uptake in astrocytes, enabling an efficient glutamatergic synapse. However, the genetic deletion of astrocytic A_{2A}R leads to a dramatic cascade of modifications in the Glu system leading to a selective up-regulation of perisynaptic NR2B-containing NMDA-R **(7)** and an adaptive increase in pre-synaptic glutamate release bolstered by neuronal A_{2A}R activation. **(8)** The increase in NR2B elicits the endocytosis of GluR1/2-containing AMPA-R, mediating the decrease in WM and an enhanced psychomotor response to MK-801, characteristic endophenotypes of schizophrenia (see **chapter 5**).

5. Glutamate transporters and adenosine $A_{2A}R$ in astrocytes as therapeutic targets

A common theme emerging from the previous chapters is that decreased expression and function of glutamate transporters may contribute to the excitotoxicity associated with many brain disorders. Therefore it is reasonable to assume that treatment aiming the up-regulation of GluTs might offer neuroprotection. Several different approaches have been used to increase GluT expression with the goal of determining their over-expression might be therapeutically effective, including transgenic mouse models over-expressing GluT, pharmacological up-regulation, and transduction of various cell types with exogenous GluTs using viral vectors (Sheldon and Robinson, 2007; Kim et al., 2011).

GLT-I-overexpressing transgenic mice display increased survival in models of ALS (Guo et al., 2003), epilepsy (Martinowich et al., 2001; McMinn et al., 2003) or hypoxia (Weller et al., 2008). Although no data is available for AD, pharmacological upregulation of GLT-I through the β-lactam class antibiotic ceftriaxone and analogues was shown to be neuroprotective in mouse models of other neurodegerative disorders such as ALS (Rothstein et al., 2005; Li et al., 2011), PD (Leung et al., 2012), HD (Miller et al., 2008) as well as in acute brain insults such as traumatic brain injury (Wei et al., 2012), stroke (Lipski et al., 2007) allodynia (Gunduz et al., 2011a) and drug addiction disorders (Gunduz et al., 2011b). Therefore, multiple studies support the notion that upregulation of GLT-I is neuroprotective in models of neurodegenerative disorders and offer a potential strategy to limit excitotoxicity in AD, where a compromised GluTs function is observed (see **Chapter 3**).

In spite of the general evidences supporting a beneficial role of overexpressing GluTs, there are still possible caveats. In particular, pharmacologic agents may have unwanted non-specific side effects, or viral-vector mediated over-expression of transporters could result in an altered trafficking of the targeted cells. For example adeno-associated viral (AAV) mediated over-expression of EAAT2 the CA1 area of the hippocampus results in an hyperactive EAAT2 activity

in neurons, a reverse glutamate release and an increased cell death following acute exposure to exogenous glutamate (Selkirk et al., 2005). In fact, although decreased GluT function has been associated with many disease processes (Benarroch, 2010), several studies have also shown that blocking glutamate uptake can also lead to significant improvement in LTP (Filosa et al., 2009), LTD (Omrani et al., 2009) and increases in peak amplitudes and decay rates of AMPA- and NMDA-receptor EPSCs (Tzingounis and Wadiche, 2007) which may underlie the cognitive disturbances observed in schizophrenia (Moghaddam and Javitt, 2012). In fact, hyperactive GluT has been consistently associated with schizophrenia (Schneider et al., 1998; Nanitsos et al., 2005; Matute et al., 2005; Rao et al., 2012). Indeed, GLT-I upregulation through ceftriaxone has been shown to impair PPI of the startle reflex in adult rats which was prevented by the GluT inhibitor DHK (Bellesi et al., 2009; Bellesi and Conti 2010). In addition, there is also evidence that over expression of GluTs might increase the susceptibility of the tissue to neuronal death (Kawahara et al., 2002; de Yebra et al., 2006; Sheldon and Robinson, 2007).

It is evident that contradictory results exist regarding the role of GluTs in neuroprotection versus contribution to neurotoxicity. It is evident that contradictory results exist regarding the role of GluT in neuroprotection *versus* their contribution to neurotoxicity. Indeed, a consensual picture is that GluT activity has to be finely adjusted to the needs of the brain circuits and either abnormally high or abnormally low activity of GluT will cause a dysfunction, probably of different nature: an increased activity of GluT will cause an impaired glutamatergic function (e.g. leading to schizophrenia-like phenotypes), whereas a decreased activity of GluT will instead lead to increased susceptibility to glutamate excitotoxicity. Normally, concentration gradients favor the transport of glutamate into astrocytes; this results in the transport of Na^+, H^+, and glutamate into the cytoplasm, and K^+ into the extracellular space (Danbolt, 2001). However, during pathophysiological events, such as ischemia, metabolic blockade, perturbed ionic conditions (e.g., increased extracellular K^+ levels) may favor transporters operating in reverse (Malarkey and Parpura, 2009), thus enhacing the risk of glutamate excitotoxicity. In fact, strategies aiming

to over-express GluTs have sometimes resulted in excitotoxicity and neuronal cell death due an increased GluT-mediated reversed glutamate release (Selkrik et al., 2005; Kawahara, et al., 2006). In schizophrenia, however, the glutamatergic dysfunction associated with increased astrocyte GluTs levels seems to result not from a reverse release of glutamate through the transporters or excitotoxicity but instead from a glutamatergic inefficiency resulting from an exacerbated GluTs-mediated removal/inactivation of glutamate (Matute et al., 2005; Spangaro et al., 2012) before it can adequately interact with the postsynaptic and perisynaptic receptores (Nanitsos et al., 2005). Our data supports the same idea, since although glutamate uptake was highly increased in gliosomes from Gfa2-A_{2A}R KO, no compensatory increase on glutamate release was observed on the glial compartment. The enhanced density of astrocytic GLT-I at astrocytic release sites may prevent activation of perisynaptic receptors and result in an abnormal activation of NR2B synaptic receptors due to a compensatory glutamate release instead by presynaptic nerve terminals. In any case, as the predisposition for schizophrenia may be, in some cases, acquired during early brain development (Ross et al., 2006) it would be of interest to examine whether the pathological upregulation of GluT could also be traced to early brain ontogeny. GluT appears to be very important during the development of brain tissue. Glutamate transporters are strongly expressed in the regions of developing brain displaying intense cell proliferation (Danbolt, 2001) and both GLT-I and GLAST - are present mainly in astrocytes in the adult brain (Rothstein et al., 1996) - have been found to be strongly expressed by immature neurons (Furness et al., 2008). Furthermore, GLAST and GLT may be critically important for the structural and functional maturation of the cerebral cortex (Voutsinos-Porsche, et al., 2003). Furthermore, it would be interesting to attempt determining whether the changes of expression, density and function of GluTs contribute to the pathogenesis or instead constitute an adaptive compensatory mechanism. GluTs are undeniably important for terminating glutamatergic transmission and preventing glutamate-induced toxicity. In some cases, alterations of GluT function, namely their upregulation, may represent a compensatory

neuroprotective mechanism; on the other hand, upregulation may contribute to and exacerbate the continuing pathology. It is apparent from our studies and previous observations that withdrawing GluT activity may be beneficial for schizophrenia while in AD the opposite is observed. Therefore, treatments should be aimed at returning glutamate transporters to normal levels of expression and function; in fact some anticonvulsants and antipsychotics appear to work in part by reversing pathologic alterations to glutamate transporters and restoring glutamatergic homeostasis (Nanitsos et al., 2005). The benefits of glutamate transporter upregulation may still outweigh the risks, especially when dealing with progressive neurodegenerative diseases such as AD.

A remarkable observation from our studies was the finding of the co-localization between the α_2 subunit of the NKA and $A_{2A}R$ in order to rapidly regulate astrocytic glutamate uptake. Our data indicate that the uptake of glutamate in astrocytes is tightly coupled to a NKA isozyme which involves the α_2 subunit which is itself regulated by $A_{2A}R$, implying a functional relationship between the glutamatergic and adenosinergic transmission through an electrochemical docking mediator. This finding is underscored by observations in the last years showing that the NKA is not only an ion pump (accounting for approximately 50% of the total body energy consumption), but is also involved in many signaling and regulatory events, most of them regulated by protein-protein interactions involving the NKA (Reinhard et al., 2013). In addition, the therapeutical relevance of the co-localization between $A_{2A}R$ and NKA-α2 is further amplified by previous observations suggesting that alterations on the function of the brain NKA have been associated with many neurological diseases (Bøttger et al., 2012) just like $A_{2A}R$ (Fredholm et al, 2005; Chen et al., 2007). Impairment in the function of the astrocyte-specific isoform NKA-α2 in particular, has been associated with an array of brain afflictions, including familial hemiplegic migraine type-2, spontaneous epileptic seizures, anxiety and impaired spatial learning (De Fusco et al., 2003; Lingrel et al., 2007; Moseley et al., 2007). In addition, the pathophysiology of some psychiatric disorders is believed to be associated with some perturbation of the ion

homeostasis, and earlier studies have shown that NKA activity is altered in patients with depression, chronic stress and various psychiatric disorders (Hokin-Neaverson and Jefferson, 1989; Gamaro et al., 2003; De Vasconcellos et al., 2005). Altered function of the NKA is also associated with memory impairment (dos Reis et al., 2002; Wyse et al., 2004; Moseley et al., 2007), and cognitive deficits have been reported in situations where NKA was impaired, such as AD (Hattori et al., 1998; Vitvitsky et al., 2012; Zhang et al., 2013). Since the NKA is crucial for maintaining ionic gradients and is reported to be critically involved in K^+ buffering after periods of hyperstimulation (Xiong & Stringer, 2000), it is well acceptable that alterations in the activity of this enzyme may impair synaptic activity and memory storage (Reinhard et al., 2013). Thus, the alteration of the NKA activity in mice lacking $A_{2A}R$, by increasing the Na^+ and K^+ electrochemical gradients, may contribute to the disturbance of the extracellular ionic homeostasis which may underlie the impairment of working memory in these mice. In this context, the mammalian hormone ouabain, an endogenous steroid derivative commonly used to treat heart and kidney failure (Aperia et al., 2006), may prove helpful in conditions of hyperactive NKA-α_2 function leading to abnormally high glutamate uptake and associated glutamatergic hypofunction, such as the ones associated with schizophrenia (Moghaddam and Javitt, 2012).

In the context of modulation of astrocyte GluT through pharmacological strategies, such as with caffeine, the most widely consumed psychoactive drug worldwide, might offer significant advantages (Fredholm et al., 1999). The observation that the consumption of caffeine (an adenosine receptor antagonist) is not only essentially safe (as confirmed by Phase-III trials with $A_{2A}R$ antagonists) but provides global beneficial effects in different neurodegenerative disorders, clearly argues against potential detrimental effects resulting from long-term consumption of $A_{2A}R$ antagonists (Gomes et al., 2010). Therefore, it is of utmost importance to clarify the functional interaction between adenosine and GluT in animal models of neurological diseases to effectively grasp if the manipulation of adenosine receptors might be envisaged as a novel reparative strategy based on the control of GluT activity.

It is now accepted that despite caffeine's similar affinities for the A_1R and the $A_{2A}R$ in brain, both pharmacological (Fredholm et al. 1999; El Yacoubi et al., 2008) and genetic knockout (Chen et al. 2000*;* Wei et al., 2011) studies have revealed that the psychostimulant effect resulting mainly from chronic caffeine intake is better correlated with its blockade of brain $A_{2A}R$ (Chen et al., 2013). Given the therapeutic potential of modulating the adenosine receptor systems in so many diseases, selective $A_{2A}R$ agonists and antagonists trials are currently in progress (Chen et al., 2013). However, a great challenge has arisen in developing adenosine receptor ligands for clinical applications: adenosine signaling is extremely widespread. Adenosine itself is present ubiquitously, adenosine receptors are widely distributed throughout the body and adenosine acting at these receptors exerts a broad spectrum of physiological and pathophysiological functions (Fredholm et al. 2005). The opposing effects of adenosine receptors in different cell types (*e.g*. astrocytes *vs*. neurons), brain regions (*e.g*. striatum *vs*. extra-striatal), opposite applications in different diseases (*e.g*. schizophrenia vs. AD) and different stages of disease (PD) clearly represent a major challenge for drug development (Gomes et al., 2010). Thus, demonstrating the effects of adenosine receptor activation or inactivation on specific systems under distinct experimental settings is not sufficient to suggest that adenosine can be delivered in a manner that is clinically effective and safe. Moreover, the presently demonstrated modulation by $A_{2A}R$ of astrocyte GluTs adds a further dimension to the role of $A_{2A}R$ in the control of brain function and dysfunction (Matos et al, 2012 a and b). Additionally, in the brain, a switch between a protective versus damaging effect of A_{2A} receptors has been shown to be associated with the local interactions between adenosine and glutamate. In response to various brain injuries, extracellular levels of adenosine as well as glutamate increase rapidly owing to their presynaptic release from neurons and possible inhibition of glutamate uptake from astrocytes (Dai et al., 2010). Remarkably, increasing the local levels of glutamate redirected $A_{2A}R$ signalling from the PKA to the PKC pathway, thus switching the effect of $A_{2A}R$ activation from anti-inflammatory to pro-inflammatory (Dai et al., 2010). Such

findings may explain - at least in part - the opposing effects of $A_{2A}R$ ligands on tissue injury by demonstrating that the effects of $A_{2A}R$ in brain injury are context-dependent as they can be influenced by local glutamate levels. Thus, a major challenge in developing effective therapeutic strategies targeting $A_{2A}R$ is to decipher these complex actions of $A_{2A}R$ at the level of cellular and tissue specificity as well as disease progression, and to define the specific interactions between $A_{2A}R$ and other critical effectors of other neurotransmitter systems, such the GluTs machinery in astrocytes. Furthermore, owing to the vast amount of functions mediated by other adenosine receptor subtypes described previously (see Chapter I. Introduction, section 3.4.) it would be interesting to investigate in more detail if their manipulation could afford therapeutic relevance and a deeper comprehension of the astrocytic biology.

Taken together, given the high density of astrocytes in the brain (~50% of human brain volume) (Volterra and Meldolesi, 2005) and the increasing evidences suggesting their involvement in many cognitive processes (Bezzi and Volterra, 2011), a deep understanding of the roles operated by $A_{2A}R$ and GluTs in astrocytes and their relationship within the tripartite synapse in different pathological models may be crucial for the development of safe and effective therapies for brain disorders (see **Fig 1**).

REFERENCES

Abbracchio MP, Ceruti S, Brambilla R, Barbieri D, Camurri A, Franceschi C (1998) Adenosine A_3 receptors and viability in astrocytes. *Drug Dev Res*. 45:379-386.

Abe K, Saito H. (2000) L-Glutamate suppresses amyloid β protein-induced stellation of cultured rat cortical astrocytes. *J Neurochem.* 74: 280-286.

Adolph O, Köster S, Räth M, Georgieff M, Weigt HU, Engele J, Senftleben U, Föhr KJ. (2007) Rapid increase of glial glutamate uptake via blockade of the protein kinase A pathway. *Glia* 55: 1699-1707.

Albasanz JL, Perez S, Barrachina M, Ferrer I, Martín M. (2008) Up-regulation of adenosine receptors in the frontal cortex in Alzheimer's disease. *Brain Pathol*. 18: 211-219.

Allaman I, Lengacher S, Magistretti PJ, Pellerin L. (2003) A2B receptor activation promotes glycogen synthesis in astrocytes through modulation of gene expression. *Am J Physiol Cell Physiol.* 284:C696-704.

Alloisio S, Cugnoli C, Ferroni S, Nobile M. (2004) Differential modulation of ATP-induced calcium signalling by A1 and A2 adenosine receptors in cultured cortical astrocytes. *Br J Pharmacol.* 141:935-942.

Amann LC, Gandal MJ, Halene TB, Ehrlichman RS, White SL, McCarren HS, Siegel SJ. (2010) Mouse behavioral endophenotypes for schizophrenia. *Brain Res Bull*. 83: 147-161.

Anderson BJ, Li X, Alcantara AA, Isaacs KR, Black JE, Greenough WT. (1994) Glial hypertrophy is associated with synaptogenesis following motor-skill learning, but not with angiogenesis following exercise. *Glia* 11:73-80.

Anderson CM, Swanson RA. (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia* 32: 1-14.

Angulo E, Casadó V, Mallol J, Canela EI, Viñals F, Ferrer I, Lluis C, Franco R. (2003) A1 adenosine receptors accumulate in neurodegenerative structures in Alzheimer disease and mediate both amyloid precursor protein processing and tau phosphorylation and translocation. *Brain Pathol.* 13:440-451.

Angulo MC, Kozlov AS, Charpak S, Audinat E. (2004) Glutamate released from glial cells synchronizes neuronal activity in the hippocampus. *J Neurosci*. 24:6920-6927.

Antolović R. (2006) Low nanomolar concentrations of ouabain may induce higher activity of the Na^+/K^+-ATPase in human erythrocytes. *Veterinarski Arhiv.* 76: 489-495.

Aperia A. (2007) New roles for an old enzyme: Na,K-ATPase emerges as an interesting drug target. *J Intern Med*. 261: 44-52.

Araque A, Parpura V, Sanzgiri RP, Haydon PG. (1999) Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci*. 22:208-215.

Arendash GW, Mori T, Cao C, Mamcarz M, Runfeldt M, Dickson A, Rezai-Zadeh K, Tane J, Citron BA, Lin X, Echeverria V, Potter H. (2009) Caffeine reverses cognitive impairment and decreases brain amyloid-beta levels in aged Alzheimer's disease mice. *J Alzheimers. Dis*. 17: 661-680.

Arnth-Jensen N, Jabaudon D, Scanziani M. (2002) Cooperation between independent hippocampal synapses is controlled by glutamate uptake. *Nat Neurosci*. 5: 325-331.

Arundine M, Tymianski M. (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity . *Cell Calcium* 34: 325-337.

Arystarkhova E, Sweadner KJ. (2005) Splice variants of the gamma subunit (FXYD2) and their significance in regulation of the Na, K-ATPase in kidney. *J Bioenerg Biomembr*. 37: 381-386.

Asztely F, Erdemli G, Kullmann DM. (1997) Extrasynaptic glutamate spillover in the hippocampus: dependence on temperature and the role of active glutamate uptake. *Neuron* 18: 281-293.

Augusto E, Matos M, Sévigny J, El-Tayeb A, Müller CE, Cunha RA, Chen JF. (2013) Ecto-5'-nucleotidase (CD73)-mediated formation of adenosine is critical for the striatal adenosine A2A receptor functions. *J Neurosci*. (in press)

Bachoo RM, Kim RS, Ligon KL, Maher EA, Brennan C, Billings N, Chan S, Li C, Rowitch DH, Wong WH, DePinho RA. (2004) Molecular diversity of astrocytes with implications for neurological disorders. *Proc Natl Acad Sci USA.* 101: 8384-8389.

Bajenaru ML, Zhu Y, Hedrick NM, Donahoe J, Parada LF, Gutmann DH. (2002) Astrocyte-specific inactivation of the neurofibromatosis 1 gene (NF1) is insufficient for astrocytoma formation. *Mol Cell Biol*. 22: 5100-5113.

Bardoni R, Goldstein PA, Lee CJ, Gu JG, MacDermott AB. (1997) ATP P2X receptors mediate fast synaptic transmission in the dorsal horn of the rat spinal cord. *J Neurosci*. 17: 5297-304.

Barres BA. (2008) The mystery and magic of glia: a perspective on their roles in health and disease. *Neuron* 60:430-440.

Bastia E, Xu YH, Scibelli AC, Day YJ, Linden J, Chen JF, Schwarzschild MA. (2005) A crucial role for forebrain adenosine A_{2A} receptors in amphetamine sensitization. *Neuropsychopharmacology* 30:891-900.

Batalha VL, Pego JM, Fontinha BM, Costenla AR, Valadas JS, Baqi Y, Radjainia H, Müller CE, Sebastião AM, Lopes LV. (2013) Adenosine A(2A) receptor blockade reverts hippocampal stress-induced deficits and restores corticosterone circadian oscillation. *Mol Psychiatry* 18:320-31.

Bauer DE, Jackson JG, Genda EN, Montoya MM, Yudkoff M, Robinson MB. (2012) The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism. *Neurochem Int.* 61: 566-574.

Beart PM, O'Shea RD. (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br J Pharmacol*. 150:5-17.

Bechtholt-Gompf AJ, Walther HV, Adams MA, Carlezon WA, Jr Ongür D, Cohen BM. (2010) Blockade of astrocytic glutamate uptake in rats induces signs of anhedonia and impaired spatial memory. *Neuropsychopharmacology* 35:2049-2059.

Bell KF, Ducatenzeiler A, Ribeiro-da-Silva A, Duff K, Bennett DA, Cuello AC. (2006) The amyloid pathology progresses in a neurotransmitter-specific manner. *Neurobiol Aging* 27: 1644-1657.

Bellesi M, Conti F. (2010) The mGluR2/3 agonist LY379268 blocks the effects of GLT-1 upregulation on prepulse inhibition of the startle reflex in adult rats. *Neuropsychopharmacology 35*, 1253-1260.

Bellesi M, Melone M, Gubbini A, Battistacci S, Conti F. (2009) GLT-1 upregulation impairs prepulse inhibition of the startle reflex in adult rats. *Glia* 57:703-713.

Ben Menachem-Zidon O, Avital A, Ben-Menahem Y, Goshen I, Kreisel T, Shmueli EM, Segal M, Ben Hur T, Yirmiya R. (2011) Astrocytes support hippocampal-dependent memory and long-term potentiation via interleukin-1 signaling. *Brain Behav Immun*. 25:1008-1016.

Benarroch EE. (2010) Glutamate transporters: diversity, function, and involvement in neurologic disease. *Neurology* 74:259-264.

Benarroch EE. (2011) Na+, K+-ATPase: functions in the nervous system and involvement in neurologic disease*. Neurology* 76: 287-293.

Bender AS, Woodbury DM, White HS. (1997) The rapid L- and D-aspartate uptake in cultured astrocytes. *Neurochem Res*. 22: 721-726.

Bergles DE. (2010) Neurotransmitter transporters. *eLS.* 1-7.

Bergles DE, Jahr CE (1997) Synaptic activation of glutamate transporters in hippocampal astrocytes. *Neuron* 19:1297-1308.

Bertorello A, Hopfield J, Aperia A, Greengard P. (1990) Inhibition by dopamine of (Na^+K^+)ATPase activity in neostriatal neurons through D1 and D2 dopamine receptor synergism. *Nature* 347: 386-394.

Bezzi P, Volterra A. (2011) Astrocytes: powering memory. *Cell* 144:644-645.

Biber K, Klotz KN, Berger M, Gebicke-Härter PJ, van Calker D. (1997) Adenosine A1 receptor-mediated activation of phospholipase C in cultured astrocytes depends on the level of receptor expression. *J Neurosci*. 17:4956-49564.

Björklund O, Shang M, Tonazzini I, Daré E, Fredholm BB (2008) Adenosine A1 and A3 receptors protect astrocytes from hypoxic damage. *Eur J Pharmacol*. 596:6-13.

Boison D, Chen JF, Fredholm BB. (2009) Adenosine signaling and function in glial cells. *Cell Death Differ.* 17: 1071-1082.

Boison D, Singer P, Shen HY, Feldon J, Yee BK. (2012) Adenosine hypothesis of schizophrenia--opportunities for pharmacotherapy. *Neuropharmacology* 62:1527-1543.

Boison D. (2007) Adenosine as modulator of brain activity. *Drug News Perspect.* 20:607-611.

Boison D. (2008) Adenosine as a neuromodulator in neurological diseases. *Curr Opin Pharmacol.* 8:2-7.

Boison D. (2011) Modulators of Nucleoside Metabolism in the Therapy of Brain Diseases. *Curr Top Med Chem*. 11: 1068-1086.

Boison D. (2012) Adenosine dysfunction in epilepsy. *Glia* 60:1234-1243.

Bøttger P, Doğanlı C, Lykke-Hartmann K. (2012) Migraine- and dystonia-related disease-mutations of Na^+/K^+-ATPases: relevance of behavioral studies in mice to disease symptoms and neurological manifestations in humans. *Neurosci Biobehav Rev*. 36: 855-871.

Boudreau AC, Reimers JM, Milovanovic M, Wolf ME. (2007) Cell surface AMPA receptors in the rat nucleus accumbens increase during cocaine withdrawal but internalize after cocaine challenge in association with altered activation of mitogen-activated protein kinases. *J Neurosci*. 27:10621-10635.

Brambilla R, Cottini L, Fumagalli M, Ceruti S, Abbracchio MP. (2003) Blockade of A2A adenosine receptors prevents basic fibroblast growth factor-induced reactive astrogliosis in rat striatal primary astrocytes. *Glia* 43:190-194.

Brockhaus J, Deitmer JW. (2002). Long-lasting modulation of synaptic input to Purkinje neurons by Bergmann glia stimulation in rat brain slices. *J Physiol*. 545(Pt 2):581-593.

Brodie C, Blumberg PM, Jacobson KA. (1998) Activation of the A2A adenosine receptor inhibits nitric oxide production in glial cells. *FEBS Lett*. 429:139-142.

Brown AM, Ransom BR (2007) Astrocyte glycogen and brain energy metabolism. *Glia* 55:1263-1271.

Brown DR. (1999) Dependence of neurones on astrocytes in a coculture system renders neurones sensitive to transforming growth factor beta1-induced glutamate toxicity. *J Neurochem*. 72:943-953.

Bullock TH, Orkand R, Grinnell AD. (1977) Introduction to Nervous System. W.H. Freeman and Company, San Francisco, *USA*, 1st edition, 1-559.

Bura SA, Nadal X, Ledent C, Maldonado R, Valverde O. (2008) A2A adenosine receptor regulates glia proliferation and pain after peripheral nerve injury. *Pain* 140:95-103.

Burnstock G (2006) Purinergic signalling - an overview. *Novartis Found Symp*. 276: 26-48.

Bushong EA, Martone ME, Jones YZ, Ellisman MH. (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. *J Neurosci*. 22:183-192.

Cajal SR. (1991) Estudios sobre la degeneración y regeneración del sistema nervioso 1913-1914. DeFelipe, J. and E.G. Jones (eds.): *Cajal's degeneration and regeneration of the nervous system*. New York: Oxford University Press, 1991.

Campbell SL, Hablitz (2004) Glutamate transporters regulate excitability in local networks in rat neocortex. *Neuroscience* 127:625-635.

Canas PM, Porciúncula LO, Cunha GMA, Silva CG, Machado NJ, Oliveira JM, Oliveira CR, Cunha RA. (2009) Adenosine A_{2A} receptor blockade prevents synaptotoxicity and memory dysfunction caused by β-amyloid peptides via p38 mitogen-activated protein kinase pathway. *J Neurosci.* 29: 14741-14751.

Cao C, Wang L, Lin X, Mamcarz M, Zhang C, Bai G, Nong J, Sussman S, Arendash G. (2011) Caffeine synergizes with another coffee component to increase plasma GCSF: linkage to cognitive benefits in Alzheimer's mice. *J Alzheimers Dis*. 25: 323-335.

Cao X, Li LP, Wang Q, Wu Q, Hu HH, Zhang M, Fang YY, Zhang J, Li SJ, Xiong WC, Yan HC, Gao YB, Liu JH, Li XW, Sun LR, Zeng YN, Zhu XH, Gao TM. (2013) Astrocyte-derived ATP modulates depressive-like behaviors. *Nat Med.* 19: 773-777.

Carmona MA, Murai KK, Wang L, Roberts AJ, Pasquale EB. (2009) Glial ephrin-A3 regulates hippocampal dendritic spine morphology and glutamate transport. *Proc Natl Acad Sci USA*. 106:12524-12529.

Carta AR, Kachroo A, Schintu N, Xu K, Schwarzschild MA, Wardas J, Morelli M. (2009) Inactivation of neuronal forebrain A_{2A} receptors protects dopaminergic neurons in a mouse model of Parkinson's disease. *J Neurochem*. 111: 1478-1489.

Castillo CA, León DA, Ballesteros-Yáñez I, Albasanz JL, Martín M. (2010) Glutamate differently modulates excitatory and inhibitory adenosine receptors in neuronal and glial cells. *Neurochem Int.* 57:33-42.

Cerqueira V, de Mendonça A, Minez A, Dias AR, de Carvalho M. (2006) Does caffeine modify corticomotor excitability? *Neurophysiol Clin*. 36: 219-226.

Chaudhry FA, Lehre KP, van Lookeren Campagne M, Ottersen OP, Danbolt NC, Storm-Mathisen J. (1995) Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry. *Neuron* 15:711-720.

Chen GJ, Harvey BK, Shen H, Chou J, Victor A, Wang Y. (2006) Activation of adenosine A3 receptors reduces ischemic brain injury in rodents. *J Neurosci Res*. 84:1848-1855.

Chen JF, Beilstein M, Xu YH, Turner TJ, MorataIla R, Standaert DG, Aloyo VJ, Fink JS, Schwarzschild MA. (2000) Selective attenuation of psychostimulant-induced behavioral responses in mice lacking A_{2A} adenosine receptors. *Neuroscience* 97:195-204.

Chen JF, Chern Y. (2011) Impacts of methylxanthines and adenosine receptors on neurodegeneration: human and experimental studies. *Handb Exp Pharmacol*. 200:267-310.

Chen JF, Eltzschig HK, Fredholm BB. (2013) Adenosine receptors as drug targets--what are the challenges? *Nat Rev Drug Discov*.12: 265-286.

Chen JF, Sonsalla PK, Pedata F, Melani A, Domenici MR, Popoli P, Geiger J, Lopes LV, de Mendonça A. (2007) Adenosine A_{2A} receptors and brain injury: broad spectrum of neuroprotection, multifaceted actions and "fine tuning" modulation. *Prog Neurobiol*. 83: 310-331.

Chen X, Gawryluk JW, Wagener JF, Ghribi O, Geiger JD. (2008) Caffeine blocks disruption of blood brain barrier in a rabbit model of Alzheimer's disease. *J Neuroinflammation* 5:1-14.

Chen JF, Xu K, Petzer JP, Staal R, Xu YH, Beilstein M, Sonsalla PK, Castagnoli K, Castagnoli N Jr, Schwarzschild MA. (2001) Neuroprotection by caffeine and A(2A) adenosine receptor inactivation in a model of Parkinson's disease. *J Neurosci.* 21:RC143.

Choi DW. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* 1: 623-634.

Cholet N, Pellerin L, Magistretti PJ, Hame lE. (2002) Similar perisynaptic glial localization for the Na+,K+-ATPase alpha 2 subunit and the glutamate transporters GLAST and GLT-1 in the rat somatosensory cortex. *Cereb Cortex* 12: 515-525.

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. (2005) Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120:421-433.

Chu K, Lee ST, Sinn DI, Ko SY, Kim EH, Kim JM, Kim SJ, Park DK, Jung KH, Song EC, Lee SK, Kim M, Roh JK. (2007) Pharmacological Induction of Ischemic Tolerance by Glutamate Transporter-1 (EAAT2) Upregulation. *Stroke* 38:177-182.

Ciccarelli R, Ballerini P, Sabatino G, Rathbone MP, D'Onofrio M, Caciagli F, Di Iorio P. (2001) Involvement of astrocytes in purine-mediated reparative processes in the brain. *Int J Dev Neurosci.*19:395-414.

Ciccarelli R, D'Alimonte I, Ballerini P, D'Auro M, Nargi E, Buccella S, Di Iorio P, Bruno V, Nicoletti F, Caciagli F. (2007) Molecular signalling mediating the protective effect of A1 adenosine and mGlu3 metabotropic glutamate receptor activation against apoptosis by oxygen/glucose deprivation in cultured astrocytes. *Mol Pharmacol*. 71:1369-1380.

Ciccarelli R, Di Iorio P, Ballerini P, Ambrosini G, Giuliani P, Tiboni GM, Caciagli F. (1994) Effects of exogenous ATP and related analogues on the proliferation rate of dissociated primary cultures of rat astrocytes. *J Neurosci Res*. 39:556-66.

Ciccarelli R, Di Iorio P, Ballerini P, Di Muzio M, Albanese C, Caciagli F. (1992) A1-mediated adenosine control and ATP P2Y site involvement in protein kinase C activity and purine release from cultured astrocytes. *Pharmacol Res*. Suppl 1:37-38.

Ciccarelli R, Di Iorio P, Bruno V, Battaglia G, D'Alimonte I, D'Onofrio M Nicoletti F, Caciagli F. (1999) Activation of A_1 adenosine or mGlu3 metabotropic glutamate receptors enhances the release of nerve growth factor and S-100β protein from cultured astrocytes. *Glia* 27:275-281.

Ciruela F, Casadó V, Rodrigues RJ, Luján R, Burgueño J, Canals M, Borycz J, Rebola N, Goldberg SR, Mallol J, Cortés A, Canela EI, López-Giménez JF, Milligan G, Lluis C, Cunha RA, Ferré S, Franco R. (2006) Presynaptic control of striatal glutamatergic neurotransmission by adenosine A_1-A_{2A} receptor heteromers. *J Neurosci*. 26: 2080-2087.

Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C. (2003) Storage and release of ATP from astrocytes in culture. *J Biol Chem*. 278:1354-1362.

Cognato GP, Agostinho PM, Hockemeyer J, Müller CE, Souza DO, Cunha RA. (2010) Caffeine and adenosine A_{2A} receptor antagonists prevent memory impairment and synaptotoxicity in adult rats triggered by a convulsive episode in early life. *J Neurochem*. 112: 453-462.

Coleman P, Federoff H, Kurlan R. (2004) A focus on the synapse for neuroprotection in Alzheimer disease and other dementias. *Neurology* 63: 1155-1162.

Cormier RJ, Mennerick S, Melbostad H, Zorumski CF. (2001) Basal levels of adenosine modulate mGluR5 on rat hippocampal astrocytes. *Glia* 33:24-35.

Costenla AR, Cunha RA, de Mendonça A. (2010) Caffeine, adenosine receptors, and synaptic plasticity. *J Alzheimers Dis.* 20 (Suppl 1): S25-S34.

Costenla AR, Diógenes MJ, Canas PM, Rodrigues RJ, Nogueira C, Maroco J, Agostinho PM, Ribeiro JA, Cunha RA, de Mendonça A. (2011) Enhanced role of adenosine A_{2A} receptors in the modulation of LTP in the rat hippocampus upon ageing. *Eur J Neurosci*. 34: 12-21.

Cristóvão-Ferreira S, Navarro G, Brugarolas M, Pérez-Capote K, Vaz SH, Fattorini G, Conti F, Lluis C, Ribeiro JA, McCormick PJ, Casadó V, Franco R, Sebastião AM. (2013) A_1R-$A_{2A}R$ heteromers coupled to G_s- and $G_{i/o}$-proteins modulate GABA transport into astrocytes. *Purinergic Signal*. [Epub ahead of print]

Cunha GM, Canas PM, Melo CS, Hockemeyer J, Müller CE, Oliveira CR, Cunha RA. (2008) Adenosine A_{2A} receptor blockade prevents memory dysfunction caused by beta-amyloid peptides but not by scopolamine or MK-801. *Exp Neurol*. 210: 776-781.

Cunha RA. (2001) Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles different sources and different receptors. *Neurochem Int.* 38:107-125.

Cunha RA. (2005) Neuroprotection by adenosine in the brain: from A1 receptor activation to A2A receptor blockade. *Purinergic Signal*. 1:111-134.

Cunha RA. (2008) Different cellular sources and different roles of adenosine: A_1 receptor-mediated inhibition through astrocytic-driven volume transmission and synapse-restricted A_{2A} receptor-mediated facilitation of plasticity. *Neurochem Int*. 52: 65-72.

Cunha RA, Agostinho PM. (2010) Chronic caffeine consumption prevents memory disturbance in different animal models of memory decline. *J Alzheimer Dis*. 20 (Suppl 1): S95-S116.

Cunha RA, Correia-de-Sá P, Sebastião AM, Ribeiro JA (1996) Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides. *Br J Pharmacol*. 119:253-260.

Dabir DV, Robinson MB, Swanson E, Zhang B, Trojanowski JQ, Lee VM, Forman MS. (2006) Impaired glutamate transport in a mouse model of tau pathology in astrocytes. *J Neurosci*. 26: 644-654.

Dai, SS, Zhou YG, Li W, An JH, Li P, Yang N, Chen XY, Xiong RP, Liu P, Zhao Y, Shen HY, Zhu PF, Chen JF. (2010) Local glutamate level dictates adenosine A_{2A} receptor regulation of neuroinflammation and traumatic brain injury. *J Neurosci*. 30: 5802-5810.

d'Alcantara P, Ledent C, Swillens S, Schiffmann SN. (2001) Inactivation of adenosine A_{2A} receptor impairs long term potentiation in the accumbens nucleus without altering basal synaptic transmission. *Neuroscience* 107:455-464.

D'Alimonte I, Ballerini P, Nargi E, Buccella S, Giuliani P, Di Iorio P, Caciagli F, Ciccarelli R. (2007) Staurosporine-induced apoptosis in astrocytes is prevented by A1 adenosine receptor activation. *Neurosci Lett*. 418:66-71.

Dall'Igna OP, Fett P, Gomes MW, Souza DO, Cunha RA, Lara DR. (2007) Caffeine and adenosine A_{2a} receptor antagonists prevent beta-amyloid (25–35)-induced cognitive deficits in mice. *Exp Neurol.* 203:241-245.

Dall'Igna OP, Porciúncula LO, Souza DO, Cunha RA, Lara DR. (2003) Neuroprotection by caffeine and adenosine A2A receptor blockade of β-amyloid neurotoxicity. *Br J Pharmacol*. 138: 1207-1209.

Dall'Igna OP, Fett P, Gomes MW, Souza DO, Cunha RA, Lara DR. (2007) Caffeine and adenosine A_{2a} receptor antagonists prevent beta-amyloid (25-35)-induced cognitive deficits in mice. *Exp Neurol*. 203: 241-245.

D'Amelio M, Cavallucci V, Middei S, Marchetti C, Pacioni S, Ferri A, Diamantini A, De Zio D, Carrara P, Battistini L, Moreno S, Bacci A, Ammassari-Teule M, Marie H, Cecconi F. (2011) Caspase-3 triggers early synaptic dysfunction in a mouse model of Alzheimer's disease. *Nat Neurosci*. 14: 69-76.

Danbolt NC. (2001) Glutamate uptake. *Prog Neurobiol*. 65: 1-105.

Daré E, Schulte G, Karovic O, Hammarberg C, Fredholm BB. (2007) Modulation of glial cell functions by adenosine receptors. *Physiol Behav*. 92:15-20.

de Bartolomeis A, Sarappa C, Magara S, Iasevoli F. (2012) Targeting glutamate system for novel antipsychotic approaches: relevance for residual psychotic symptoms and treatment resistant schizophrenia. *Eur J Pharmacol*. 682:1-11.

De Fusco M, Marconi R, Silvestri L, Atorino L, Rampoldi L, Morgante L, Ballabio A, Aridon P, Casari G. (2003) Haploinsufficiency of ATP1A2 encoding the Na^+/K^+ pump alpha2 subunit associated with familial hemiplegic migraine type 2. *Nat Genet*. 33: 192-196.

de Mendonça A, Cunha RA. (2010) Therapeutic opportunities for caffeine in Alzheimer's disease and other neurodegenerative disorders. *J Alzheimers Dis*. 20 (Suppl 1): S1-2.

De Mendonca A, Ribeiro, JA. (2000) Long-term potentiation observed upon blockade of adenosine A(1) receptors in rat hippocampus is N-methyl-Daspartate receptor-dependent. *Neurosci Lett*. 291:81-84.

de Vasconcellos AP, Zugno AI, Dos Santos AH, Nietto FB, Crema LM, Gonçalves M, Franzon R, de Souza Wyse AT, da Rocha ER, Dalmaz C. (2005) Na+,K(+)-ATPase activity is reduced in hippocampus of rats submitted to an experimental model of depression: effect of chronic lithium treatment and possible involvement in learning deficits. *Neurobiol Learn Mem*. 84:102-110.

Dellu F, Fauchey V, LeMoal M, Simon H. (1997) Extension of a new two-trial memory task in the rat: influence of environmental context on recognition processes. *Neurobiol Learn Mem*. 67: 112-120.

Deng X, Shibata H, Ninomiya H, Tashiro N, Iwata N, Ozaki N, Fukumaki Y. (2004) Association study of polymorphisms in the excitatory amino acid transporter 2 gene (SLC1A2) with schizophrenia. *BMC Psychiatry* 4:21.

Dennis SH, Jaafari N, Cimarosti H, Hanley JG, Henley JM, Mellor JR. (2011) Oxygen/glucose deprivation induces a reduction in synaptic AMPA receptors on hippocampal CA3 neurons mediated by mGluR1 and adenosine A3 receptors. *J Neurosci*. 31:11941-11952.

Desfrere L, Olivier P, Schwendimann L, Verney C, Gressens P. (2007) Transient inhibition of astrocytogenesis in developing mouse brain following postnatal caffeine exposure. *Pediatr Res*. 62:604-609.

de Yebra L, Malpesa Y, Ursu G, Pugliese M, Lievens JC, Goff LK, Mahy N. (2006) Dissociation between hippocampal neuronal loss, astroglial and microglial reactivity after pharmacologically induced reverse glutamate transport. *Neurochem Int*. 49:691-697.

Di Castro MA, Chuquet J, Liaudet N, Bhaukaurally K, Santello M, Bouvier D, Tiret P, Volterra A. (2011) Local Ca^{2+} detection and modulation of synaptic release by astrocytes. *Nat Neurosci*. 14:1276-1284.

Di Lorio P, Kleywegt S, Ciccarelli R, Traversa U, Andrew CM, Crocker CE, Werstiuk ES, Rathbone MP. (2002) Mechanisms of apoptosis induced by purine nucleosides in astrocytes. *Glia* 38:179-190.

Doengi M, Deitmer JW, Lohr C. (2008) New evidence for purinergic signaling in the olfactory bulb: A2A and P2Y1 receptors mediate intracellular calcium release in astrocytes. *FASEB J*. 22:2368-2378.

Domercq M, Etxebarria E, Pérez-Samartín A, Matute C. (2005) Excitotoxic oligodendrocyte death and axonal damage induced by glutamate transporter inhibition. *Glia* 52: 36-46.

Dong Y, Benveniste EN. (2001) Immune function of astrocytes. *Glia* 36:180-190.

dos Reis-Lunardelli EA, Ramirez MR, Castro CC, Coitinho AS, Bavaresco C, da Trindade LS, Perrenoud MF, Wyse AT, Sarkis JJ, Izquierdo I. (2007) Effects of an acute treatment with L-

thyroxine on memory, habituation, danger avoidance, and on Na+, K+ -ATPase activity in rat brain. *Curr Neurovasc Res.* 4:259-267.

Du J, Wei Y, Liu L, Wang Y, Khairova R, Blumenthal R, Tragon T, Hunsberger JG, Machado-Vieira R, Drevets W, Wang YT, Manji HK. (2010) A kinesin signaling complex mediates the ability of GSK-3beta to affect mood-associated behaviors. *Proc Natl Acad Sci USA*. 107: 11573-11578.

Duarte JM, Agostinho PM, Carvalho RA, Cunha RA. (2012) Caffeine consumption prevents diabetes-induced memory impairment and synaptotoxicity in the hippocampus of NONcZNO10/LTJ mice. *PLoS One* 7: e21899.

Duarte JM, Carvalho RA, Cunha RA, Gruetter R. (2009) Caffeine consumption attenuates neurochemical modifications in the hippocampus of streptozotocin-induced diabetic rats. *J Neurochem.* 111: 368-79.

Dunkley PR, Jarvie PE, Robinson PJ. (2008) A rapid Percoll gradient procedure for preparation of synaptosomes. *Nat Protoc*. 3: 1718-1728.

Dunlop J. (2006) Glutamate-based therapeutic approaches: targeting the glutamate transport system. *Curr Opin Pharmacol*. 6:103-107.

Dunwiddie TV, Masino SA. (2001) The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci*. 24:31-55.

El Yacoubi M, Ledent C, Parmentier M, Costentin J, Vaugeois JM. (2008) Evidence for the involvement of the adenosine A_{2A} receptor in the lowered susceptibility to pentylenetetrazol-induced seizures produced in mice by long-term treatment with caffeine. *Neuropharmacology* 55: 35-40.

Eskelinen MH, Ngandu T, Tuomilehto J, Soininen H, Kivipelto M. (2009) Midlife coffee and tea drinking and the risk of late-life dementia: a population-based CAIDE study. *J Alzheimers Dis*. 16: 85-91.

Farina C, Aloisi F, Meinl E. (2007) Astrocytes are active players in cerebral innate immunity. *Trends Immunol*. 28:138-145.

Fattorini G, Melone M, Bragina L, Candiracci C, Cozzi A, Pellegrini-Giampietro DE, Torres-Ramos, M, Pérez-Samartín A, Matute C, Conti F. (2008) GLT-1 expression and Glu uptake in rat cerebral cortex are increased by phencyclidine. *Glia* 56: 1320-1327.

Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. (2004) Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron* 43:729-743.

Fernández-Tomé, P, Brera, B, Arévalo, M, Ceballos, ML. (2004) β-Amyloid 25-35 inhibits glutamate uptake in cultured neurons and astrocytes: modulation of uptake as a survival mechanism. *Neurobiol Dis*. 15: 580-589

Ferrarese C, Begni B, Canevari C, Zoia C, Piolti R, Frigo M, Appollonio I, Frattola L. (2000) Glutamate uptake is decreased in platelets from Alzheimer's disease patients. *Ann Neurol*. 47: 641-643.

Ferré S, Ciruela F, Quiroz C, Luján R, Popoli P, Cunha RA, Agnati LF, Fuxe K, Woods AS, Lluis C, Franco R. (2007) Adenosine receptor heteromers and their integrative role in striatal function. *ScientificWorldJournal* 7: 74-85.

Ferré S, Lluís C, Justinova Z, Quiroz C, Orru M, Navarro G, Canela EI, Franco R, Goldberg SR. (2010) Adenosine-cannabinoid receptor interactions Implications for striatal function. *Br J Pharmacol.* 160:443-453.

Ferré S. (2008) An update on the mechanisms of the psychostimulant effects of caffeine. *J. Neurochem.* 105: 1067-1079.

Fiacco T, Casper K, Sweger E, Agulhon C, Taves S , Kurtzer-Minton S, McCarthy KD. (2009) Molecular Approaches for Studying Astrocytes. V. Parpura and P.G. Haydon (eds.), *Astrocytes in (Patho)Physiology of the Nervous System.* Springer Science + Business Media, LLC (2009). 14: 383-405.

Field JR, Walker AG, Conn PJ. (2011) Targeting glutamate synapses in schizophrenia. *Trends Mol Med.* 17: 689-698.

Fields RD, Burnstock G. (2006) Purinergic signaling in neuron-glia interactions. *Nat Rev Neurosci.* 7:423-436.

Figiel M, Maucher T, Rozyczka J, Bayatti N, Engele J. (2003) Regulation of glial glutamate transporter expression by growth factors. *Exp Neurol*. 183: 124-135.

Filosa A, Paixão S, Honsek SD, Carmona MA, Becker L, Feddersen B, Gaitanos L, Rudhard Y, Schoepfer R, Klopstock T, Kullander K, Rose CR, Pasquale EB, Klein R. (2009) Neuron-glia communication via EphA4/ephrin-A3 modulates LTP through glial glutamate transport. *Nat. Neurosci.* 12: 1285-1292.

Flajolet M, Wang Z, Futter M, Shen W, Nuangchamnong N, Bendor J, Wallach I, Nairn AC, Surmeier DJ, Greengard P. (2008) FGF acts as a co-transmitter through adenosine A_{2A} receptor to regulate synaptic plasticity. *Nat Neurosci*. 11: 1402-1409.

Franco R, Fuxe K, Bader M. (2007) Working memory deficits in transgenic rats overexpressing human adenosine A_{2A} receptors in the brain. *Neurobiol Learn Mem*. 87:42-56.

Franke H, Verkhratsky A, Burnstock G, Illes P. (2012) Pathophysiology of astroglial purinergic signalling. *Purinergic Signal*. 8:629-657.

Fredholm BB, Bättig K, Holmén J, Nehlig A, Zvartau EE. (1999) Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev*. 51: 83-133.

Fredholm BB, Chen JF, Cunha RA, Svenningsson P, Vaugeois JM. (2005) Adenosine and brain function. *Int Rev Neurobiol*. 63:191-270.

Furness DN, Dehnes Y, Akhtar AQ, Rossi DJ, Hamann M, Grutle NJ, Gundersen V, Holmseth S, Lehre KP, Ullensvang K, Wojewodzic M, Zhou Y, Attwell D, Danbolt NC. (2008) A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a neuronal role for excitatory amino acid transporter 2 EAAT2. *Neuroscience* 157: 80-94.

Gamaro GD, Streck EL, Matté C, Prediger ME, Wyse AT, Dalmaz C. (2003) Reduction of hippocampal Na+, K+-ATPase activity in rats subjected to an experimental model of depression. *Neurochem Res*. 28:1339-1344.

Gao J, Wymore RS, Wang Y, Gaudette GR, Krukenkamp IB, Cohen IS, Mathias RT. (2002) Isoform-specific stimulation of cardiac Na/K pumps by nanomolar concentrations of glycosides. *J Gen Physiol.* 119*:* 297-312*.*

Garção P, Oliveira CR, Agostinho P. (2006) Comparative study of microglia activation induced by amyloid-beta and prion peptides: role in neurodegeneration. *J Neurosci Res*. 84: 182-193.

Garcia-Marin V, Garcia-Lopez P, Freire M. (2007) Cajal's contributions to glia research. *Trends Neurosci.* 30:479-487.

Gardoni F, Di Luca M. (2006) New targets for pharmacological intervention in the glutamatergic synapse. *Eur J Pharmacol*. 545:2-10.

Ge Y, Dong Z, Bagot RC, Howland JG, Phillips AG, Wong TP, Wang YT. (2010) Hippocampal long-term depression is required for the consolidation of spatial memory. *Proc Natl Acad Sci USA.* 107:16697-16702.

Gegelashvili G, Danbolt NC, Schousboe A. (1997) Neuronal soluble factors differentially regulate the expression of the GLT1 and GLAST glutamate transporters in cultured astroglia. *J. Neurochem.* 69: 2612-2615.

Gegelashvili G, Dehnes Y, Danbolt NC, Schousboe A. (2000) The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms. *Neurochem Int.* 37: 163-170.

Gegelashvili M, Rodriguez-Kern A, Sung L, Shimamoto K, Gegelashvili G (2007) Glutamate transporter GLAST/EAAT1 directs cell surface expression of FXYD2/gamma subunit of Na, K-ATPase in human fetal astrocytes. *Neurochem Int*. 50: 916-920.

Gehring EM, Zurn A, Klaus F, Laufer J, Sopjani M, Lindner R, Strutz-Seebohm N, Tavaré JM, Boehmer C, Palmada M, Lang UE, Seebohm G, Lang F. (2009) Regulation of the glutamate transporter EAAT2 by PIKfyve. *Cell Physiol Biochem*. 24: 361-368.

Gelber RP, Petrovitch H, Masaki KH, Ross GW, White LR. (2011) Coffee intake in midlife and risk of dementia and its neuropathologic correlates. *J Alzheimers Dis*. 23: 607-615.

Genda EN, Jackson JG, Sheldon AL, Locke SF, Greco TM, O'Donnell JC, Spruce LA, Xiao R, Guo W, Putt M, Seeholzer S, Ischiropoulos H, Robinson MB. (2011) Co-compartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria. *J Neurosci.* 31:18275-18288.

Genoud C, Quairiaux C, Steiner P, Hirling H, Welker E, Knott GW. (2006) Plasticity of astrocytic coverage and glutamate transporter expression in adult mouse cortex. *PLoS Biol*. 4:e343.

Gether U, Andersen PH, Larsson OM, Schousboe A. (2006) Neurotransmitter transporters: molecular function of important drug targets. *Trends Pharmacol*. Sci. 27:375-383.

Gibbs ME, Anderson DG, Hertz L. (2006) Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. *Glia* 54:214-222.

Gibbs ME, Hertz L. (2008) Inhibition of astrocytic energy metabolism by D-lactate exposure impairs memory. *Neurochem Int*. 52:1012-1018.

Giménez-Llort L, Schiffmann SN, Shmidt T, Canela L, Camón L, Wassholm M, Canals M, Terasmaa A, Fernández-Teruel A, Tobeña A, Popova E, Ferré S, Agnati L, Ciruela F, Martínez E, Scheel-Kruger J, Lluis C, Franco R, Fuxe K, Bader M. (2007) Working memory deficits in transgenic rats overexpressing human adenosine A2A receptors in the brain. *Neurobiol Learn Mem*. 87:42-56.

Goff DC, Lamberti JS, Leon AC, Green MF, Miller AL, Patel J, Manschreck T, Freudenreich O, Johnson SA. (2008) A placebo-controlled add-on trial of the ampakine, CX516, for cognitive deficits in schizophrenia*. Neuropsychopharmacology* 33:465-472.

Gomes CV, Kaster MP, Tomé AR, Agostinho PM, Cunha RA. (2011) Adenosine receptors and brain diseases: Neuroprotection and neurodegeneration. *Biochim Biophys Acta*. 1808: 1380-1399.

Gomez-Pinilla F, So V, Kesslak JP. (1998) Spatial learning and physical activity contribute to the induction of fibroblast growth factor: neural substrates for increased cognition associated with exercise. *Neuroscience* 85:53-61.

Gordon GR, Baimoukhametova DV, Hewitt SA, Rajapaksha WR, Fisher TE, Bains JS. (2005) Norepinephrine triggers release of glial ATP to increase postsynaptic efficacy. *Nat Neurosci*. 8: 1078-1086.

Goubard V, Fino E, Venance L. (2011) Contribution of astrocytic glutamate and GABA uptake to corticostriatal information processing. *J Physiol*. 589:2301-2319.

Guillet BA, Velly LJ, Canolle B, Masmejean FM, Nieoullon AL, Pisano P. (2005) Differential regulation by protein kinases of activity and cell surface expression of glutamate transporters in neuron-enriched cultures. *Neurochem. Int*. 46**:** 337-346.

Gunduz O, Oltulu C, Buldum D, Guven R, Ulugol A. (2011a) Anti-allodynic and anti-hyperalgesic effects of ceftriaxone in streptozocin-induced diabetic rats. *Neurosci Lett*. 491:23-25.

Gunduz O, Oltulu C, Ulugol A. (2011b) Role of GLT-1 transporter activation in prevention of cannabinoid tolerance by the β-lactam antibiotic, ceftriaxone, in mice. *Pharmacol Biochem Behav.* 99:100-103.

Guo H, Lai L, Butchbach ME, Stockinger MP, Shan X, Bishop GA, Lin CL. (2003) Increased expression of the glial glutamate transporter EAAT2 modulates excitotoxicity and delays the onset but not the outcome of ALS in mice. *Hum Mol Genet*. 12:2519-2532.

Guthrie PB, Knappenberger J, Segal M, Bennett MV, Charles AC, Kater SB. (1999) ATP released from astrocytes mediates glial calcium waves. *J Neurosci*. 19:520-528.

Halassa MM, Fellin T, Haydon PG. (2007a) The tripartite synapse: roles for gliotransmission in health and disease. *Trends Mol Med*. 13:54-63.

Halassa MM, Fellin T, Haydon PG. (2009) Tripartite synapses: roles for astrocytic purines in the control of synaptic physiology and behavior. *Neuropharmacology* 57: 343-346.

Halassa MM, Fellin T, Takano H, Dong JH, Haydon PG. (2007b) Synaptic islands defined by the territory of a single astrocyte. *J Neurosci*. 27:6473-6477.

Halassa MM, Florian C, Fellin T, Munoz JR, Lee SY, Abel T, Haydon PG, Frank MG. (2009) Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. *Neuron* 61:213-219.

Halassa MM, Haydon PG. (2010) Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annu Rev Physiol*. 72: 335-355.

Hambry ME, Uliasz TF, Hewett SJ, Hewett JA. (2006) Characterization of an improved procedure for the removal of microglia from confluent monolayers of primary astrocytes. J *Neurosci Meth.* 150: 128-137.

Hamby ME, Sofroniew MV. (2010) Reactive astrocytes as therapeutic targets for CNS disorders. *Neurotherapeutics* 2010:494-506.

Hammer J, Qu H, Håberg A, Sonnewald U. (2001) In vivo effects of adenosine A_2 receptor agonist and antagonist on neuronal and astrocytic intermediary metabolism studied with ex vivo ^{13}C MR spectroscopy. *J Neurochem*. 79: 885-892.

Han J, Kesner P, Metna-Laurent M, Duan T, Xu L, Georges F, Koehl M, Abrous DN, Mendizabal-Zubiaga J, Grandes P, Liu Q, Bai G, Wang W, Xiong L, Ren W, Marsicano G, Zhang X. (2012) Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. *Cell* 48: 1039-1050.

Hansson E, Rönnbäck L. (2003) Glial neuronal signaling in the central nervous system. *FASEB J.* 17:341-348.

Hardy J, Cowburn R, Barton A, Reynolds G, Lofdahl E, O'Carroll AM, Wester P, Winblad B. (1987) Region-specific loss of glutamate innervation in Alzheimer's disease. *Neurosci Lett*. 73: 77-80.

Hardy JA, Higgins GA. (1992) Alzheimer's disease: The amyloid cascade hypothesis. *Science* 256: 184-185.

Harkany T, Abrahám I, Timmerman W, Laskay G, Tóth B, Sasvári M, Kónya C, Sebens JB, Korf J, Nyakas C, Zarándi M, Soós K, Penke B, Luiten PG. (2000) beta-amyloid neurotoxicity is mediated by a glutamate-triggered excitotoxic cascade in rat nucleus basalis. *Eur J Neurosci*. 12: 2735-2745.

Harris ME, Wang Y, Pedigo NW Jr, Hensley K, Butterfield DA, Carney JM. (1996) Amyloid beta peptide (25-35) inhibits Na+-dependent glutamate uptake in rat hippocampal astrocyte cultures. *J Neurochem*. 67: 277-286.

Hartman RE, Wozniak DF, Nardi A, Olney JW, Sartorius L, Holtzman DM. (2001) Behavioral phenotyping of GFAP-apoE3 and -apoE4 transgenic mice: apoE4 mice show profound working memory impairments in the absence of Alzheimer's-like neuropathology. *Exp Neurol*. 170:326-344.

Hattori N, Kitagawa K, Higashida T, Yagyu K, Shimohama S, Wataya T, Perry G, Smith MA, Inagaki C. (1998) CI-ATPase and Na+/K(+)-ATPase activities in Alzheimer's disease brains. *Neuroscience Letters* 254:141-144

Haydon PG. (2001) GLIA: listening and talking to the synapse. *Nat Rev Neurosci*. 2:185-293.

Haydon PG, Carmignoto, G. (2006) Astrocyte control of synaptic transmission and neurovascular coupling. *Physiol Rev*. 86: 1009-1031.

Hazell AS, Itzhak Y, Liu H, Norenberg MD (1997) 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) decreases glutamate uptake in cultured astrocytes. *J Neurochem*. 68: 2216-2219.

Hazelwood L, Free R, Cabrera D, Skinbjerg M, Sibley D. (2008) Reciprocal modulation of function between the D1 and D2 dopamine receptors and the Na^+,K^+-ATPase. *J Biol Chem*. 283:36441-36494.

Headley PM, Grillner S. (1990) Excitatory amino acids and synaptic transmission: the evidence for a physiological function. *Trends Pharmacol Sci*. 11: 205-211.

Henneberger C, Papouin T, Oliet SH, Rusakov DA. (2010) Long-term potentiation depends on release of D-serine from astrocytes. *Nature* 463:232-236.

Hertz L, Hansson E, Rönnbäck L. (2001) Signaling and gene expression in the neuron-glia unit during brain function and dysfunction: Holger Hydén in memoriam. *Neurochem Int*. 39:227-252.

Hertz L. (1978) Kinetics of adenosine uptake into astrocytes. *J Neurochem* 31: 55-62.

Hettinger BD, Lee A, Linden J, Rosin DL. (2001) Ultrastructural localization of adenosine A2A receptors suggests multiple cellular sites for modulation of GABAergic neurons in rat striatum. *J Comp Neurol.* 431: 331-346.

Hindley S, Herman MA, Rathbone MP. (1994) Stimulation of reactive astrogliosis in vivo by extracellular adenosine diphosphate or an adenosine A2 receptor agonist. *J Neurosci Res.* 38:399-406.

Hines DJ, Schmitt LI, Hines RM, Moss SJ, Haydon PG. (2013) Antidepressant effects of sleep deprivation require astrocyte-dependent adenosine mediated signaling. *Transl Psychiatry* 3:e212.

Hokin-Neaverson M, Jefferson JW. (1989) Erythrocytes sodium pump activity in bipolar affective disorder and other psychiatry disorders. *Neuropsychobiology* 22:1-7.

Hösli E, Hösli L**.** (1988) Autoradiographic studies on the uptake of adenosine and on binding of adenosine analogues in neurons and astrocytes of cultured rat cerebellum and spinal cord. *Neuroscience* 24:621-628.

Huang AM, Lee EH. (1997) Identification of a novel glial fibrillary acidic protein mRNA isotype related to memory retention in rats. *Neuroreport* 8:1619-1624.

Huang YH, Bergles DE. (2004) Glutamate transporters bring competition to the synapse. *Curr Opin Neurobiol.* 14: 346-352.

Huang YH, Lin Y, Mu P, Lee BR, Brown TE, Wayman G, Marie H, Liu W, Yan Z, Sorg BA, Schlüter OM, Zukin RS, Dong Y. (2009) In vivo cocaine experience generates silent synapses. *Neuron* 63: 40-47.

Huang YH, Sinha SR, Tanaka K, Rothstein JD, Bergles DE. (2004) Astrocyte glutamate transporters regulate metabotropic glutamate receptor-mediated excitation of hippocampal interneurons. *J Neurosci*. 24:4551-4559.

Hydén H, Egyhazi E. (1963) Glial RNA changes during a learning experiment in rats. *Proc Natl Acad Sci USA*. 49:618-624.

Hynd MR, Scott HL, Dodd PR. (2004) Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. *Neurochem Int*. 45: 583-595.

Iadecola C, Nedergaard M (2007) Glial regulation of the cerebral microvasculature. *Nat Neurosci.* 10:1369-1376.

Illarionova NB, Gunnarson E, Li Y, Brismar H, Bondar A, Zelenin S, Aperia A. (2010) Functional and molecular interactions between aquaporins and Na, K-ATPase. *Neuroscience* 168:915-925.

Inoue K, Kato F, Tsuda M. (2010) The modulation of synaptic transmission by the glial purinergic system. *The Open Neuroscience Journal*. 4: 84-92.

Jacob CP, Koutsilieri E, Bartl J, Neuen-Jacob E, Arzberger T, Zander N, Ravid R, Roggendorf W, Riederer P, Grünblatt E. (2007) Alterations in expression of glutamatergic transporters and receptors in sporadic Alzheimer's disease. *J Alzheimers Dis*. 11: 97-116.

James VA, Sharma RP, Walker RJ, Wheal HV. (1980) Actions of glutamate kainate dihydrokainate and analogues on leech neurone acidic amino acid receptors. *Eur J Pharmacol*. 62: 35-39.

John CS, Smith KL, Van't Veer A, Gompf HS, Carlezon WA Jr, Cohen BM, Öngür D, Bechtholt-Gompf AJ. (2012) Blockade of astrocytic glutamate uptake in the prefrontal cortex induces anhedonia. *Neuropsychopharmacology* 37:2467-2475.

Johnson JW, Ascher P. (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. *Nature* 325:529-531.

Jones TA, Hawrylak N, Greenough WT. (1996) Rapid laminar-dependent changes in GFAP immunoreactive astrocytes in the visual cortex of rats reared in a complex environment. *Psychoneuroendocrinology* 21:189-201.

Jourdain P, Bergersen LH, Bhaukaurally K, Bezzi P, Santello M, Domercq M, Matute C, Tonello F, Gundersen V, Volterra A. (2007) Glutamate exocytosis from astrocytes controls synaptic strength. *Nat Neurosci*. 10:331-339.

Kálmán M*,* Hajós F. (1989*)* Distribution of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes in the rat brain. I. Forebrain*. Exp Brain Res*. 78*:* 147-163*.*

Kanai Y, Hediger MA. (1992) Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* 360: 467-471.

Kanai Y, Hediger MA. (2004) The glutamate/neutral amino acid transporter family SLC1: molecular, physiological and pharmacological aspects. *Pflugers Arch*. 447: 469-479.

Kang J, Jiang L, Goldman SA, Nedergaard M. (1998) Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat Neurosci*. 1:683-692.

Kanno T, Nishizaki T. (2012) A(2a) adenosine receptor mediates PKA-dependent glutamate release from synaptic-like vesicles and Ca(2+) efflux from an IP(3)- and ryanodine-insensitive intracellular calcium store in astrocytes. *Cell Physiol Biochem*. 30:1398-1412.

Kashani A, Lepicard E, Poirel O, Videau C, David JP, Fallet-Bianco C, Simon A, Delacourte, A Giros, B Epelbaum, J, Betancur C, El Mestikawy S. (2008) Loss of VGLUT1 and VGLUT2 in the prefrontal cortex is correlated with cognitive decline in Alzheimer disease. *Neurobiol Aging*. 29: 1619-1630.

Katagiri H*,* Tanaka K*,* Manabe T*. (*2001*)* Requirement of appropriate glutamate concentrations in the synaptic cleft for hippocampal LTP induction. *Eur J Neurosci.* 14*:* 547-553*.*

Kawahara K, Hosoya R, Sato H, Tanaka M, Nakajima T, Iwabuchi, S. (2002) Selective blockade of astrocytic glutamate transporter GLT-1 with dihydrokainate prevents neuronal death during ouabain treatment of astrocyte/neuron cocultures. *Glia* 40:337-349.

Kawamura M, Gachet C, Inoue K, Kato F. (2004) Direct excitation of inhibitory interneurons by extracellular ATP mediated by P2Y1 receptors in the hippocampal slice. *J Neurosci*. 24: 10835-10845.

Ke RH, Xiong J, Liu Y, Ye ZR. (2009) Adenosine A2a receptor induced gliosis via Akt/NF-kappaB pathway in vitro. *Neurosci Res*. 65:280-5

Ke RH, Xiong J, Liu Y. (2012) Adenosine A2a receptor induces GDNF expression by the Stat3 signal in vitro. *Neuroreport*. 23:958-962.

Kessey K, Mogul DJ. (1997) NMDA-Independent LTP by adenosine A2 receptormediated postsynaptic AMPA potentiation in hippocampus. *J Neurophysiol*. 78:1965-1972.

Kim J, Lee S, Park K, Hong I, Song B, Son G, Park H, Kim WR, Park E, Choe HK, Kim H, Lee C, Sun W, Kim K, Shin KS, Choi S. (2007) Amygdala depotentiation and fear extinction. *Proc Natl Acad Sci USA*. 104: 20955-20960.

Kim K, Lee SG, Kegelman TP, Su ZZ, Das SK, Dash R, Dasgupta S, Barral PM, Hedvat M, Diaz P, Reed JC, Stebbins JL, Pellecchia M, Sarkar D, Fisher PB. (2011) Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: opportunities for developing novel therapeutics. *J Cell Physiol*. 226:2484-2493.

Kirischuk S, Parpura V, Verkhratsky A. (2012) Sodium dynamics: another key to astroglial excitability? *Trends Neurosci*. 35: 497-506.

Kirvell SL, Esiri M, Francis PT. (2006) Down-regulation of vesicular glutamate transporters precedes cell loss and pathology in Alzheimer's disease. *J Neurochem*. 98: 939-950.

Köfalvi A, Rodrigues RJ, Ledent C, Mackie K, Vizi ES, Cunha RA, Sperlágh B. (2005) Involvement of cannabinoid receptors in the regulation of neurotransmitter release in the rodent striatum: a combined immunochemical and pharmacological analysis. *J Neurosci*. 25: 2874-2884.

Kondo K, Hashimoto H, Kitanaka J, Sawada M, Suzumura A, Marunouchi T, Baba A. (1995) Expression of glutamate transporters in cultured glial cells. *Neurosci Lett*. 188: 140-142.

Kondziella D, Brenner E, Eyjolfsson EM, Sonnewald U. (2007) How do glial-neuronal interactions fit into current neurotransmitter hypotheses of schizophrenia? *Neurochem Int*. 50:291-301.

Korn T, Magnus T, Jung S. (2005) Autoantigen specific T cells inhibit glutamate uptake in astrocytes by decreasing expression of astrocytic glutamate transporter GLAST: a mechanism mediated by tumor necrosis factor-alpha. *FASEB J*. 13:1878-1880.

Kreutzberg G W, Barron K D, Schubert P. (1978) Cytochemical localization of 5'-nucleotidase in glial plasma membranes. *Brain Res.* 158: 247-257.

Kristiansen LV, Huerta I, Beneyto M, Meador-Woodruff JH. (2007) NMDA receptors and schizophrenia. *Curr Opin Pharmacol*. 7:48-55.

Kurihara K, Nakanishi N, Ueha T. (2000) Regulation of Na^+-K^+-ATPase by cAMP-dependent protein kinase anchored on membrane via its anchoring protein. *Am J Physiol Cell Physiol*. 279: C1516-C1527.

Kurokawa M, Koga K, Kase H, Nakamura J, Kuwana Y. (1996) Adenosine A2a receptor-mediated modulation of striatal acetylcholine release in vivo. *J Neurochem*. 66:1882-1888.

Landolt HP. (2008) Sleep homeostasis: a role for adenosine in humans? *Biochem Pharmacol*. 75:2070-2079.

Lara DR, Souza DO. (2000) Schizophrenia: a purinergic hypothesis. *Med Hypotheses* 54:157-166.

Lara DR. (2010) Caffeine, mental health, and psychiatric disorders. *J Alzheimers* Dis. 20:S239-48.

Latini S, Pedata F. (2001) Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem*. 79: 463-484.

Lauderback CM, Harris-White ME, Wang Y, Pedigo NW Jr, Carney JM, Butterfield DA. (1999) Amyloid beta-peptide inhibits Na^+-dependent glutamate uptake. *Life Sci*. 65: 1977-1981.

Lee HU, Yamazaki Y, Tanaka KF, Furuya K, Sokabe M, Hida H, Takao K, Miyakawa T, Fujii S, Ikenaka K. (2013) Increased astrocytic ATP release results in enhanced excitability of the hippocampus. *Glia* 61:210-224.

Lee Y, Messing A, Su M, Brenner M. (2008) GFAP promoter elements required for region-specific and astrocyte-specific expression. *Glia* 56: 481-493.

Lee Y, Su M, Messing A, Brenner M. (2006) Astrocyte heterogeneity revealed by expression of a GFAP-LacZ transgene. *Glia* 53:677-687.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC. (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. *J Neurosci*. 15:1835-1853.

Leite MR, Wilhelm EA, Jesse CR, Brandão R, Nogueira CW. (2011) Protective effect of caffeine and a selective A_{2A} receptor antagonist on impairment of memory and oxidative stress of aged rats. *Exp Gerontol*. 46: 309-315.

Leung TC, Lui CN, Chen LW, Yung WH, Chan YS, Yung KK. (2012) Ceftriaxone ameliorates motor deficits and protects dopaminergic neurons in 6-hydroxydopamine-lesioned rats. *ACS Chem Neurosci.* 2012 3:22-30.

Levison SW, de Vellis J, Goldman JE. (2005) Astrocyte Development. Mahendra S. Rao and Marcus Jacobson (eds), *Developmental Neurobiology*, Kluwer Academic / Plenum Publishers, New York (2005) 7: 197-222.

Li S, Mallory M, Alford M, Tanaka S, Masliah E. (1997). Glutamate transporter alterations in Alzheimer disease are possibly associated with abnormal APP expression. *J Neuropathol Exp Neurol*. 56: 901-911.

Li XX, Nomura T, Aihara H, Nishizaki T. (2001) Adenosine enhances glial glutamate efflux via A2a adenosine receptors. *Life Sci*. 68:1343-1350.

Li Y, Sattler R, Yang EJ, Nunes A, Ayukawa Y, Akhtar S, Ji G, Zhang PW, Rothstein JD. (2011) Harmine, a natural beta-carboline alkaloid, upregulates astroglial glutamate transporter expression. *Neuropharmacology* 60:1168-1175.

Li YK, Wang F, Wang W, Luo Y, Wu PF, Xiao JL, Hu ZL, Jin Y, Hu G, Chen JG. (2012) Aquaporin-4 deficiency impairs synaptic plasticity and associative fear memory in the lateral amygdala: involvement of downregulation of glutamate transporter-1 expression. *Neuropsychopharmacology* 37:1867-1878.

Li YK, Wang F, Wang W, Luo Y, Wu PF, Xiao JL, Hu ZL, Jin Y, Hu G, Chen JG. (2012) Aquaporin-4 deficiency impairs synaptic plasticity and associative fear memory in the lateral amygdala: involvement of downregulation of glutamate transporter-1 expression. *Neuropsychopharmacology* 37: 1867-1878.

Liang Z, Valla J, Sefidvash-Hockley S, Rogers J, Li R. (2002) Effects of estrogen treatment on glutamate uptake in cultured human astrocytes derived from cortex of Alzheimer's disease patients. *J Neurochem*. 80:807-814.

Lichtstein D, Samuelov S, Bourrit A. (1985) Characterization of the stimulation of neuronal Na^+, K^+-ATPase activity by low concentrations of ouabain. *Neurochem Int*. 7: 709-715.

Lievens JC , Woodman B , Mahal A , Spasic-Boscovic O , Samuel D , Kerkerian-Le Goff L , Bates GP (2001) Impaired glutamate uptake in the R6 Huntington"s disease transgenic mice. *Neurobiol Dis*. 8:807-821.

Lim DK, Kim HS. (2003) Opposite modulation of glutamate uptake by nicotine in cultured astrocytes with/without cAMP treatment. *Eur J Pharmacol*. 476: 179-184.

Lingrel JB, Williams MT, Vorhees CV, Moseley AE. (2007) Na,K-ATPase and the role of alpha isoforms in behavior. *J Bioenerg Biomembr*. 39: 385-389.

Lipski J, Wan CK, Bai JZ, Pi R, Li D, Donnelly D. (2007) Neuroprotective potential of ceftriaxone in in vitro models of stroke. *Neuroscience* 146:617-629.

Lipton SA, Rosenberg PA. (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *N Engl J Med*. 330: 613-622.

Lisman JE, Coyle JT, Green RW, Javitt DC, Benes FM, Heckers S, Grace AA. (2008) Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends Neurosci*. 31:234-242.

Lisman JE, Coyle JT, Green RW, Javitt DC, Benes, FM, Heckers S, Grace AA. (2008) Circuit-based framework for understanding neurotransmitter and risk gene interactions in schizophrenia. *Trends Neurosci*. *31*: 234-242.

Liu QS, Xu Q, Arcuino G, Kang J, Nedergaard M. (2004a) Astrocyte-mediated activation of neuronal kainate receptors. *Proc Natl Acad Sci USA*. 101:3172-3177.

Liu QS, Xu Q, Kang J, Nedergaard M. (2004b) Astrocyte activation of presynaptic metabotropic glutamate receptors modulates hippocampal inhibitory synaptic transmission. *Neuron Glia Biol*. 1:307-316.

Liu XB, Murray KD, Jones EG. (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *J Neurosci*. 24:8885-8895.

Longuemare MC, Rose CR, Farrell K, Ransom BR, Waxman SG, Swanson RA. (1999) K(+)-induced reversal of astrocyte glutamate uptake is limited by compensatory changes in intracellular Na+. *Neuroscience* 93:285-292.

Lopes LV, Cunha RA, Kull B, Fredholm BB, Ribeiro JA. (2002) Adenosine A(2A) receptor facilitation of hippocampal synaptic transmission is dependent on tonic A(1) receptor inhibition. *Neuroscience* 112: 319-329.

Lopes LV, Halldner L, Rebola N, Johansson B, Ledent C, Chen JF, Fredholm BB, Cunha RA. (2004) Binding of the prototypical adenosine A_{2A} receptor agonist CGS 21680 to the cerebral cortex of adenosine A_1 and A_{2A} receptor knockout mice. *Br J Pharmacol*. 141: 1006-1014.

Lopes LV, Sebastião AM, Ribeiro JA. (2011) Adenosine and related drugs in brain diseases: present and future in clinical trials. *Curr Top Med Chem*. 11:1087-1101.

Luscher C, Malenka RC, Nicoll RA. (1998) Monitoring glutamate release during LTP with glial transporter currents. *Neuron* 21: 435-441.

Magistretti PJ, Ransom BR. (2002) Astrocytes. Kenneth L. Davis, Dennis Charney, Joseph T. Coyle, and Charles Nemeroff (eds.), *Neuropsychopharmacology: The Fifth Generation of Progress.* American College of Neuropsychopharmacology (2002). 10:133-146.

Maia L, De Mendonça A. (2002). Does caffeine intake protect from Alzheimer's disease? *Eur J Neurol.* 9: 377-382.

Maienschein V, Marxen M, Volknandt W, Zimmermann H. (1999) A plethora of presynaptic proteins associated with ATP-storing organelles in cultured astrocytes. *Glia* 26: 233-244.

Malarkey EB, Parpura V. (2009) Mechanisms of Transmitter Release from Astrocytes. V. Parpura and P.G. Haydon (eds.), *Astrocytes in (Patho)Physiology of the Nervous System.* Springer Science + Business Media, LLC (2009). 12:301-350.

Malenka RC, Bear MF. (2004) LTP and LTD: an embarrassment of riches. *Neuron* 44:5-21.

Maleszka R*,* Helliwell P*,* Kucharski R*. (*2000*)* Pharmacological interference with glutamate re-uptake impairs long-term memory in the honeybee*,* Apis mellifera*. Behav Brain Res.* 115*:* 49-53*.*

Manzoni OJ, Manabe T, Nicoll RA. (1994) Release of adenosine by activation of NMDA receptors in the hippocampus. *Science* 265:2098-2101.

Mao LM, Wang W, Chu XP, Zhang GC, Liu XY, Yang YJ, Haines M, Papasian CJ, Fibuch EE, Buch S, Chen JG, Wang JQ. (2009) Stability of surface NMDA receptors controls synaptic and behavioral adaptations to amphetamine. *Nat Neurosci*. 12: 602-610.

Maragakis NJ, Rothstein JD (2004) Glutamate transporters: animal models to neurologic disease. *Neurobiol Dis*. 15: 461-473.

Marcaggi P, Billups D, Attwell D. (2003) The role of glial glutamate transporters in maintaining the independent operation of juvenile mouse cerebellar parallel fibre synapses. *J Physiol*. 552:89-107.

Martin ED, Fernandez M, Perea G, Pascual O, Haydon PG, Araque A, Cena V. (2007) Adenosine released by astrocytes contributes to hypoxia-induced modulation of synaptic transmission. *Glia* 55: 36-45.

Martin LJ , Brambrink AM , Lehmann C , Portera-Cailliau C , Koehler R , Rothstein J , Traystman RJ (1997) Hypoxia–ischemia causes abnormalities in glutamate transporters and death of astroglia and neurons in newborn striatum. *Ann Neurol*. 42:335- 348.

Martinowich K, Mendelowitz DS, Williams SH, Sutherland ML. (2001) EAAT2 overexpression plays a neuroprotective role in models of temporal lobe epilepsy. Society for Neuroscience Abstract.

Masliah E, Alford M, DeTeresa R, Mallory M, Hansen L. (1996) Deficient glutamate transport is associated with neurodegeneration in Alzheimer's disease. *Ann Neurol*. 40:759-766.

Masliah E, Alford M, Mallory M, Rockenstein E, Moechars D, Leuven FV. (2000) Abnormal Glutamate Transport Function in Mutant Amyloid Precursor Protein Transgenic Mice. *Exp Neurol.* 163: 381-387.

Matos M, Augusto E, Agostinho P, Cunha, RA., Chen JF. (2013) Antagonistic interaction between adenosine A_{2A} receptors and Na^+/K^+-ATPase-α_2 controlling glutamate uptake in astrocytes. *J Neurosci*. (accepted)

Matos M, Augusto E, Machado NJ, dos Santos-Rodrigues A, Cunha RA, Agostinho P. (2012a) Astrocytic adenosine A_{2A} receptors control the amyloid-β peptide-induced decrease of glutamate uptake*. J Alzheimers Dis.* 31: 555-567.

Matos M, Augusto E, Oliveira CR, Agostinho P. (2008) Amyloid-beta peptide decreases glutamate uptake in cultured astrocytes: involvement of oxidative stress and mitogen-activated protein kinase cascades. *Neuroscience* 156: 898-910.

Matos M, Augusto E, Santos-Rodrigues A, Schwarzschild MA, Chen JF, Cunha RA, Agostinho P. (2012b) Adenosine A_{2A} receptors modulate glutamate uptake in cultured astrocytes and gliosomes. *Glia* 60: 702-716.

Matute C, Melone M, Vallejo-Illarramendi A, Conti F. (2005) Increased expression of the astrocytic glutamate transporter GLT-1 in the prefrontal cortex of schizophrenics. Glia. 49:451-500.

Matyash V, Kettenmann H. (2010) Heterogeneity in astrocyte morphology and physiology. *Brain Res Rev*. 63:2-10.

McMinn MT, Williams SH, Sutherland ML. (2003) The role of enhanced glutamate transport in a pilocarpine model of temporal lobe epilepsy. Society for Neuroscience Abstract 688.2.

Melone M, Bellesi M, Conti F. (2009) Synaptic localization of GLT-1a in the rat somatic sensory cortex. *Glia* 57:108-117.

Melone M, Bragina L, Conti F. (2003) Clozapine-induced reduction of glutamate transport in the frontal cortex is not mediated by GLAST and EAAC1. *Mol Psychiatry* 8: 12-13.

Mennerick S, Zorumski CF. (1994). Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature* 368, 59-62.

Middeldorp J, Hol EM. (2011) GFAP in health and disease. *Prog Neurobiol*. 93: 421-443.

Miguel-Hidalgo JJ (2009) The role of glial cells in drug abuse. *Curr Drug Abuse Rev*. 2:72-82

Milanese M, Bonifacino T, Zappettini S, Usai C, Tacchetti C, Nobile M, Bonanno G. (2009) Glutamate release from astrocytic gliosomes under physiological and pathological conditions. *Int Rev Neurobiol*. 85: 295-318.

Miller BR, Dorner JL, Shou M, Sari Y, Barton SJ, Sengelaub DR, Kennedy RT, Rebec GV. (2008) Up-regulation of GLT1 expression increases glutamate uptake and attenuates the Huntington's disease phenotype in the R6/2 mouse. *Neuroscience* 153:329-337.

Miller HP, Levey AI, Rothstein JD, Tzingounis AV, Conn PJ. (1997) Alterations in glutamate transporter protein levels in kindling-induced epilepsy. *J Neurochem*. 68:1564-1570.

Miller RH, Raff MC. (1984) Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. *J Neurosci*. 4:585-592.

Min MY, Rusakov DA, Kullmann DM. (1998) Activation of AMPA, kainate, and metabotropic receptors at hippocampal mossy fiber synapses: role of glutamate diffusion. *Neuron* 21*:* 561-570.

Minelli A, Barbaresi P, Reimer RJ, Edwards RH, Conti F. (2001) The glial glutamate transporter GLT-1 is localized both in the vicinity of and at distance from axon terminals in the rat cerebral cortex. *Neuroscience* 108:51-59.

Miralles VJ, Martínez-López I, Zaragozá R, Borrás E, García C, Pallardó FV, Viña JR. (2001) Na^+ dependent glutamate transporters EAAT1, EAAT2, and EAAT3 in primary astrocyte cultures: effect of oxidative stress. *Brain Res*. 922: 21-29.

Mishima T, Hirase H. (2010) In vivo intracellular recording suggests that gray matter astrocytes in mature cerebral cortex and hippocampus are electrophysiologically homogeneous. *J Neurosci*. 30:3093-3100.

Mitterauer BJ. (2010) The syncytiopathy hypothesis of depression: downregulation of glial connexins may protract synaptic information processing and cause memory impairment. *Med Hypotheses* 74:497-502.

Moghaddam B, Javitt D. (2012) From revolution to evolution: the glutamate hypothesis of schizophrenia and its implication for treatment. *Neuropsychopharmacology* 37: 4-15.

Moidunny S, Vinet J, Wesseling E, Bijzet J, Shieh CH, van Ijzendoorn SC, Bezzi P, Boddeke HW, Biber K. (2012) Adenosine A(2B) receptor-mediated leukemia inhibitory factor release from astrocytes protects cortical neurons against excitotoxicity. *J Neuroinflammation* 9:1-18.

Montana V, Malarkey EB, Verderio C, Matteoli M, Parpura V. (2006) Vesicular transmitter release from astrocytes. *Glia* 54: 700-715.

Mookherjee P, Green PS, Watson GS, Marques MA, Tanaka K, Meeker KD, Meabon JS, Li N, Zhu P, Olson VG, Cook DG. (2011) GLT-1 loss accelerates cognitive deficit onset in an Alzheimer's disease animal model. *J Alzheimers Dis*. 26: 447-455.

Morelli M, Blandini F, Simola N, Hauser RA. (2012) A(2A) Receptor Antagonism and Dyskinesia in Parkinson's Disease. *Parkinsons Dis*. 2012: 1-8.

Morelli M, Carta AR, Kachroo A, Schwarzschild MA. (2010) Pathophysiological roles for purines: adenosine, caffeine and urate. *Prog Brain Res*. 183:183-208.

Moseley AE, Williams MT, Schaefer TL, Bohanan CS, Neumann JC, Behbehani MM, Vorhees CV, Lingrel JB (2007) Deficiency in Na, K-ATPase alpha isoform genes alters spatial learning, motor activity, and anxiety in mice. *J Neurosci*. 27:616-626.

Mothet JP, Parent AT, Wolosker H, Brady RO, Jr, Linden DJ, Ferris CD, Rogawski MA, Snyder SH. (2000) D-serine is an endogenous ligand for the glycine site of the N-methyl-D-aspartate receptor. *Proc Natl Acad Sci USA*. 97:4926-4931.

Mothet JP, Pollegioni L, Ouanounou G, Martineau M, Fossier P, Baux G. (2005) Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine. *Proc Natl Acad Sci USA*. 102:5606-5611.

Mothet JP, Rouaud E, Sinet PM, Potier B, Jouvenceau A, Dutar P, Videau C, Epelbaum J, Billard JM. (2006) A critical role for the glial-derived neuromodulator D-serine in the age-related deficits of cellular mechanisms of learning and memory. *Aging Cell* 5:267-274.

Mulligan SJ, MacVicar BA. (2004) Calcium transients in astrocyte endfeet cause cerebrovascular constrictions. *Nature* 431:195–99.

Murphy MG, Moak CM, Byczko Z, MacDonald WF. (1991) Adenosine-dependent regulation of cyclic AMP accumulation in primary cultures of rat astrocytes and neurons. *J Neurosci Res*. 30:631-640.

Nagele RG, Wegiel J, Venkataraman V, Imaki H, Wang KC, Wegiel J.(2004) Contribution of glial cells to the development of amyloid plaques in Alzheimer's disease. *Neurobiol Aging* 25: 663-674.

Nakagawa T, Otsubo Y, Yatani Y, Shirakawa H, Kaneko S. (2008) Mechanisms of substrate transport-induced clustering of a glial glutamate transporter GLT-1 in astroglial-neuronal cultures. *Eur J Neurosci*. 28: 1719-1730.

Nakamura Y, Iga K, Shibata T, Shudo M, Kataoka K. (1993) Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. *Glia* 9: 48-56.

Namura S, Maeno H, Takami S, Jiang XF, Kamichi S, Wada K, Nagata I. (2002) Inhibition of glial glutamate transporter GLT-1 augments brain edema after transient focal cerebral ischemia in mice. *Neurosci Lett*. 324:117-120.

Nanitsos EK, Nguyen KT, St'astný F, Balcar VJ. (2005) Glutamatergic hypothesis of schizophrenia: involvement of Na+/K+-dependent glutamate transport. *J Biomed Sci*. 12: 975-984.

Navarrete M, Araque A. (2010) Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. *Neuron* 68:113-126.

Navarrete M, Araque A. (2011) Basal Synaptic Transmission: Astrocytes Rule! *Cell* 146:675-677.

Nedergaard M, Rodríguez JJ, Verkhratsky A. (2010) Glial calcium and diseases of the nervous system. *Cell Calcium* 47:140-149.

Newman EA. (2003) Glial cell inhibition of neurons by release of ATP. *J Neurosci.* 23:1659-1666.

Newman LA, Korol DL, Gold PE. (2011) Lactate produced by glycogenolysis in astrocytes regulates memory processing. *PLoS One* 6:e28427.

Nguyen KT, Buljan V, Else PL, Pow DV, Balcar VJ. (2010) Cardiac glycosides ouabain and digoxin interfere with the regulation of glutamate transporter GLAST in astrocytes cultured from neonatal rat brain. *Neurochem Res*. 35:2062-2069.

Nicholls DG. (2003) Bioenergetics and transmitter release in the isolated nerve terminal *Neurochem Res*. 28:1433-1441.

Nikbakht MR, Stone TW. (2001) Suppression of presynaptic responses to adenosine by activation of NMDA receptors. *Eur J Pharmacol*. 427:13-25.

Nishizaki T, Nagai K, Nomura T, Tada H, Kanno T, Tozaki H, Li XX, Kondoh T, Kodama N, Takahashi E, Sakai N, Tanaka K, Saito N. (2002) A new neuromodulatory pathway with a glial contribution mediated via A(2a) adenosine receptors. *Glia* 39:133-147.

Nishizaki T. (2004) ATP- and adenosine-mediated signaling in the central nervous system: adenosine stimulates glutamate release from astrocytes via A2a adenosine receptors. *J Pharmacol Sci.* 94: 100-102.

Obara M, Szeliga M, Albrecht J. (2008) Regulation of pH in the mammalian central nervous system under normal and pathological conditions: facts and hypotheses. *Neurochem Int*. 52: 905-19.

Oberheim NA, Goldman SA, Nedergaard M. (2012) Heterogeneity of astrocytic form and function. *Methods Mol Biol*. 814:23-45.

Oberheim NA, Tian GF, Han X, Peng W, Takano T, Ransom B, Nedergaard M. (2008) Loss of astrocytic domain organization in the epileptic brain. *J Neurosci*. 28: 3264-3276.

Oberheim NA, Wang X, Goldman S, Nedergaard M. (2009) Astrocytic complexity distinguishes the human brain. *Trends Neurosci*. 29:547-553.

Ogata T, Nakamura Y, Schubert P. (1996) Potentiated cAMP rise in metabotropically stimulated rat cultured astrocytes by a Ca2+-related A1/A2 adenosine receptor cooperation. *Eur J Neurosci.* 8:1124-1131.

Ogata T, Nakamura Y, Tsuji K, Shibata T, Kataoka K, Schubert P. (1994) Adenosine enhances intracellular Ca2+ mobilization in conjunction with metabotropic glutamate receptor activation by t-ACPD in cultured hippocampal astrocytes. *Neurosci Lett*. 170:5-8.

Oliveira JF, Krügel U, Köles L, Illes P, Wirkner K. (2008) Blockade of glutamate transporters leads to potentiation of NMDA receptor current in layer V pyramidal neurons of the rat prefrontal cortex via group II metabotropic glutamate receptor activation. *Neuropharmacology* 2008 55:447-453.

Omrani A, Melone M, Bellesi M, Safiulina V, Aida T, Tanaka K, Cherubini E, Conti F. (2009) Up-regulation of GLT-1 severely impairs LTD at mossy fibre--CA3 synapses. *J Physiol*. 587:4575-4588.

Panatier A, Theodosis DT, Mothet JP, Touquet B, Pollegioni L, Poulain DA, Oliet SH. (2006) Glia-derived D-serine controls NMDA receptor activity and synaptic memory. *Cell* 125:775-784.

Panatier A, Vallée J, Haber M, Murai KK, Lacaille JC, Robitaille, R. (2011) Astrocytes are endogenous regulators of basal transmission at central synapses. *Cell* 146: 785-798.

Pappas TC, Alagarsamy S, Pollard RB, Nokta M. (1998) HIV decreases glutamate transport in SK-N-MC neuroblastoma cells. *J Neurovirol.* 4:69-79.

Parkinson FE, Sinclair CJ, Othman T, Haughey NJ, Geiger JD. (2002) Differences between rat primary cortical neurons and astrocytes in purine release evoked by ischemic conditions. *Neuropharmacology* 43:836-46.

Parpura V, Basarsky TA, Liu F, Jeftinija K, Jeftinija S, Haydon PG. (1994) Glutamate-mediated astrocyte-neuron signalling. *Nature* 369:744-747.

Parpura V, Heneka MT, Montana V, Oliet SH, Schousboe A, Haydon PG, Stout RF Jr, Spray DC, Reichenbach A, Pannicke T, Pekny M, Pekna M, Zorec R, Verkhratsky A. (2012) Glial cells in (patho)physiology. *J Neurochem*. 121:4-27.

Parpura V, Zorec R. (2010) Gliotransmission: Exocytotic release from astrocytes. *Brain Res Rev.* 63: 83-92.

Parpura-Gill A, Beitz D, Uemura E. (1997) The inhibitory effects of beta-amyloid on glutamate and glucose uptakes by cultured astrocytes. *Brain Res*. 754: 65-71.

Pascual O, Casper KB, Kubera C, Zhang J, Revilla-Sanchez R, Sul JY, Takano H, Moss SJ, McCarthy K, Haydon PG. (2005) Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310: 113-116.

Pasti L, Volterra A, Pozzan T, Carmignoto G. (1997) Intracellular calcium oscillations in astrocytes: a highly plastic, bidirectional form of communication between neurons and astrocytes in situ. *J Neurosci*. 17:7817-7830.

Pasti L, Zonta M, Pozzan T, Vicini S, Carmignoto G. (2001) Cytosolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate. *J Neurosci*. 21:477-484.

Peakman MC, Hill SJ. (1994) Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. *Br J Pharmacol*. 111:191-198.

Peakman MC, Hill SJ. (1996) Adenosine A1 receptor-mediated inhibition of cyclic AMP accumulation in type-2 but not type-1 rat astrocytes. *Eur J Pharmacol*. 306:281-289.

Pekny M, Nilsson M. (2005) Astrocyte activation and reactive gliosis. *Glia* 50:427-434.

Pellerin L, Magistretti PJ. (1997) Glutamate uptake stimulates Na^+, K^+-ATPase activity in astrocytes via activation of a distinct subunit highly sensitive to ouabain. *J Neurochem*. 69: 2132-2137.

Peng L, Huang R, Yu AC, Fung KY, Rathbone MP, Hertz L. (2005) Nucleoside transporter expression and function in cultured mouse astrocytes. *Glia* 52: 25-35.

Perea G, Araque A. (2007). Astrocytes potentiate transmitter release at single hippocampal synapses. *Science* 317: 1083-1086.

Perea G, Navarrete M, Araque A: (2009) Tripartite synapses: astrocytes process and control synaptic information. *Trends Neurosci.* 32: 421-431.

Pereira GS, Rossato JI, Sarkis JJ, Cammarota M, Bonan CD, Izquierdo I. (2005) Activation of adenosine receptors in the posterior cingulate cortex impairs memory retrieval in the rat. *Neurobiol Learn Mem*. 83:217-223.

Phillis JW. 2004. Adenosine and adenine nucleotides as regulators of cerebral blood flow: roles of acidosis, cell swelling, and KATP channels. *Crit Rev Neurobiol*. 16: 237-270.

Pickel VM, Chan J, Linden J, Rosin DL. (2006) Subcellular distributions of adenosine A1 and A2A receptors in the rat dorsomedial nucleus of the solitary tract at the level of the area postrema. *Synapse* 60:496-509.

Pilitsis JG, Kimelberg HK. (1998) Adenosine receptor mediated stimulation of intracellular calcium in acutely isolated astrocytes. *Brain Res*. 798:294-303.

Pines G , Danbolt NC , Bjoras M , Zhang Y , Bendahan A , Eide L , Koepsell H , Storm-Mathisen J , Seeberg E , Kanner BI. (1992) Cloning and expression of a rat brain l -glutamate transporter. *Nature* 360: 464-467.

Pintor A, Galluzzo M, Grieco R, Pèzzola A, Reggio R, Popoli P. (2004) Adenosine A_{2A} receptor antagonists prevent the increase in striatal glutamate levels induced by glutamate uptake inhibitors. *J Neurochem*. 89: 152-156.

Pita-Almenar JD, Collado MS, Colbert CM, Eskin A. (2006) Different mechanisms exist for the plasticity of glutamate reuptake during early long-term potentiation (LTP) and late LTP. *J Neurosci.* 26:10461-1071.

Pita-Almenar JD, Zou S, Colbert CM, Eskin A. (2012) Relationship between increase in astrocytic GLT-1 glutamate transport and late-LTP. *Learn Mem*. 19:615-626.

Plachez C, Martin A, Guiramand J, Récasens M. (2004) Astrocytes repress the neuronal expression of GLAST and GLT glutamate transporters in cultured hippocampal neurons from embryonic rats. *Neurochem Int*. 45: 1113-1123.

Ponzio TA, Wang YF, Hatton GI. (2006) Activation of adenosine A2A receptors alters postsynaptic currents and depolarizes neurons of the supraoptic nucleus. *Am J Physiol Regul Integr Comp Physiol*. 291:R359-366.

Popoli P, Betto P, Reggio R, Ricciarello G. (1995) Adenosine A2A receptor stimulation enhances striatal extracellular glutamate levels in rats. *Eur J Pharmacol*. 287:215-217.

Popoli P, Frank C, Tebano MT, Potenza RL, Pintor A, Domenici MR, Nazzicone V, Pèzzola A, Reggio R. (2003).Modulation of glutamate release and excitotoxicity by adenosine *A2A* receptors. *Neurology* 61: S69-71.

Porter JT, McCarthy KD (1995) Adenosine receptors modulate $[Ca^{2+}]i$ in hippocampal astrocytes in situ. *J Neurochem.* 65:1515-1523.

Potier B, Billard JM, Rivière S, Sinet PM, Denis I, Champeil-Potokar G, Grintal B, Jouvenceau A, Kollen M, Dutar P. (2010) Reduction in glutamate uptake is associated with extrasynaptic NMDA and metabotropic glutamate receptor activation at the hippocampal CA1 synapse of aged rats. *Aging Cell* 9:722-735.

Powell EM, Geller HM. (1999) Dissection of astrocyte-mediated cues in neuronal guidance and process extension. *Glia* 26:73-83.

Prediger RD, Batista LC, Takahashi RN. (2005a) Caffeine reverses age-related deficits in olfactory discrimination and social recognition memory in rats. Involvement of adenosine A1 and A2A receptors. *Neurobiol Aging*. 26:957-964.

Pugliese AM, Traini C, Cipriani S, Gianfriddo M, Mello T, Giovannini MG, Galli A, Pedata F. (2009) The adenosine A2A receptor antagonist ZM241385 enhances neuronal survival after oxygen-glucose deprivation in rat CA1 hippocampal slices. *Br J Pharmacol*. 157:818-30.

Querfurth, HW, LaFerla, FM. (2007) Alzheimer's disease. *N Engl J Med*. 362:329-344.

Rajkowska G, Stockmeier CA. (2013) Astrocyte pathology in major depressive disorder: insights from human postmortem brain tissue. *Curr Drug Targets* 7. [Epub ahead of print]

Ramon y Cajal, S. (1995) Histology of the nervous system, translated by N.Swanson and L. Swanson, Oxford University Press (1995).

Ranganath C, Minzenberg MJ, Ragland JD. (2008) The cognitive neuroscience of memory function and dysfunction in schizophrenia. *Biol Psychiatry* 64: 18-25.

Rao JS, Kellom M, Reese EA, Rapoport SI, Kim HW. (2012) Dysregulated glutamate and dopamine transporters in postmortem frontal cortex from bipolar and schizophrenic patients. *J Affect Disord. 136*: 63-71.

Rao-Ruiz P, Rotaru DC, van der Loo RJ, Mansvelder HD, Stiedl O, Smit AB, Spijker S. (2011) Retrieval-specific endocytosis of GluA2-AMPARs underlies adaptive reconsolidation of contextual fear. *Nat Neurosci.* 14: 1302-1308.

Räth M, Föhr KJ, Weigt HU, Gauss A, Engele J, Georgieff M, Köster S, Adolph O. (2008) Etomidate reduces glutamate uptake in rat cultured glial cells: involvement of PKA. *Br J Pharmacol.* 155: 925-933.

Rathbone MP, Middlemiss PJ, DeLuca B, Jovetich M. (1991) Extracellular guanosine increases astrocyte cAMP: inhibition by adenosine A2 antagonists. *Neuroreport.* 2:661-664.

Rebola N, Canas PM, Oliveira CR, Cunha RA. (2005) Different synaptic and subsynaptic localization of adenosine A_{2A} receptors in the hippocampus and striatum of the rat. *Neuroscience* 132: 893-903.

Rebola N, Lujan R, Cunha RA, Mulle C (2008) Adenosine A_{2A} receptors are essential for long-term potentiation of NMDA-EPSCs at hippocampal mossy fiber synapses. *Neuron* 57:121-34.

Rebola N, Pinheiro PC, Oliveira CR, Malva JO, Cunha RA. (2003) Subcellular localization of adenosine A_1 receptors in nerve terminals and synapses of the rat hippocampus. *Brain Res*. 987: 49-58.

Rebola N, Rodrigues RJ, Lopes LV, Richardson PJ, Oliveira CR, Cunha RA. (2005) Adenosine A_1 and A_{2A} receptors are co-expressed in pyramidal neurons and co-localized in glutamatergic nerve terminals of the rat hippocampus. *Neuroscience* 133: 79-83.

Rebola N, Simões AP, Canas PM, Tomé AR, Andrade GM, Barry CE, Agostinho PM, Lynch MA, Cunha RA. (2011) Adenosine A_{2A} receptors control neuroinflammation and consequent hippocampal neuronal dysfunction. *J Neurochem*. 117: 100-111.

Reichelt W, Knöpfel T. (2002) Glutamate uptake controls expression of a slow postsynaptic current mediated by mGluRs in cerebellar Purkinje cells. *J Neurophysiol*. 87:1974-1980.

Reichenbach A, Derouiche A, Kirchhoff F. (2010) Morphology and dynamics of perisynaptic glia. *Brain Res Rev*. 63: 11-25.

Reichenbach A, Wolburg H. (2005) Astrocytes and ependymal glia. H. Kettenmann, B.R. Ransom (Eds.), *Neuroglia 2nd edition*, Oxford Univ. Press (2005), 19-36.

Reinhard L, Tidow H, Clausen MJ, Nissen P (2013) Na^+,K^+-ATPase as a docking station: protein-protein complexes of the Na^+,K^+-ATPase. *Cell Mol Life Sci*. 70: 205-222.

Ribeiro JA, Sebastião AM. (2010) Caffeine and adenosine. *J Alzheimers Dis*. 20 Suppl 1:S3-15.

Rimondini R, Ferré S, Ogren SO, Fuxe K. (1997) Adenosine A_{2A} agonists: a potential new type of atypical antipsychotic. *Neuropsychopharmacology* 17: 82-91.

Ritchie K, Carrière I, de Mendonca A, Portet F, Dartigues JF, Rouaud O, Barberger-Gateau P, Ancelin ML. (2007) The neuroprotective effects of caffeine: a prospective population study (the Three City Study). *Neurology* 69: 536-545.

Robinson MB. (2006) Acute regulation of sodium-dependent glutamate transporters: a focus on constitutive and regulated trafficking. *Handb Exp Pharmacol*. 175: 251-275.

Rodrigues RJ, Alfaro TM, Rebola N, Oliveira CR, Cunha RA. (2005) Co-localization and functional interaction between A_{2A} and mGluR5 in glutamatergic nerve terminals of the rat striatum. *J Neurochem*. 92: 433-441.

Rodrigues RJ, Canas PM, Lopes LV, Oliveira CR, Cunha RA. (2008) Modification of adenosine modulation of acetylcholine release in the hippocampus of aged rats. *Neurobiol Aging* 29: 1597-1601.

Rodriguez-Kern A, Gegelashvili M, Schousboe A, Zhang J, Sung L, Gegelashvili G. (2003) Beta-amyloid and brain-derived neurotrophic factor, BDNF, up-regulate the expression of glutamate transporter GLT-1/EAAT2 via different signaling pathways utilizing transcription factor NF-kappaB. *Neurochem Int*. 43: 363-370.

Rose EM, Koo JC, Antflick JE, Ahmed SM, Angers S, Hampson DR. (2009) Glutamate transporter coupling to Na,K-ATPase. *J Neurosci*. 29:8143-8155.

Rosenberg PA, Aizenman E. (1989) Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. *Neurosci Lett*. 103:162-168.

Ross CA, Margolis RL, Reading SA, Pletnikov M, Coyle JT. (2006). Neurobiology of schizophrenia. *Neuron* 5: 139-153.

Rothstein JD , Van Kammen M , Levey AI , Martin LJ , Kuncl RW (1995) Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis . *Ann Neurol.* 38: 73-84.

Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, Kanai Y, Hediger MA, Wang Y, Schielke JP, Welty DF. (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* 16:675-686.

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB. (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. *Nature* 433:73-77.

Rouach N, Koulakoff A, Abudara V, Willecke K, Giaume C. (2008) Astroglial metabolic networks sustain hippocampal synaptic transmission. *Science* 322:1551-1555.

Santos C, Costa, J Santos J, Vaz-Carneiro A, Lunet N. (2010) Caffeine intake and dementia: systematic review and meta-analysis. *J Alzheimers Dis.* 20 (Suppl 1): S187-204.

Santos C, Lunet N, Azevedo A, de Mendonça A, Ritchie K, Barros H. (2010) Caffeine intake is associated with a lower risk of cognitive decline: a cohort study from Portugal. *J Alzheimers Dis.* 1:S175-185.

Sarkar PK. (2002) A quick assay for Na+-K+-ATPase specific activity. *Z Naturforsch C*. 57: 562-564.

Sasaki T, Kuga N, Namiki S, Matsuki N, Ikegaya Y. (2011) Locally synchronized astrocytes. *Cereb Cortex*. 21:1889-1900.

Sattler R, Rothstein JD. (2006) Regulation and dysregulation of glutamate transporters. *Handb Exp Pharmacol.* 175: 277-303.

Saura J. (2007) Microglial cells in astroglial cultures: a cautionary note. *J Neuroinflammation* 15: 1-26.

Sawada K, Echigo N, Juge N, Miyaji T, Otsuka M, Omote H, Yamamoto A, Moriyama Y. (2008) Identification of a vesicular nucleotide transporter. *Proc Natl Acad Sci USA*. 105:5683-5686.

Schaddelee MP, Voorwinden HL, van Tilburg EW, Pateman TJ, Ijzerman AP, Danhof M, de Boer AG. (2003) Functional role of adenosine receptor subtypes in the regulation of blood-brain barrier permeability: possible implications for the design of synthetic adenosine derivatives. *Eur J Pharm Sci.* 19:13-22.

Schallier A, Smolders I, Van Dam D, Loyens E, De Deyn PP, Michotte A, Michotte Y, Massie A. (2011) Region- and age-specific changes in glutamate transport in the AβPP23 mouse model for Alzheimer's disease. *J Alzheimers Dis*. 24: 287-300.

Scheff SW, Price DA, Schmitt FA, Mufson EJ. (2006) Hippocampal synaptic loss in early Alzheimer's disease and mild cognitive impairment. *Neurobiol Aging* 27: 1372-1384.

Schenk U, Verderio C, Benfenati F, Matteoli M. (2003) Regulated delivery of AMPA receptor subunits to the presynaptic membrane. *EMBO J.* 22: 558-568.

Schiffmann SN, Fisone G, Moresco R, Cunha RA, Ferré S. (2007) Adenosine A_{2A} receptors and basal ganglia physiology. *Prog Neurobiol*. 83:277-292.

Schmittgen TD, Livak KJ. (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*. 3: 1101-1108.

Schneider JS, Wade T, Lidsky TI. (1998) Chronic neuroleptic treatment alters expression of glial glutamate transporter GLT-1 mRNA in the striatum. *Neuroreport*. 9:133-136.

Schnieder TP, Dwork AJ. (2011) Searching for neuropathology: gliosis in schizophrenia. *Biol Psychiatry* 69:134-139.

Schoen SW, Kreutzberg GW. (1997) 5'-nucleotidase enzyme cytochemistry as a tool for revealing activated glial cells and malleable synapses in CNS development and regeneration. *Brain Res Protoc.* 1:33-43.

Schousboe A, Sickmann HM, Bak LK, Schousboe I, Jajo FS, Faek SA, Waagepetersen HS. (2011) Neuron-glia interactions in glutamatergic neurotransmission: roles of oxidative and glycolytic adenosine triphosphate as energy source. *J Neurosci Res*. 89:1926-1934.

Schwaninger M, Neher M, Viegas E, Schneider A, Spranger M. (1997) Stimulation of interleukin-6 secretion and gene transcription in primary astrocytes by adenosine. *J Neurochem*. 69:1145-1150.

Schwarz MJ, Myint A-M. (2011) Astrocytes in Major Depressive Disorder and Schizophrenia. David C . Scemes and Eliana Spray (eds.), *Astrocytes - Wiring the Brain*. CRC Press (2011) 14:357-386.

Schwarzschild MA, Agnati L, Fuxe K, Chen JF, Morelli M. (2006) Targeting adenosine A2A receptors in Parkinson's disease. *Trends Neurosci.* 29:647-654.

Scott HA, Gebhardt FM, Mitrovic AD, Vandenberg RJ, Dodd PR. (2011) Glutamate transporter variants reduce glutamate uptake in Alzheimer's disease. *Neurobiol Aging* 32: 553.e1-11.

Scott HL, Tannenberg AE, Dodd PR. (1995) Variant forms of neuronal glutamate transporter sites in Alzheimer's disease cerebral cortex. *J Neurochem*. 64: 2193-2202.

Sebastião AM, Ribeiro JA (2009) Adenosine Receptors and the Central Nervous System. *Handb Exp Pharmacol*. 193:471-534.

Seifert G, Schilling K, Steinhauser C. (2006) Astrocyte dysfunction in neurological disorders: a molecular perspective. *Nat Rev Neurosci.* 7:194-206.

Selkoe DJ. (2002) Alzheimer's disease is a synaptic failure. *Science* 298: 789-791.

Selkirk JV, Stiefel TH, Stone IM, Naeve GS, AC Foster, Poulsen DJ. (2011) Over-expression of the human EAAT2 glutamate transporter within neurons of mouse organotypic hippocampal slice cultures leads to increased vulnerability of CA1 pyramidal cells. *Eur J Neurosci.* 21:2291-2296

Serrano A, Haddjeri N, Lacaille JC, Robitaille R. (2006) GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. *J Neurosci.* 26: 5370-5382.

Shao L, Vawter MP. (2008) Shared gene expression alterations in schizophrenia and bipolar disorder. *Biol Psychiatry.* 64:89-97.

Sheldon AL, Robinson MB. (2007) The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention. *Neurochem Int*. 51:333-355.

Shen HY, Coelho JE, Ohtsuka N, Canas PM, Day YJ, Huang QY, Rebola N, Yu L, Boison D, Cunha RA, Linden J, Tsien JZ, Chen JF. (2008) A critical role of the adenosine A_{2A} receptor in extrastriatal neurons in modulating psychomotor activity as revealed by opposite phenotypes of striatum and forebrain A_{2A} receptor knock-outs. *J Neurosci*. 19: 2970-2975.

Silva CG, Porciúncula LO, Canas PM, Oliveira CR, Cunha RA. (2007) Blockade of adenosine A_{2A} receptors prevents staurosporine-induced apoptosis of rat hippocampal neurons. *Neurobiol Dis*. 27: 182-189.

Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M. (2003) Signaling at the gliovascular interface. *J Neurosci.* 23: 9254-9262.

Simard M, Nedergaard M. (2004) The neurobiology of glia in the context of water and ion homeostasis. *Neuroscience* 129: 877-896.

Simpson MD, Slater P, Deakin JF. (1998) Comparison of glutamate and gamma-aminobutyric acid uptake binding sites in frontal and temporal lobes in schizophrenia. *Biol Psychiatry*. 15: 423-427.

Simpson MD, Slater P, Royston MC, Deakin JF. (1992) Regionally selective deficits in uptake sites for glutamate and gamma-aminobutyric acid in the basal ganglia in schizophrenia. *Psychiatry Res*. 42:273-282.

Singer P*,* McGarrity S, Shen HY, Boison D, Yee BK. (2012) Working memory and the homeostatic control of brain adenosine by adenosine kinase. *Neuroscience* 213:81-92.

Sirevaag AM, Greenough WT. (1987) Differential rearing effects on rat visual cortex synapses. III. Neuronal and glial nuclei, boutons, dendrites, and capillaries. *Brain Res*. 424: 320-332.

Skucas VA, Mathews IB, Yang J, Cheng Q, Treister A, Duffy AM, Verkman AS, Hempstead BL, Wood MA, Binder DK, Scharfman HE. (2011) Impairment of select forms of spatial memory and neurotrophin-dependent synaptic plasticity by deletion of glial aquaporin-4. *J Neurosci*. 31: 6392-6397.

Smith AP. (2009) Caffeine, cognitive failures and health in a non-working community sample. *Hum Psychopharmacol*. 24: 29-34.

Smith RE, and Meador-Woodruff JH. (2002) Striatal excitatory amino acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder. *Neuropsychopharmacology* 26: 368-375.

Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH. (2001) Expression of excitatory amino acid transporter transcripts in the thalamus of subjects with schizophrenia. *Am J Psychiatry* 158: 1393-1399.

Smith T, Weston C, Lieberman J. (2010) Schizophrenia (maintenance treatment). *Am Fam Physician*. 82: 338-339.

Snyder SH. (1976) The dopamine hypothesis of schizophrenia: focus on the dopamine receptor. *Am J Psychiatry*. 133:197-202.

Snyder, SH. (1976) The dopamine hypothesis of schizophrenia*:* focus on the dopamine receptor. Am J Psychiatry. 133:197-202.

Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ , Jarvius J, Wester K, Hydbring P, Bahram F, Larsson L-G, Landegren U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods*. 3: 995-1000.

Sofroniew MV, Vinters HV. (2012) Astrocytes: biology and pathology. *Acta Neuropathol*. 119:7-35.

Spangaro M, Bosia M, Zanoletti A, Bechi M, Cocchi F, Pirovano A, Lorenzi C, Bramanti P, Benedetti F, Smeraldi E, Cavallaro R. (2012) Cognitive dysfunction and glutamate reuptake - effect of EAAT2 polymorphism in schizophrenia. *Neurosci Lett*. 522: 151-155.

Squire LR, Schacter DL. (2003). Studies of memory in rodents and birds. LR Squire and DL Schacter (eds). *The Neuropsychology of Memory*. Guilford Press. (2003) 3: 309-508.

Stehberg J, Moraga-Amaro R, Salazar C, Becerra A, Echeverría C, Orellana JA, Bultynck G, Ponsaerts R, Leybaert L, Simon F, Sáez JC, Retamal MA. (2012) Release of gliotransmitters through astroglial connexin 43 hemichannels is necessary for fear memory consolidation in the basolateral amygdala. *FASEB J*. 26:3649-3657.

Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA. (2007) The classical complement cascade mediates CNS synapse elimination. *Cell* 131:1164-1178.

Stigliani S, Zappettini S, Raiteri L, Passalacqua M, Melloni E, Venturi C, Tacchetti C, Diaspro A, Usai C, Bonanno G. (2006) Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate. *J Neurochem*. 96: 656-668.

Stone TW, Ceruti S, Abbracchio MP. (2009) Adenosine receptors and neurological disease: neuroprotection and neurodegeneration. *Handb Exp Pharmacol*. 193:535-587.

Streit, WJ, Walter SA, Pennell NA. (1999) Reactive Microgliosis. *Prog Neurobiol*. 57:563-581.

Suchak SK, Baloyianni NV, Perkinton MS, Williams RJ, Meldrum BS, Rattray M. (2003) The 'glial' glutamate transporter, EAAT2 GLT-I accounts for high affinity glutamate uptake into adult rodent nerve endings. *J Neurochem*. 84: 522-532.

Suzuki A, Stern SA, Bozdagi O, Huntley GW, Walker RH, Magistretti PJ, Alberini CM. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell* 144: 810-823.

Swanson RA, Liu J, Miller JW, Rothstein JD, Farrell K, Stein BA, Longuemare MC. (1997) Neuronal regulation of glutamate transporter subtype expression in astrocytes. *J Neurosci*. 17: 932-940.

Swanson RA. (2005) Astrocyte neurotransmitter uptake. H. Kettenmann and B. R. Ransom (eds.), *Neuroglia*, *2nd edition*, Oxford University Press (2005). 27:346-350.

Taft JR, Vertes RP, Perry GW. (2005) Distribution of GFAP+ astrocytes in adult and neonatal rat brain. *Int J Neurosci*. 115: 1333-1343.

Takahashi N, Sakurai T. (2012) Roles of glial cells in schizophrenia: Possible targets for therapeutic approaches. *Neurobiol Dis*. 53:49-60.

Takahashi RN, Pamplona FA, Prediger RD. (2008) Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. *Front Biosci*. 13: 2614-2632.

Takano T, Tian GF, Peng W, Lou N, Libionka W, Han X, Nedergaard M. (2006) Astrocyte-mediated control of cerebral blood flow. *Nat Neurosci*. 9:260-267.

Takayasu Y, Iino M, Shimamoto K, Tanaka K, Ozawa S. (2006) Glial glutamate transporters maintain one-to-one relationship at the climbing fiber-Purkinje cell synapse by preventing glutamate spillover. *J Neurosci*. 26:6563-6572.

Tanaka K, Watase K, Manabe T, Yamada K, Watanabe M, Takahashi K, Iwama H, Nishikawa T, Ichihara N, Kikuchi T, Okuyama S, Kawashima N, Hori S, Takimoto M, Wada K. (1997) Epilepsy and exacerbation of brain injury in mice lacking glutamate transporter GLT-1. *Science* 276: 1699-1702.

Theis M , Jauch R , Zhuo L , Speidel D , Wallraff A , Doring B , Frisch C , Sohl G , Teubner B , Euwens C , Huston J , Steinhauser C , Messing A , Heinemann U , Willecke K (2003)

Accelerated hippocampal spreading depression and enhanced locomotory activity in mice with astrocyte-directed inactivation of connexin 43. *J Neurosci*. 23: 766-776.

Tigaret CM, Thalhammer A, Rast GF, Specht CG, Auberson YP, Stewart MG, Schoepfer R. (2006) Subunit dependencies of N-methyl-D-aspartate (NMDA) receptor-induced alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor internalization. *Mol Pharmacol*. 69:1251-1259.

Todd KJ, Darabid H, Robitaille R. (2010) Perisynaptic glia discriminate patterns of motor nerve activity and influence plasticity at the neuromuscular junction. *J Neurosci*. 30: 11870-11882.

Toms NJ, Roberts PJ. (1999) Group 1 mGlu receptors elevate [Ca2+]i in rat cultured cortical type 2 astrocytes: [Ca2+]i synergy with adenosine A1 receptors. *Neuropharmacology* 38:1511-1517.

Tong G, Jahr CE. (1994) Block of glutamate transporters potentiates postsynaptic excitation. *Neuron* 13: 1195-1203.

Tozzi A, de Iure A, Di Filippo M, Tantucci M, Costa C, Borsini F, Ghiglieri V, Giampà C, Fusco FR, Picconi B, Calabresi P. (2011) The distinct role of medium spiny neurons and cholinergic interneurons in the D_2/A_2A receptor interaction in the striatum: implications for Parkinson's disease. *J Neurosci.* 31:1850-1862.

Trifilieff P, Rives ML, Urizar E, Piskorowski RA, Vishwasrao HD, Castrillon J, Schmauss C, Slättman M, Gullberg M, Javitch JA. (2011) Detection of antigen interactions ex vivo by proximity ligation assay: endogenous dopamine D_2-adenosine A_{2A} receptor complexes in the striatum. *Biotechniques* 51: 111-118.

Trotti D , Rolfs A , Danbolt NC , Brown RH , Jr. , Hediger MA (1999) SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter. *Nat Neurosci*. 2:427-433.

Tsukada S, Iino M, Takayasu Y, Shimamoto K, Ozawa S. (2005) Effects of a novel glutamate transporter blocker, (2S, 3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate (TFB-TBOA), on activities of hippocampal neurons. *Neuropharmacology* 48: 479-491.

Tsvetkov E, Shin RM, Bolshakov VY. (2004) Glutamate uptake determines pathway specificity of long-term potentiation in the neural circuitry of fear conditioning. *Neuron* 41:139-151.

Türközkan N, Bilighan A, Cayci B, Doğulu F, Aykol S. (1996) The effects of 2-chloroadenosine and deoxycoformycin on the ATP level, Na-K ATPase activity in experimental brain ischemia of gerbil. *Neurol Res*. 18: 345-348.

Tzingounis AV, Wadiche JI. (2007) Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat Rev Neurosci*. 8:935-947.

Vallejo-Illarramendi A, Torres-Ramos M, Melone M, Conti F, Matute C. (2005) Clozapine reduces GLT-1 expression and glutamate uptake in astrocyte cultures. *Glia* 50: 276-279.

van Calker, D., Muller, M. and Hamprecht, B. (1979) Adenosine regulates via two different types of receptors, the accumulation of cyclic AMP in cultured brain cells. *J Neurochem*. 33, 999-1005.

van Os J, Kapur S. (2009) Schizophrenia. *Lancet* 374: 635-645.

Van Snellenberg JX. (2009) Working memory and long-term memory deficits in schizophrenia: Is there a common substrate? *Psychiatry Res*. 174:89-96.

Vazdarjanova A, Bunting K, Muthusamy N, Bergson C. (2011) Calcyon upregulation in adolescence impairs response inhibition and working memory in adulthood. *Mol Psychiatry*. 16:672-684.

Velligan DI, Prihoda TJ, Ritch JL, Maples N, Bow-Thomas CC, Dassori A. (2002) A randomized single-blind pilot study of compensatory strategies in schizophrenia outpatients. *Schizophr Bull.* 28: 283-292.

Ventura R, Harris KM. (1999) Three-dimensional relationships between hippocampal synapses and astrocytes. *J Neurosci*. 19:6897-6906.

Verkhratsky A, Krishtal OA, Burnstock G. (2009) Purinoceptors on neuroglia. *Mol Neurobiol*. 39:190-208.

Verkhratsky A, Parpura V. (2010) Recent advances in (patho)physiology of astroglia. *Acta Pharmacol Sin.* 31:1044-1054.

Verkhratsky A, Toescu EC. (2006) Neuronal-glial networks as substrate for CNS integration. *J Cell Mol Med*. 10: 826-836.

Volterra A, Meldolesi J. (2005) Astrocytes, from brain glue to communication elements: the revolution continues. *Nat Rev Neurosci*. 6:626-640.

Vitvitsky VM, Garg SK, Keep RF, Albin RL, Banerjee R. (2012) Na+ and K+ ion imbalances in Alzheimer's disease. *Biochim Biophys Acta.* 1822:1671-1681.

von Engelhardt J, Doganci B, Jensen V, Hvalby Ø, Göngrich C, Taylor A, Barkus C, Sanderson DJ, Rawlins JN, Seeburg PH, Bannerman DM, Monyer H. (2008) Contribution of hippocampal and extra-hippocampal NR2B-containing NMDA receptors to performance on spatial learning tasks. *Neuron* 60: 846-860.

Voutsinos-Porsche B, Knott G, Tanaka K, Quairiaux C, Welker E, Bonvento G. (2003) Glial glutamate tranporters and maturation of the mouse somatosensory cortex. *Cereb. Cortex* 13: 1110-112.

Wadiche JI, Arriza JL, Amara SG, Kavanaugh MP. (1995) Kinetics of a human glutamate transporter. *Neuron* 14:1019-1027.

Wang F, Smith NA, Xu Q, Fujita T, Baba A, Matsuda T, Takano T, Bekar L, Nedergaard M. (2012) Astrocytes modulate neural network activity by Ca^{2+}-dependent uptake of extracellular K+. *Sci Signal.* 5: ra26.

Wang JH, Ma YY, van den Buuse M. (2006) Improved spatial recognition memory in mice lacking adenosine A2A receptors. *Exp Neurol.* 199: 438-445.

Wang M, Yang Y, Wang CJ, Gamo NJ, Jin LE, Mazer JA, Morrison JH, Wang XJ, Arnsten AF. (2013) NMDA Receptors Subserve Persistent Neuronal Firing during Working Memory in Dorsolateral Prefrontal Cortex. *Neuron* 77:736-749.

Wang ZY, Zhang YQ, Zhao ZQ. (2006) Inhibition of tetanically sciatic stimulation-induced LTP of spinal neurons and Fos expression by disrupting glutamate transporter GLT-1. *Neuropharmacology* 51:764-772.

Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, Hori S, Takimoto M, Wada K, Tanaka K. (1998) Motor discoordination

and increased susceptibility to cerebellar injury in GLAST mutant mice. *Eur J Neurosci*. 10:976-988.

Webster MJ, O'Grady J, Kleinman JE, Weickert CS. (2005) Glial fibrillary acidic protein mRNA levels in the cingulate cortex of individuals with depression, bipolar disorder and schizophrenia. *Neuroscience* 133:453-461.

Wei CJ, Li W, Chen JF. (2011) Normal and abnormal functions of adenosine receptors in the central nervous system revealed by genetic knockout studies. *Biochim Biophys Acta.*1808:1358-1379.

Wei CJ, Singer P, Coelho J, Boison D, Feldon J, Yee BK, Chen JF. (2011) Selective inactivation of adenosine A(2A) receptors in striatal neurons enhances working memory and reversal learning. *Learn Mem.* 18:459-474.

Wei J, Pan X, Pei Z, Wang W, Qiu W, Shi Z, Xiao G. (2012) The beta-lactam antibiotic, ceftriaxone, provides neuroprotective potential via anti-excitotoxicity and anti-inflammation response in a rat model of traumatic brain injury. *J Trauma Acute Care Surg.* 73:654-660.

Weibrecht I, Leuchowius KJ, Clausson CM, Conze T, Jarvius M, Howell WM, Kamali-Moghaddam M, Soderberg O (2010) Proximity ligation assays: a recent addition to the proteomics toolbox. *Expert Rev Proteomics. 7*: 401-409.

Weller ML, Stone IM, Goss A, Rau T, Rova C, Poulsen DJ. (2008) Selective overexpression of excitatory amino acid transporter 2 (EAAT2) in astrocytes enhances neuroprotection from moderate but not severe hypoxia-ischemia. *Neuroscience* 155:1204-1211.

Wenzel J, Lammert G, Meyer U, Krug M. (1991) The influence of long term potentiation on the spatial relationship between astrocyte processes and potentiated synapses in the dentate gyrus neuropil of rat brain. *Brain Res.* 560: 122-131.

Westphalen RI, Scott HL, Dodd PR (2003) Synaptic vesicle transport and synaptic membrane transporter sites in excitatory amino acid nerve terminals in Alzheimer disease. *J Neural Transm.* 110: 1013-1027.

Wilhelm A, Volknandt W, Langer D, Nolte C, Kettenmann H, Zimmermann H. (2004) Localization of SNARE proteins and secretory organelle proteins in astrocytes in vitro and in situ. *Neurosci Res*. 48: 249-257.

Wyse AT, Bavaresco CS, Reis EA, Zugno AI, Tagliari B, Calcagnotto T, Netto CA. (2004) Training in inhibitory avoidance causes a reduction of Na+,K+-ATPase activity in rat hippocampus. *Physiol Behav.* 80:475-479.

Wong ML, Medrano JF. (2005) Real-time PCR for mRNA quantitation. *Biotechniques* 39: 75-85.

Wong TP, Howland JG, Robillard JM, Ge Y, Yu W, Titterness AK, Brebner K, Liu L, Weinberg J, Christie BR, Phillips AG, Wang YT. (2007) Hippocampal long-term depression mediates acute stress-induced spatial memory retrieval impairment. *Proc Natl Acad Sci USA*. 104:11471-11476.

Wu J, Lee MR, Choi S, Kim T, Choi DS. (2010) ENT1 regulates ethanol-sensitive EAAT2 expression and function in astrocytes. *Alcohol Clin Exp Res*. 34: 1110-1117.

Wu J, Lee MR, Kim T, Johng S, Rohrback S, Kang N, Choi DS. (2011) Regulation of ethanol-sensitive EAAT2 expression through adenosine A1 receptor in astrocytes. *Biochem Biophys Res Commun*. 406: 47-52.

Wu ZQ, Li M, Chen J, Chi ZQ, Liu JG. (2006) Involvement of cAMP/cAMP-dependent protein kinase signaling pathway in regulation of Na+,K+-ATPase upon activation of opioid receptors by morphine. *Mol Pharmacol*. 69: 866-876.

Wyss-Coray T. (2006) Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat Med*. 12: 1005-1015.

Xiong ZQ, Stringer JL. (2000) Sodium pump activity, not glial spatial buffering, clears potassium after epileptiform activity induced in the dentate gyrus. *J Neurophysiol.* 83:1443-1451.

Xu K, Bastia E, Schwarzschild MA. (2005) Therapeutic potential of adenosine A_{2A} receptor antagonists in Parkinson's disease. *Pharmacol Ther*. 105: 267-310

Xu NJ, Bao L, Fan HP, Bao GB, Pu L, Lu YJ, Wu CF, Zhang X, Pei G. (2003) Morphine withdrawal increases glutamate uptake and surface expression of glutamate transporter GLT1 at hippocampal synapses. *J Neurosci*. 23:4775-4784.

Yamagata K, Hakata K, Maeda A, Mochizuki C, Matsufuji H, Chino M, Yamori Y. (2007) Adenosine induces expression of glial cell line-derived neurotrophic factor (GDNF) in primary rat astrocytes. *Neurosci Res*. 59:467-474.

Yang CH, Huang CC, Hsu KS. (2005) Behavioral stress enhances hippocampal CA1 long-term depression through the blockade of the glutamate uptake. *J Neurosci*. 25:4288-4293.

Yang Y, Ge W, Chen Y, Zhang Z, Shen W, Wu C, Poo M, Duan S. (2003) Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. *Proc Natl Acad Sci USA*. 100:15194-15199.

Ye ZC, Rothstein JD, Sontheimer H. (1999) Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. *J Neurosci*. 19: 10767-10777.

Yu C, Gupta J, Chen JF, Yin HH. (2009) Genetic deletion of A2A adenosine receptors in the striatum selectively impairs habit formation. *J Neurosci*. 29:15100-15103.

Yu L, Shen HY, Coelho JE, Araújo IM, Huang QY, Day YJ, Rebola N, Canas PM, Rapp EK, Ferrara J, Taylor D, Müller CE, Linden J, Cunha RA, Chen JF. (2008) Adenosine A_{2A} receptor antagonists exert motor and neuroprotective effects by distinct cellular mechanisms. *Ann Neurol*. 63: 338-346.

Zhang D, Hou Q, Wang M, Lin A, Jarzylo L, Navis A, Raissi A, Liu F, Man HY. (2009) Na, K-ATPase activity regulates AMPA receptor turnover through proteasome-mediated proteolysis. *J Neurosci.* 29:4498-4511.

Zhang LN, Sun YJ, Pan S, Li JX, Qu YE, Li Y, Wang YL, Gao ZB. (2013) Na^+-K^+-ATPase, a potent neuroprotective modulator against Alzheimer disease. *Fundam Clin Pharmacol*. 27:96-103.

Zhang G, Raol YS, Hsu FC, Brooks-Kayal AR. (2004) Long-term alterations in glutamate receptor and transporter expression following early-life seizures are associated with increased seizure susceptibility. *J Neurochem*. 88, 91-101.

Zhang H, Xin W, Dougherty PM. (2009) Synaptically evoked glutamate transporter currents in Spinal Dorsal Horn Astrocytes. *Mol Pain* 5:36.

Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZL, Wu CP, Poo MM, Duan S. (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. *Neuron* 40:971-982.

Zhou SJ, Zhu ME, Shu D, Du XP, Song XH, Wang XT, Zheng RY, Cai XH, Chen JF, He JC. (2009) Preferential enhancement of working memory in mice lacking adenosine A(2A) receptors. *Brain Res.* 1303:74-83.

Zimmermann H, Braun N. (1996) Extracellular metabolism of nucleotides in the nervous system. *J Auton Pharmacol.* 16: 397-400.

Zoia CP, Tagliabue E, Isella V, Begni B, Fumagalli L, Brighina L, Appollonio I, Racchi M, Ferrarese C. (2005) Fibroblast glutamate transport in aging and in AD: correlations with disease severity. *Neurobiol Aging.* 26: 825-832.

Zylka MJ. (2011) Pain-relieving prospects for adenosine receptors and ectonucleotidases. *Trends Mol Med.* 17:188-196.